*Biochemical Pharmacology*, Vol. 43, No. 3, pp. 625–633, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00
© 1992. Pergamon Press plc

# MODULATION OF LIPOPROTEIN PRODUCTION IN HEP G2 CELLS BY FENOFIBRATE AND CLOFIBRATE

SUSAN E. HAHN and DAVID M. GOLDBERG*†

Department of Biochemistry, Hospital for Sick Children, Toronto, Ontario M5G 1X8, Canada; and
*Department of Clinical Biochemistry, University of Toronto, Toronto, Ontario M5G 1L5, Canada

(*Received* 6 *May* 1991; *accepted* 6 *September* 1991)

**Abstract**—Fenofibrate and other fibrate derivatives are commonly used to treat hyperlipidemia. It is not yet clear how they exert their modulatory effects on plasma lipoproteins. To investigate whether these drugs act on the liver to primarily inhibit very low density lipoprotein production, we utilized the highly differentiated human hepatoma cell line, Hep G2. At concentrations greater than 15 μg/mL, fenofibrate caused a 30% decrease in secreted apolipoprotein B (apo B) after 4 days of treatment. Pulse-chase studies demonstrated that this was not due to inhibition of apo B synthesis. Triglyceride synthesis by fenofibrate-treated Hep G2 cells was decreased by 30%, and the amount secreted into the medium was reduced by 50%. At a low concentration of drug (5 μg/mL), triglyceride secretion was reduced markedly while apo B secretion remained unchanged. Thus, apo B secretion is less sensitive to fenofibrate than the synthesis and secretion of triglyceride, and may be secondary to changes in the latter. Fenofibrate has also been shown to raise plasma high density lipoprotein concentrations. We found that low concentrations of fenofibrate caused a 20–101% increase in secreted apolipoprotein AI (apo AI), and pulse-chase immunoprecipitation studies showed that this was due to an increase in apo AI synthesis. Fenofibrate was compared to clofibrate to investigate whether their relative effects on lipoprotein production in Hep G2 cells were comparable to their relative effects on plasma lipoproteins. Both fibrates decreased the secretion of apo B to the same extent, but only fenofibrate increased apo AI secretion. Fenofibrate was more effective than clofibrate in inhibiting the secretion of lipids by these cells. Thus, the known effects of fenofibrate on plasma lipoproteins can be attributed to its direct modulation of lipoprotein synthesis in the liver cell. Hep G2 cells may thus be useful in testing the relative efficacy of fibric acid derivatives *in vitro*.

Atherosclerosis is the most ommon cause of death in North America. High plasma concentrations of low density lipoprotein (LDL‡) cholesterol and low concentrations of high density lipoprotein (HDL) cholesterol increase the risk of coronary heart disease [1]. The Coronary Primary Prevention Trial [2] showed that drug intervention to reduce plasma cholesterol and triglycerides can reduce the incidence of death due to atherosclerosis. Several classes of drugs are used for this purpose, one of the most common being the fibric acid derivatives.

Clofibrate was first released as a hypolipidemic agent in 1965; since then it has been shown to markedly reduce plasma triglyceride concentrations in all types of hyperlipidemia except Fredericksen Type I [3]. It has a more variable effect on LDL cholesterol, which increased in some studies or decreased in others, and it has no effect on HDL cholesterol [4]. Clofibrate is a potent peroxisomal proliferator with a high incidence of serious side-effects, including liver cancer [5]. The lack of consistent changes in plasma cholesterol levels and the adverse effects associated with long-term clofibrate treatment have limited its use. Newer fibrate derivatives, such as gemfibrozil and fenofibrate, were introduced. Besides causing a 25–60% decrease in plasma triglyceride concentration, they significantly reduce LDL cholesterol and raise HDL cholesterol concentrations; these changes in plasma lipids are accompanied by a 3–38% increase in apolipoprotein AI (apo AI) and a 10–37% decrease in apoliprotein B (apo B) [6]. Their incidence of side-effects is also much lower; European clinical trials indicate that only 6% of fenofibrate-treated patients experience adverse effects, and these are mostly gastrointestinal [5, 6].

Despite their long use, the mechanisms by which fibrates manifest their effects on plasma lipids are not yet clear. Those suggested include induction of lipoprotein lipase to speed lipoprotein removal [7], binding of drug to lipoprotein particles with inhibition of their uptake [8], inhibition of hydroxy methyl glutaryl coenzyme A (HMG-CoA) reductase [9], and increasing the clearance of LDL from plasma [10]. Studies in the rat using etofibrate revealed a possible direct effect on hepatic lipoprotein production [11]. However, etofibrate is a derivative of clofibrate and nitonic acid, which is another hypolipidemic agent. Thus, the effect of fibrate alone has yet to be elucidated.

The Hep G2 cell line has been widely employed

† Corresponding author: Dr. David M. Goldberg, Department of Clinical Biochemistry, University of Toronto, 100 College St., Toronto, Ontario M5G 1L5 Canada. Tel. (416) 978-2638; FAX (416) 978-5650.

‡ Abbreviations: LDL, low density lipoprotein(s); VLDL, very low density lipoprotein(s); HDL, high density lipoprotein(s); apo AI, apolipoprotein AI; apo B, apolipoprotein B; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; ABTS, 2,2'-azino-di-[3-ethyl-benzthiazoline-6-sulphonate]; MEM, minimal essential medium; FBS, fetal bovine serum; LD-FBS, lipoprotein-deficient fetal bovine serum; and SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

to study VLDL and HDL biosynthesis. It secretes all the major apolipoproteins with their lipid components [12], although the secreted lipoproteins, especially VLDL, show some density and size differences by comparison with mature human plasma lipoproteins [13]. Lipoprotein metabolism in Hep G2 cells has been shown to respond to various drug and hormonal stimuli [14, 15]. We have therefore used Hep G2 cells to investigate the effects of fibrates on several aspects of lipoprotein production. Specifically, we studied the synthesis and secretion of the major apolipoproteins of VLDL (apoliprotein B) and HDL (apolipoprotein AI), as well as of triglyceride and cholesterol which are the principle targets for the hypolipidemic drugs. Fenofibrate was used for these investigations since it is a potent modulator of plasma lipoproteins, and among the most effective of five fibrates currently in clinical use [6]. Finally, to determine whether the relative efficacies of the fibrate derivatives *in vivo* could be explained by a difference in their modulation of lipoprotein synthesis and secretion *in vitro*, we compared fenofibrate with clofibrate, which is the least potent of the fibrate derivaties.

