Hypolipidemic activity of select fibrates correlates to changes in hepatic apolipoprotein C-III expression: a potential physiologic basis for their mode of action

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Abstract For the last 30 years fibrates have been widely prescribed to treat human dyslipidemia. However, the primary mechanism by which they lower plasma lipid levels is still unknown. Studies with transgenic mice have suggested that changes in apoC-III expression levels have a dramatic influence on plasma triglyceride levels. These results suggested that fibrates could reduce lipid levels by lowering apoC-III gene expression. In the current studies, we sought to determine whether the selected fibrates, bezafibrate, clofibrate, fenofibrate, and gemfibrozil, could reduce hepatic apoC-III mRNA and plasma apoC-III levels. Chow-fed rats were orally gavaged daily with a dosing vehicle alone or with 100 mg/kg of each of the fibrates for 1 week and in addition with gemfibrozil for 2 weeks. Bezafibrate and fenofibrate lowered plasma triglyceride by approximately half and dramatically reduced hepatic apoC-III mRNA and plasma apoC-III levels. In contrast, clofibrate did not reduce plasma triglyceride levels and only partially reduced apoC-III mRNA and plasma protein levels. Gemfibrozil strongly reduced plasma triglyceride levels and had an intermediate but significant effect on apoC-III mRNA and plasma apoC-III levels. Some of the fibrates, especially gemfibrozil also reduced plasma apoC-II levels, an effect that could contribute to the observed triglyceride-lowering effect. In addition, the ratio of plasma apoE to plasma apoC-II plus apoC-III was strongly and inversely correlated with plasma triglyceride levels. As plasma apoE levels were not reduced in gemfibrozil-treated animals, this could also have contributed to the triglyceride-lowering effect of this fibrate. Fibrate-mediated triglyceride lowering was not the result of a decreased apoB or VLDL production and, therefore, suggested an enhanced VLDL remnant catabolism. Our results suggest that the mechanism by which fibrates lower plasma triglycerides is by reducing the level of hepatic apoC-III expression.—**Haubenwallner, S., A. D. Essenburg, B. C. Barnett, M. E. Pape, R. B. DeMattos, B. R. Krause, L. L. Minton, B. J. Auerbach, R. S. Newton, T. Leff, and C. L. Bisgaier.** Hypolipidemic activity of select fibrates correlates to changes in hepatic apolipoprotein C-III expression: a potential physiologic basis for their mode of action. *J. Lipid Res.* 1995. **36:** 2541–2551.

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Hypertriglyceridemia and low levels of HDL may each be independent risk factors for cerebrovascular and coronary heart disease (CHD) (1–3). Conventional therapy for hypertriglyceridemia includes dietary weight loss, and drug intervention if appropriate, which might include fibrate therapy, and gemfibrozil when HDL levels are also diminished, nicotinic acid, fish oils, and under certain conditions, reductase inhibitors and bile acid sequestrants (4–7). A primary prevention clinical trial has shown that gemfibrozil lowers the incidence of CHD in large part by elevating HDL (8). Surprisingly, however, little is known of the mechanisms by which fibrates lower triglycerides and in the case of gemfibrozil, the mechanism that leads to HDL elevation.

Although some results have shown an effect of fibrates on hepatic (HL) and lipoprotein lipase (LPL) activity and/or expression, no consensus has emerged from these studies. Similarly, both in vitro and in vivo studies on apolipoprotein levels and expression have suggested that fibrates have differential effects on

Abbreviations: apo, apolipoprotein; CHD, coronary heart disease; HL, hepatic lipase; HPGC, high performance gel chromatography; LPL, lipoprotein lipase; PPAR, peroxisome proliferator activated receptor; PPRE, peroxisome proliferator activated receptor response element; HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein.

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apolipoprotein synthesis. For example, clofibrate, fenofibrate, and gemfibrozil in rats do not affect hepatic apoE mRNA levels but these compounds either reduce (clofibrate and fenofibrate) or elevate (gemfibrozil) plasma apoE levels (9). Similarly, in the cholesterol-fed rat, only gemfibrozil, but neither ciprofibrate, fenofibrate, clofibrate, nor bezafibrate elevated plasma apoE (10). Studies of Staels et al. (9) have shown rat hepatic apoA-I mRNA levels diminish after clofibrate, fenofibrate, or gemfibrozil treatment, which is consistent with diminished plasma levels observed with clofibrate and fenofibrate, but not with gemfibrozil where plasma apoA-I levels were unchanged. In the cholesterol-fed rat, gemfibrozil actually elevates plasma apoA-I (10). Thus, causal relationships between apolipoprotein production and plasma levels may be inconclusive and can simply reflect a complex relationship between apolipoprotein synthesis, intracellular degradation, secretion, redistribution between plasma lipoproteins, and clearance.

Previous observations have suggested that reduced plasma apoC-III levels are associated with reduced triglycerides (11). ApoC-III inhibits hepatic clearance of triglyceride-rich remnants (12) and lipoprotein lipase activity in vitro (13). Genetic studies have demonstrated a strong association of a polymorphism in the 3′ untranslated region of the apoC-III gene with hypertriglyceridemia in several distinct populations (14). Similarly, transgenic mice overexpressing apoC-III are hypertriglyceridemic (15–17), while mice genetically deficient in apoC-III are hypotriglyceridemic (18). In the current study we report that apoC-III expression is reduced during fibrate therapy, suggesting that a possible mechanism for the hypolipidemic action of these compounds is the reduction of apoC-III gene expression.

MATERIALS AND METHODS

Animal diets and treatments

Male Sprague-Dawley rats (100–200 g) were obtained from Charles River Laboratories and housed 4–6 rats per cage. All animals were allowed normal rat chow (Ralston-Purina) and water ad libitum in temperature-controlled rooms, under a 12-h light, 12-h dark cycle beginning with lights on at 6 AM. Rats were dosed daily between 6 and 9 AM by oral gavage using a suspension vehicle (0.2% Tween-20 plus 1.5% carboxymethylcellulose). Control animals received vehicle alone. Vehicle volume represented 0.25% of body weight. Fibrates were administered at 100 mg/kg per day for 1 or 2 weeks, as indicated for the specific experiments. For all but the production rate studies, non-fasted rats were anesthetized by ether inhalation 10–12 h post-dosing, weighed, bled by cardiac puncture, and subjected to hepatectomy for liver weight determination and sampling for RNA isolation. Blood was transfered to ethylenediaminetetraacetic acid-containing Vacutainer tubes for plasma isolation.

