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Decreased susceptibility to copper-induced oxidation of rat-lipoproteins after fibrate treatment: influence of fatty acid composition

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1 The effect of clofibrate (CFB), bezafibrate (BFB), and gemfibrozil (GFB) on plasma lipoprotein (VLDL and LDL) concentration, composition and resistance to copper-induced oxidation has been studied in male Sprague-Dawley rats after a 15 day treatment.

2 Plasma triglyceride levels were reduced by CFB (41%) and BFB (39%). This effect was related to a significant reduction (67% for CFB and 56% for BFB) in the amount of circulating VLDL-protein.

3 Plasma total cholesterol was reduced by 28% and 45% in CFB- and BFB-treated animals, respectively, mainly by modification of the cholesteryl ester fraction. In contrast, GFB significantly increased total cholesterol (27%). No modification in the LDL protein or lipid content was introduced by fibrates, although GFB decreased the proportion of LDL-triglycerides, at the expense of an increase in total cholesterol.

4 The fatty acid species carried by VLDL and LDL were affected after fibrate treatment. In general, both particles showed an increase in monounsaturated fatty acids (MUFA) (18:1) and a decrease in polyunsaturated fatty acids (PUFA) species (18:2 n-6, 20:4 n-6, 18:3 n-3, 20:5 n-3). As a consequence, the ratio of PUFA/(SFA+MUFA) for the whole lipoproteins was markedly reduced.

5 The degree of copper-induced VLDL- and LDL-oxidation was assessed by means of the analysis of lysine content, thiobarbituric acid reactive substances (TBARS) production and conjugated dienes formation. Lipoproteins obtained from fibrate-treated rats were more resistant to the oxidative challenge. For each lipoprotein, BFB was the most effective drug, followed by CFB and GFB.

6 The observed *antioxidant* effect can be ascribed to two independent phenomena produced by fibrates: the reduction of the amount of substrate for the oxidation process due to their hypolipidemic activity, and the alteration in the type of fatty acids transported by the lipoproteins towards an enrichment in species resistant to the oxidation process.

7 As similar lipoprotein fatty acid changes have been reported after fibrate treatment in human subjects, an *antioxidant* effect of fibrates in human therapy, independent of their well known hypolipidaemic activity, should be expected.

Keywords: Clofibrate; bezafibrate; gemfibrozil; rat low density lipoprotein (LDL); rat VLDL; copper oxidation, fatty acids

Introduction

In the pathogenesis of atherosclerosis there is growing evidence that polyunsaturated fatty acids (PUFA) are the substrate of the lipid peroxidation of low density lipoproteins (LDL) (Esterbauer *et al.*, 1992). The replacement of some of these PUFA by either monounsaturated (MUFA) or saturated fatty acids (SFA), which are more resistant to oxidation, can also increase the resistance of lipoproteins to oxidation (Berry *et al.*, 1991; Scaccini *et al.*, 1992).

Fibric acid derivatives can promote peroxisome proliferation in rodent liver (Sirtori *et al.*, 1992). Further, fibrate-treated animals show a marked increase in the activity of enzymes related to fatty acid metabolism. For example, the administration of fibrates to rats or mice produces a marked increase in the activities of both stearoyl-CoA desaturase and palmitoyl-CoA chain elongation in hepatic microsomes (Alegret *et al.*, 1995). These enzymes have an important role in the modification of the pool of fatty acids used for the biosynthesis of glycerolipids and, as a result, the acyl composition of liver microsomal glycerolipids is modified (Vázquez *et al.*, 1995). As hepatic lipoproteins are synthesized from microsomal glycerolipids (Vance, 1989; Vance & Vance, 1990), changes in the acyl composition of lipoproteins could be expected after fibrate treatment. The quality of these fatty acids (chain length and number of unsaturations) has an important role in the control of lipoprotein metabolism (Wang *et al.*, 1992; Hida *et al.*, 1993) and, as mentioned, in the oxidative modification of lipoproteins.

The aim of this work was to study the effect of three fibric acid derivatives, clofibrate (CFB), bezafibrate (BFB) and gemfibrozil (GFB) on the acyl composition of plasma lipoproteins (VLDL and LDL) in rats. Moreover, the relevance of these changes for peroxidation of lipoproteins was also examined. The changes found in the fatty acyl composition of lipoproteins can be related in part to the proliferation of peroxisomes. Nevertheless, since GFB modifies the acyl composition of microsomal phospholipids in guinea-pigs (Vázquez *et al.*, 1993), and fibrate treatment alters the acyl composition of lipoproteins in human subjects (Jurand & Oliver, 1963; Vessby *et al.*, 1980a,b; Agheli & Jacotot, 1991; Tavella *et al.*, 1993), both species being resistant to the peroxisomal induction (Hawkins *et al.*, 1987), we cannot rule out changes in the peroxidation of lipoproteins from these species after fibrate treatment.

Methods

Animals and treatments

Four treatments were performed. For each treatment, 24 male Sprague-Dawley rats from Letica (Barcelona, Spain) weighing

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110–120 g were maintained under conditions of constant humidity and temperature (22±2°C) and a 12 h light-dark cycle (20 h 00 min/08 h 00 min). They were fed standard diet (Panlab, Barcelona, Spain) for five days before the beginning of the studies. The animals were distributed randomly into four groups of 6 rats. Each group was fed, respectively, a control diet or a diet containing equimolar doses of CFB (0.3 w/w), BFB (0.45%) or GFB (0.3%), prepared as described previously (Alegret *et al.*, 1994), for 15 days. Throughout the study, the weight and daily food intake of the animals were measured. After 16 h fast the animals were killed under pentobarbitone anaesthesia by exsanguination.