## MATERIALS AND METHODS

### *Materials*

Hep G2 cells were kindly provided by Dr. Barbara K. Knowles (Wistar Institute of Anatomy and Biology, Philadelphia, PA). Sheep anti-human apolipoproteins AI and B were a gift from Boehringer–Mannheim (Dorval, Canada), from whom we also purchased 2,2'-azino-di-[3-ethyl-benzthiazoline-6-sulphonate] (ABTS) and horse-radish peroxidase. Fetal bovine serum (FBS) and penicillin–streptomycin were obtained from Gibco BRL (Burlington, Canada); Trans $^{35}$S-label™ (1120 Ci/mmol) was from ICN Biochemicals (Quebec, Canada) and [2-$^{14}$C]acetate (55.0 mCi/mmol) was from New England Nuclear (Toronto, Canada). Clofibric acid was purchased from the Sigma Chemical Co. (St. Louis, MO). Fenofibric acid was a gift from Fournier Laboratories (Paris, France).

### *Cell culture*

Hep G2 cells were grown at 37° with 5% $CO_2$ in $\alpha$-Minimum Essential Medium (MEM) supplemented with 10% (v/v) FBS and penicillin-streptomycin (100 U/mL). Clofibric acid or fenofibric acid dissolved in ethanol (130 mM final concentration) was added in various concentrations for various periods to monolayers, with appropriate controls. Treatment was started when cells were 60% confluent, for reasons that will be explained. Since the half-life of fenofibrate in plasma is approximately 21 hr [10], and fibrate drugs are usually given 1–3 times per day [6], cell cultures were supplemented with drugs every 24 hr during all experiments. When apolipoproteins were measured in the medium, cells were grown in MEM supplemented with 10% (v/v) lipoprotein-deficient fetal bovine serum (LD-FBS). This was prepared by treating FBS with Cab-o-sil (Research Products International Corp., Elk Grove, IL) as described by Weinstein [16].

### *Anti-apolipoprotein antibodies*

Antibodies to human apo AI and apo B were purified on affinity columns consisting of LDL-Sepharose for anti-apo B antibodies and apo AI-Sepharose for anti-apo AI antibodies. The columns were prepared according to the manufacturer's instructions (Pharmacia, Dorval, Canada), and the affinity chromatography was carried out according to the method of Gibson *et al.* [17].

### *Enzyme-linked immunosorbent assay (ELISA)*

To establish quantitative ELISAs for apo AI and apo B, purified apolipoproteins were prepared as standards and for making immunoaffinity columns. Apo AI was isolated from human HDL ($1.063 < d < 1.21$) [18]. It was delipidated with cold ethanol/ether [19], and the apo HDL was separated by gel exclusion chromatography [20]. The composition of each fraction was checked by 3–20% (w/v) gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [21], and the purity was verified by amino acid analysis [22]. Human LDL ($1.03 < d < 1.05$) was used as a source of standard apo B [23] and was prepared by ultracentrifugation of human plasma. The material from this density region cointains a single major protein, apo B, as judged by SDS–PAGE.

Sandwich enzyme assays were carried out essentially as described by Koren *et al.* [24], using ABTS [25] as the substrate for horse-radish peroxidase. The sensitivity of the apo AI assay was 4 ng/mL (standard curve 0–100 ng/mL) and for the apo B assay was 50 ng/mL (standard curve 0–1000 ng/mL). The coefficients of variation for replicate assays were 4.6% at 27 ng/mL and 3.6% at 900 ng/mL for apo AI and apo B, respectively. Affinity-purified antibodies were used as capture antibodies, and indicator antibodies were made by cross-linking affinity-purified antibodies to horse-radish peroxidase [26]. To measure extracellular (secreted) apolipoproteins, solutions of the apolipoprotein standards diluted in lipoprotein free medium, and appropriately diluted conditioned medium were added to the wells. Intracellular apolipoproteins were determined by scraping the cells into cold phosphate-buffered saline (10 mM phosphate, pH 7.4, 150 mM NaCl; PBS), and collecting the cell pellets by centrifugation at 200 *g* for 5 min. These were then resuspended in 1 mL of 10 mM Tris (pH 7.4), and disrupted by sonication for 2 × 5 sec at 75 W. Further details of the individual experiments are provided in the figure legends.

### *Apolipoprotein synthesis*

After treatment with the appropriate drug, medium was removed and the cells were grown in methionine-free MEM for 2 hr. [$^{35}$S]Methionine (100 $\mu$Ci/plate) was added for 10 min, and then removed. Plates were washed with PBS, and medium containing 15 $\mu$g/mL of L-methionine was added for 10 min. The cells were washed with PBS and disrupted as described, and the apolipoproteins were immunoprecipitated using affinity-purified antibodies according to the method of Wettesten *et al.* [27]. Preliminary studies with $^{125}$I-labeled HDL

and LDL were carried out to adjust the antibody concentration to the point where 100% apo AI and apo B were precipitated from cell lysates to which 1 μg of labeled protein was added. This is 1–2 orders of magnitude greater than the amounts endogenously present in these extracts. A portion of each was used for radioactivity determination, and another portion was subjected to PAGE, followed by autoradiography. This showed that >95% of the radioactivity in the immunoprecipitates was in the band corresponding to apo B or apo AI according to the antibody used (data not shown).