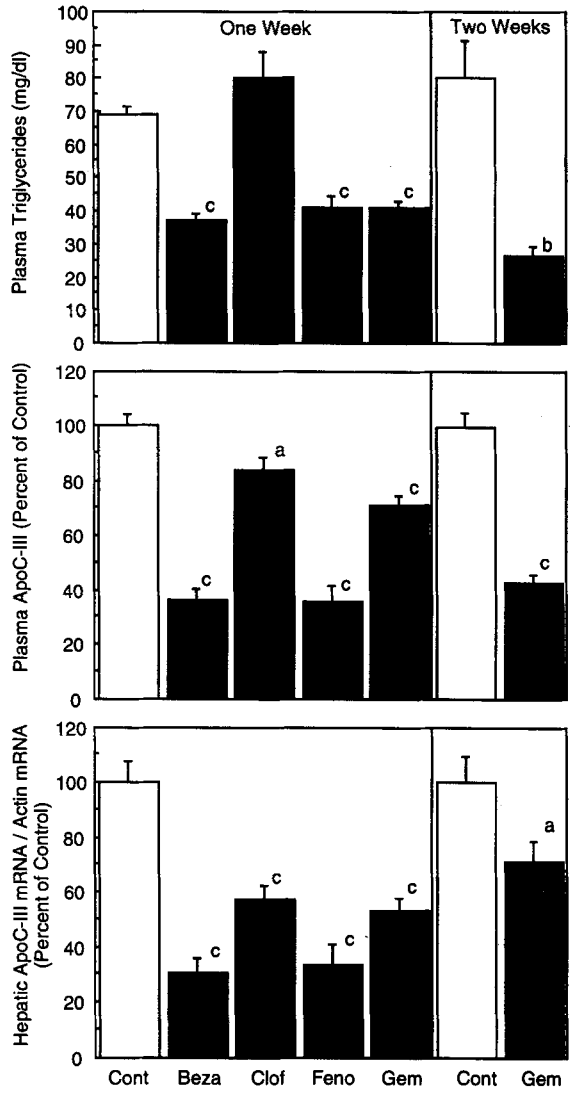

Fig. 1. Plasma triglycerides, plasma apoC-III and hepatic apoC-III mRNA/actin mRNA were determined in rats treated for 1 week with the dosing vehicle (Cont) or 100 mg/kg per day bezafibrate (Beza), clofibrate (Clof), fenofibrate (Feno), or gemfibrozil (Gem), or for 2 weeks with the dosing vehicle or gemfibrozil. For gemfibrozil, data were collected from up to eight separate 1-week experiments (n = 5 or 6 rats/group per experiment), and a single 2-week experiment (n = 8 rats/group). Data for the other fibrates (two experiments) and gemfibrozil (one of the two experiments) were also obtained from animals treated with fibrates for 1 week (n = 4–6 rats/group). Plasma apoC-III and hepatic apoC-III mRNA/actin mRNA data were compared by assigning a value of 100 to the average of the control-treated animals for each experiment. Data were analyzed by ANOVA and Fisher PLSD post hoc analysis for determination of significant mean difference compared to controls (1-week experiments) or by an unpaired *t*-test (2-week experiment); a, $P < 0.05$; b, $P < 0.001$; c, $P < 0.0001$.

Table 1.

Parameter	Units Treatment (dose in mg/kg/day)	One-Week Studies[a,b] Vehicle Control n = 53	Bezafibrate (100) n = 11	Clofibrate (100) n = 10	Fenofibrate (100) n = 10	Gemfibrozil (100) n = 47	Two-Week Study[a,b] Vehicle Control n = 8	Gemfibrozil (100) n = 8
Plasma triglycerides	mg/dl ± SEM (% of control)	69 ± 3 (100)	37 ± 2 (54)[f]	80 ± 8 (116)	41 ± 4 (59)[f]	41 ± 2 (59)[f]	80 ± 11 (100)	27 ± 3 (33)[e]
ApoC-III	% of control ± SEM	100 ± 5 (n = 22)	36 ± 4[f]	83 ± 5[c]	36 ± 5[f]	71 ± 3 (n = 17)[f]	100 ± 5 (n = 7)	43 ± 3[f]
Hepatic apoC-III mRNA/actin mRNA	% of control ± SEM	100 ± 8 (n = 16)	31 ± 5 (n = 10)[f]	57 ± 5 (n = 8)[f]	34 ± 7[f]	53 ± 5 (n = 12)[f]	100 ± 9	71 ± 8[c]
Plasma lipid parameters								
Total cholesterol	mg/dl ± SEM (% of control)	55 ± 1 (100)	52 ± 3 (94)	46 ± 2 (84)[c]	46 ± 3 (84)[c]	49 ± 2 (89)[d]	46 ± 2 (100)	46 ± 4 (99)
VLDL cholesterol	mg/dl ± SEM (% of control)	4.5 ± 0.2 (100)	2.3 ± 0.3 (50)[f]	4.8 ± 0.6 (106)	2.3 ± 0.3 (50)[f]	2.4 ± 0.2 (54)[f]	5.2 ± 0.8 (100)	1.8 ± 0.3 (35)[d]
LDL cholesterol	mg/dl ± SEM (% of control)	12.4 ± 0.5 (100)	15.3 ± 1.0 (123)[d]	9.2 ± 0.8 (74)[d]	13.6 ± 0.7 (109)	6.5 ± 0.4 (52)[f]	9.5 ± 0.8 (100)	4.8 ± 0.5 (50)[e]
HDL cholesterol	mg/dl ± SEM (% of control)	38 ± 1 (100)	34 ± 2 (90)	32 ± 1 (84)	30 ± 2 (80)[c]	40 ± 2 (106)	32 ± 2 (100)	39 ± 4 (124)
HDL/(VLDL +LDL)	Ratio ± SEM (% of control)	2.30 ± 0.07 (100)	1.98 ± 0.15 (86)	2.41 ± 0.19 (104)	1.95 ± 0.16 (85)	5.35 ± 0.43 (232)[f]	2.23 ± 0.18 (100)	6.45 ± 0.79(290)[f]
Plasma apolipoprotein parameters								
ApoB	% of control ± SEM	100 ± 2 (n = 46)	102 ± 7	106 ± 5	103 ± 4	57 ± 3 (n = 41)[f]	100 ± 11	59 ± 4[d]
ApoA-I	% of control ± SEM	100 ± 1	81 ± 6[f]	90 ± 3[d]	83 ± 2[f]	102 ± 2	100 ± 1	93 ± 2[d]
ApoE	% of control ± SEM	100 ± 2	89 ± 5	84 ± 4[c]	84 ± 6[c]	102 ± 3	100 ± 6	141 ± 8[d]
ApoC-II	% of control ± SEM	100 ± 3 (n = 22)	67 ± 3[f]	94 ± 4	71 ± 4[f]	59 ± 3 (n = 17)[f]	100 ± 5 (n = 7)	56 ± 3[f]
ApoA-I/apoB	% of control ± SEM	100 ± 3 (n = 46)	75 ± 5	79 ± 6	74 ± 4	192 ± 18 (n = 35)[f]	100 ± 7	154 ± 12[d]
ApoE/(apoC-II + apoC-III)	Ratio ± SEM	0.50 ± 0.02 (n = 22)	0.88 ± 0.04[f]	0.47 ± 0.02	0.80 ± 0.06[f]	0.85 ± 0.03(n = 17)[f]	0.49 ± 0.03 (n = 7)	1.43 ± 0.06[f]

