

Fibrates Downregulate Apolipoprotein C-III Expression Independent of Induction of Peroxisomal Acyl Coenzyme A Oxidase

A Potential Mechanism for the Hypolipidemic Action of Fibrates

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Abstract

Epidemiological and transgenic animal studies have implicated apo C-III as a major determinant of plasma triglyceride metabolism. Since fibrates are very efficient in lowering triglycerides, it was investigated whether fibrates regulate apo C-III gene expression. Different fibrates lowered rat liver apo C-III mRNA levels up to 90% in a dose- and time-dependent manner, whereas intestinal apo C-III mRNA remained constant. This decrease in liver apo C-III mRNA was rapid (1 d) and reversible, since it was restored to control levels within 1 wk after cessation of treatment. In addition, fenofibrate treatment abolished the developmental rise of hepatic apo C-III mRNA observed during the suckling-weaning period. Administration of fibrates to rats induced liver and intestinal expression of the acyl CoA oxidase gene, the rate-limiting enzyme for peroxisomal β -oxidation of fatty acids. In primary cultures of rat and human hepatocytes, fenofibric acid lowered apo C-III mRNA in a time- and dose-dependent manner. This reduction in apo C-III mRNA levels was accompanied by a decreased secretion of apo C-III in the culture medium of human hepatocytes. In rat hepatocytes fenofibric acid induced acyl CoA oxidase gene expression, whereas acyl CoA oxidase mRNA remained unchanged in human hepatocytes. Nuclear run-on and transient transfection experiments of a reporter construct driven by the human apo C-III gene promoter indicated that fibrates downregulate apo C-III gene expression at the transcriptional level. In conclusion, these studies demonstrate that fibrates decrease rat and human liver apo C-III gene expression. In humans the mechanism appears to be independent of the induction of peroxisomal enzymes. This downregulation of liver apo C-III gene expression by fibrates may contribute to the hypotriglyceridemic action of these drugs. (*J. Clin. Invest.* 1995. 95:705–712.) **Key words:** gene regulation • hypolipidemic drugs • hyperlipidemia • peroxisome proliferation • nuclear hormone receptors

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Introduction

Fibrates are efficient drugs to treat diet-resistant hypertriglyceridemia, a relatively common disorder (1). Classically, the decrease in plasma triglyceride concentrations upon fibrate treatment are thought to be the result of a decreased hepatic secretion of VLDL accompanied by an enhanced plasma triglyceride clearance, possibly due to the induction of lipoprotein lipase (LPL)¹ activity in peripheral tissues (2–5). Although several studies have demonstrated increases in postheparin plasma LPL activity after fibrate treatment, no clear effects on adipose tissue or muscle LPL activity and mRNA could be demonstrated either in humans or laboratory animals, such as the rat (6, 7). Conversely, the triglyceride lowering action of fibrates might be mediated either by a decrease in hepatic triglyceride secretion or by changes in plasma concentrations of (co-)factors interfering with hydrolysis and/or clearance of triglyceride-rich particles in plasma, such as the different C apolipoproteins.

Several lines of evidence have implicated apo C-III in plasma triglyceride metabolism. Apo C-III, a 79-amino acid glycoprotein, synthesized in liver and small intestine, is found predominantly in chylomicrons, VLDL, and HDL. Apo C-III levels have been correlated with triglyceride concentrations in plasma of hypertriglyceridemic patients, as well as in the normal population (8–11). Subjects deficient in apo C-III exhibit an increased catabolism of VLDL, whereas elevated apo C-III synthetic rates have been observed in hypertriglyceridemic patients (12, 13). Furthermore, genetic studies have identified several apo C-III gene polymorphisms which may be associated with increased plasma apo C-III levels and hypertriglyceridemia (14, 15). Previous reports have indicated that apo C-III inhibits triglyceride hydrolysis by LPL and hepatic lipase in vitro and impairs the uptake of triglyceride-rich lipoproteins by the liver (13, 16–18). Transgenic animal studies, showing that plasma triglyceride levels are proportional to plasma apo C-III concentrations and liver apo C-III gene expression, provided more direct evidence for the causal involvement of apo C-III in hypertriglyceridemia (19). Metabolic studies in these animals indicated the primary abnormality to be an impaired clearance of triglyceride-rich lipoproteins due to interference with apo E-mediated uptake of these particles possibly by cellular receptors (20–22).

Recent studies in rodents have shown that fibrates modulate lipid metabolism by regulating the expression of several genes coding for enzymes involved in the peroxisomal β -oxidation of

1. *Abbreviations used in this paper:* ACO, acyl CoA oxidase; CAT, chloramphenicol acetyl transferase; LPL, lipoprotein lipase; PPAR, peroxisome proliferator-activated receptor; RSV, Rous sarcoma virus.

fatty acids, as well as proteins involved in plasma lipid and lipoprotein metabolism (23, 24). Therefore, we hypothesized that fibrates might act on plasma triglyceride metabolism by changing the expression of the apo C-III gene. Consequently the effects of different fibrates on apo C-III gene regulation were studied *in vivo* in the rat and *in vitro* using primary cultures of human and rat hepatocytes. The results from these studies demonstrate that fibrates downregulate human and rat apo C-III gene expression, which may contribute to the lipid-lowering action of these drugs. Furthermore, these compounds abolish the developmental rise in liver apo C-III gene expression, which suggests that their natural analogues may play an important role in the developmental regulation of lipid-related genes.

