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Selective modification of rat hepatic microsomal fatty acid chain elongation and desaturation by fibrates: relationship with peroxisome proliferation

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1 The time-course of the effect of clofibrate (CFB), bezafibrate (BFB) and gemfibrozil (GFB) on lipid plasma levels and palmitoyl-, palmitoleoyl- and γ-linolenoyl-CoA elongase, Δ-9, Δ-6 and Δ-5 desaturase activities, and microsomal electron transport chains, as well as the correlation with the peroxisomal proliferation phenomenon have been studied in male Sprague-Dawley rats.

2 As reported in our previous work, the three drugs behave as peroxisomal proliferators (the order of potency was BFB > CFB ≥ GFB) and induced a clear reduction in both plasma cholesterol and triglyceride levels.

3 Palmitoyl-CoA elongation activity was increased by the three drugs (BFB = GFB > CFB), whereas palmitoleoyl-CoA elongation activity was only enhanced by GFB. Elongation activity was not modified by fibrates when γ-linolenoyl-CoA was used as substrate. These results are in accordance with the existence of three different elongation systems for saturated, mono- and polyunsaturated fatty acids.

4 Δ-9, Δ-6 and Δ-5 desaturase activities were increased by the three fibrates, with an order of potency BFB > CFB = GFB for Δ-9 and Δ-5, and GFB > BFB = CFB for Δ-6.

5 Of the enzyme activities integrated in the microsomal electron transport chains, NADH cytochrome b_5 reductase was not affected by fibrate treatment, NADPH cytochrome c reductase activity was enhanced (BFB = GFB > CFB), whereas NADH cytochrome c reductase activity was reduced by CFB and BFB.

6 The increase in Δ-9 and Δ-5 desaturase activities was highly dependent on the peroxisomal proliferation phenomena, whereas the increase in Δ-6 desaturase activity and the decrease in NADH cytochrome c reductase was mainly independent. The modifications of palmitoyl-CoA elongase and NADPH cytochrome c reductase activities, as well as plasma lipid levels, were partially correlated with peroxisomal β-oxidation, but the r^2 values obtained point to the existence of additional independent mechanisms.

7 As man is assumed to be a species refractory to peroxisomal proliferation, only those fibrate effects not absolutely related to this phenomenon are expected to appear after fibrate therapy.

Keywords: Fibrates; peroxisomal proliferation; β-oxidation; elongases; desaturases

Introduction

Fibric acid derivatives constitute a well known group of hypolipidemic drugs, mainly used in the treatment of mixed hyperlipoproteinemia and hypertriglyceridemia (Shepherd *et al.*, 1991). They share three characteristic features: (a) they behave as potent peroxisome proliferators in mice and rats (Sirtori *et al.*, 1992); (b) their hypolipidemic mechanism of action is not well established (Catapano, 1992), and (c) they increase the content of mono-unsaturated fatty acids (mainly palmitoleic and oleic) and decrease the content of polyunsaturated fatty acids (mainly linoleic) in the cholesteryl ester, triglyceride, and phospholipid plasma fractions from hyperlipidemic-treated subjects (Vessby & Lithell, 1990; Agheli & Jacotot, 1991; Tavella *et al.*, 1993). The mechanism underlying this latter effect is, at present, unknown.

In the last decade, a considerable body of information has been gathered regarding the influence of the different species of fatty acid containing glycerolipid molecules, on lipoprotein metabolism. We are aware that the type of fatty acid, especially the length and number of unsaturations present in their structure, modulates the enzyme activities directly related to the handling of lipoproteins in the organism, e.g. lipoprotein lipase (Wang *et al.*, 1992) or lecithin-cholesterol acyltransferase (Hida *et al.*, 1993). In consequence, we have been interested in the effect of fibric acid derivatives on the enzyme activities related to hepatic fatty acid synthesis, either *in vitro* (Alegret *et al.*, 1991; Sánchez *et al.*, 1992a,b; 1993a,b) or *in vivo* (Vázquez *et al.*, 1993; Alegret *et al.*, 1994). We have also studied the possible relationship between these effects and peroxisome proliferation; given that man is assumed to be resistant to the induction of these cellular organelles (Lake & Gray, 1985; Hawkins *et al.*, 1987). Many of the studies about the effects of fibrates on fatty acid metabolism are based on only one drug, clofibrate, accepted as a fibrate prototype, but which is gradually being replaced by more efficient drugs, like bezafibrate and gemfibrozil. Thus, here we compare the effect of three fibric acid derivatives, clofibrate (CFB), bezafibrate (BFB) and gemfibrozil (GFB) on the fatty acid chain elongation and desaturation systems *in vivo*. The microsomal electron transport chains were also studied, as they are directly involved in providing reducing power to such systems (Keyes & Cinti, 1980). Further, we have studied the temporal evolution of these activities and their relationship with peroxisome induction. The results obtained provide evidence of a selective modification of the elongation activity produced by fibrate treatment in rats and also an increase of Δ-5, Δ-6, and Δ-9 desaturase activities. In the case of Δ-6 desaturase, the mechanism may be indepen-

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dent of the peroxisomal proliferation. These modifications may help to explain some of the alterations in plasma fatty acid composition observed in humans after fibrate therapy.

The abbreviations used in this paper are as follows: CFB, clofibrate; BFB, bezafibrate; GFB, gemfibrozil; 16:0, palmitic acid; 16:1, palmitoleic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, γ-linolenic acid; 20:3, dihomo-γ-linolenic acid.