Lipoprotein isolation

Blood samples were collected in EDTA tubes (1 mg ml^{-1}) by cannulation of the carotid artery. Plasma was obtained by centrifugation at 2200 *g* for 10 min at 4°C. For each treatment, the pooled plasma fractions from the 6 rats of each group were used for lipoprotein fractionation.

Plasma lipoproteins were fractionated into VLDL ($d < 1.006$ g ml^{-1}) and LDL ($d = 1.006-1.063$ g ml^{-1}) by sequential ultracentrifugation (Mills *et al.*, 1984). Briefly, 6 ml of plasma was overlayed with 3 ml isotonic saline containing EDTA 0.037% ($d = 1.006$ g ml^{-1}) in 10 × 1.0 ml thick-walled polycarbonate tubes and centrifuged (18 h, 20°C, 160,000 *g*) in a Beckman L8-M ultracentrifuge with fixed angle rotor (Ty 65). VLDL in the top 2 ml of each tube was harvested by aspiration and the density of the remaining plasma was adjusted to 1.063 g ml^{-1} by addition of 0.07648 g of solid NaBr per ml of plasma. This plasma was overlayed with 3 ml of a solution of 7.6% NaBr ($d = 1.063$ g ml^{-1}) and centrifuged as before to isolate the LDL fraction in the 2 ml supernatant of each tube. Lipoprotein fractions were dialyzed against a 200 fold volume of degassed phosphate-buffered saline (PBS, 0.01 M phosphate, 0.15 M NaCl, pH 7.4) at 4°C in the dark for 48 h. The dialysis buffer was changed four times during this period.

The protein content of plasma and isolated lipoproteins was determined by the method of Bradford (1976), with BSA used as standard. Total cholesterol (ref. 144235) and free cholesterol (ref. 310328), phospholipid (ref. 691844) and triglyceride (ref. 701904) values were determined by Boehringer Mannheim enzymatic tests.

Lipoprotein fatty acid analysis

Lipids were extracted from each lipoprotein fraction (about 0.2–0.3 mg of protein for VLDL and LDL) following Bligh & Dyer (1959). The organic fraction was applied to Silica gel 60 F_{254} plates (20 × 20 cm, E. Merck, Barcelona, Spain) for the separation of phospholipids, triglycerides and cholesteryl esters. Development was performed with petroleum ether/diethyl ether/acetic acid (90:30:1 by vol.). The plates were sprayed lightly with 0.2% ethanolic dichlorofluorescein and spots were visualized by comparison with authentic standards under fluorescent light at 254 nm. All solvent systems used contained 0.01% (w/v) of butylhydroxytoluol. Silica gel corresponding to each lipid was scraped off and extracted twice with 5 ml Bligh and Dyer reagent, and dried under a stream of N_2. Fatty acid analysis, after formation of methyl esters, was performed by capillary-gas chromatography, as described elsewhere (Vázquez *et al.*, 1993).

Measurement of lipid peroxidation

Assay for thiobarbituric acid-reacting substances (TBARS) The malondialdehyde (MDA) content of lipoprotein samples was determined spectrophotometrically by the method described by Mao *et al.* (1991). LDL (50 μg of protein) or VLDL (25 μg of protein) were brought to a volume of 1 ml with PBS (pH 7.4). Lipid peroxidation was initiated by the addition of $CuSO_4.5H_2O$ to a final concentration of 5 μM, followed by incubation at 37°C for 2 h (VLDL) or 3 h (LDL). The reaction was stopped by adding 100 μl of a solution containing 50 mM Na_2EDTA and 50 μM BHT; 1.5 ml of 20% trichloroacetic acid was added to the reaction mixture, which was then vortexed. Finally, 1.5 ml of 0.67% thiobarbituric acid (TBA) in 0.05 N NaOH was added and the mixture was incubated at 90°C for 30 min. Samples were centrifuged in a table-top centrifuge at 1500 r.p.m. for 10 min and the absorbance of the supernatant was determined at 532 nm in a Perkin-Elmer u.v.-Vis spectrophotometer. A standard curve (1–5 nmol) of MDA was generated by using malonaldehyde bis(dimethyl acetal) as a reference to determine the lipid peroxidation content after copper oxidation.

Measurement of conjugated dienes The appearance of conjugated dienes (CD) was monitored by recording the u.v. absorbance at 234 nm of LDL (50 μg protein) or VLDL (25 μg protein) suspension in PBS at 37°C, in the presence or absence of freshly prepared $CuSO_4.5H_2O$ (5 μM) (Esterbauer *et al.*, 1989). Lag time, maximal CD formation, and maximal rate of CD production were calculated as described by Kleinveld *et al.* (1992).