Methods

Animals and treatments. Adult male Wistar rats were treated for different periods of time with fenofibrate (Laboratories Fournier, Daix, France), clofibrate (Sigma Chemical Co., St. Louis, MO), or gemfibrozil (Lopid®; Warner-Lambert Pharmaceutical Co., Ann Arbor, MI) mixed at the indicated concentrations (wt/wt) in standard rat chow. None of the treatments caused major changes in the amount of food consumed by the animals. At the end of the experiments animals were fasted overnight and killed by exsanguination under ether anesthesia. Liver and intestinal epithelium were removed immediately and frozen in liquid nitrogen.

Male Wistar rats of different ages (at least four per age group) were killed and livers were pooled to determine the developmental expression of the rat apo C-III and acyl CoA oxidase (ACO) genes. Since fibric acid derivatives are active in fetal tissues after maternal administration (25), treatment of timed pregnant rats with fenofibrate (0.5% wt/wt, mixed in rat chow) was started on day 15 after conception. Control mothers received normal rat chow. Pups born between the morning of one day and the morning of the next were considered 0 d old. On day 5 postnatally, each litter was reduced to nine pups per mother. One-third ($n = 3$) of control and fenofibrate-treated pups were killed on days 13, 20, and 30 after birth, respectively.

Isolation and culture of rat and human hepatocytes. Rat hepatocytes were isolated by collagenase perfusion (26) of livers from male rats weighing between 150 and 250 g (cell viability higher than 85% by the Trypan blue exclusion test). The hepatocytes were cultured in monolayer (1.5×10^5 cells/cm²) in Leibovitz-15 medium (GIBCO BRL, Paisley, United Kingdom) supplemented with FCS (10% vol/vol), fatty acid-free bovine serum albumin (0.2% wt/vol), NaHCO₃ (26 mM), L-glutamine (2 mM), glucose (3 g/l), dexamethasone (10^{-7} M) and antibiotics at 37°C in a humidified atmosphere of 5% CO₂/95% air.

Treatments with fenofibric acid (in DMSO, 0.1% vol/vol final concentration) at the indicated doses and periods of time were started immediately after seeding.

Human liver specimens were collected from physically healthy multiorgan donors for transplantation at the Moscow Medical Center, who died after severe traumatic brain injury. Permission to use the remaining, nontransplanted part of donor liver for scientific research was obtained from the Ministry of Health of the Russian Federation. Hepatocytes were obtained by a two-step collagenase perfusion as previously described (27). Cells were resuspended in minimal essential medium with Earl's salts (GIBCO BRL) supplemented with 10% FCS, 2 mM L-glutamine, 50 mg/ml gentamycin, seeded at a density of 1.5×10^5 cells/cm² in 60 mm plastic culture dishes, coated with 20 mg rat tail collagen type I (Sigma Chemical Co.), and incubated in a humidified atmosphere of 5% CO₂/95% air at 37°C. Medium was renewed after a 4-h adhesion period. After 20 h the medium was discarded and fenofibric acid (in DMSO, final concentration 0.5% vol/vol) was added at the indicated concentrations in serum-free medium.

No morphological differences in cell adhesion or cell toxicity (determined by the MTT [tetrazolium] colorimetric test [28]) were observed between control and fenofibric acid-treated human and rat hepatocytes. At the end of the experiments, medium was removed and frozen, cells were washed three times with ice-cold PBS, and solubilized by addition of 1 ml of 4 M guanidinium isothiocyanate (29).

RNA analysis. Total cellular RNA was prepared by the guanidinium isothiocyanate/cesium chloride procedure (liver and intestine) or by the acid guanidinium thiocyanate/phenol-chloroform method (primary hepatocyte cultures) (29, 30). Northern and dot blot hybridizations of total cellular RNA were performed as described previously (6). A rat apo C-III cDNA fragment spanning nucleotides +54 to +356 (31) was cloned from rat liver by reverse transcription and PCR-amplification (sense primer: ATG CAG CCC CGA ATG CTC CTC ATC GTG GCC; antisense primer: TCA CGG CTC AAG AGT TGG TGT TGT TAG TTG GTC CTC AGG). The resulting PCR fragment was cloned into pUC18 and sequence analysis revealed complete identity to the previously reported rat apo C-III cDNA sequence (31). A rat ACO cDNA probe was used as a positive control for fibrate action in all rat experiments (32). A 440-bp human apo C-III cDNA fragment was obtained by PstI digestion of the pCIII-606 plasmid (33). A GAPDH probe was used as a control probe (34). All cDNA probes were labeled by random primed labeling (Boehringer Mannheim Biochemicals, Indianapolis, IN). Filters were hybridized to 1.5×10^6 cpm/ml of each probe as described (35). They were washed once in $0.5 \times$ SSC and 0.1% SDS for 10 min at room temperature and twice for 30 min at 65°C and subsequently exposed to x-ray film (X-OMAT-AR; Eastman Kodak Co., Rochester, NY). Autoradiograms were analyzed by quantitative scanning densitometry (GS670 Densitometer; Bio-Rad Laboratories, Richmond, CA) as described (35).