Methods

Animals

Male Sprague-Dawley rats (150) from Letica (Barcelona, Spain), weighing around 120 g, were maintained in a 12 h light-dark cycle under conditions of constant humidity and temperature (22 ± 2°C) and were fed standard Panlab diet (Barcelona, Spain) for five days prior to the beginning of the studies. The animals were distributed randomly according to their weight in four groups of 36. Each group was fed, respectively, a control diet or a diet containing 0.3% w/w CFB, 0.45% w/w BFB or 0.3% w/w GFB (equimolar doses). The six remaining rats were used as controls on day 0 of treatment to establish basal values. Throughout the study the weight and daily food intake of the animals was measured. The 36 rats of each group were killed in random groups of 6 after 1, 2, 4, 7, 10 and 15 days of treatment. The diets were prepared as described previously (Alegret *et al.*, 1994).

Experimental

The animals were killed on the assigned day by decapitation between 08 h 00 min and 09 h 00 min, to avoid circadian variations of the enzyme activities (Dato *et al.*, 1972). Samples of blood were collected from the neck and plasma was obtained by centrifugation at 3000 *g* for 10 min. Livers were removed, perfused with ice-cold 0.9% NaCl, weighed and homogenized in eight volumes of 0.25 M sucrose, 50 mM Tris-HCl buffer, pH 7.4. Microsomal and cytosolic fractions were obtained by differential centrifugations as described previously (Nagi *et al.*, 1989), and the protein content was determined by the method of Bradford (1976) with bovine serum albumin used as standard. Liver subcellular distribution was determined by assaying marker enzyme activities, i.e. NADPH cytochrome c reductase for microsomes, succinate dehydrogenase for mitochondria and glucose-6-phosphate for cytosol (Alegret *et al.*, 1994).

Synthesis of γ-linolenoyl-CoA and [1-^{14}C]-stearoyl-CoA

18:3 CoA and [1-^{14}C]-18:0 CoA were synthesized by the mixed anhydride procedure as described elsewhere (Sánchez *et al.*, 1993b). Concentrations of the CoA derivatives were determined by the method of Ellman (1959) after cleavage of the thioester bond with hydroxylamine.

Enzyme assays

Microsomal fatty acid elongation was assayed by the measurement of [2-^{14}C]-malonyl-CoA incorporation into exogenous acyl-CoAs essentially as described previously (Sánchez *et al.*, 1992b), with some modifications. Briefly, the assay medium contained: 100 mM Tris-HCl, pH 7.4, 500 μM NADPH, 40 μM acyl-CoA and 200 μg of microsomal protein, freshly obtained, all in a final volume of 0.5 ml. After 2 min preincubation at 37°C, the reaction was started by adding 60 μM malonyl-CoA (containing 0.037 μCi [2-^{14}C]-malonyl-CoA). 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 2 ml hexane (total volume 6 ml). The pooled hexane fractions were placed in scintillation vials and dried under nitrogen, and after addition of 7 ml scintillation mixture, the radioactivity incorporated was counted in a scintillator counter. Blanks were carried out by adding KOH before malonyl-CoA. Under our assay conditions, fatty acid elongation activity was proportional to the amount of protein added (up to 800 μg ml^{-1}), and was linear for at least 15 min.

Δ9 desaturase activity was determined according to Laguna *et al.* (1989), with 200 μg of microsomal protein, freshly obtained, in a final volume of 0.5 ml. Blanks were performed by adding 18:0 CoA after stopping the reaction.

To determine the Δ6 desaturase activity, the assay medium contained, in a final volume of 0.5 ml, 100 mM Tris-HCl buffer pH 7.4, 150 μM GSH, 7.5 mM $MgCl_2$, 5 mM ATP, 200 μM CoA, 500 μM NADH and 500 μg of microsomal protein and 125 μg of cytosolic protein, freshly obtained. The reaction was started, after 2 min preincubation, by adding an ethanolic solution of 18:2 (containing 0.05 μCi of [1-^{14}C]-18:2) to a final concentration of 25 μM. The volume of ethanol added did not exceed 1% of the total volume. The assay continued as described (Sánchez *et al.*, 1993b), except for the content of $AgNO_3$ of the thin layer chromatography plates, which was 20%, and the solvent system used to develop them, which was diethyl ether/petroleum ether 1:1 (v/v).

Δ5 desaturase activity was determined essentially as described (Sánchez *et al.*, 1993b), but using 20:3 (containing 0.05 μCi of [1-^{14}C]-20:3) in a final concentration of 50 μM as a substrate, and 20% $AgNO_3$ plates in order to achieve better resolution.

The enzyme activities related to the microsomal electron transport chains, namely NADH and NADPH cytochrome c reductase and NADH cytochrome b_5 reductase, and also the peroxisomal β-oxidation activity were determined as described in Alegret *et al.* (1991, 1994).

Chemicals

Malonyl-CoA, palmitoyl-CoA (16:0 CoA), palmitoleoyl-CoA (9–16:1 CoA), γ-linolenic acid (6,9,12–18:3), stearoyl-CoA (18:0 CoA), linoleic acid (6,9–18:2), dihomo-γ-linolenic acid (8,11,14–20:3), NAD(P)H, fatty acid-free bovine serum albumin (BSA), GSH, ATP, CoA and Trizma were obtained from Sigma (St. Louis, MO, U.S.A.). [2-^{14}C]-malonyl-CoA (50 mCi mmol^{-1}), [1-^{14}C]-stearic acid (18:0) (58 mCi mmol^{-1}), [1-^{14}C]-linoleic acid (18:2) (50 mCi mmol^{-1}) and [1-^{14}C]-dihomo-γ-linolenic acid (20:3) (47 mCi mmol^{-1}) were purchased from New England Nuclear (Boston, MA, U.S.A.) and Amersham Iberica (Madrid, Spain). Scintillation fluids (Co 136 and Co 36) were from Scharlau Co. (Barcelona, Spain). $AgNO_3$ (20%) silica gel G thin layer chromatography plates were from Alltech (Deerfield, IL, U.S.A.). General chemicals were obtained from commercial sources and were of analytical grade.