[a]Data calculated from up to 10 separate 1-week studies (n = 5 control animals for 7 studies, n = 6 control animals for 3 studies) and a 2-week study. In each study, apolipoprotein and mRNA data were normalized to those of the control animals. Lipids are given as absolute values. The numbers within parentheses are percent of the controls for all studies of the same duration.

[b]ANOVA, Fisher PLSD post hoc analysis used for determination of significant mean difference compared to controls for 1-week studies. Unpaired *t*-test (two-sided) for the 2-week study.

[c]$P < 0.05$.

[d]$P < 0.01$.

[e]$P < 0.001$.

[f]$P < 0.0001$.

Triglyceride and apoB production rates

Triglyceride and apoB production rates were determined in rats fasted overnight that were treated with fibrates for 8 days. Triton WR 1339 administration blocks the catabolism of triglyceride-rich lipoproteins; therefore, the net plasma accumulation after administration is representative of production. After 8 days of fibrate treatment (100 mg/kg per day), a baseline tail-vein blood sample was drawn after an overnight fast which was also 2 h post the last fibrate dose. Triton WR 1339 (Tyloxapol, Sigma Chemical Co., St. Louis, MO) was then administered intravenously (600 mg/kg in normal saline) (19–21) and additional blood samples were obtained 2 and 4 h post-Triton WR 1339 administration. Triglyceride production was linear up to 4 h. Plasma samples were analyzed for triglyceride and apoB determinations as described below. The difference between the basal and the 2-h post-Triton WR1339 samples was used to determine triglyceride and apoB production rates. For data analysis, plasma volume was assumed to be 3.86 ml/100 g body weight (20).

Analytical methods for plasma lipids

Triglycerides were determined using commercially available kits (Trigli-cinet 2, Sclavo, Siena, Italy or Triglyceride G, Wako Pure Chemical Industries, Ltd. Osaka, Japan). Total plasma cholesterol concentrations were determined enzymatically (22). Plasma total cholesterol lipoprotein profiles were determined by on-line post column analysis on Superose 6HR high performance gel chromatography (HPGC) on a high pressure liquid chromatograph (Rainin Instrument Co., Inc., Woburn, MA) as previously described (21, 23). Lipoprotein cholesterol was determined from independent total cholesterol determinations and percent area distribution of cholesterol was determined by HPGC. Peaks were collected and areas were integrated and averaged using Dynamax and Compare Module Software (Rainin Instrument Co., Inc., Woburn, MA) developed for Macintosh computers. In rats, unlike other species on chow diets, the presence of the large sized HDL_1 does not allow a complete distinct separation of LDL from HDL by the HPGC technique. However, although the