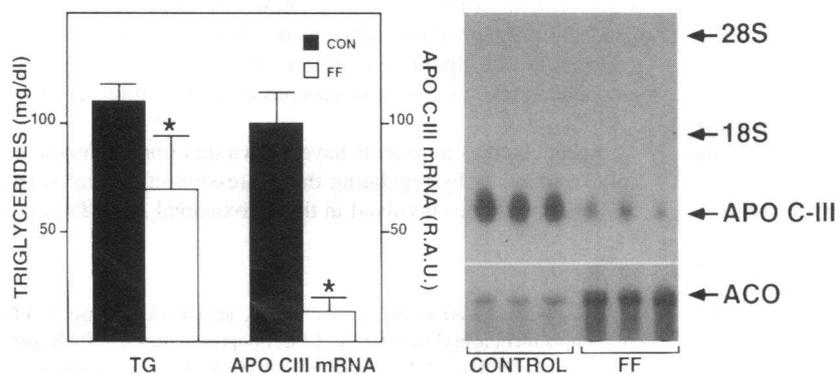


Figure 1. Fenofibrate decreases plasma triglycerides and liver apo C-III mRNA levels in rats. Adult male rats were treated for 14 d with fenofibrate (FF, 0.5%, wt/wt, mixed in rat chow). Plasma triglyceride concentrations were measured as described (35). Total RNA was extracted and apo C-III and ACO mRNA levels were measured as described in Methods. (A) Plasma triglycerides and hepatic apo C-III mRNA levels in control and fenofibrate-treated rats. Values represent the mean \pm SD of three animals. Statistically (ANOVA, $P < 0.05$) significant differences are indicated by an asterisk. (B) Northern blot analysis. 10 μ g of total RNA was subjected to electrophoresis, transferred to a nylon membrane, and hybridized to rat apo C-III (top) or ACO (bottom) cDNA as described in Methods. The position of the 18S and 28S rRNA bands are indicated on the right of the top panel.

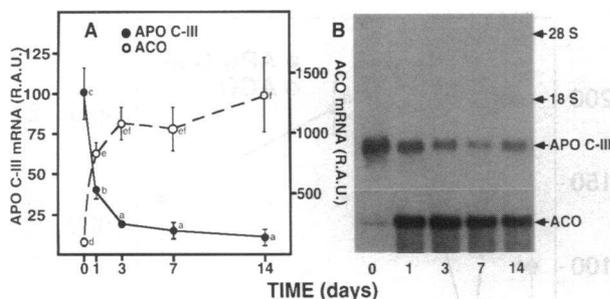


Figure 2. Kinetics of rat liver apo C-III and ACO gene regulation by fenofibrate. Adult male rats were treated during the indicated number of days with fenofibrate (0.5%, wt/wt, mixed in rat chow). Total RNA was extracted and apo C-III and ACO mRNA levels were measured as described in Methods. (A) Quantitative dot blot analysis. Values represent the mean \pm SD of three animals. Statistically (ANOVA, $P < 0.001$) significant differences are observed between values followed by different letters. (B) Northern blot analysis. 10 μ g of total RNA was subjected to electrophoresis, transferred to a nylon membrane, and hybridized to rat apo C-III (top) or ACO (bottom) cDNA as described in Methods. The position of the 18S and 28S rRNA bands is indicated on the right of the top panel.

Isolation of nuclei and transcriptional rate assay. Nuclei were prepared from primary rat hepatocytes treated with fenofibric acid (500 μ M) or vehicle and transcription run-on assays were performed as described by Nevins (36). Equivalent counts of nuclear RNA labeled with α - 32 P UTP (3,000 Ci/mmol) were hybridized for 36 h at 42°C to 5 μ g of apo C-III cDNA, ACO cDNA (32), and vector DNA immobilized on Hybond-C Extra filters (Amersham Corp., Arlington Heights, IL). After hybridization, filters were washed at room temperature for 10 min in 0.5 \times SSC and 0.1% SDS and twice for 30 min at 65°C and subsequently exposed to x-ray film (Eastman Kodak Co.). Quantitative analysis was performed by scanning densitometry (Bio-Rad Laboratories).

Transient transfection experiments. A genomic fragment spanning nucleotides -1415 to +24 and containing the human apo C-III gene promoter was subcloned in the polylinker of pBLCAT5 (37). Human HepG2 cells (passage number < 15) were maintained in DME (GIBCO BRL) supplemented with 10% (vol/vol) heat-inactivated dextran charcoal-stripped FCS, 2-mM glutamine, 10⁻⁶ M dexamethasone, and antibiotics. Cells were transfected at 60% confluence by the calcium phosphate coprecipitation procedure with a mixture containing either pCIII-CAT or Rous Sarcoma virus (RSV)-CAT and a cytomegalovirus-driven β -galactosidase vector used as an internal control for transfection efficiency. After 10 h of incubation, cells were changed to fresh medium

containing fenofibric acid or solvent and incubated for a further 38 h. CAT activity was determined on cell extracts as described by Gorman et al. (38). Transfection efficiency was normalized for β -galactosidase activity and cellular protein concentration (measured using the Coomassie protein assay reagent; Pierce Chemical Co., Rockford, IL). Transfection experiments were performed at least three times.

Triglyceride and apo C-III ELISA. Plasma triglyceride concentrations were measured as described previously (35). Human apo C-III, in culture medium of primary human hepatocytes, was measured by a noncompetitive ELISA as described previously (39). Briefly, polystyrene microtiter plates were coated with affinity-purified polyclonal antibodies to human apo C-III or human apo E. Triplicate medium culture samples were diluted 1:5 and 1:10 with PBS, and were added to the wells along with the standards and controls. After incubation, apo C-III or apo E antibodies, conjugated to peroxidase, were added. Color development was performed with the addition of substrate (*o*-phenylenediamine dichloride). The plates were read at 492 nm on a microtiter plate reader.