Drugs

Clofibrate (CFB) was a generous gift from ICI-Farma (Pontevedra, Spain), bezafibrate (BFB) from Boehringer Mannheim (Barcelona, Spain) and gemfibrozil (GFB) from Parke-Davis (Barcelona, Spain).

Plasma cholesterol and triglyceride concentration

Cholesterol was determined by the colorimetric test Monotest Cholesterol CHODPAP No 290319, and triglyceride concentration was assayed by means of a Peridochrom Triglyceride GPO-PAP No 701882 test, both from Boehringer Mannheim (Barcelona, Spain).

Statistical evaluation

Results are expressed as means ± s.e.mean of six experiments performed in duplicate. Statistical differences were established by a two way ANOVA test and the *a posteriori* multiple comparison Duncan test, using the FOUNDS computer programme. Differences with $P<0.05$ were considered significant. Correlations between two variables were performed by linear regression using the GPIP computer programme.

Results

Non-enzymatic parameters

Body weight and daily food intake of the rats were measured throughout the treatment. The body weight of treated rats was similar to that of control rats up to the seventh day of treatment. From that time on, treated rats tended to gain weight slower than control rats (data not shown). Daily food intake was not significantly changed by the treatment, although BFB- and GFB-treated animals showed a tendency to consume more than the corresponding controls (e.g. on day 14 the daily food intake was 32.6 ± 1.4 g day^{-1} for control animals, and 32.3 ± 0.9, 36.2 ± 2.7, and 36.2 ± 2.3 g day^{-1} for CFB-, BFB- and GFB-treated animals, respectively) in accordance with our previous paper (Alegret *et al.*, 1994).

Liver/body weight ratio and peroxisomal β-oxidation activity are suitable markers for the peroxisomal proliferation phenomena (Vázquez *et al.*, 1993; Alegret *et al.*, 1994; Lake & Gray, 1985). As expected, and in agreement with our previous results (Alegret *et al.*, 1994), both parameters were greatly increased in rats treated with CFB, BFB and GFB. The increase in liver/body weight ratio began early, on the second day of treatment with each of the three drugs (Figure 1). In rats treated with BFB, the most potent drug in this respect, a maximal ratio was achieved on day 10 of treatment (77% increase).

As can be seen in Figure 2, BFB was also the most potent inducer of peroxisomal β-oxidation activity, followed by CFB and GFB. Differences with respect to control β-oxidation activity were significant from day 2 of treatment (day 1 in the case of the BFB group). Maximal increase in β-oxidation activity was achieved on day 7 for BFB-treated (9 fold induction), on day 7 for GFB-treated rats (3.3 fold induction) and on day 15 for CFB-treated rats (7.3 fold induction).

Treatment of rats with fibric acid derivatives led to a marked hypolipidemic effect, which is reflected in the reduction of plasma cholesterol and triglyceride levels (Figure 3a and b). The hypocholesterolemic effect was statistically significant on the first day of treatment. While CFB and BFB exhibited similar potency in reducing cholesterol levels (maximal reductions about 40% on days 10–15 of treatment), the effect of GFB was less significant. In fact, on days 10 and 15 there was no difference between the values obtained in GFB-treated and control animals (Figure 3a). Regarding the reduction of plasma triglyceride levels (Figure 3b), the most effective drugs were BFB and GFB, while the decreases attributable to CFB were slighter, although still significant. The hypotriglyceridemic effect was significant from the first day of treatment with all three drugs. Maximal effect (about 60% reduction after treatment with BFB or GFB) was reached on day 7 and maintained for the following days.

Microsomal electron transport chains

The three enzyme activities assayed, namely NADH and NADPH cytochrome c reductase and NADH cytochrome b_5 reductase, behaved differently after fibrate administration. NADH cytochrome b_5 reductase was unaffected throughout fibrate treatment (data not shown). For example, on day 15,

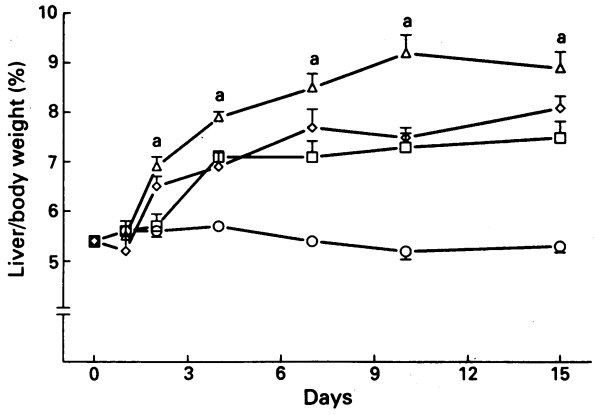

Figure 1 Time-course of the effect of a standard diet (○) or a diet supplemented with 0.3% w/w CFB (□), 0.45% w/w BFB (△), or 0.3% w/w GFB (◇) on liver/body weight ratio (%) of male Sprague-Dawley rats. Results are means ± s.e.mean of 6 experiments performed in duplicate. [a]Values for CFB, BFB and GFB are all significantly different from controls, $P<0.05$.