Analysis of lysine content LDL and VLDL apoprotein reactive amino groups were determined by the TNBS (trinitrobenzensulphonic acid) method, as described by Aviram *et al.* (1992). Briefly, LDL (50 μg protein) or VLDL (25 μg protein) were brought to a final volume of 1 ml in PBS (pH 7.4) and incubated with $CuSO_4.5H_2O$ 5 μM for 2–3 h (see Table 9). The reaction was stopped by adding 100 μl of a 50 mM EDTA, 50 μM BHT solution. One ml of 4% $NaHCO_3$ pH 8.4 and 50 μl 0.1% TNBS were added. After incubation for 1 h at 37°C, 100 μl of 1 N HCl and 100 μl of 10% sodium dodecyl sulphate were added, and the absorbance of the final solution at 340 nm was measured. For each assay, a freshly prepared standard curve was obtained with DL-lysine, in order to produce quantitative results.

Due to an accident, lipoprotein samples for oxidation assays corresponding to the last treatment were lost. Thus, only the values corresponding to three treatments are reported.

Chemicals

Na_2EDTA was obtained from Merck (Barcelona, Spain), butylhydroxytoluol (BHT), thiobarbituric acid (TBA), and malonaldehyde *bis*(dimethyl acetal) from Fluka (Madrid, Spain), sodium dodecyl sulphate (SDS) from Carlo Erba (Barcelona, Spain), trinitrobenzenesulphonic acid (TNBS), DL-lysine, bovine serum albumin (BSA), sodium pentobarbitone, TLC lipid standards (phospholipids, triglycerides and cholesteryl esters) from Sigma Chemical Co. (Madrid, Spain); and sodium methylate from Supelco (Barcelona, Spain). Fibrates were generous gifts from several companies, CFB was from ICI Farma (Pontevedra, Spain), BFB from Boehringer Mannheim (Barcelona, Spain) and GFB from Parke-Davis (Barcelona, Spain). Other general chemicals were obtained from commercial sources and were of the highest purity available.

Statistical analysis

Plasma and lipoprotein fatty acid results are expressed as mean±s.d. of four experiments performed in duplicate. For plasma and lipoprotein composition values, statistical differences were established by a one-way ANOVA test (FOUNDS computer programme); when differences were found, comparisons between treatment groups were performed with Duncan's test. Differences with $P < 0.05$ were considered significant.

Results

Plasma lipids and lipoprotein composition

Rat daily food intake and body weight gain behaved as described previously (Alegret *et al.*, 1994; 1995) (data not shown). Plasma lipids levels after fibrate administration are shown in Table 1. Levels of plasma triglycerides (TG) were significantly reduced by CFB (41%) and BFB (39%), while the reduction caused by GFB (15%) was not significant. Plasma total cholesterol (TC) was reduced by 28% in the CFB group and by 45% in the BFB group. In contrast, GFB significantly increased total cholesterol (27%). CFB and BFB also reduced cholesteryl esters (CE) in plasma (39% by CFB and 53% by BFB), while GFB-treated animals showed a 16% increase. Plasma phospholipids were modified only by CFB and BFB, showing a 17% and 34% decrease, respectively.

VLDL content in lipids and total protein is shown in Table 2. CFB and BFB induced a significant reduction (67 and 56% respectively) in the amount of circulating VLDL protein. No other compositional change was induced by the drugs, except that VLDL from fibrate-treated rats showed a marked reduction in the proportion of polyunsaturated fatty acids, as reflected by the ratio PUFA/(SFA + MUFA) values (36, 49, and 30% reductions for CFB, BFB, and GFB-treated animals).

Percentage molar compositions of FA in VLDL-TG and VLDL-PL are shown in Tables 3 and 4 (in our conditions, VLDL-CE content was not sufficient for FA analysis). The TG fraction was the most affected by fibrates, with a clear increase in the proportion of saturated fatty acids (mainly 16:0) and a decrease in PUFA, of both the n-6 (mainly 18:2) and the n-3 (mainly 18:3 and 20:5) series. In VLDL-PL, the clearest effect of fibrates (BFB and GFB) was to increase the proportion of MUFA (basically 18:1).

No modification in the LDL protein or lipid content was introduced by fibrates (Table 5). LDL from GFB-treated animals showed a tendency to contain fewer triglycerides and more proteins than control, but these differences were not significant. Nevertheless, when percentages (weight basis) of the different constituents were evaluated, a significant decrease in the proportion of triglycerides was found. This decrease was balanced by a significant increase in the proportion of total cholesterol, and a non significant increase (probably due to higher variability) in the proportion of proteins. As occurred in VLDL, the ratio of PUFA/(SFA + MUFA) in the whole LDL decreased, due both to a fall in the proportion of PUFA and to a rise in SFA and MUFA after fibrate administration (27, 39, and 22% for CFB, BFB and GFB, respectively).