*Triacyglycerol synthesis*

Hep G2 monolayers were washed with PBS and fresh medium was added, supplemented for 6 hr with 4 μCi/plate of [2-$^{14}$C]acetate, corresponding to 72.7 nmol acetate/plate or approximately 12 nmol/mL medium. The rate of incorporation of the $^{14}$C-label into all the lipid classes examined was linear with time to at least 24 hr for secreted lipids and 6.5 hr for intracellular lipids. Cells were collected and homogenized as described. VLDL were prepared from cell medium by ultracentrifugation in 1.063 g/mL KBr. These particles, which contain all the secreted apo B, have the same size as LDL, $d < 1.063$, and are relatively lipid-poor in contrast to mature plasma VLDL of $d < 1.006$ [13]; with these qualifications the term VLDL will continue to be used in this paper. Cell extracts and VLDL were extracted with chloroform–methanol (2:1; v/v) by the method of Folch *et al.* [28]. Total lipid extracts were separated by thin-layer chromatography to resolve neutral lipids using a two-solvent system. Plates were first developed in chloroform–methanol–acetic acid (93:2:0.1; by vol.) and then developed in hexane–diethyl ether–acetic acid (89:6:0.1; by vol.). Triglycerides, free cholesterol esters and free fatty acids were identified using appropriate standards. The silica was then scraped from the plate and the radioactivity was counted in 10 mL of Aquasol.

*Total lipid analysis*

The mass of secreted triglycerides, cholesterol, and cholesteryl esters was determined by gas-liquid chromatography (GLC). Conditioned media from four pooled plates were lyophilized and delipidated using chloroform–methanol (2:1; v/v) by the method of Folch *et al.* [28]. Total lipid extracts were applied to a Sep Pak column (Waters, Mississauga, Ontario) and neutral lipids were eluted with 4 mL of chloroform. They were then dried under $N_2$ and the GLC analyses were performed as described by Myher and Kuksis [29] with tridecanoylglycerol as an internal standard.

*Other methods*

All results were corrected for cell density as expressed by total cell protein for each plate [30]. Good correlations were obtained between the cellular protein concentration, the number of cells as counted using a hemocytometer, and the cellular DNA as measured by a diphenylamine method [31] and a fluorimetric method [32]. Since the protein results were the most precise, these are given in this paper. Statistical analyses were performed using Student's *t*-test.

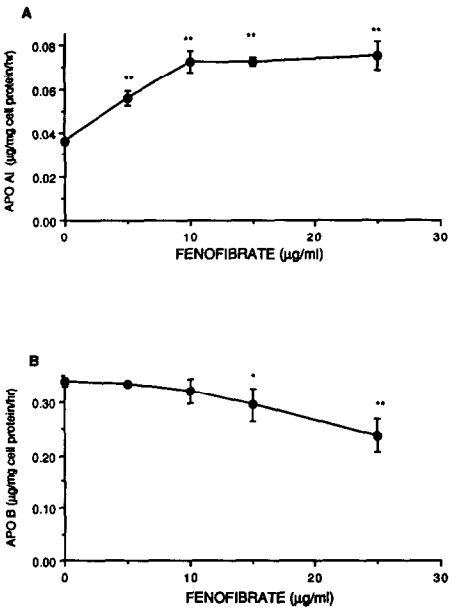


Fig. 1. Secretion of apolipoproteins AI and B. Hep G2 cells were grown until 60% confluent, and then supplemented with 0–25 μg/mL fenofibrate dissolved in ethanol (130 mM) every 24 hr for 4 days. Monolayers were washed with PBS, and 2 mL of MEM supplemented with LD-FBS was added for 2 hr. The media were collected and apolipoprotein AI (panel A) and apolipoprotein B (panel B) were measured by ELISA. Each point is the mean ± SD of 4 plates, assayed in duplicate (* $P < 0.05$, and ** $P < 0.01$; significance of difference between data for drug concentration and ethanol control).

## RESULTS

Both ethanol and dimethyl sulfoxide (DMSO) were tested as possible solvents for fenofibrate by examining their effects on the secretion of apolipoproteins. After 4 days of treatment, ethanol (130 mM) had no effect on apo AI secretion, whereas DMSO (62 mM) caused a 200% increase. Both solvents caused a small increase (<10%) in apo B secretion. Since DMSO had such a large effect independently on apo AI secretion, ethanol was used for all experiments.

Preliminary studies revealed the advantages of treating cells at 60% confluency. At this point, they become confluent in 2 days and they produce lipoproteins at a stable rate for a further 4 days. In this way, all experiments were carried out under stable and optimal conditions.

*Secreted apolipoproteins*

*Concentration–response.* We first examined the effect of fenofibrate on apolipoprotein secretion. Concentration–response studies were carried out for apo AI (Fig. 1A) and apo B (Fig. 1B). Apo AI secretion increased in a concentration-dependent manner and reached a maximum at 20 μg/mL

Table 1. Summary of experiments to determine the effect of fenofibrate (20 μg/mL for 4 days) on apolipoprotein secretion by Hep G2 cells

| | Control | Fenofibrate | N* | P† |
|---|---|---|---|---|
| Apo AI (ng/mg cell protein/hr) | 46.0 ± 16.8‡ | 65.0 ± 17.8 | 8 (32) | <0.001 |
| Apo B (μg/mg cell protein/hr) | 0.44 ± 0.17 | 0.32 ± 0.13 | 9 (36) | <0.001 |

* Number of individual experiments (open figures) each with 3–5 plates for control and fenofibrate-treated cells assayed in duplicate; total number of plates, each assayed in duplicate, is given in parentheses.

† Paired *t*-test employing mean of the 3–5 plates assayed in duplicate for each individual experiment. In all, the apo AI secretion was higher and the apo B secretion was lower than the control.

‡ Mean ± SD.

fenofibrate. After 4 days of treatment, cells showed a concentration-dependent decrease in apo B secretion. This decrease was only significant when concentrations of fenofibrate reached 15 μm/mL. Apo AI secretion thus appeared to be more sensitive to fenofibrate because lower concentrations were required to increase apo AI than to lower apo B levels. Since 20 μg/mL of fenofibrate maximally increased apo AI secretion, and was close to the highest fenofibrate concentration decreasing apo B secretion in these initial studies, this concentration was used in all further experiments. Table 1 presents the results of 8–9 individual experiments in each of which 3–5 control and 3–5 fenofibrate-treated cultures were assayed in duplicate. In every instance, apo AI secretion was higher and apo B secretion was lower than the control cultures at a significance level of at least $P < 0.05$ for each individual experiment. However, the increase in apo A1 showed no correlation with decrease in apo B ($r = 0.013$).