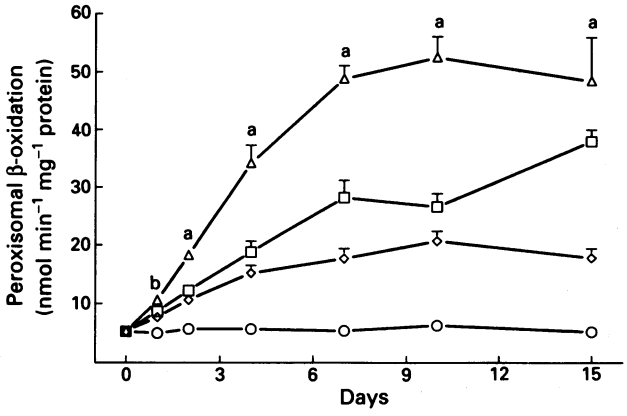

Figure 2 Time-course of the effect of a standard diet (○) or a diet supplemented with 0.3% w/w CFB (□), 0.45% w/w BFB (△), or 0.3% w/w GFB (◇) on cyanide-insensitive peroxisomal β-oxidation determined in the postnuclear fraction of livers from male Sprague-Dawley rats. Results are means ± s.e.mean of 6 experiments performed in duplicate. [a]Values for CFB, BFB and GFB are all significantly different from controls, $P<0.05$; [b]values for BFB are significantly different from controls, $P<0.05$.

the activity values were 3.3 ± 0.2, 3.5 ± 0.1, 3.4 ± 0.2 and 3.5 ± 0.2 μmol min^{-1} mg^{-1} microsomal protein for control, CFB, BFB and GFB-treated animals. NADH cytochrome c reductase activity was significantly reduced from the second day of treatment with CFB and BFB, as shown in Figure 4 (mean reductions of 37 and 39% respectively). In contrast, GFB administration resulted in no modification of this activity. NADPH cytochrome c reductase activity was increased by the administration of all three drugs, and reached maximal values at day 7 (Figure 5). BFB and GFB produced the same degree of increase (79% and 77%, mean increase versus control values); CFB was somewhat less effective (64% mean increase).

Microsomal chain elongation

Microsomal chain elongation was assayed using exogenously added CoA esters of saturated, monounsaturated and polyunsaturated fatty acids as substrates, namely 16:0, 16:1, and 18:3 CoAs. The variations of the elongase activity upon drug treatment depended on the substrate used. As shown in Figure 6a, 16:0 CoA elongation activity was significantly increased from days 1–2 of treatment by the three drugs

assayed. BFB and GFB exhibited similar potency, with about 4 fold increases from day 4. CFB was less effective, leading to maximal induction (2.3 fold) on day 4.

16:1 CoA elongation activity (Figure 6b) was less affected in comparison to the changes in the activity using the saturated substrate (i.e. maximal induction, caused by GFB after 4 days of treatment, was about 2 fold). GFB treatment significantly induced 16:1 CoA chain elongation activity from day 2 of treatment, while CFB failed to cause any significant induction. BFB showed intermediate behaviour and caused a slight increase in activity after day 4.

On the other hand, when 18:3 CoA was used, none of the drugs assayed caused significant changes in the elongase activity (Figure 6c).

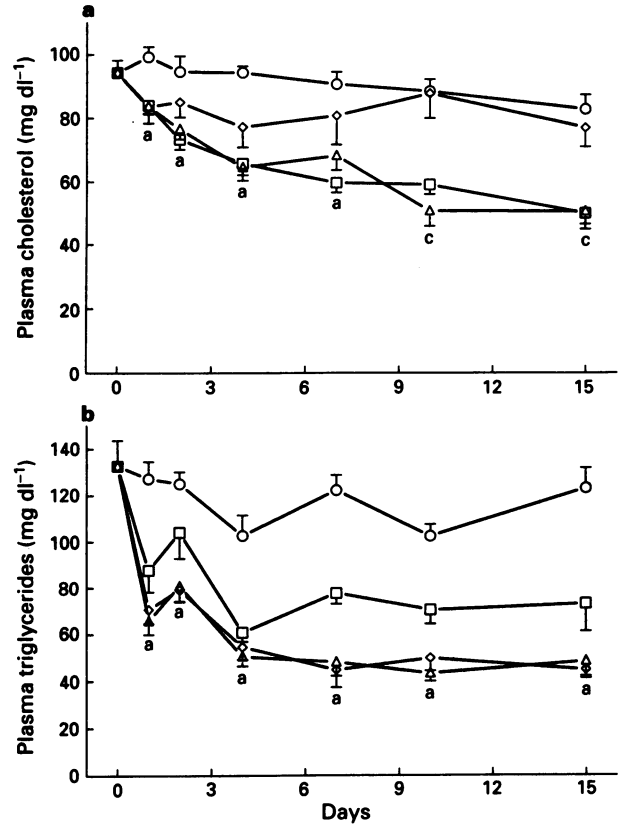

Figure 3 Time-course of the effect of a standard diet (○) or a diet supplemented with 0.3% w/w CFB (□), 0.45% w/w BFB (△), or 0.3% w/w GFB (◇) on (a) plasma cholesterol levels, and (b) plasma triglyceride levels of male Sprague-Dawley rats. Results are means ± s.e.mean of 6 experiments performed in duplicate. [a]Values for CFB, BFB and GFB are all significantly different from controls, $P<0.05$; [c]values for CFB and BFB are significantly different from controls, $P<0.05$.