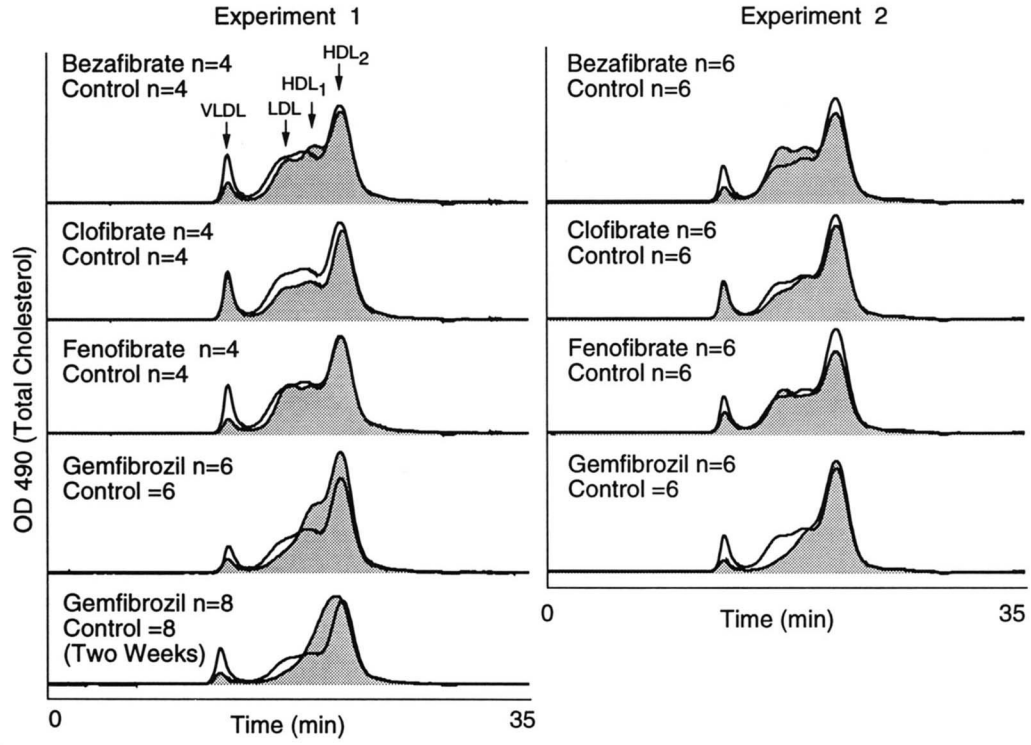

Fig. 2. Representative plasma lipoprotein cholesterol determination in rats treated with the dosing vehicle or 100 mg/kg per day of the indicated fibrates by high performance gel chromatography (HPGC). Data shown were collected from 1- or 2-(indicated) week experiments. In each case, the shaded areas represent averaged profiles of all fibrate-treated rats, while the open profiles represent the averaged profile of vehicle-treated rats for a specific experiment. Plasma samples (20 µL) from each rat were individually analyzed although each profile shown represents an averaged composite composed from 4–8 profiles as indicated. Methods used to integrate area of VLDL, LDL and the combined HDL_1 plus HDL_2 peak are given in Materials and Methods.

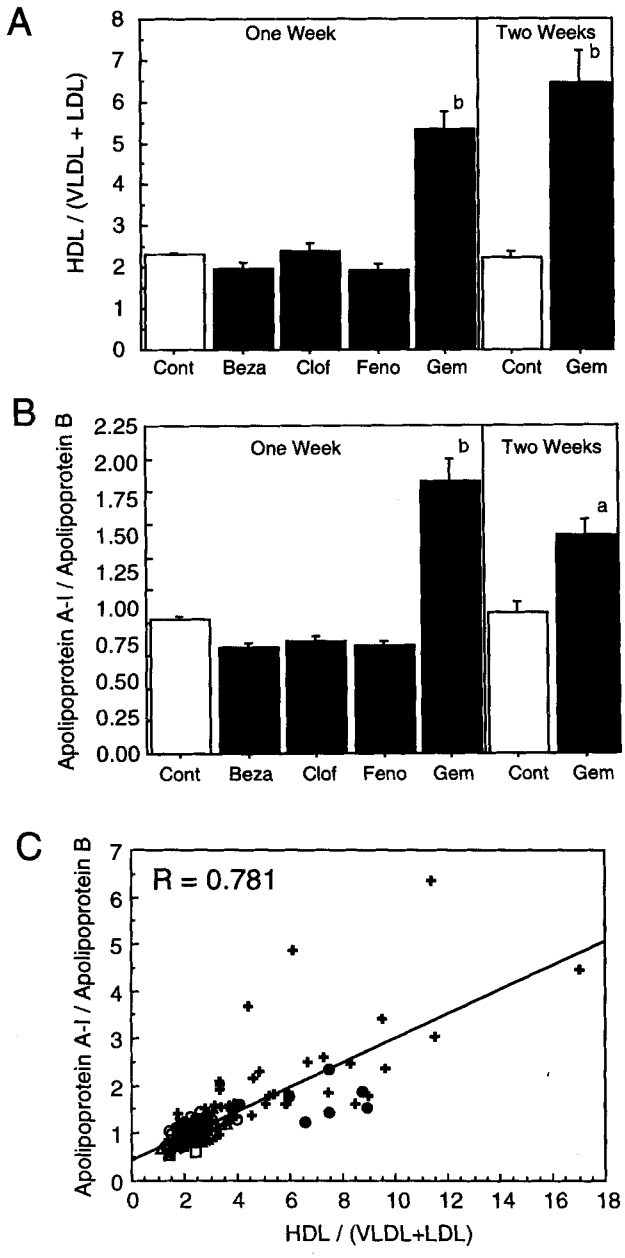

Fig. 3. (A) Plasma HDL to VLDL plus LDL cholesterol ratios and (B) plasma apoA-I to apoB ratios in chow-fed rats treated with for 1 week with the dosing vehicle (Cont) or 100 mg/kg per day bezafibrate (Beza), clofibrate (Clof), fenofibrate (Feno), or gemfibrozil (Gem), or for 2 weeks with the dosing vehicle or gemfibrozil. For gemfibrozil, data were collected from up to eight separate 1-week experiments (n = 5 or 6 rats/group per experiment), and a single 2-week experiment (n = 8 rats/group). Data for the other fibrates (two experiments) and gemfibrozil (one of the two experiments) were also obtained from animals treated with fibrates for 1 week (n = 4 to 6 rats/group). Plasma apoA-I and apoB levels were compared by assigning a value of 100 to the average of the control-treated animals for each experiment. Data were analyzed by ANOVA and Fisher PLSD post hoc analysis for determination of significant mean difference compared to controls (1-week experiments) or by an unpaired *t*-test (2-week experiment); a, $P < 0.01$; b, $P < 0.0001$. (C) Regression analysis of HDL/(VLDL + LDL) to that of apoA-I/apoB in rats treated daily (oral gavage) with 100 mg/kg bezafibrate (□), clofibrate (△), fenofibrate (◇), gemfibrozil (✚) or control vehicle (○) for 1 week or 100 mg/kg gemfibrozil (●) or control vehicle (✖) for 2 weeks.

LDL and HDL peaks are merged, their relative contribution to peak area can be estimated. The area representing the distinct VLDL peak can be easily integrated. LDL area in the remaining merged area was resolved by assuming a symmetrical elution of the LDL peak and estimating its total area by doubling the integrated ascending half of the LDL peak. HDL in the remaining area (1/2 LDL plus HDL_1 and HDL_2) was determined by subtraction of estimated 1/2 LDL area.