We then compared the effects of both fenofibrate and clofibrate on apolipoprotein secretion. After 4 days of treatment, both drugs decreased apo B secretion to the same extent at concentrations up to 20 μg/mL (Fig. 2B), but at the highest concentration (30 μg/mL), fenofibrate had a much greater effect. However, the two drugs had differential effects on the secretion of apo AI (Fig. 2A). While fenofibrate significantly increased apo AI secretion at both 10 and 20 μg/mL, clofibrate treatment had no such effect.

*Time dependence.* The time required for fenofibrate to modulate apolipoprotein secretion can be seen in Fig. 3. Increases in apo AI secretion were pronounced even after 2 days of treatment and remained increased approximately 30% over control values for the duration of the experiment. Although small decreases in apo B were observed after 2 days, these differences were not significant until day 4. Total cell protein was not affected by fenofibrate for up to 6 days (data not shown). Since the increase in apo AI secretion and the decrease in apo B secretion were well established by 4 days, this time was used for all further experiments. Earlier studies over a 7-day period showed that the effect of clofibrate upon apo B secretion was also maximal after 4 days of treatment (data not shown).

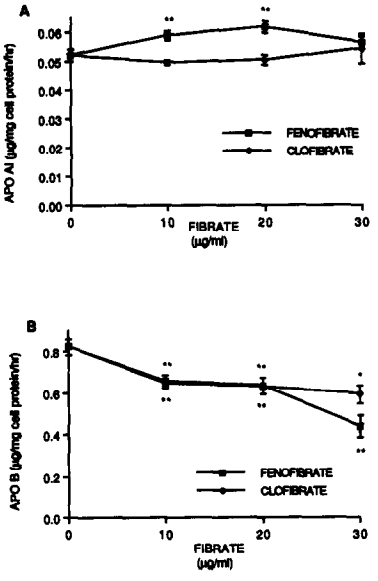


Fig. 2. Comparative effects of clofibrate and fenofibrate on apolipoprotein secretion. Hep G2 cells were grown until 60% confluent and then supplemented every 24 hr with clofibrate or fenofibrate (0–30 μg/mL) dissolved in ethanol (130 mM). After 4 days of treatment, medium was removed and cells were washed with PBS. Two milliliters of MEM supplemented with LD-FBS was added, and collected after 2 hr. Apolipoprotein AI (panel A) and apolipoprotein B (panel B) were measured by ELISA. Each point is the mean ± SD of 4 plates assayed in duplicate (* $P < 0.05$, and ** $P < 0.01$; significance of difference between data for drug concentration and ethanol control).

*Intracellular apolipoproteins*

Intracellular concentrations of both apo AI and apo B in response to various concentrations of fenofibrate are shown in Table 2. The intracellular content of both apolipoproteins is about one-fifth of that secreted by the cells in 1 hr, suggesting that their storage within the cell is very limited. There was a concentration-dependent increase in apo AI and a corresponding decrease in apo B. These reflect the changes seen in the extracellular apolipoproteins. Apo AI concentrations increased about 50%, while

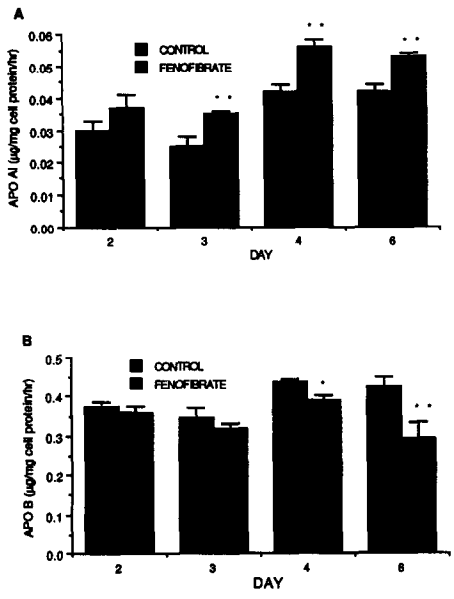


Fig. 3. Effect of fenofibrate on extracellular apolipoproteins after various times of treatment. Hep G2 cells were grown until 60% confluent and then supplemented every 24 hr with either fenofibrate (20 μg/mL in ethanol) or ethanol alone (130 mM). After 2–6 days of treatment, monolayers were washed with PBS and 2 mL of medium supplemented with 10% LD-FBS was added for 2 hr. The medium was collected and apolipoproteins were assayed by ELISA. Panel A shows the results for apo AI and panel B shows the results for apo B. Each value is the mean ± SD for 3 plates assayed in duplicate (* $P < 0.05$, and ** $P < 0.01$; significance of difference between fenofibrate-treated and ethanol control at each time).

Table 2. Effect of fenofibrate on intracellular apolipoproteins*

| Fenofibrate (μg/mL) | Apo AI (ng/mg cell protein) | Apo B (μg/mg cell protein) |
|---|---|---|
| 0 | 10.16 ± 0.10 | 0.207 ± 0.008 |
| 5 | 12.59 ± 0.57† | 0.187 ± 0.006† |
| 10 | 13.94 ± 1.41† | 0.189 ± 0.012‡ |
| 15 | 14.20 ± 0.72† | 0.150 ± 0.007† |
| 25 | 15.42 ± 1.16† | 0.158 ± 0.008† |

* Hep G2 cells were grown in fenofibrate-supplemented media for 4 days as described in Fig. 1. After this time cells were washed twice with PBS, and cell pellets were collected and sonicated as described in Materials and Methods. Intracellular apo AI and Apo B were assayed by ELISA. Each value is the mean ± SD of 4 plates assayed in duplicate.

† $P < 0.01$.

‡ $P < 0.05$.

apo B decreased 25% at 25 μg/mL fenofibrate. The lower drug concentrations caused a significant decrease in intracellular apo B (Table 2) while they caused no significant change in secreted apo B (Fig. 1B).