Microsomal desaturases

The three desaturases here studied, Δ9, Δ5 and Δ6, showed marked increases in their specific activities in response to the treatment of rats with fibric acid derivatives. However, the potency order of the three drugs assayed was not the same in all cases.

Δ9 desaturase activity was increased by the treatment of rats with CFB, BFB and GFB (Figure 7a). The increase caused by CFB and GFB was similar, while BFB was slightly more potent. Thus, maximal effect of the drugs was observed on day 7, with 2.3, 2.4, and 3 fold increase of Δ9 desaturase activity in CFB-, GFB-, and BFB-treated rats, respectively. Δ5 desaturase response to drug treatment (Figure 7b) was similar to that of Δ9; this enzyme activity was clearly enhanced and the order of potency of the drugs for this effect was also the same, i.e. BFB > CFB = GFB. Inductions were significant from day 2 with BFB or GFB (day 4 for the CFB group), and maximal effect was achieved after 15 days of treatment with BFB (about 3 fold induction).

In the case of Δ6 desaturase, GFB showed clearly higher inductive ability than CFB and BFB, which were of similar potency. Values from GFB-treated rats were statistically different from control values from the first day of treatment, and maximal increase, found on day 10, was about 4 fold (Figure 7c).

Discussion

The three drugs studied clearly acted as peroxisome inducers in rats, producing strong hepatomegaly and an evident increase in the peroxisomal β-oxidation activity. The order of potency as peroxisomal inducers (BFB > CFB ≥ GFB) agrees with the reported structural requisites for the manifestation

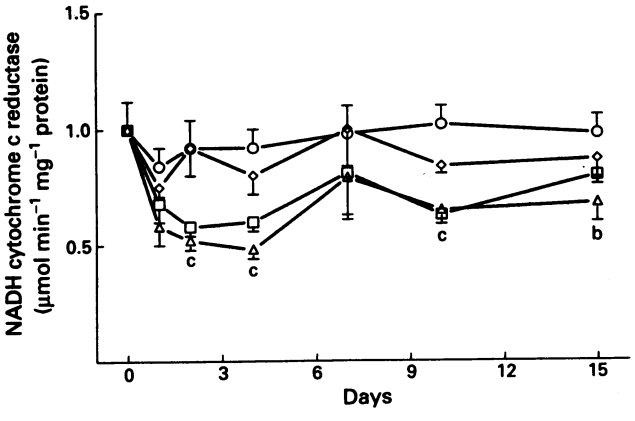

Figure 4 Time-course of the effect of a standard diet (○) or a diet supplemented with 0.3% w/w CFB (□), 0.45% w/w BFB (△), or 0.3% w/w GFB (◇) on NADH cytochrome c reductase activity in the microsomal fraction of livers from male Sprague-Dawley rats. Results are means ± s.e.mean of 6 experiments performed in duplicate. [b]Values for BFB are significantly different from controls, $P<0.05$; [c]values for CFB and BFB are significantly different from controls, $P<0.05$.

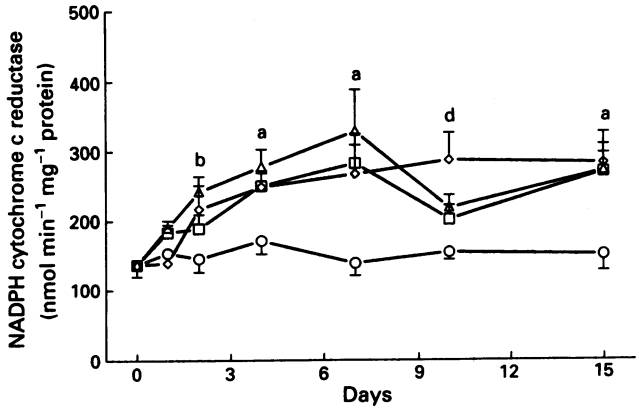

Figure 5 Time-course of the effect of a standard diet (○) or a diet supplemented with 0.3% w/w CFB (□), 0.45% w/w BFB (△), or 0.3% w/w GFB (◇) on NADH cytochrome c reductase activity in the microsomal fraction of livers from male Sprague-Dawley rats. Results are means ± s.e.mean of 6 experiments performed in duplicate. [a]Values for CFB, BFB and GFB are all significantly different from controls, $P<0.05$; [b]values for BFB are significantly different from controls, $P<0.05$; [d]values for GFB are significantly different from controls, $P<0.05$.