Analytical methods for plasma apolipoproteins

Apolipoproteins A-I, C-II, C-III, and E in whole plasma were quantitated by rocket immunoelectrophoresis (24) using antibodies raised in a rabbit against rat apoA-I and in goats against rat apoE (from Dr. Patrick Tso, LSU Medical Center, Shreveport, LA) or apoC-II and C-III (from Dr. Peter J. Dolphin, Dalhousie University, Halifax, Nova Scotia, Canada). Plasma samples were diluted in 4 M urea, 1% Triton X-100, 12 mM Tricine, 40 mM Tris, 0.6 mM calcium lactate, 0.01% sodium azide, pH 8.2 (apoA-I and apoE) or 7 M urea, 3 mM Tricine, 10 mM Tris, 0.15 mM calcium lactate, 0.0025% sodium azide, pH 8.2 (apoC-II and apoC-III) and incubated for 60 min at 52°C prior to immunoelectrophoresis. Immunoelectrophoresis was carried out on GelBond film (Cat 53748, FMC Bioproducts, Rockland, ME) usually containing either 4% rabbit anti-rat apoA-I, 2% goat anti-rat apoE, 0.25% goat anti-rat apoC-II, or 0.25% goat anti-rat apoC-III antiserum in 1% agarose, 2% polyethylene glycol 6000 in 24 mM Tricine, 80 mM Tris, 1.2 mM calcium lactate (apoA-I and apoE) or 4.8 mM calcium lactate (apoC-II and C-III), 0.02% sodium azide, pH 8.2, from 6 to 20 h at 0.5–1 mA/cm. Rocket height was determined on amino black-stained gels. For data analysis, apolipoproteins in plasma from animals in the control groups were arbitrarily set to 100. For all immunoelectrophoretic assays, appropriate dilutions of rat plasma were made so that apolipoproteins were in the linear range of the assay. The inter- (n = 10) and intra- (n = 3) assay coefficients of variation for the immunoelectrophoretic assays were; 6% and 10% for apoA-I, 6% and 7% for apoE, 6% and 13% for apoC-II, 6% and 4% for apoC-III, respectively.

Rat apoB was assessed in microtiter plates with minor modifications of a previously described immunoturbidometric method (25) utilizing antibodies to mouse apoB raised in sheep that cross-react with rat apoB. Plasma samples from experimental animals (5, 10, 20, and 30 µL) or a pooled rat plasma standard (0–50 µL) were combined with 2 M urea, 10% sheep anti-mouse apoB serum, 1.6% polyethylene glycol 8000 (final concentrations) in a total volume of 200 µl phosphate-buffered saline. Turbidity (OD = 340 nm) was determined initially and after an overnight incubation at room tem-

perature utilizing a Titertek Multiscan MCC/340MK II (Flow Laboratories, Inc. McLean, VA) 96-well absorbance spectrophotometer. An appropriate dilution of rat plasma (usually 10 μL) was used to determine apoB in the linear range of the assay. The inter- (n = 10) and intra- (n = 3) assay coefficients of variation for the apoB immunoturbidometric assay were 5% and 12%, respectively. For data analysis, apoB levels in the plasma of drug-treated animals were compared to those obtained in the control group which was arbitrarily set to 100 for each experiment. For apoB production studies, the change in apoB mass prior to and after Triton WR1339 administration is represented as arbitrary optical density units.

Hepatic mRNA analysis

Hepatic RNA was isolated with RNAzol (Cinna/Biotecx Inc., Houston, TX) and assessed for any degradation by agarose electrophoresis (26). Rat mRNA specific probes and internal standards for rat HL, apoE, and apoA-I mRNA were synthesized from pBluescript II SK (+) plasmids containing partial rat cDNAs for the genes of interest. Cloned cDNAs were prepared in polymerase chain reactions using the conserved primers described elsewhere (27). The identity of the amplified products and cloned cDNAs was confirmed by DNA sequencing. mRNA levels for the genes of interest were determined by an internal standard/RNAse protection assay essentially as described previously in detail (27, 28). Each protection assay contained either 5 μg (apoE) or 30 μg (apoA-I and HL) of total liver RNA and 30 pg (apoE), 20 pg (apoA-I), or 10 pg (HL) internal standard plus 200,000 cpm (2×10^8 cpm/μg) of radiolabeled RNA probe. Autoradiographic images were analyzed using the Molecular Dynamics 400 E PhosphorImager (Molecular Dynamics, Sunnyvale, CA). In some internal standard/RNAse protection assays (apoA-I for example), protected probe fragments migrated as multiple bands upon electrophoretic separation on polyacrylamide. We have observed this phenomena previously; it is most likely due to secondary structure of the DNA fragments, which could be related to resistance of the probe to RNase activity (27, 28).

Hepatic apoC-III and actin mRNA were determined by Northern blot analysis. Briefly, 20 μg of total liver RNA samples was subjected to gel electrophoresis on a