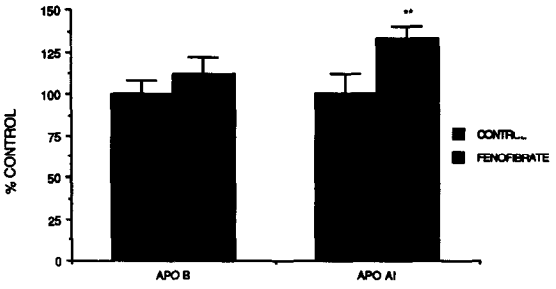


Fig. 4. Effect of fenofibrate on apolipoprotein synthesis. Hep G2 cells were grown in 35 mm plates until 60% confluent, and then supplemented every 24 hr for 4 days with fenofibrate (20 μg/mL in ethanol) or ethanol (130 mM). The monolayers were then washed with PBS and pretreated for 2 hr with methionine-free MEM supplemented with 10% FBS. This was removed and cells were pulsed with 100 μCi/dish of [$^{35}$S]methionine for 10 min. Cells were washed twice with PBS, and then MEM containing 15 μg/mL cold methionine was added for 10 min. This was removed and cells were washed extensively with PBS, collected and sonicated as described. Apolipoproteins were immunoprecipitated and quantitated as described in Materials and Methods. Data are expressed as the cpm in fenofibrate samples as a percentage of the cpm in control samples, corrected for cell protein (4720 ± 580 cpm/mg cell protein for apo AI and 10,120 ± 820 cpm/mg cell protein for apo B). Each point is the average ± SD of 4 plates (** $P < 0.01$; significance of difference between fenofibrate-treated and control cells).

*Apolipoprotein synthesis*

To determine whether the observed effects on the apolipoproteins could be explained by a change in synthesis, pulse/chase immunoprecipitation studies were performed (Fig. 4). Apo AI synthesis was increased significantly with fenofibrate (33% increase over control), and thus the increases in intracellular apo AI content and apo AI secretion due to this drug can be attributed to an increase in the synthesis of this protein. There was no significant change in the synthesis of apo B with 20 μg/mL fenofibrate. The fibrate-induced decreases in intracellular apo B and in apo B secretion cannot therefore be attributed to a decrease in apo B synthesis. This is consistent with the fact that fenofibrate only causes a decrease in apo B secretion after 4 days; direct inhibition of protein synthesis would be expected to cause changes much earlier.

Clofibrate (up to 30 μg/mL) had no effect on the synthesis of either apolipoprotein AI or B (data not shown). Thus, these two fibrate derivatives have differential effects on apo AI synthesis and secretion.

*Lipid secretion*

The effect of fenofibrate on the mass of triglycerides secreted by Hep G2 cells is seen in Table 3, which presents the triglyceride concentrations in unfractionated conditioned medium. Since almost no triglyceride is associated with HDL [13], this can be considered as VLDL triglyceride. A decrease to approximately 60% of control values occurred with

Table 3. Effect of fenofibrate on the mass of secreted triglyceride*

| Fenofibrate (μg/mL) | Triglycerides (μg/mg cell protein/hr) | % Control |
|---|---|---|
| 0 | 0.820 | 100 |
| 5 | 0.437 | 59 |
| 10 | 0.504 | 66 |
| 15 | 0.500 | 61 |
| 25 | 0.449 | 55 |

* Monolayers of Hep G2 cells were treated for 4 days with the indicated concentrations of fenofibrate (dissolved in ethanol) or ethanol alone (130 mM). Media from 4 replicate plates were pooled and prepared for GLC as described in Materials and Methods, and values given are the average of two experiments in which the results were in good agreement. The cholesterol esters secreted in these experiments at 0, 10 and 20 μg/mL of fenofibrate were, respectively, 0.88, 0.35 and 0.33 μg/mg cell protein/hr.


Fig. 5. Comparative effects of fibrates on secreted lipids. Cells were treated as described in Fig. 2, with either clofibrate (20 μg/mL), fenofibrate (20 μg/mL) or ethanol (130 mM). Lipids were isolated from cell media pooled from 4 replicate plates, and triglycerides, free cholesterol and cholesteryl esters were measured by GLC.

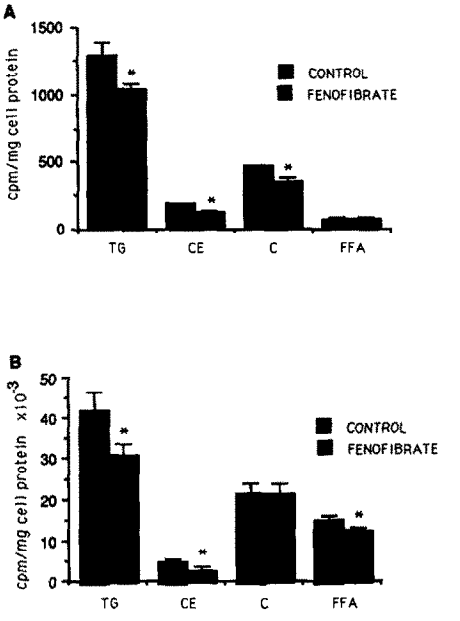


Fig. 6. Effect of fenofibrate on [$^{14}$C]acetate incorporation into lipids of Hep G2 cells. Hep G2 cells were grown on 10 cm plates until 60% confluent. Cells were then supplemented every 24 hr with fenofibrate (20 μg/mL in ethanol) or ethanol (130 mM) for 4 days. [2-$^{14}$C]Acetate (4 μCi/dish) was added for 6 hr. Media and cells were then collected, and VLDL were isolated from cell medium as described in Materials and Methods. Lipids were extracted from cell pellets and VLDL and separated by thin-layer chromatography. Radioactivity in the bands corresponding to triglycerides (TG), free cholesterol (C), cholesteryl esters (CE), and free fatty acids (FFA) was measured. Each value is the mean ± SD of 3 VLDL fractions (panel A) or 6 cell pellets (panel B) (* $P < 0.05$; significance of difference between fenofibrate-treated and control cells).

only 5 μg/mL fenofibrate treatment, and remained unchanged as the concentration was increased. The rate of secretion of cholesteryl esters was also greatly reduced (>50%) by fenofibrate. A corresponding decrease in secreted apo B did not occur with this low concentration (Fig. 1B), although there was a significant reduction in intracellular apo B. Apo B secretion is therefore less sensitive to fenofibrate than the secretion of triglyceride. The relative ability of the two fibrates to reduce lipid secretion by Hep G2 cells is shown in Fig. 5. Both reduced triglyceride and cholesteryl ester secretion, but fenofibrate did so to a greater extent. Fenofibrate also decreased free cholesterol secretion, while clofibrate did not. Thus, fenofibrate was more effective at reducing lipid secretion by Hep G2 cells.