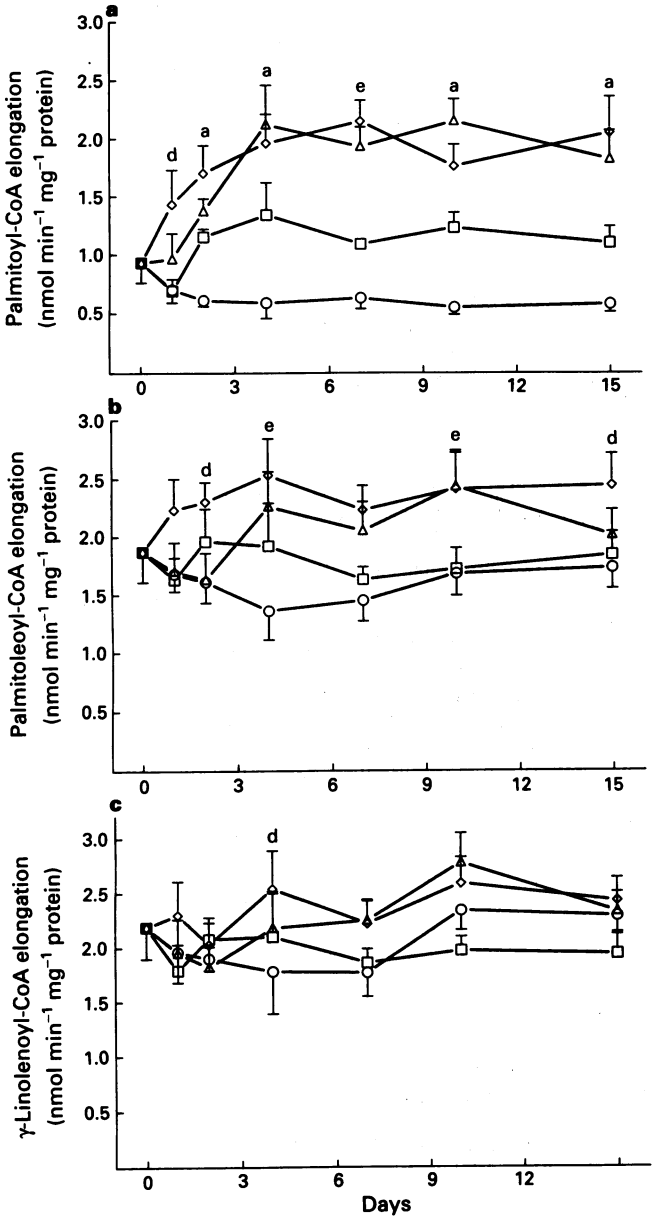

Figure 6 Time-course of the effect of a standard diet (○) or a diet supplemented with 0.3% w/w CFB (□), 0.45% w/w BFB (△), or 0.3% w/w GFB (◇) on (a) palmitoyl-CoA elongation, (b) palmitoleoyl-CoA elongation, and (c) γ-linolenoyl-CoA elongation activities on the microsomal fraction of livers from male Sprague-Dawley rats. Results are means ± s.e.mean of 6 experiments performed in duplicate. [a]Values for CFB, BFB and GFB are all significantly different from controls, $P<0.05$; [d]values for GFB are significantly different from controls, $P<0.05$; [e]values for BFB and GFB are significantly different from controls, $P<0.05$.

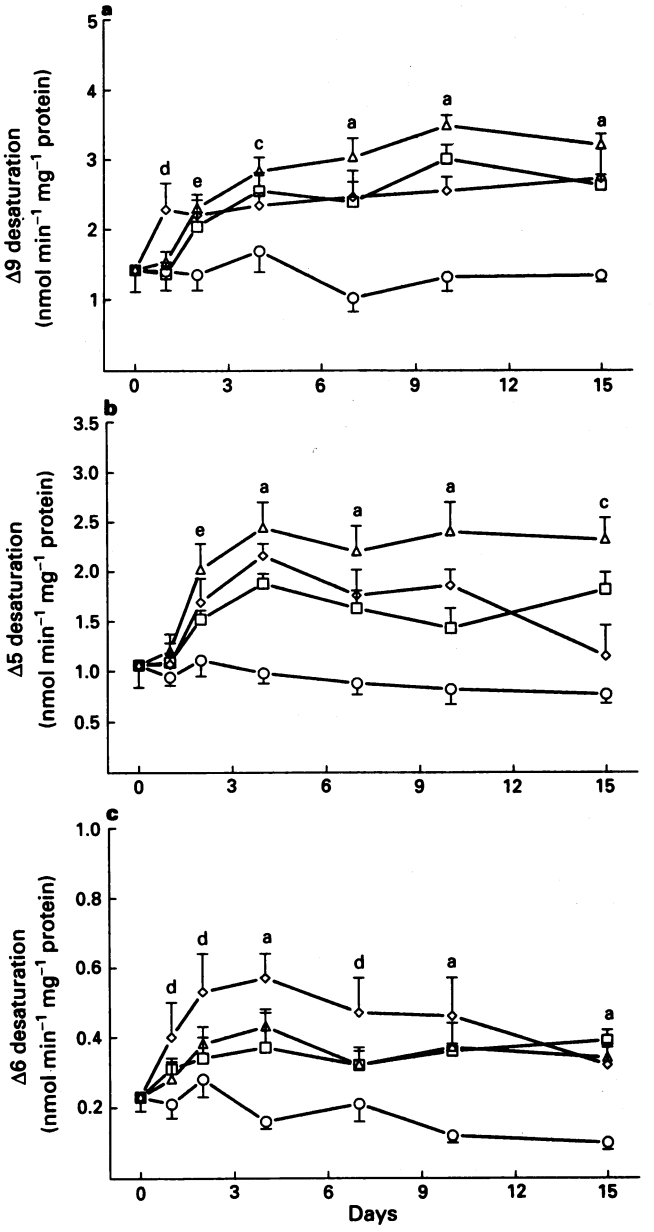

Figure 7 Time-course of the effect of a standard diet (○) or a diet supplemented with 0.3% w/w CFB (□), 0.45% w/w BFB (△), or 0.3% w/w GFB (◇) on (a) Δ9 desaturase, (b) Δ5 desaturase, and (c) Δ6 desaturase activities on the microsomal fraction of livers from male Sprague-Dawley rats. Results are means ± s.e.mean of 6 experiments performed in duplicate. [a]Values for CFB, BFB and GFB are all significantly different from controls, $P<0.05$; [c]values for CFB and BFB are significantly different from controls, $P<0.05$; [d]values for GFB are significantly different from controls, $P<0.05$; [e]values for BFB and GFB are significantly different from controls, $P<0.05$.