Results

Fibrates lower rat apo C-III gene expression in a reversible manner. Since apo C-III has been implicated in plasma triglyceride metabolism, it might be a potential target for the lipid-lowering action of fibrates. Therefore, the influence of fenofibrate was studied on apo C-III gene expression in the major apo C-III-producing tissues in rats, and its effects were correlated to the changes observed in plasma triglyceride concentrations. Treatment of adult male rats for 14 d with 0.5% fenofibrate mixed in standard rat chow resulted in a significant decrease in plasma triglyceride concentrations, which was accompanied by a more than sixfold decrease in liver apo C-III mRNA levels (Fig. 1). The decrease in liver apo C-III mRNA levels was already evident within 1 d after fenofibrate treatment and a further decrease was observed within 14 d (Fig. 2). Since fibrates are potent peroxisomal proliferators in rodents due to the induction of enzymes involved in the β -oxidation of fatty acids (24, 32, 40), liver ACO gene expression was measured as a positive control for fibrate action and compared to apo C-III gene regulation by fibrates in all rat experiments. Liver ACO gene expression exhibited similar kinetics of regulation as apo C-III: a drastic increase was already observed within one day of fenofibrate and a maximal effect was attained between 3 and 14 d after treatment (Figs. 1 and 2). When rats were treated with different doses of fenofibrate for 14 d hepatic apo C-III

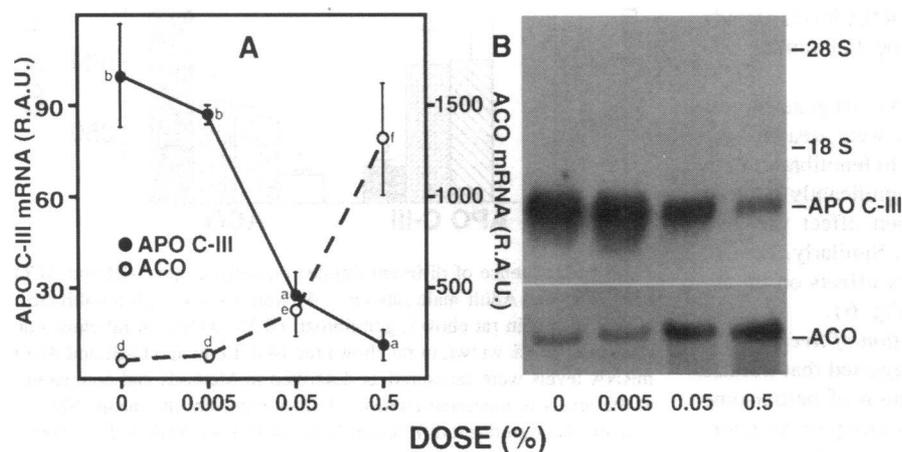


Figure 3. Dose-dependent regulation of rat liver apo C-III and ACO mRNA by fenofibrate. Adult male rats ($n = 3$) were treated for 14 d with the indicated doses of fenofibrate (wt/wt, mixed in rat chow). Total RNA was extracted and apo C-III and ACO mRNA levels were quantified by dot (A) and Northern (B) blot analysis as described under Fig. 2. Statistically (ANOVA, $P < 0.001$) significant differences are observed between values followed by different letters.

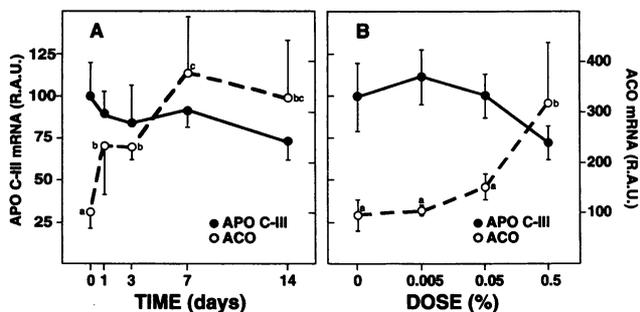


Figure 4. Time- (A) and dose-dependent (B) effects of fenofibrate on intestinal apo C-III and ACO mRNA levels. Animals, treatments, and RNA analysis were exactly as described under Figs. 1 and 2. Statistically significant differences (ANOVA, ACO: $P < 0.005$, apo C-III: NS) are observed between values followed by different letters.

mRNA levels already decreased near-maximally at an intermediary dose of 0.05% (Fig. 3). ACO mRNA induction showed a similar dose-response compared to apo C-III, although the effects were less pronounced at the intermediary dose (Fig. 3). In contrast, intestinal apo C-III mRNA levels did not change significantly after treatment with fenofibrate (Fig. 4, A and B), thereby indicating that apo C-III gene regulation is regulated in a tissue-specific manner by fibrates. Intestinal ACO mRNA levels displayed similar kinetics and dose-dependency of induction after fenofibrate when compared to liver (Fig. 4). Although the overall induction was less pronounced in the intestine, these results indicate that rodent intestine is responsive to peroxisomal proliferation.