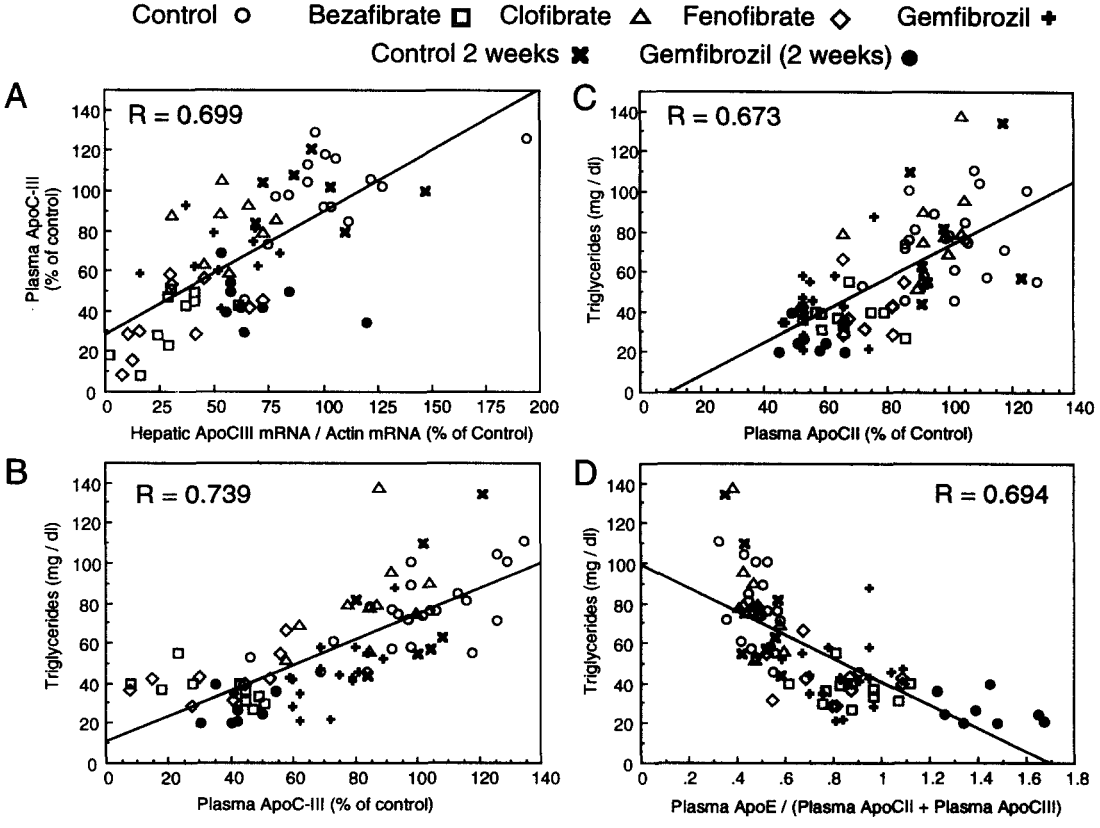

Fig. 4. Regression analysis of (A) plasma apoC-III as a function of hepatic apoC-III mRNA/actin mRNA, and plasma triglycerides as a function of (B) plasma apoC-III, (C) plasma apoC-II, and (D) as the ratio of plasma apoE to plasma apoC-II plus apoC-III in rats treated daily (oral gavage) with 100 mg/kg bezafibrate (□), clofibrate (△), fenofibrate (◇), gemfibrozil (✚) or control vehicle (○) for 1 week or 100 mg/kg gemfibrozil (●) or control vehicle (✖) for 2 weeks.

1.2% agarose gel (6% formaldehyde) and blotted onto nitrocellulose (Schleicher & Schuell, Keene, NH). Blots were baked for 2 h at 80°C and prehybridized for 1 h in 2× prehybridization/hybridization solution (Gibco BRL, Grand Island, NY). Blots were simultaneously probed with [^{32}P]UTP-(Amersham Inc., Arlington Heights, IL) labeled riboprobes generated from a mouse apoC-III cDNA (200 ng of a 269 bp fragment) and mouse actin cDNA (200 ng of a 249 bp fragment, Ambion Inc., Austin, TX). Hybridization was conducted overnight at 65°C with 500,000 cpm/ml of each probe. Blots were washed under stringent conditions at 65°C. Northern blots were also analyzed on the Phosphorimager.

Data represent area units of the specific signals for authentic/internal standard mRNA (internal standard/RNAse protection assays) or apoC-III mRNA/actin mRNA (Northern blots). To normalize the apoC-III mRNA/actin mRNA ratio between experiments, a value of 100 was assigned to the average of the control-treated animals for each experiment.

Statistical analyses

When multiple groups were compared in the 1-week experiments, analysis of variance and Fisher PLSD post hoc analysis was used for determination of significant mean difference compared to controls. In the 2-week experiment, when only gemfibrozil was compared to a control group, an unpaired *t*-test (two-sided) was used to determine significant difference between means. All numerical data are given as the mean ± standard error of the mean. All statistical data were analyzed on Statview Version 4.01 developed for Macintosh computers.

RESULTS

To determine the relationship between apoC-III production and plasma triglyceride levels after fibrate treatment, chow-fed male rats were orally dosed daily with 100 mg/kg of select fibrates or the dosing vehicle for 1 or 2 weeks. Body weight gains were similar in all fibrate-treated rats compared to controls (data not shown). After the treatment period, animals were killed and blood and tissue samples were collected for analysis. Liver to body weight ratios were significantly elevated in all fibrate-treated rats (data not shown). Plasma triglycerides and apoC-III and hepatic apoC-III mRNA levels were determined and are shown in **Fig. 1** and **Table 1.** With all treatments, except clofibrate, plasma triglycerides were significantly reduced. For all treatments, significant reductions in plasma apoC-III and hepatic apoC-III mRNA were observed.

For all fibrates except gemfibrozil in the 1-week experiments, the degree of triglyceride reduction was roughly correlated to the degree of apoC-III mRNA reduction (Fig. 1). These results demonstrate that one of the actions of fibrates is to lower hepatic apoC-III mRNA levels, and suggests that this may be the primary mechanism by which fibrates lower plasma triglyceride levels. It is notable that with gemfibrozil, in spite of a dramatic reduction in triglyceride levels, plasma and hepatic apoC-III reduction is not as profound as with bezafibrate or fenofibrate. This suggests that gemfibrozil may have additional effects that contribute to the observed changes in plasma lipoprotein levels. In fact, examination of lipid parameters (Table 1) reveals that gemfibrozil has several distinct effects when compared to the other fibrates. Chief among these are lower levels of apoC-II and higher levels of apoE (Table 1). Both of these differences would have the effect of raising the apoE/apoC-II and C-III ratio which may contribute to the lowering of plasma triglyceride (discussed below).

Representative averaged lipoprotein cholesterol profiles from 1-week experiments in which bezafibrate, clofibrate, fenofibrate, and gemfibrozil were compared to control groups are shown in **Fig. 2.** In addition, a representative 2-week experiment with gemfibrozil alone is also shown. With treatments, total plasma cholesterol was slightly reduced in all groups except bezafibrate-treated animals at 1 week and gemfibrozil-treated rats at 2 weeks (Table 1). Under these conditions, all fibrates, with the exception of clofibrate, caused a marked reduction in VLDL cholesterol (Fig. 2 and Table 1). The lipoprotein cholesterol profiles from each treatment group also revealed distinct effects of gemfibrozil. A consistent finding with gemfibrozil was a significant lowering of both VLDL and LDL cholesterol and a tendency to maintain or elevate HDL cholesterol. Although gemfibrozil effects on HDL elevation approached but did not reach statistical significance in the 1-($P = 0.24$) or 2-week ($P = 0.08$) studies, its unique effect on lipoprotein cholesterol can be appreciated as the ratio of HDL to VLDL plus LDL (Table 1 and **Fig. 3**). In addition, apoB levels were reduced by 41–43% with gemfibrozil treatment, but unchanged in all other fibrate-treated rats (1- and 2-week experiments, Table 1). ApoA-I levels were unchanged with gemfibrozil treatment, but reduced in all other fibrate-treated rats (1-week experiments). In the 2-week gemfibrozil experiment, apoA-I was reduced by 7%; however, apoE levels were elevated (Table 1) and likely reflect the amplification of the HDL_1 peak (Fig. 2). The effect that fibrates had on the ratio of apoA-I to apoB correlated with the ratio of HDL to VLDL plus LDL cholesterol for all treatment groups (Fig. 3 and Table 1). For gemfibrozil,