of this effect (McGuire *et al.*, 1991). Moreover, there is a strong direct correlation between liver/body weight ratio and the increase in peroxisomal activity ($r^2=0.66$, $P<0.001$; Table 1). Regarding the hypolipidemic activity, the correlation values for cholesterol or triglyceride levels and peroxisomal β-oxidation activity (Table 1), point to some involvement of peroxisomal proliferation in the hypolipidemic activity of fibrates in rats, although the r^2 values found (0.26 and 0.33 for triglycerides and cholesterol respectively) leave a major part of the hypolipidemic response unexplained. Thus, the proliferation of peroxisomes and their associated enzyme activities can be viewed as an additional contribution to an independent hypolipidemic mechanism. Moreover, it should be borne in mind that in guinea-pigs (Vázquez *et al.*, 1993) and presumably in man (Lake & Gray, 1985; Hawkins *et al.*, 1987), the hypolipidemic response to treatment with fibrates is not accompanied by peroxisomal proliferation.

The results obtained in this study concerning the weight evolution, daily food intake, liver/body weight ratio, peroxisomal β-oxidation activity and hypolipidemic profile are in accordance with those reported in our previous study *in vivo* (Alegret *et al.*, 1994), where a more extensive discussion of the modifications of these parameters can be found.

Kawashima *et al.* (1983; 1986; 1989) demonstrated that microsomal cytochrome b_5 levels are not modified by the treatment of rats with different peroxisome proliferators. Further, the same authors reported that NADH cytochrome

b_5 reductase activity was not modified, while NADH cytochrome c reductase was decreased in rats after feeding a 0.5% w/w CFB-diet (Kawashima *et al.*, 1983; 1989; 1990). Our results with the three fibrates agree with those previous reports. The decrease in NADH cytochrome c reductase activity does not appear to be related to the proliferation of peroxisomes, as there is no significant correlation between the reductase and the peroxisomal β-oxidation activities (Table 1). The decrease in activity could be related to the hindering of electron flow from the flavoprotein to the cytochrome b_5 produced by fibrates *in vitro* (Alegret *et al.*, 1991). In contrast to NADH cytochrome c reductase, the NADPH-dependent reductase activity is increased in the fibrate-treated rats. Although the r^2 value for the correlation between the reductase and the peroxisomal β-oxidation activities is low (Table 1), the induction of the NAPDH cytochrome c reductase activity may be directly associated to the activation of cytochrome P450IVA1 genes (Milton *et al.*, 1990) produced by fibrates, like other cytochrome P450 inducers, such as phenobarbitone (Waxman & Azaroff, 1992). Moreover, the increase in activity cannot be ascribed to a direct interaction of the drugs with the reductase, as the activity of this enzyme is not modified *in vitro* by the addition of any of the three fibrates studied (Alegret *et al.*, 1991).

It should be pointed out that the activities of reductases present in microsomes from control or treated-animals are about two orders of magnitude greater than those of elongases or desaturases, more than sufficient to supply reducing power to support these enzyme activities.

The microsomal fatty acid chain elongation system is composed of four independent enzyme activities, of which the first, the condensing enzyme, is the rate limiting step (Bernert & Sprecher, 1979). As far as we know, this protein has not been purified to homogeneity from mammalian sources (Cinti *et al.*, 1992). Nevertheless, Sprecher (Bernert & Sprecher, 1977), and Cinti's group in particular (Prasad *et al.*, 1986), have provided indirect evidence for the existence of at least three different condensation/elongation systems responsible for the elongation of saturated, monounsaturated and polyunsaturated fatty acids. Accordingly, we have assayed the microsomal elongation activity using three different substrates, 16:0, 16:1, and 18:3 CoAs. The differentiated effects produced by the fibrates on these three activities are in good agreement with Cinti's proposal (Prasad *et al.*, 1986). Thus, CFB induced the elongation of 16:0, while it was ineffective in modifying 16:1 elongation, supporting the hypothesis of two, differentiated enzymes for saturated and monounsaturated fatty acids. Moreover, GFB induced 16:1 elongation while not affecting 18:3 elongation, suggesting the existence of two different enzymes for monounsaturated and polyunsaturated fatty acids.

Conflicting results appeared in the literature concerning the effect of fibric acid derivatives on fatty acid chain elongation activity. For example, Landriscina *et al.* (1977), found a 47% decrease in elongation activity after 14 days of CFB treatment. In contrast, Kawashima *et al.* (1984) described an induction of palmitoyl-CoA elongation activity after CFB treatment, ascribing the discrepancy with Landriscina's results to the fact that the latter had used acetyl-CoA and not 16:0 CoA as a primer. Our results are in good agreement with those of Kawashima *et al.* (1990), not only because 16:0 CoA elongation activity is increased, but also because they found that 18:3 CoA elongation activity was not modified by CFB treatment. As far as we know, no studies have been carried out *in vivo* on the effect of other fibric acid derivatives, such as BFB and GFB, on microsomal chain elongation activity. From the data presented here, it is clear that these two drugs are more potent than CFB as chain elongation inducers. Kawashima *et al.* (1990) reported that the induction of palmitoyl-CoA elongation produced by CFB was directly related to the proliferation of peroxisomes. Nevertheless, the proliferation of peroxisomes explains, at most, only 29% of the increase in 16:0 CoA elongation (Table 1). Moreover, GFB, which is the weakest peroxisome proliferator of the three fibrates tested, is the only one capable of inducing the 16:1 CoA elongation activity. In addition, no correlation was found between the values for this activity in GFB-treated rats and the corresponding peroxisomal β-oxidation activities. Thus, from our own results, the induction of the elongation system of fibrates is mainly independent of the activity of these compounds as peroxisome proliferators.