*Lipid synthesis*

Since fenofibrate decreased the secretion of triglycerides by Hep G2 cells, we studied its effect on lipid synthesis by these cells. The incorporation of $^{14}$C-labeled neutral lipids into VLDL prepared from conditioned medium was decreased after 4 days of 20 μg/mL fenofibrate (Fig. 6A). The secretion of newly synthesized triglyceride, free cholesterol, and cholesteryl ester all decreased around 30%. The synthesis of intracellular lipids by Hep G2 cells (Fig. 6B) was also decreased 30% by fenofibrate in the case of triglycerides, cholesteryl esters and free fatty acids. The decrease in apo B secretion may be a consequence of the marked inhibition of triglyceride or cholesteryl ester synthesis and secretion. There appeared to be no effect on the synthesis of free cholesterol.

## DISCUSSION

Fibrate administration is known to cause several beneficial alterations in the plasma lipoprotein profile. The most striking is decreased plasma triglyceride concentration, which is accompanied by decreased concentrations of LDL cholesterol and apo B, and increased plasma concentrations of HDL cholesterol and apo AI [6]. The magnitude of these changes varies according to the fibrate used. Fenofibrate and gemfibrozil are the most effective, whereas clofibrate only has a consistent effect on plasma triglycerides [6].

Fibrates are metabolized by the microsomal cytochrome P450 system. They have been shown to induce peroxisomal proliferation in the liver, and to induce the production of the isozyme cytochrome P452 [33]. A putative cytosolic receptor for clofibrate has recently been isolated, and identified as a member of the heat-shock protein family, HSP70 [34]. Whether the hypolipidemic effects of all fibrates are mediated through this protein remains to be elucidated.

The plasma lipoprotein profile is maintained through a balance between hepatic synthesis and secretion, intravascular metabolism, and cellular uptake and degradation. Indirectly, through *in vivo* studies, fibrates have been shown to alter all of these processes. A decrease in hepatic triglyceride synthesis and secretion in humans was reported by Kesaniemi and Grundy [35] who also calculated that the transport rate of VLDL triglyceride out of the liver was reduced by 28%. HMG-CoA reductase, the key regulatory enzyme of cholesterol metabolism, is inhibited by clofibrate in chick liver [9]. *In vivo* studies in male rats demonstrated that fenofibrate inhibits the incorporation of [$^{14}$C]acetate into cholesterol [36]. However, experiments with solubilized HMG-CoA reductase showed that fenofibrate is not a potent inhibitor, except at concentrations much higher than those likely to be achieved in the body [10]. Cholesterol esterification was also shown to be reduced [37]. The catabolism of VLDL may be affected by fibrate treatment. These drugs are known to increase the activity of lipoprotein lipase [7] which converts VLDL to LDL. Fibrates also increase the rate of LDL degradation by enhancing its endocytosis, possibly through an increased number of cell surface LDL receptors [10].

Most *in vitro* studies of fibrate derivatives have used rat models [4]. Rats have a different lipoprotein profile from humans in that they have very little LDL [38], they metabolize fenofibrate differently from humans [10], and obese rats have been shown to respond differently to fenofibrate than do obese humans [39]. For these reasons, it is preferable to use human hepatocytes. Although Hep G2 is a transformed cell line, it is highly differentiated and synthesizes and secretes plasma lipoproteins [12–15].

Fenofibrate-induced increases in apo AI production by Hep G2 cells are in good agreement with fenofibrate-induced increases in plasma apo AI and HDL concentrations in human subjects [6]. Apolipoprotein AI secretion and intracellular concentration increased in a concentration-dependent manner after exposure to fenofibrate, and these changes were accompanied by increased synthesis of apo AI. Thus, it is possible that fenofibrate or a metabolite of the parent drug is able to stimulate apo AI gene expression. Plasma HDL concentrations are augmented by other inducers of cytochrome P450, including ethanol [40], phenobarbital and phenytoin [41]. Apo AI mRNA levels also increased 10-fold in rats after 16 hr of phenobarbital treatment [42]. Thus, the enhancement of apo AI synthesis by fenofibrate may be a consequence of its induction of cytochrome P450. This, in turn, could increase production of a metabolite which enhances the transcription of apo AI mRNA (Tam S-P and Deeley RG, personal communication, cited with permission). However, clofibrate is also a potent cytochrome P450 inducer, and it had no effect on apo AI synthesis or secretion (Fig. 2A). This discrepancy may be attributed to the induction of different isozymes which may stimulate different regulatory elements, only one of which may increase apo AI gene transcription.

In contrast to apo AI, the secretion of apo B was decreased in a concentration-dependent manner by both fenofibrate and clofibrate. The reduction of secreted and intracellular apo B requires a higher concentration and a longer time than the augmentation of apo AI. This suggests a different mechanism than that responsible for the increase in apo AI. We also observed no decrease in apolipoprotein B synthesis; in contrast, there appeared to be a small but nonsignificant increase (Fig. 4). The lack of coordination between apolipoprotein B secretion and synthesis is consistent with recent findings. Pullinger *et al.* [43] have shown that over a 7-fold variation in apo B secretion, there is no change in apo B mRNA transcription or translation. Apo B is synthesized constitutively, and its rate of secretion is regulated by some other mechanism, probably degradation. This degradation is believed to be controlled by the availability of core lipids. If lipid synthesis is reduced, the excess apo B becomes exposed to degradative enzymes on the cytoplasmic face of the endoplasmic reticulum [44]. Both triglyceride [45] and cholesteryl ester [46] have been suggested as the lipid which primarily regulates this event.

Thus, fenofibrate could exert its control on apo B secretion by limiting the synthesis of triglyceride or cholesteryl ester, thereby increasing apo B degradation. Our results are consistent with this possibility. Fenofibrate inhibited the synthesis of cellular cholesteryl ester and triglyceride as judged by [$^{14}$C]acetate incorporation (Fig. 6B). [$^{14}$C]Acetate was used in most of our experiments so that we could simultaneously measure the synthesis of both triglyceride and cholesterol derivatives, but similar results for the former were obtained when [$^{3}$H]-glycerol was employed (data not shown).