LDL-TG from fibrate-animals mainly contributed to the increase in saturated species (16:0 and 18:0) (Table 6), while LDL-PL showed a marked increase in MUFA (18:1) (Table 7). Both LDL-glycerolipids presented a decreased proportion of PUFA after fibrate administration, but while 18:2(n-6) was the PUFA most affected in LDL-TG, 20:4(n-6) was mainly responsible for the decrease in PUFA proportion in LDL-PL. FA were only slightly affected by fibrates in the LDL-CE

Table 1 Effect of fibrate treatment on plasma lipid levels

	Control	*CFB*	*BFB*	*GFB*
TG (mg dl^{-1})	91.6±15.1	53.9±2.3* (–41%)	55.4±2.8* (–39%)	78.0±9.5 (–15%)
TC (mg dl^{-1})	91.1±6.8	65.8±6.8* (–28%)	49.7±6.7* (–45%)	115.5±5.3* (+27%)
CE (mg dl^{-1})	66.9±8.6	41.0±3.7* (–39%)	31.6±5.0* (–53%)	77.8±6.6* (+16%)
FC (mg dl^{-1})	24.3±5.6	24.8±7.7 (+2%)	17.8±4.3 (–27%)	37.7±4.7* (+55%)
PL (mg dl^{-1})	139.5±16.3	115.6±9.2* (–17%)	91.8±14.2* (–34%)	153.0±13.4 (+10%)

Results are means±s.d. of four independent treatments. *Values different from controls ($P<0.05$). TG: triglycerides; TC: total cholesterol; CE: cholesteryl ester, FC: free cholesterol; PL: phospholipids.

Table 2 Effect of fibrate treatment on VLDL composition

	Control	*CFB*	*BFB*	*GFB*
TP (mg)	1.12±0.48	0.37±0.04*	0.49±0.11*	1.12±0.38
Prot.(mg dl^{-1} pl.)	18.8±8.0	6.2±0.6*	8.3±1.8*	18.7±6.2
TG (mg mg^{-1} p.)	5.35±1.70	3.34±1.26	3.59±1.49	3.83±1.61
TC (mg mg^{-1} p.)	0.42±0.10	0.23±0.12	0.47±0.25	0.46±0.15
FC (mg mg^{-1} p.)	0.21±0.09	0.26±0.20	0.36±0.40	0.23±0.15
CE (mg mg^{-1} p.)	0.21±0.14	0.09±0.18	0.24±0.37	0.25±0.24
PL (mg mg^{-1} p.)	0.59±0.30	0.54±0.42	0.75±1.04	0.59±0.15
% (w/w)				
TG	71.8±6.5	64.7±8.6	61.2±13.6	64.4±10.2
TC	5.8±1.1	6.3±0.8	8.7±2.1	8.1±3.1
CE	2.9±2.0	1.8±3.6	3.5±5.2	4.6±4.3
FC	2.9±1.0	4.9±3.0	5.1±3.9	3.5±1.2
PL	8.2±4.3	8.2±10.0	11.1±14.8	17.1±3.9
Prot.	14.1±3.1	20.8±5.6	19.0±8.4	17.1±3.9
PUFA/(SFA + MUFA)	0.77±0.03	0.49±0.07*	0.39±0.03*	0.54±0.04*

Results are means±s.d. of four different VLDL preparations and expressed as mg per dl of plasma (pl.) or mg per mg of VLDL protein (mg p.). *Values different from controls ($P<0.05$). TP: total VLDL-protein; TG: triglycerides; TC: total cholesterol; CE: cholesteryl ester; FC: free cholesterol; PL: phospholipids; PUFA: polyunsaturated; MUFA: monounsaturated and SFA: saturated fatty acids.

fraction. Only BFB was able to increase the proportion of MUFA (18:1) at the expense of PUFA, with a marked reduction in the proportion of 18:2(n-6) and 20:4 (n-6) (see Table 8).

Lipoprotein oxidation

Rat VLDL/LDL oxidative parameters are shown in Table 9. Although there is high variability between treatments, the whole trend shows a protective effect of fibrates on lipoprotein oxidation.

For VLDL, all parameters studied seemed to be improved by fibrate administration, at least in the case of CFB and BFB. Thus, TBARS formation, maximal CD formation, and maximal rate of CD production were reduced with respect to control values, whereas lysine content and lag time values were increased. VLDL oxidation parameters obtained from GFB-treated animals showed the same tendency, although the modifications were less marked.

LDL-oxidation parameters showed similar behaviour. Nevertheless, in this case the protection afforded by fibrate administration seemed to be weaker than in the case of VLDL.

Table 3 Effect of fibrate treatment on VLDL-TG fatty acid molar composition (%)

	Control	*CFB*	*BFB*	*GFB*
14:0	0.65±0.49	1.8±0.8*	1.3±0.4	0.69±0.45
16:0	25.0±0.9	30.7±2.9*	32.5±1.2*	31.0±1.1*
16:1	3.9±0.3	2.7±0.3*	2.9±0.2*	4.0±0.5
18:0	2.8±0.41	3.1±0.37	3.4±0.6	2.5±0.4
18:1	23.9±1.1	26.4±3.8	31.6±2.2*	27.2±1.4
18:2 (n-6)	29.2±0.7	20.3±2.1*	15.5±1.0*	23.9±1.8*
18:3 (n-6)	0.49±0.61	2.3±1.6*	1.2±0.5	0.52±0.41
18:3 (n-3)	1.3±0.2	0.60±0.22*	0.37±0.04*	0.65±0.14*
20:1 (n-9)	0.16±0.05	0.17±0.04	0.16±0.04	0.13±0.01
20:3 (n-9)	0.54±0.07	0.63±0.12	0.91±0.09*	0.38±0.12
20:3 (n-6)	0.22±0.02	0.37±0.04*	0.32±0.06*	0.18±0.03
20:4 (n-6)	8.4±0.7	8.7±2.0	7.8±1.1	6.7±0.5
20:5 (n-3)	0.78±0.14	0.32±0.26*	0.29±0.32*	0.28±0.05*
22:4 (n-6)	0.68±0.13	0.28±0.12*	0.37±0.16	0.76±0.31
22:5 (n-3)	0.43±0.06	0.16±0.07*	0.22±0.05*	0.34±0.11
22:6 (n-3)	1.6±0.2	1.5±0.9	1.2±0.5	0.85±0.17
SFA	28.4±1.1	35.6±2.3*	37.2±1.2*	34.1±0.9*
MUFA	27.9±1.3	29.3±3.6	35.8±1.4*	31.3±1.2
PUFA	43.6±1.4	35.1±5.9*	28.2±2.5*	34.5±1.2*
n-6	39.0±1.2	32.0±4.6*	25.2±1.9*	32.0±1.2*
n-3	4.1±0.4	2.5±1.31*	2.6±1.2*	2.1±0.21*
PUFA/ (SFA+MUFA)	0.77±0.05	0.55±0.15*	0.39±0.04*	0.53±0.03*