Next it was investigated whether the decreased liver apo C-III gene expression is associated to general, irreversible changes in liver structure and function or whether the downregulation of apo C-III expression is reversible upon cessation of therapy. Therefore, rats were treated for 14 d after which fenofibrate was withdrawn. Liver apo C-III mRNA levels were measured at different time-points after withdrawal and compared to the levels in untreated, as well as in fenofibrate-treated, rats. As expected, apo C-III mRNA levels dropped to only 10% of the untreated controls after 14 d of fenofibrate (Fig. 5, compare C with day 0). Apo C-III mRNA levels already started to increase within 3 d after interruption of fenofibrate treatment. Interestingly, after 7–14 d, apo C-III mRNA levels were ~ twofold higher than in untreated control liver, returning slowly to control levels after 28 d (Fig. 5). Similarly, ACO mRNA levels already started to decrease within 3 d after stopping fenofibrate, and reached control levels after 7 d (Fig. 5).

To investigate whether the decreased apo C-III gene expression is a general property of fibrates, rats were treated with different fibrates and their effects compared to fenofibrate. Both treatment with clofibrate and gemfibrozil significantly reduced liver apo C-III mRNA levels, although their effect was less pronounced relative to fenofibrate (Fig. 6). Similarly, both fibrates exhibited significant, but intermediary effects on the induction of hepatic ACO gene expression (Fig. 6).

Fibrates abolish the development induction of liver apo C-III gene expression. Recent studies have suggested that fibrates modulate gene transcription through activation of peroxisome proliferator-activated receptors or PPARs, a group of receptors belonging to the nuclear hormone receptor superfamily (41–

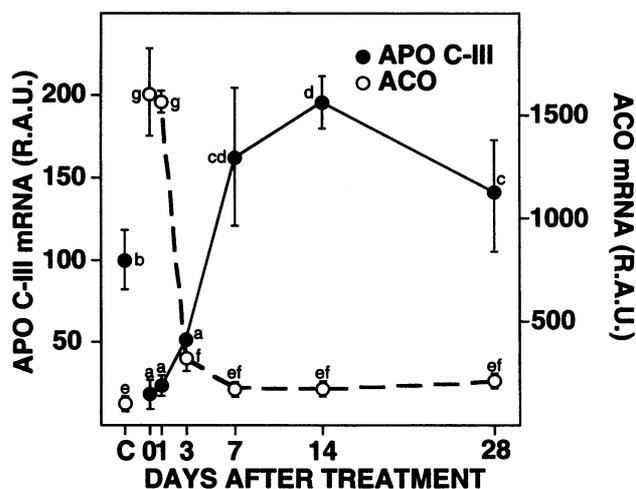


Figure 5. Influence of cessation of treatment with fenofibrate on liver apo C-III and ACO mRNA levels. Adult male rats ($n = 3$) were given fenofibrate (0.5% wt/wt, mixed in rat chow) for 14 d. Treatment with fenofibrate was stopped on day 0. Apo C-III and ACO mRNA levels were measured in livers of rats 0, 1, 3, 7, 14, and 28 d after cessation of fenofibrate treatment and compared to the levels in untreated controls (C). Statistically (ANOVA, $P < 0.001$) significant differences are observed between values followed by different letters.

44). After binding to specific ligands (such as corticosteroids, thyroid hormone, and retinoic acid) these receptors are activated and play important roles not only in physiological, but also in developmental processes. Therefore, it was hypothesized that fibrates may also modulate the developmental regulation of apo C-III gene expression. Apo C-III mRNA levels are very low in fetal rat liver, start to rise after birth, and reach a first peak between day 10 and 15 after birth (Fig. 7 A). They subsequently

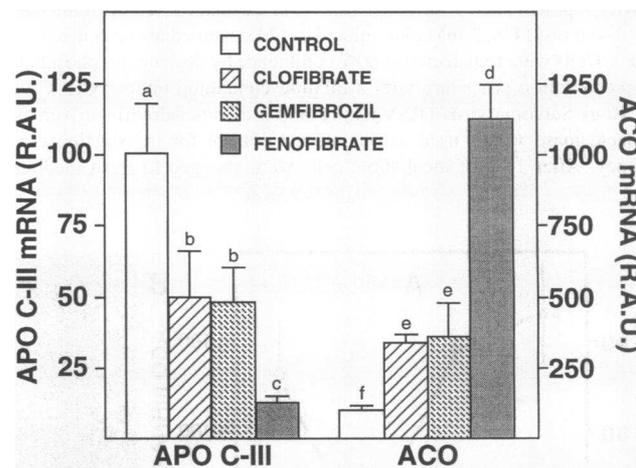


Figure 6. Influence of different fibrates on hepatic apo C-III and ACO mRNA levels. Adult male rats ($n = 4$) were treated with fenofibrate (0.5% wt/wt, in rat chow), gemfibrozil (0.3% wt/wt, in rat chow) or clofibrate (0.3% wt/wt, in rat chow) for 14 d. Liver apo C-III and ACO mRNA levels were measured as described in Methods and compared to the levels in untreated controls. Values represent the mean \pm SD. Statistically significant differences from controls (ANOVA, $P < 0.001$) are observed between values followed by different letters.