Fenofibrate, but not clofibrate, caused a reduction in the secretion of free cholesterol by Hep G2 cells (Fig. 5). This is consistent with clinical studies showing that fenofibrate invariably decreases plasma free cholesterol, whereas clofibrate has a more variable effect [6]. We have shown by both mass measurement and by isotopic labeling studies that fenofibrate decreased the secretion of free cholesterol by 30%; however, there was no change in intracellular free cholesterol synthesis (Fig. 6B). This is consistent with the results of Stahlberg *et al.* [37], who demonstrated that rats, after 2 weeks of fibrate feeding, had no significant changes in hepatic microsomal HMG-CoA reductase activity. However, Castillo *et al.* [9] reported that clofibrate inhibits chick liver HMC-CoA reductase. A possible reason for this discrepancy is that they added clofibrate directly to chick liver microsomes containing the enzyme. They also used extremely high concentrations (270–1070 μg/mL) of drug. Our intact

cells treated with much lower drug concentrations (10–30 μg/mL) did not exhibit inhibition of free cholesterol synthesis. The decreased synthesis and secretion of cholesteryl esters is therefore not caused by a decrease in free cholesterol synthesis. Cholesteryl ester synthesis is controlled by acyl cholesterol:acyl transferase (ACAT), and the activity of this enzyme is reduced markedly by fibrate treatment in rats [37].

The decreases in cellular cholesteryl ester and triacyglycerol synthesis could be a consequence of a decrease in free fatty acid synthesis (see Fig. 6). Fibrates, as well as other acidic drugs, form CoA-thioesters when they enter the cell [10, 47]. This may sequester CoA and prevent its use for the activation of acetate, which would inhibit the production of fatty acids [10].

In the VLDL fraction, the secretion of newly synthesized triglyceride, cholesteryl ester and free cholesterol was reduced 30% by fenofibrate (Fig. 6A). Preliminary results (not presented) showed that the secretion of newly synthesized phosphatidyl choline, which comprises two-thirds of VLDL phospholipids, was also reduced by 30%. Since the inhibition of secretion of all the lipid components and of apo B is approximately the same, the VLDL particles secreted by fenofibrate-treated cells probably have a normal size and composition, but are secreted in smaller numbers.

Hep G2 cells may be a useful model to further characterize how fibrate derivatives affect each stage of lipoprotein production, and especially the possible relationship between their mechanism of action and cytochrome P450 induction. Since Hep G2 cells have been shown to undergo induction of cytochrome P450 [48], they can be utilized for these studies. In addition, the relative effects of clofibrate and fenofibrate on lipoprotein production in Hep G2 cells are similar to their relative effects *in vivo*. These cells may therefore be useful as a general tool for measuring the biological effectiveness of lipid-lowering drugs, and for determining the biochemical basis of their actions.

*Acknowledgements*—We are grateful to Dr. A. Kuksis for performing the GLC analysis. We thank Dr. M. A. Moscarello for his advice and generous access to laboratory equipment. Dr. J. G. Parkes and Dr. M. Paquet also helped with valuable suggestions. This work was supported by the Medical Research Council of Canada and a Research Training Fellowship to S. E. Hahn from the Hospital for Sick Children.

## REFERENCES

1. Naito HK, The association of serum lipids, lipoproteins and apolipoproteins with coronary artery disease assessed by coronary arteriography. *Ann NY Acad Sci* **454**: 230–238, 1985.
2. Superko HR, Drug therapy and the prevention of atherosclerosis in humans. *Am J Cardiol* **64**: 31G–38G, 1989.
3. Davignon J, Xhignesse M and Roederer F, Identification of the patient at risk in the physician's office and drug management of dyslipoproteinemia. *Can J Cardiol* **4**: 36A–47A, 1988.
4. Havel RJ and Kane JP, Drugs and lipid metabolism. *Annu Rev Pharmacol* **13**: 287–308, 1973.
5. Roberts WC, Safety of fenofibrate—US and worldwide experience. *Cardiology* **76**: 169–179, 1989.
6. Blane GF, Review of European clinical experience with fenofibrate. *Cardiology* **76**(Suppl 1): 1–13, 1989.
7. Heller F and Harvengt C, Effects of clofibrate, bezafibrate, fenofibrate and probucol on plasma lipolytic enzymes in normolipidemic subjects. *Eur J Clin Pharmacol* **25**: 57–63, 1983.
8. Olivier JL, Chachaty C, Wolf C, Salmon S and Bereziat G, Binding of spin-labelled clofibrate to lipoproteins. *Biochim Biophys Acta* **963**: 515–524, 1988.
9. Castillo M, Burgos C, Rodriguez-Vico F, Zafra MF and Garcia-Peregrin E, Effects of clofibrate on the main regulatory enzymes of cholesterogenesis. *Life Sci* **46**: 397–403, 1990.
10. Caldwell J, The biochemical pharmacology of fenofibrate. *Cardiology* **76**(Suppl 1): 33–44, 1989.
11. Herrera E, Lasuncion MA, Castro M, Gomez-Coronado D, Martin A and Quack G, Studies with etofibrate in the rat. Part I: Effects on glycerol, free fatty acid and triacyglycerol metabolism. *Biochim Biophys Acta* **963**: 42–52, 1989.
12. Zannis VI, Breslow JL, SanGiacomo TR, Aden DP and Knowles BB, Characterization of the major apolipoproteins secreted by two human hepatoma cell lines. *Biochemistry* **20**: 7089–7096, 1981.
13. Thrift RN, Forte TM, Cahoon BE and Shore VG, Characterization of lipoproteins produced by the human liver cell line, Hep G2, under defined conditions. *J Lipid Res* **27**: 236–250, 1986.
14. Tam S-P, Archer TK and Deeley RG, Effects of estrogen on apolipoprotein secretion by the human hepatocarcinoma cell line, Hep G2. *J Biol Chem* **260**: 1670–1675, 1985.
15. Wong S and Nestel PJ, Eicosapentaenoic acid inhibits the secretion of triacyglycerol and of apolipoprotein B and the binding of LDL to Hep G2 cells. *Atherosclerosis* **64**: 139–146, 1987.
16. Weinstein DB, A single-step adsorption method for removal of lipoproteins and preparation of cholesterol-free serum. *Circulation* **60**: 11–54, 1979.
17. Gibson JC, Rubinstein A, Ginsberg HN and Brown WV, Isolation of apolipoprotein E-containing lipoproteins by immunoaffinity chromatography. *Methods Enzymol* **139**: 186–198, 1986.
18. Havel RJ, Eder HA and Bradgon JH, The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J Clin Invest* **34**: 1345–1354, 1955.
19. Scanu AM and Edelstein C, Solubility in aqueous solutions of ethanol of the small molecular weight peptides of the serum very low density and high density lipoproteins: Relevance to the recovery problem during delipidation of serum lipoproteins. *Anal Biochem* **44**: 576–588, 1974.
20. Dallinga-Thie GM, Groot PHE and Van Tol A, Electroimmunoassay of rat apolipoproteins AI, AII and E. A procedure for sample treatment to increase the sensitivity in diluted fractions. *J Lipid Res* **26**: 889–892, 1985.
21. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage TG4. *Nature* **227**: 680–685, 1970.
22. Bidlingmeyer BA, Cohen SA and Tarvin TL, Rapid analysis of amino acids using pre-column derivitization. *J Chromatogr* **336**: 93–104, 1984.
23. Rosseneu M, Vercaemst R, Steinberg KK and Cooper GR, Some considerations of methodology and standardization of apolipoprotein B immunoassays. *Clin Chem* **29**: 427–433, 1983.
24. Koren E, Puchois P, McConathy WJ, Fesmire JD and Alaupovic P, Quantitative determination of human plasma apolipoprotein A-I by a noncompetitive