Results (expressed as molar %) are means±s.d. of four different VLDL preparations. *Values different from controls ($P<0.05$). PUFA: polyunsaturated, MUFA: monounsaturated and SFA: saturated fatty acids.

Table 4 Effect of fibrate treatment on VLDL-PL fatty acid molar composition (%)

	Control	*CFB*	*BFB*	*GFB*
14:0	0.92±0.40	3.8±1.4*	4.3±1.9*	1.3±0.7
16:0	25.4±3.1	29.7±0.1	30.9±2.0*	33.0±2.1*
16:1	0.90±0.06	0.71±0.02*	0.76±0.10*	0.91±0.04
18:0	23.2±1.5	16.5±0.5*	14.2±1.1*	15.7±0.6*
18:1	7.0±1.6	8.7±0.1	11.1±0.6*	10.0±0.3*
18:2 (n-6)	11.1±0.2	10.0±0.5	9.2±0.6*	12.0±1.6
18:3 (n-6)	0.44±0.16	2.7±2.1*	2.5±0.8*	0.91±0.70
18:3 (n-3)	0.05±0.04	0	0	0
20:1 (n-9)	0.13±0.05	0.45±0.21*	0.29±0.13	0.13±0.07
20:3 (n-9)	0.11±0.03	0.51±0.06*	0.86±0.08*	0.14±0.07
20:3 (n-6)	0.33±0.03	0.93±0.01*	0.71±0.12*	0.36±0.10
20:4 (n-6)	23.5±2.5	21.8±1.5*	18.4±2.4*	21.7±1.9
20:5 (n-3)	0	0	0	0
22:4 (n-6)	0.54±0.06	0.23±0.18*	0.17±0.13*	0.70±0.19
22:5 (n-3)	0.40±0.06	0.25±0.12	0.23±0.04*	0.41±0.15
22:6 (n-3)	4.1±0.9	3.7±1.9	4.6±2.0	2.8±0.7
SFA	50.3±3.2	50.0±2.0	49.3±1.7	50.0±2.0
MUFA	8.1±1.5	9.8±0.2	12.1±0.6*	11.0±0.3*
PUFA	41.6±2.0	40.1±2.1	38.6±1.9	39.0±2.1
n-6	37.0±1.2	35.7±0.2	32.9±1.4*	35.8±1.3
n-3	4.5±0.9	4.0±2.0	4.8±2.0	3.2±0.8
PUFA/ (SFA+MUFA)	0.71±0.06	0.67±0.06	0.63±0.05	0.64±0.06

Results (expressed as molar %) are means±s.d. of four different VLDL preparations. *Values different from controls ($P<0.05$). PUFA: polyunsaturated; MUFA: monounsaturated and SFA: saturated fatty acids.

Table 5 Effect of fibrate treatment on LDL composition

	Control	*CFB*	*BFB*	*GFB*
PT (mg)	1.64±0.74	1.47±0.57	1.22±0.38	2.50±1.35
Prot. (mg dl^{-1} pl.)	27.3±8.0	24.5±9.5	20.4±6.3	41.8±22.5
TG (mg mg^{-1} p.)	1.75±0.56	1.53±0.06	1.62±0.63	0.87±0.47
TC (mg mg^{-1} p.)	1.12±0.27	1.05±0.25	1.22±0.38	1.25±0.44
FC (mg mg^{-1} p.)	0.31±0.11	0.38±0.15	0.52±0.27	0.35±0.19
CE (mg mg^{-1} p.)	0.80±0.27	0.68±0.23	0.86±0.33	0.89±0.33
PL (mg mg^{-1} p.)	0.92±0.32	0.97±0.33	0.95±0.51	1.00±0.42
% (W/W)				
TG	35.8±5.8	32.6±7.2	32.3±5.8	20.3±5.3*
TC	23.8±3.0	23.3±2.7	27.7±2.2	29.8±2.2*
CE	17.0±3.9	15.0±4.1	17.2±3.7	21.4±3.5
FC	6.7±2.2	8.4±2.7	10.5±3.7	8.5±2.6
PL	19.2±3.9	20.9±4.6	18.4±7.9	23.5±5.3
Prot.	22.1±5.1	23.2±6.7	21.7±7.2	26.5±9.6
PUFA/ (SFA+MUFA)	0.90±0.07	0.66±0.06*	0.55±0.05*	0.70±0.03*

Results are means±s.d. of four different LDL preparations. *Values different from controls ($P<0.05$). TP: total VLDL-protein; TG: triglycerides; TC: total cholesterol; CE: cholesteryl ester; FC: free cholesterol; PL: phospholipids. PUFA: polyunsaturated, MUFA: monounsaturated and SFA: saturated fatty acids.