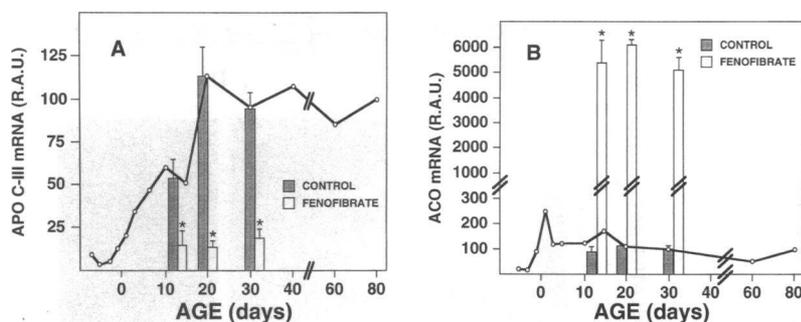


Figure 7. Influence of maternal fenofibrate administration on the developmental regulation of hepatic apo C-III- and ACO-gene expression. RNA was prepared from pooled livers ($n > 4$) of male rats of different ages or from individual livers of pups ($n = 3$) from control or fenofibrate-treated (0.5% wt/wt, in rat chow) timed-pregnant female rats and killed 13, 20, or 30 d after birth. Apo C-III (A) and ACO (B) mRNA levels were determined as described in Methods. Values are expressed relative to the levels in 80-d-old adult rats. Statistically (t test; $P < 0.05$) significant differences from controls are indicated by an asterisk.

attain adult levels around the suckling/weaning transition period, results which are in line with previous observations (31). To investigate the effects of fibrates on the developmental expression of the apo C-III gene, treatment with fenofibrate was started in utero and continued until killing at days 13, 20, and 30 of life. At all time-points fenofibrate treatment resulted in a dramatic reduction of liver apo C-III mRNA levels compared with untreated controls, thereby abolishing completely the developmental rise in hepatic apo C-III gene expression (Fig. 7 A). Hepatic ACO mRNA levels are low in fetal liver, peak around birth, and then decrease to reach adult levels 3 d after birth (Fig. 7 B). At 13, 20, and 30 d after birth, treatment with fenofibrate resulted in a dramatic increase in liver ACO gene expression, which was even more pronounced than in adult animals (Fig. 7 B).

Fenofibrates decrease apo C-III gene expression in primary rat hepatocyte cultures. To study whether the regulation of apo C-III gene expression observed in vivo could also be seen in vitro, primary cultures of adult rat hepatocytes were treated with fenofibrates. Addition of different concentrations of fenofibrates for 24 h to the culture medium resulted in a dose-dependent reduction and induction of apo C-III and ACO mRNA levels, respectively (Fig. 8 A). The decrease in apo C-III mRNA was of relatively slow onset: apo C-III mRNA levels remained fairly constant after 6 h of fenofibrates at 500 μ M, started decreasing after 12 h and reached minimal levels after 24 h (Fig. 8 B). By contrast, ACO mRNA is already maximally induced after 6 h and remain elevated thereafter, results which confirm previous reports studying the effects of different peroxi-

some proliferators, such as clofibrate, on ACO gene expression (45).

To analyze whether apo C-III gene regulation by fenofibrates occurred at the transcriptional level, RNA polymerase nuclear run-on assays were performed next on primary cultures of adult rat hepatocytes treated with 500 μ M fenofibrates for 9 h, a time when apo C-III mRNA steady-state levels started to decrease (Fig. 8 B). The rate of apo C-III gene transcription was \sim twofold lower in nuclei isolated from fenofibrates treated cells compared with control cells, whereas ACO gene transcription increased more than fivefold (Fig. 9). This decrease in apo C-III gene transcription was comparable to the decrease in apo C-III mRNA levels obtained after 24 h of fenofibrates (Fig. 8 B). These results suggest, therefore, that fibrates affect apo C-III expression mainly at the transcriptional level.

Human apo C-III gene expression is transcriptionally down-regulated by fenofibrates. Addition of fenofibrates to culture medium of primary hepatocytes isolated from human liver resulted after 24 h in a decrease of human apo C-III gene expression (Fig. 10 A). This reduction, which was already maximal at a dose of 500 μ M (Fig. 10 A), was accompanied within 24 h by a dose-dependent decrease in apo C-III secretion in the culture medium (Fig. 10 B). In contrast, apo E secretion in the culture medium remained constant under these conditions (DMSO: 457 ± 41 ng/ml; fenofibrates, 500 μ M: 495 ± 49 ng/ml).

These results in human hepatocytes prompted us to clone the human apo C-III gene promoter in front of the CAT reporter gene (Fig. 11 A). This construction was transfected in the human hepatoma cell line Hep-G2 and cells were treated with different doses of fenofibrates. A viral promoter (RSV)-

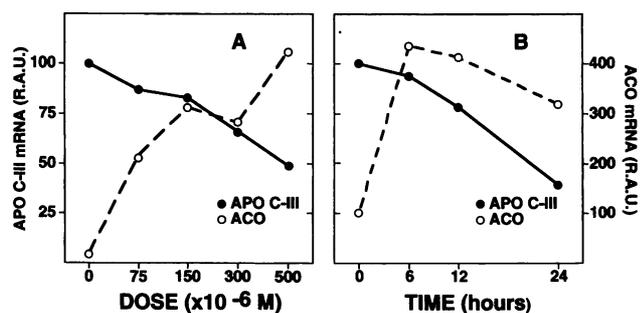


Figure 8. Dose- (A) and time- (B) dependent effects of fenofibrates on apo C-III and ACO mRNA levels in primary cultures of adult rat hepatocytes. Rat hepatocytes were isolated, treated either with the indicated doses for 24 h (A) or with 500 μ M of fenofibrates for the indicated periods of time (B). Apo C-III and ACO mRNA levels were quantified as described in Methods.