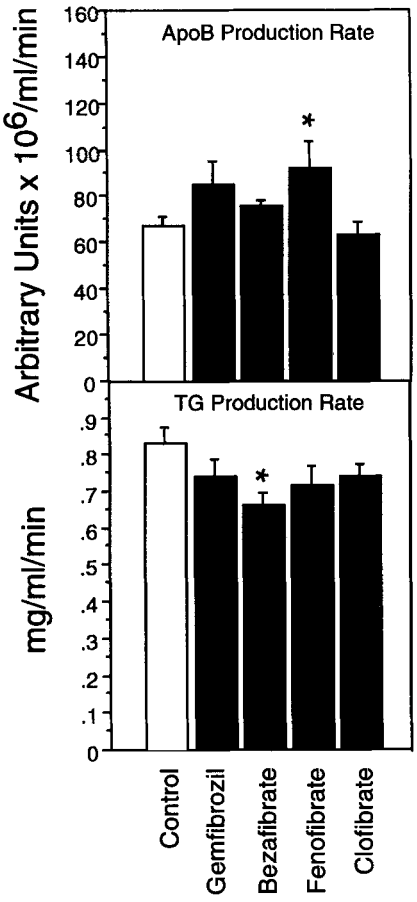

Fig. 5. Triglyceride and apoB production rates were determined in rats treated with vehicle alone or the indicated fibrates at 100 mg/kg per day for 8 days. Rats were fasted overnight, then dosed in the morning on day 8. Two hours post-fibrate dosing, a basal tail-vein blood sample was drawn. Triton WR 1339 (600 mg/kg in normal saline) was then administered intravenously and additional blood samples were obtained 2 and 4 h post-Triton WR 1339 administration. Triglyceride production was linear over 4 h. Triglycerides and apoB were determined as described in Materials and Methods. Data represent the mean ± SEM of for 10 (control group) or 5 (all other treatments) rats per group. ANOVA, Fisher PLSD post hoc analysis was used to determine significant mean difference compared to controls; *$P < 0.05$.

in particular, both the apoA-I to apoB and HDL to VLDL plus LDL ratios were elevated (Fig. 3).

In the 1-week experiments, apoE levels were either unchanged (bezafibrate and gemfibrozil) or significantly reduced (clofibrate and fenofibrate). In a 2-week experiment, the apoE levels were elevated by 41% with gemfibrozil and may reflect substitution of HDL apoA-I by apoE. In contrast, bezafibrate treatment for 2 weeks in chow-fed rats, at dosages of 100 or 300 mg/kg per day, reduced plasma apoE by approximately 20 percent (B. R. Krause and C. L. Bisgaier, unpublished observations). The ratio of apoE to that of apoC-II plus apoC-III was markedly elevated for all fibrate-treated rats, with the exception of clofibrate (Table 1). Linear regression analyses of select data are shown in **Fig. 4.** Plasma apoC-III was highly and positively correlated with hepatic apoC-III mRNA levels (Fig. 4, panel A). In addition, plasma triglycerides were highly and positively correlated with plasma apoC-III (Fig. 4, panel B) or apoC-II (Fig. 4, panel C) and inversely correlated to the ratio of plasma apoE to apoC-II plus apoC-III (Fig. 4, panel D). We observed no correlation (r = 0.100) between triglycerides and apoE (not shown). These results further support the relationship between apoC-III and plasma triglyceride levels. The strong correlation between plasma triglycerides and the apoE/(apoC-II + apoC-III) raises the possibility that the ratios of these apolipoproteins on lipoproteins are a key parameter that regulates plasma triglyceride levels.

As 2-week treatment with gemfibrozil resulted in plasma apoE elevation and a small reduction in plasma apoA-I, we investigated whether hepatic mRNA levels reflected the plasma levels. We also sought to determine whether increased expression of HL could account for the hypolipidemic effects of gemfibrozil. After 2 weeks of 100 mg/kg per day gemfibrozil treatment, total liver RNA was prepared from individual animals for determination of apoA-I, apoE, or HL mRNA by an internal standard/RNase protection assay. Relative to the signal of the mRNA internal standards (i.e., authentic mRNA/internal standard mRNA signal), apoE mRNA levels were similar in control (28.2 ± 3.5, n = 8) compared to gemfibrozil-treated rats (25.2 ± 4.0, n = 8). Control HL mRNA levels (6.8 ± 0.8, n = 8) were similar to those of gemfibrozil-treated rats (5.3 ± 0.5, n = 8). ApoA-I mRNA levels were reduced 24% in the gemfibrozil-treated rats (24.7 ± 3.0, n = 8, control vs. 18.7 ± 3.1, n = 8, gemfibrozil), however this decrease was not statistically significant.

To determine whether changes in triglyceride production rates play a role in the action of fibrates, VLDL production was assessed in Triton WR 1339-administered rats. There was no decrease in the apoB production in the fibrate-treated rats. In fact, with fenofibrate, apoB production was significantly elevated ($P < 0.05$) (**Fig. 5**). Triglyceride production was either not reduced (clofibrate, fenofibrate, and gemfibrozil) or minimally reduced by 20% (bezafibrate) with treatment (Fig. 5). The finding that bezafibrate minimally reduced triglyceride production rates ($P < 0.05$) in this study should be taken with reservation in that other studies in our laboratory (B. R. Krause and C. L. Bisgaier, unpublished observations) and elsewhere (29) have failed to demonstrate a bezafibrate-specific effect on this parameter. Overall, these data suggest that the predominant effect of fibrates in rats is to enhance triglyceride-rich lipoprotein clearance without affecting VLDL produc-

tion rates. The increase in triglyceride-rich lipoprotein clearance is presumably due to a reduction in the amount of apoC-III protein on the surface of the particles.