Table 6 Effect of fibrate treatment on LDL-TG fatty acid molar composition (%)

	Control	*CFB*	*BFB*	*GFB*
14:0	1.5±1.3	1.4±0.8	1.3±0.6	1.4±0.9
16:0	22.2±1.4	26.2±0.8*	27.4±1.1*	27.5±0.7*
16:1	3.4±0.4	1.9±0.05*	2.4±0.2*	3.6±0.5
18:0	2.8±0.4	2.3±0.2*	2.4±0.2	2.3±0.3*
18:1	23.0±0.7	30.7±1.2*	34.0±2.1*	27.4±1.3*
18:2 (n-6)	26.9±1.5	17.5±1.0*	13.9±2.2*	22.4±1.9*
18:3 (n-6)	1.6±1.6	1.3±0.8	1.5±1.1	2.1±1.5
18:3 (n-3)	1.1±0.2	0.41±0.13*	0.29±0.04*	0.61±0.15*
20:1 (n-9)	0.16±0.02	0.16±0.06	0.19±0.05	0.17±0.03
20:3 (n-9)	0.86±0.19	1.8±0.3*	2.3±0.1*	0.67±0.18
20:3 (n-6)	0.23±0.03	0.38±0.05*	0.30±0.03*	0.21±0.03
20:4 (n-6)	12.0±1.6	12.9±1.7	11.5±1.9	8.8±0.7*
20:5 (n-3)	1.0±0.16	0.32±0.09*	0.26±0.05*	0.41±0.06*
22:4 (n-6)	0.86±0.19	0.78±0.15	0.59±0.11	1.0±0.5
22:5 (n-3)	0.43±0.09	0.27±0.08*	0.27±0.02*	0.33±0.11
22:6 (n-3)	2.1±0.2	1.7±0.1*	1.3±0.3*	1.2±0.4*
SFA	26.5±1.5	29.9±1.3*	31.2±1.3*	31.2±0.6*
MUFA	26.5±1.1	32.8±1.2*	36.6±2.2*	31.1±0.9*
PUFA	47.0±1.8	37.3±2.2*	32.2±3.4*	37.7±0.9*
n-6	41.5±1.4	32.9±2.0*	27.8±3.1*	34.5±0.9*
n-3	4.7±0.2	2.6±0.2*	2.1±0.3*	2.6±0.5*
PUFA/ (SFA+MUFA)	0.89±0.06	0.60±0.06*	0.48±0.08*	0.60±0.02*

Results (expressed as molar %) are means±s.d. of four different LDL preparations. *Values different from controls ($P<0.05$). PUFA: polyunsaturated, MUFA: monounsaturated and SFA: saturated fatty acids.

Thus, BFB is the only fibrate that showed an appreciable improvement in all parameters, except in the case of TBARS production.

Discussion

CFB and BFB induced a strong hypolipidemic effect in normolipidaemic rats, reducing plasma levels of cholesterol (basically cholesteryl esters), triglycerides and phospholipids. Surprisingly, GFB-treated animals showed no reduction in plasma triglycerides, and even a significant increase in both free and esterified cholesterol levels (see Table 1). This behaviour differs from that reported in previous studies where GFB showed a strong hypotriglyceridaemic effect and no modification of plasma cholesterol levels in practically identical experimental conditions (Alegret *et al.*, 1994; 1995), except for the fact that, in the present study, the animals were fasted for 16 h before exsanguination, in order to avoid the confounding presence of chylomicrons, and in previous studies (Alegret *et al.*, 1994; 1995) they were not. Thus, GFB seems to be mainly effective in reducing postprandrial lipaemia, at least in normolipidaemic animals.

The reduction in plasma triglyceride levels induced by CFB and BFB could be attributed to a reduction in the hepatic output of VLDL particles. As can be seen in Table 2, neither fibrate modified the quantity of triglycerides carried per milligram of VLDL protein, while both strongly reduced the amount of VLDL protein present in plasma, indicating a decrease in the number of circulating VLDL particles rather than a reduction in the amount of triglyceride carried per VLDL lipoprotein.

Cholesterol content per milligram of lipoprotein protein was not modified after CFB or BFB treatment, either in VLDL or in LDL (see Tables 2 and 5). Given that the total amount of cholesterol carried in VLDL particles was very low (7.9, 1.4,

Table 7 Effect of fibrate treatment on LDL-PL fatty acid molar composition (%)