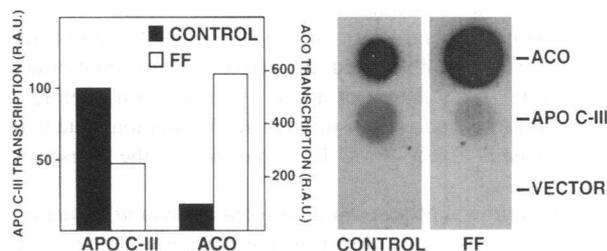


Figure 9. Fenofibrates regulate apo C-III and ACO gene expression at the transcriptional level. Nuclear run-on assays were performed on adult rat hepatocytes treated for 9 h with 500 μ M of fenofibrates (FF) or vehicle (CONTROL) as described under Methods and analyzed by quantitative scanning densitometry.

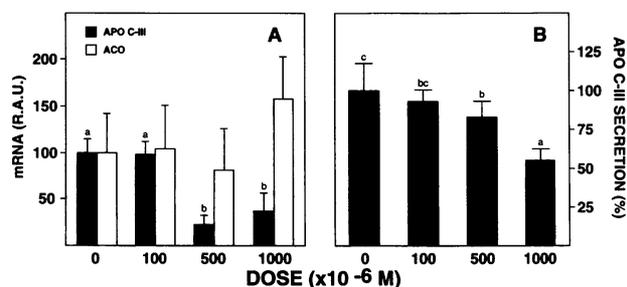


Figure 10. Dose-dependent effects of fenofibrate on apo C-III and ACO mRNA (A) and apo C-III secretion (B) in primary cultures of adult human hepatocytes. Human hepatocytes were isolated, treated for 24 h with the indicated doses of fenofibric acid. Apo C-III and ACO mRNA levels were measured and normalized to GAPDH mRNA, and apo C-III protein concentrations were quantified as described in Methods. Values are expressed relative to controls and represent the mean \pm SD of three (A) and six (B) points. Statistically significant differences (ANOVA, Apo C-III: $P < 0.001$; ACO: NS) are observed between values followed by different letters.

driven CAT plasmid was transfected as a control. The apo C-III promoter-driven CAT activity decreased \sim fourfold compared to solvent, both at 250 and 500 μ M of fenofibric acid (Fig. 11 B and C). By contrast, the control RSV-driven CAT activity remained unchanged (Fig. 11 D). These results, therefore, indicate that fibrates decrease apo C-III gene expression in human liver at the transcriptional level.

Discussion

Fibrates are potent hypolipidemic drugs both in humans, as well as in laboratory animals, such as the rat. One mechanism through which these drugs may exert their effects is by modulating the expression of specific proteins involved in lipoprotein metabolism. Since apo C-III is implicated in the metabolism of triglycerides, the effect of fibrates on apo C-III expression was studied. Our results show that treatment of adult rats in vivo with fibrates results in lowered plasma triglyceride concentrations, which is accompanied by a tissue-specific decrease of liver apo C-III gene expression. Since all three fibrates (fenofibrate, clofibrate, and gemfibrozil) tested lower hepatic apo C-III mRNA levels, it appears to be a general effect of fibrates. The decrease in hepatic apo C-III gene expression is dose dependent, already evident after 1 d and maximal within 3 d of in vivo administration of fenofibrate. These changes in apo C-III expression after fenofibrate are a result of fibrate action per se, and not merely a consequence of alterations in plasma lipid and lipoprotein concentrations, since treatment of isolated rat hepatocytes with fenofibric acid, the active form of fenofibrate, results in a down-regulation of apo C-III expression after 12–24 h. Furthermore, nuclear run-on experiments demonstrate that these compounds affect apo C-III expression at the transcriptional level.

After withdrawal of fenofibrate in vivo a rebound induction of apo C-III expression is observed. Furthermore, the decrease in apo C-III mRNA is relatively slow, compared to the induction of the ACO gene, a peroxisomal marker enzyme. This suggests that, at the cellular level, fibrates act indirectly, probably by affecting intermediary steps of intracellular lipid metabolism, as has been suggested previously (24). Fibrates are potent in-

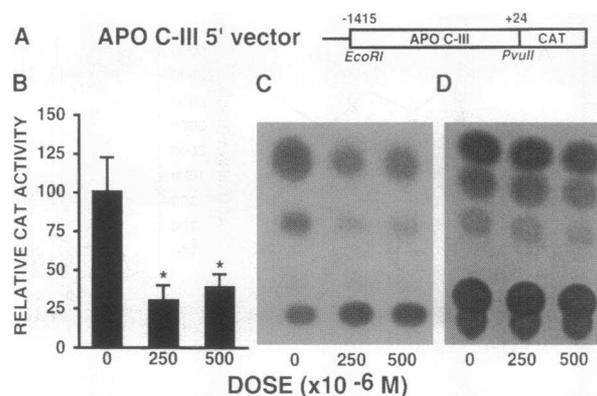


Figure 11. Downregulation of the human apo C-III promoter by fenofibrate. Hep-G2 cells were transiently transfected with plasmids containing a human apo C-III promoter fragment (–1415 to +24) fused to the CAT gene (A) or a control RSV-driven CAT vector and treated with the indicated doses of fenofibric acid in DMSO. Apo C-III (B, C) and RSV-(D) driven CAT activity was measured and expressed as described under Methods. Statistically (ANOVA, $P < 0.001$) significant differences from controls are indicated by an asterisk (B).

ducers of the β -oxidation system of fatty acids in liver and, to a lesser extent, in intestine and kidney in rodents (24, 46, 47). In rodents these compounds act by inducing the expression of several genes coding for peroxisomal enzymes, which results in a strong proliferation of peroxisomes and an extreme hepatomegaly (23, 32, 40, 48). However, since the effects of fibrates are reversible within 7 d after withdrawal, it seems unlikely that their effects on apo C-III gene expression are linked to irreversible changes in liver structure, morphology, or function. Moreover, in contrast to rodents, the expression of the ACO gene remains constant in human hepatocytes treated with fenofibrate, thereby indicating that fibrates have no or only a very limited capacity to induce peroxisomal enzymes in humans, as has been suggested previously by several authors (24, 46, 47, 49). Thus, our results suggest that the downregulation of human apo C-III gene expression occurs by a distinct mechanism and is independent of the induction of peroxisomal enzymes.