DISCUSSION

A hallmark of the biological activity of fibric acid compounds is their ability to lower plasma triglyceride levels in patients and in animal models. The mechanism by which these compounds have their hypolipidemic effect is unclear. Here we report that four different fibrates caused a significant lowering of hepatic apoC-III mRNA levels and apoC-III protein concentration in plasma and that, in general, these changes correlate with reduced plasma triglyceride levels. These findings suggest that fibrates lower plasma triglyceride levels by repressing apoC-III gene expression. Several lines of evidence support the model that fibrates lower plasma triglycerides by reducing apoC-III gene expression. ApoC-III has been shown to inhibit the processes that remove triglyceride from the circulation. Triglyceride carried in VLDL is hydrolyzed by lipoprotein lipase LPL at the vascular endothelium allowing the liberated free fatty acids to be absorbed by the neighboring tissue. Previous studies have demonstrated that LPL activity is inhibited by high amounts of apoC-III (13).

The catabolism of VLDL and VLDL remnants by LPL (30, 31) and HL (32, 33) yields smaller remnant particles that are removed from the circulation by a receptor-mediated process. Although this process is poorly understood, it appears to require the presence of apoE on the surface of the particle, suggesting that it is the ligand for a receptor-mediated uptake mechanism. It is well known that apoE enrichment of triglyceride-rich remnants enhances their clearance whereas the C apolipoproteins impede particle clearance (12, 34–37). In transgenic mice, overexpression of apoC-III alone causes hypertriglyceridemia (15–17) apparently by reducing the rate of triglyceride clearance (16), while the absence of expression by apoC-III gene knockout (18) induces rapid clearance of triglyceride and very low plasma triglyceride levels. Overexpression of apoE alone causes enhanced triglyceride clearance rates when challenged with lipid-rich diets (38, 39). In addition, the hypertriglyceridemia caused by apoC-III overexpression can be corrected by co-expression of apoE (17, 40). A reasonable explanation for the opposing effects of apoE and apoC-III is that apoE is required for catabolism of triglyceride-rich lipoproteins and that high levels of apoC-III, and possibly apoC-I and C-II as well (12, 40, 41), mask or block the apoE effects. In this model, the ratio of apoE to apoC-III on the surface of VLDL particles would have a major influence on the rate of receptor-mediated uptake of remnant particles and possibly also on the activity of LPL. Taken together, these results indicate that a reduction in plasma apoC-III levels by fibrate treatment would produce triglyceride-rich particles that have reduced amounts of apoC-III relative to apoE, and that these particles would be rapidly catabolized resulting in lower plasma triglyceride levels.

This specific model of fibrate action is supported by several of the findings reported in this study. A comparison of the effects of bezafibrate, clofibrate, and fenofibrate show a strong correlation between the triglyceride-lowering and apoC-III-lowering effects. The compounds that reduce apoC-III levels the most also show the most potent lipid-lowering effects (Fig. 1). Gemfibrozil is an exception in that it lowers triglyceride levels as well as fenofibrate and bezafibrate but only partially reduces apoC-III levels (Fig. 1). This can be explained by the fact that gemfibrozil-treated animals had higher apoE levels then animals treated with the other fibrates (Table 1). In fact, the apoE/apoC-II + C-III ratio in the 1-week gemfibrozil study is comparable to the ratio for bezafibrate and fenofibrate (Table 1). These data support the model stated above that the lipoprotein parameter that most directly controls plasma triglyceride level is the apoE/apoC ratio. In fact, as seen in Fig. 4D, there is a strong correlation between the apoE/apoC-III + C-II ratios and plasma triglyceride levels among all of the treatment groups. Also supporting the role of apoC-III reduction in the hypolipidemic effects of fibrates are the results demonstrating that the observed reductions in plasma triglyceride levels could not be explained by changes in triglyceride production rate (Fig. 5).

It is notable that gemfibrozil appears to have some distinct additional effects as compared to the other fibrates. In our studies, we also observed that gemfibrozil caused significant elevation of both HDL/(VLDL + LDL) and maintenance (1-week studies) or elevation (2-week studies) of apoE in the chow-fed rat. This property was unique to gemfibrozil when compared to bezafibrate in 1- (Table 1 and Fig. 3) and 2-week studies (B. R. Krause and C. L. Bisgaier, unpublished observations), despite triglyceride-lowering effects observed with both compounds. Compared to vehicle-treated rats, 2-week gemfibrozil treatment did not induce a difference in hepatic apoA-I, apoE or HL mRNA levels. The elevation of the HDL/(VLDL + LDL) may be related to the mechanism by which triglyceride-rich lipoproteins are metabolized in gemfibrozil-treated rats. Perhaps, with gemfibrozil, sufficient redundant surface derived from triglyceride-rich lipoproteins is generated prior to removal of the remnant, thereby allowing greater transfer to the HDL pool. Possibly an enhanced margination of the remnants in the extrahepatic pool would allow this,

especially if the hepatic uptake of the remnant was partially impeded.

The results presented in this study, together with previously reported studies by Staels et al. (42), suggest that the hypolipidemic action of fibrates is due to a direct effect on apoC-III gene expression. A molecular mechanism by which fibrates could affect gene expression was suggested by the identification of a new member of the nuclear receptor family of transcription factors that is activated by fibrates (43). This receptor, known as the peroxisome proliferator activated receptor (PPARα) regulates transcription of target genes in response to fibrates. One of the best characterized PPAR responsive promoters is in the gene encoding the peroxisomal enzyme acyl CoA oxidase. When a cell is exposed to fibrates (or other molecules that cause peroxisome proliferation) the compound interacts with and activates the PPARα protein which then binds to a PPAR response element (PPRE) in acyl-CoA oxidase gene promoter and activates transcription. In some rodent species fibrates act as peroxisome proliferators (42, 44), probably activating transcription of peroxisomal genes by a PPARα-mediated mechanism similar to that described above for acyl-CoA oxidase. It is significant that the apoC-III promoter contains a regulatory element that is closely related to the acyl-CoA oxidase PPRE (11 out of 13 match). The presence of this element, identified as C3P in previous studies (45), presents the strong possibility that the transcriptional activity of the apoC-III gene is regulated by PPARα or a related protein. This possibility is supported by a recent report demonstrating that the C3P element can bind to PPARα in vitro (46). These authors also reported that the hypolipidemic drug Medica 16, which appears to have similar effects as fibrates, represses apoC-III transcription in HepG2 cells by a PPAR-dependent mechanism. Experiments are currently underway to determine the molecular mechanism by which fibrates modulate apoC-III expression.

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