	Control	*CFB*	*BFB*	*GFB*
14:0	0.47±0.28	1.3±0.7	1.1±0.7	0.56±0.33
16:0	25.3±1.9	33.2±1.1*	34.4±2.1*	34.3±1.0*
16:1	0.67±0.07	0.49±0.02*	0.57±0.09*	0.76±0.05
18:0	25.5±1.6	18.3±0.6*	16.2±1.2*	16.8±0.5*
18:1	7.1±1.2	10.9±0.6*	13.8±0.6*	10.8±0.9*
18:2 (n-6)	12.0±0.4	11.9±0.8	11.5±0.4	14.0±1.4*
18:3 (n-6)	0.30±0.14	1.3±0.8	1.1±1.2	0.50±0.41
18:3 (n-3)	0	0	0	0.02±0.01
20:1 (n-9)	0.12±0.05	0.16±0.10	0.11±0.05	0.12±0.02
20:3 (n-9)	0.13±0.00	0.67±0.15*	0.96±0.33*	0.18±0.03
20:3 (n-6)	0.45±0.05	1.1±0.1*	0.95±0.06*	0.39±0.06
20:4 (n-6)	20.9±1.9	17.2±0.7*	15.6±2.3*	17.4±1.3*
20:5 (n-3)	0.02±0.03	0	0	0
22:4 (n-6)	0.65±0.12	0.22±0.05*	0.29±0.05*	0.80±0.14
22:5 (n-3)	0.56±0.09	0.29±0.06*	0.38±0.04*	0.52±0.15
22:6 (n-3)	4.7±0.7	2.9±0.1*	3.0±0.7*	2.8±0.5*
SFA	52.0±1.2	52.8±1.1	51.7±1.8	51.7±0.5
MUFA	7.9±1.2	11.5±0.5*	14.5±0.7*	11.7±0.8*
PUFA	40.3±1.6	35.6±1.2*	33.8±2.2*	36.6±0.9*
n-6	34.9±1.1	31.7±1.1*	29.5±1.8*	33.1±0.4
n-3	5.3±0.7	3.2±0.1*	3.4±0.8*	3.4±0.7*
PUFA/ (SFA+MUFA)	0.67±0.04	0.55±0.03*	0.51±0.05*	0.58±0.02*

Results (expressed as molar %) are means±s.d. of four different LDL preparations. *Values different fromn controls ($P<0.05$). PUFA: polyunsaturated, MUFA: monounsaturated and SFA; saturated fatty acids.

Table 8 Effect of fibrate treatment on LDL-CE fatty acid molar composition (%)

	Control	*CFB*	*BFB*	*GFB*
14:0	3.2±1.8	5.9±2.3	4.6±1.1	3.2±1.0
16:0	13.7±1.7	16.6±1.0*	16.4±1.9	14.5±2.0
16:1	4.3±0.6	2.6±0.5*	4.3±0.2	5.7±1.1*
18:0	1.0±0.1	0.95±0.11	1.8±0.3*	0.68±0.22*
18:1	12.8±2.2	11.4±2.4	24.9±1.2*	17.3±4.5
18:2 (n-6)	15.5±2.1	9.0±1.5*	8.9±0.8*	16.7±1.7
18:3 (n-6)	8.4±5.5	12.3±4.0	8.8±1.9	5.4±1.0
18:3 (n-3)	0.6±0.1	0.07±0.09*	0.17±0.03*	0.28±0.11*
20:1 (n-9)	0.10±0.11	0.14±0.17	0.08±0.1	0.04±0.08
20:3 (n-9)	0.13±0.06	0.35±0.06*	0.53±0.08*	0.10±0.01
20:3 (n-6)	0.24±0.04	0.41±0.05*	0.28±0.05	0.17±0.04
20:4 (n-6)	37.0±6.2	39.0±2.2	27.6±3.6*	34.8±5.3
20:5 (n-3)	0.17±0.13	0.04±0.04*	0.06±0.05	0.11±0.02
22:4 (n-6)	0	0	0	0
22:5 (n-3)	0	0	0	0
22:6 (n-3)	1.4±0.6	1.3±0.2	1.5±0.7	0.88±0.15
SFA	19.4±4.8	23.4±2.9	22.9±2.1	18.4±1.9
MUFA	17.2±2.4	14.2±2.7	29.3±1.2*	23.0±5.3*
PUFA	63.4±3.4	62.5±1.0	46.7±3.5*	58.9±6.2
n-6	61.1±3.2	60.7±1.3	45.6±3.0*	57.5±6.2
n-3	2.2±0.4	1.4±0.3*	1.8±0.7	1.3±0.1*
PUFA/ (SFA+MUFA)	1.75±0.26	1.66±0.07	0.90±0.11*	1.45±0.33

Results (expressed as molar %) are means±s.d. of four different LDL preparations. *Values different from controls ($P<0.05$). PUFA: polyunsaturated, MUFA: monounsaturated and SFA: saturated fatty acids.

3.9 and 8.6 mg dl^{-1} of plasma for control, CFB, BFB, and GFB treated animals, respectively), the reduction in plasma cholesterol values produced by CFB and BFB could be attributed to a reduction in HDL-cholesterol. It should be borne in mind that HDL is the main cholesterol-carrying particle in rodents (Suckling & Jackson, 1993) (for example, in controls, VLDL+LDL cholesterol represents only 42% of the total cholesterol circulating in plasma).

The opposite situation should be expected for the increase in plasma cholesterol induced by GFB. Nevertheless, despite the possible increase in HDL-cholesterol produced by GFB (Maxwell *et al.*, 1983), it also appears to increase the proportion of cholesterol carried by LDL particles, at the expense of a reduction in the proportion of triglycerides (see Table 5).