Table 1 Correlation between enzymatic and non enzymatic parameters and peroxisomal proliferation (measured as peroxisomal β-oxidation activity)

	Peroxisomal β-oxidation	
	n	r^2
Liver/body weight	142	0.664*
Plasma triglycerides	142	0.260*†
Plasma cholesterol	142	0.331*†
Palmitoyl-CoA elongase	141	0.292*
Palmitoleoyl-CoA elongase	33	0.000
Δ9 desaturase	141	0.443*
Δ9 desaturase (only for bezafibrate-treated animals)	34	0.580*
Δ5 desaturase	134	0.425*
Δ6 desaturase	136	0.014†
NADH cytochrome c reductase	149	0.023†
NADPH cytochrome c reductase	149	0.286*

Enzymatic and non enzymatic parameters were measured as described under Methods. *n* are the number of pairs of data analysed. *r^2 values statistically significant ($P<0.001$). †Negative correlation.

We have previously shown that fibrates (Sánchez *et al.*, 1993b), especially GFB (Sánchez *et al.*, 1992b), behave as potent inhibitors of the microsomal fatty acid chain elongation *in vitro*, irrespective of the degree of unsaturation of the substrate used. Therefore, the induction elicited by fibrates *in vivo* should be regarded as a compensatory response by the cell, in order to override the drug inhibition. This behaviour has been reported for other drugs acting as inhibitors of key lipogenic enzymes, such as statins for hydroxymethylglutaryl-CoA reductase (Endo, 1986).

It is known that feeding a fat-free, carbohydrate rich diet to 24 h starved rats greatly increases the activity of the elongation system (Cinti *et al.*, 1992). With such dietary manipulation, in our laboratory we have obtained activities between 2–2.4 nmol min^{-1} mg^{-1}, irrespective of the substrate used (Sánchez *et al.*, 1992b), which means that, in our experimental conditions, 18:3 CoA elongation is already fully expressed in control animals (see values in Figure 6c). Further, the 'fat-free diet activities' practically matched the induced activities for saturated and monounsaturated substrates obtained after fibrate treatment. Taking all these data together, it seems that, at least with respect to GFB, the selectivity for the three elongation systems is a consequence of the different basal levels of expression of these systems.

The three fibrates tested here acted as inducers of the three desaturase activities assayed, namely Δ9, Δ5, and Δ6 desaturase. Nevertheless, from the results shown in Figure 7, it seems that the behaviour of Δ9 and Δ5 desaturase consistently differs from that of Δ6 desaturase. Kawashima *et al.* (1986, 1989) reported that not only CFB, but other structurally unrelated peroxisome proliferators such as tiadenol, are capable of inducing the Δ9 desaturase activity. Indeed, the same authors (Kawashima *et al.*, 1990) relate the induction of Δ9 directly to peroxisome proliferation. Our results seem to confirm Kawashima's hypothesis: First, the order of potency in inducing Δ9 desaturase activity (BFB > CFB ≥ GFB) matched the order of peroxisome inductive activity. Second, no inhibition of Δ9 desaturase can be obtained *in vitro*, even with supraphysiological concentrations of fibrates

(Sánchez *et al.*, 1993b). Third, there is a fairly good correlation between Δ9 desaturase activity and peroxisomal β-oxidation ($r^2 = 0.44$; $P < 0.001$). When analysing the data for BFB, the most potent peroxisomal inducer, the correlation is even better ($r^2 = 0.58$). The same points outlined for Δ9 desaturase can be argued for Δ5 desaturase behaviour after fibrate treatment. Although in this case there is some inhibition of Δ5 desaturase activity in the presence of fibrates *in vitro* (Sánchez *et al.*, 1993b), the effect is obtained at such high concentrations that a compensatory response *in vivo* appears unlikely.

Although the three fibrates evoked an induction of Δ6 desaturase activity, in this case GFB, the strongest inducer of this desaturase is the least potent in inducing peroxisome proliferation. Consistently, there is a lack of correlation between Δ6 desaturase and peroxisomal β-oxidation activities (see Table 1). Thus, for this enzyme activity, the effect of fibrates seems to be independent of the peroxisomal proliferation.

The modification of fatty acid elongation or desaturation activities by fibrates may be reflected in changes in the nature of acyl chains in the microsomal phospholipid fraction or, further, in plasma lipoproteins (Kawashima *et al.*, 1984; Agheli & Jacotot, 1991). Those modifications that are unrelated to peroxisomal proliferation, such as the increase in Δ6 desaturase activity shown in the present work, are more likely to occur in man and may be responsible for the decrease in linoleic acid content in lipoproteins reported for human subjects after fibrate treatment (Vessby & Lithell, 1990; Agheli & Jacotot, 1991; Tavella *et al.*, 1993). Nevertheless, the same authors also report an increase in monoenoic species. In view of the results reported here (Δ9 desaturase is dependent of peroxisomal proliferation), these data can be explained in two ways. First, although human subjects are generally considered refractory to peroxisomal proliferation (Lake & Gray, 1985; Hawkins *et al.*, 1987), this phenomenon has been identified to a certain extent in several studies (Hanefeld *et al.*, 1983; Reddy *et al.*, 1984). Second, the increase in substrates for the Δ9 desaturase provided by the induction of the elongation of saturated fatty acids, which is independent of peroxisomal proliferation, could result in an increased proportion of monoenoic products. This second possibility is currently under investigation.

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