One potential mechanism by which fibric acid derivatives may exert their effects on apo C-III expression is through activation of specific receptors, called PPARs, which belong to the nuclear hormone receptor gene superfamily (41, 42). Upon activation, these receptors bind to response elements consisting of a direct repeat spaced by one nucleotide in the promoter regions of target genes (50). Although their natural ligands are presently unknown, PPARs have been shown to be activated by peroxisome proliferators, such as fibrates, as well as by various fatty acids (42, 44). These nuclear receptors are suggested to be important in mediating nutritional control of gene expression. Our results indicate that fibrates downregulate apo C-III gene expression at the transcriptional level and that this effect is mediated by sequences located within 1 kb upstream of the transcription initiation site of the apo C-III gene. Promoter elements implicated in the basal and tissue-specific expression of the apo C-III gene have been extensively characterized both by transient transfection, as well as by transgenic animal experiments (19, 51–53). Interestingly, one of these *cis*-elements contains a direct repeat spaced by one nucleotide sequence, which mediates transregulation by other members of the nuclear

receptor superfamily, such as HNF-4, Arp1, EAR-2, and EAR-3 (54, 55) and therefore may be a potential target site for PPARs. If PPARs prove to mediate the effects of fibrates on apo C-III expression, then one would expect that apo C-III expression would be regulated both in vivo and in vitro by dietary factors, such as changes in fat content and/or composition. Studies to identify the promoter region implicated in apo C-III gene regulation by fibrates, as well as studies relating to nutritional control of apo C-III gene expression are currently under way in our laboratory.

Liver apo C-III expression is heavily regulated during development in rats: being extremely low in fetal liver, it rises rapidly after birth during the neonatal period and attains maximal, adult levels during the suckling/weaning transition period. During this period major developmental changes occur in the expression of several genes playing a role in lipoprotein metabolism. Compared to apo C-III, liver LPL expression shows an opposite developmental profile: being high in fetal and neonatal liver, it decreases rapidly during the suckling/weaning transition period and becomes undetectable in adult liver, a pattern which closely resembles liver α -fetoprotein expression (6). During fetal and early neonatal development, several physiological changes occur, such as alterations in dietary composition (fat:carbohydrate ratio) and in circulating plasma concentrations of several hormones (e.g., insulin, glucagon, glucocorticoids, and thyroid hormone), all of which may modulate developmental gene expression. Similarly, fibrates appear to be potent regulators during development not only of lipid-related genes, such as apo C-III and LPL, but also of hepato-specific genes, such as α -fetoprotein (6). Indeed, treatment with fibric acid derivatives perturbs the developmental regulation of the LPL, α -fetoprotein, and apo C-III genes and selectively reinduces the expression of extinguished genes, such as LPL, in adult rat liver (6). Therefore, it appears that fibric acid derivatives not only regulate physiological processes, such as lipid and energy metabolism, but also mediate alterations in developmental patterns of gene expression. If these effects are mediated through activation of PPAR, the natural analogues of fibrates and ligands or activators of PPARs should be important mediators of both physiological as well as developmental processes, as has been demonstrated for other activators of receptors belonging to this superfamily (e.g., retinoic acid, thyroid hormone, and glucocorticoids).

Treatment of hypertriglyceridemic patients with fibrates results in the reduction of plasma triglyceride concentrations. Previous clinical reports have indicated that the hypertriglyceridemia in these patients is accompanied by increases in plasma concentration and synthesis rate of apo C-III (12). These abnormalities can be normalized after treatment with fenofibrate (12). Our results on primary cultures of human hepatocytes demonstrate that fibrates decrease apo C-III synthesis and secretion by the liver, through a direct action on apo C-III gene expression. This lowered secretion of apo C-III, along with unchanged apo E secretion, by the liver may lead to a decreased apo C-III/apo E ratio of triglyceride-rich particles, which then could be more efficiently cleared from plasma. Indeed, studies on transgenic mice have shown that overexpression of apo C-III leads to a hypertriglyceridemia due to a diminished clearance rate of triglyceride-rich lipoproteins, containing increased amounts of apo C-III and reduced apo E on the particles (20, 22). This apo C-III-induced hypertriglyceridemia could be corrected by simultaneous overexpression of apo E, indicating that apo C-III may be implicated in modulating the apo E-mediated

lipoprotein clearance (20). In addition, apo C-III has been suggested to be an inhibitor of LPL, and decreases in plasma apo C-III concentrations may therefore contribute to an improved lipolysis of triglyceride-rich lipoproteins in plasma (17). Consequently, treatment with fibrates will lead to alterations in plasma apolipoprotein composition, favoring the clearance of triglyceride-rich lipoproteins. Therefore, the decrease in apo C-III gene expression by fibrates provides a potential mechanism by which these drugs induce a less atherogenic plasma lipoprotein profile.

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