Previous studies have shown modifications of rat microsomal phospholipid acyl-composition induced by fibrate treatment (Vázquez *et al.*, 1995). As a whole, the fatty acid species carried by VLDL and LDL from fibrate-treated rats reflected the alterations detected in the microsomal fraction. Thus, in general both particles showed an increase in MUFA and a decrease in PUFA species, affecting n-6 and n-3 series, as found in microsomal phospholipids from treated rats. Further,

Table 9 Effect of fibrate treatment on rat VLDL/LDL oxidative parameters

VLDL	*Control*	*CFB*	*BFB*	*GFB*
$V_{max}CD$	868 ± 140	673 ± 215	321 ± 161	757 ± 143
MaxCD	1155 ± 301	739 ± 361	446 ± 204	1147 ± 173
LT	27 ± 0	68 ± 21	43 ± 9	29 ± 0
TBARS	366 ± 47	101 ± 100	153 ± 76	291 ± 45
LYS	342 ± 39	1290 ± 315	1005 ± 582	659 ± 139
LDL	*Control*	*CFB*	*BFB*	*GFB*
$V_{max}CD$	485 ± 93	351 ± 45	356 ± 73	282 ± 75
MaxCD	973 ± 242	819 ± 226	684 ± 153	685 ± 114
LT	15 ± 0	23 ± 6	27 ± 5	15 ± 1
TBARS	322 ± 51	301 ± 59	234 ± 91	238 ± 25
LYS	563 ± 141	600 ± 123	694 ± 153	436 ± 130

Results shown are the mean ± s.e.mean from 3 different lipoprotein pools obtained in duplicate. Results are expressed as: $V_{max}CD$: nmol CD min^{-1} mg^{-1} protein; MaxCD: nmol CD mg^{-1} protein; LT: min; TBARS: nmol MDA mg^{-1} protein 2 h^{-1}-VLDL or 3 h^{-1}-LDL; LYS: nmol mg^{-1} protein 2 h^{-1}-VLDL or 3 h^{-1}-LDL.

CFB and BFB generally increased the proportion of 20 : 3(n-9), as a reflection of the strong induction of peroxisomal proliferation produced by these drugs. These results are in accordance with those reported by Vance's group (1989; 1990), showing that VLDL assembly requires *de novo* synthesis of the glycerolipids for incorporation in its structure.

The results shown in Table 9 point to a protective effect of fibrate treatment for rat VLDL against an oxidative challenge. Thus, the three fibrates afforded an increase in the content of lysine residues and the lag time for conjugated diene formation, and a reduction in the values of TBARS, maximal rate of CD production and maximal CD formation. For each parameter, BFB was the most effective drug, followed by CFB and GFB.

There is no evidence of redox properties attributable to the molecular structure of fibrates (Hoffman *et al.*, 1992). More important, fibrate administration to rats seems to leave unchanged or even to reduce the plasma levels of vitamin E (Weiss & Bianchine, 1970; Glauert *et al.*, 1992; Ashby *et al.*, 1994). Thus, the observed *antioxidant* effect should be ascribed to two different phenomena produced by these drugs.

The reduction in the amount of substrate for the oxidation process due to the hypolipidaemic activity of fibrates. The 25 μg of VLDL-protein ml^{-1} of reaction medium (see Methods, section), used for the determination of the oxidation parameters equals 133, 83, 90 and 96 μg of TG ml^{-1} added, for control, CFB, BFB, and GFB groups, respectively. This effect is inherent to any hypolipidaemic activity.

Fibrates produce an alteration in the type of fatty acids transported by VLDL, with a reduction in the proportion of PUFA, the main substrate of oxidation, and an increase in the proportion of MUFA and SFA, which are more resistant to the oxidative process (1). This effect seems to be a special property of fibrates, and is suppored by (a) the significant reduction in the ratio PUFA/(SFA + MUFA) produced by the three drugs for the whole VLDL particle, and (b) when oxidative values are expressed per μmol of fatty acids present in the reaction (data not shown), the parameters became independent of the quantity of oxidizable substrate present. In this situation, values for VLDL obtained from treated animals still showed increased protection against copper oxidation.

The situation for LDL oxidation is very close to that of VLDL. The main difference is that the intensity of the protective effect detected in this case is, roughly, half that observed in the case of VLDL. This situation should be not surprising, given that, as stated in Table 5, fibrates did not modify the amount of lipid substrates carried per mg of LDL protein. Thus, in the case of LDL, the *antioxidant* effect of fibrates could be attributed exclusively to the reduction of the degree of unsaturation for the whole LDL particle (see Table 5) produced by these drugs.

Thus globally, the results presented in this paper strongly support an antioxidant effect of fibrates in rats, which is not related to their well known hypolipidaemic activity but rather derived from their ability to modify fatty acid lipoprotein composition. Although caution should be exercised in extrapolating these results to man, from the information available human lipoproteins seem to be altered by fibrates in a similar way to rat lipoproteins. Thus, several authors have reported increases in MUFA and decreases in the proportion of PUFA transported in either plasma or VLDL/LDL after treatment with CFB (Jurand & Oliver, 1963; Vessby *et al.*, 1980a;), BFB (Vessby *et al.*, 1980b), fenofibrate (Agheli & Jacotot, 1991), and, to a lesser extent, GFB (Tavella *et al.*, 1993). Further, Hoffman *et al.* (1992), have reported an antioxidant effect of BFB in man independent of its hypolipidaemic activity and related to unexplained changes in the lipoprotein structure induced by this drug.

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