enzyme-linked immunosorbent assay. *Clin Chim Acta* **147**: 85–95, 1985.
25. Porstmann B, Comparison of chromogens for the determination of horseradish peroxidase as a marker in enzyme immunoassay. *J Clin Chem Clin Biochem* **19**: 435–439, 1981.
26. Avrameas S, Coupling of enzymes to proteins with glutaraldehyde: Use of the conjugates for the detection of antigens and antibodies. *Immunochemistry* **6**: 43–52, 1969.
27. Wettesten M, Boström K, Bondjers G, Jarfeldt M, Norfeldt P-I, Carrella M, Wiklund O, Borén J and Olofsson S-O, Pulse-chase studies of the synthesis of apolipoprotein B in a human hepatoma cell line, Hep G2. *Eur J Biochem* **149**: 461–466, 1985.
28. Folch J, Lees M and Sloane Stanley GH, A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* **226**: 497–509, 1957.
29. Myher JJ and Kuksis A, Determination of plasma total lipid profiles by capillary gas-liquid chromatography. *J Biochem Biophys Methods* **10**: 13–23, 1984.
30. Peterson GL, A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. *Anal Biochem* **83**: 346–356, 1977.
31. Leyva A and Kelley WN, Measurement of DNA in cultured human cells. *Anal Biochem* **62**: 173–179, 1974.
32. Labarca C and Paigen K, A simple, rapid and sensitive DNA assay procedure. *Anal Biochem* **102**: 344–352, 1980.
33. Sharma R, Lake BG, Foster J and Gibson GG, Microsomal cytochrome P-452 induction and peroxisome proliferation by hypolipidaemic agents in rat liver: A mechanistic inter-relationship. *Biochem Pharmacol* **37**: 1193–1201, 1988.
34. Alvares K, Carrillo A, Yuan PM, Kawano H, Morimoto RJ and Reddy JK, Identification of cytosolic peroxisome proliferator binding protein as a member of the heat shock protein HSP70 family. *Proc Natl Acad Sci USA* **87**: 5293–5297, 1990.
35. Kesaniemi YA and Grundy SM, Influence of gemfibrozil and clofibrate on metabolism of cholesterol and plasma triglycerides in man. *JAMA* **251**: 2241–2246, 1984.
36. Kritchevsky D, Tepper SA and Story JA, Influence of procetofen on lipid metabolism in normocholesterolemic rats. *Pharmacol Res Commun* **11**: 635–641, 1979.
37. Stahlberg D, Angelin B and Einarsson K, Effects of treatment with clofibrate, bezafibrate and ciprofibrate on the metabolism of cholesterol in rat liver microsomes. *J Lipid Res* **30**: 953–958, 1989.
38. Chapman MJ, Comparative analysis of mammalian plasma lipoproteins. *Methods Enzymol* **128**: 70–143, 1986.
39. Olivier P, Plancke MO, Theret N, Marzin D, Clavey V and Fruchart JC, Effects of fenofibrate on lipoprotein metabolism and fatty acid distribution in Zucker rats. *Atherosclerosis* **74**: 15–21, 1988.
40. Taskinen M-R, Nikkila EA, Valimaki M, Sane T, Kuusi T, Kesaniemi YA and Ylikahri R, Alcohol-induced changes in serum lipoproteins and their metabolism. *Am Heart J* **113**: 458–464, 1987.
41. Luoma PV, Sotaniemi EA, Pelkonen RO and Myllyla VV, Plasma high-density lipoprotein cholesterol and hepatic cytochrome P-450 concentrations in epileptics undergoing anticonvulsant treatment. *Scand J Clin Lab Invest* **40**: 163–167, 1980.
42. Chao Y-S, Pickett CB, Yamin T-T, Guo LSS, Alberts AW and Kroon PA, Phenobarbital induces rat liver apolipoprotein A-I mRNA. *Mol Pharmacol* **27**: 394–398, 1985.
43. Pullinger CR, North JD, Teng B-B, Rifici VA, de Brito AER and Scott J, The apolipoprotein B gene is constitutively expressed in Hep G2 cells: Regulation of secretion by oleic acid, albumin, and insulin, and measurement of the mRNA half-life. *J Lipid Res* **30**: 1065–1077, 1989.
44. Sato R, Imanaka T, Takatsuki A and Takano T, Degradation of newly synthesized apolipoprotein B-100 in a pre-Golgi compartment. *J Biol Chem* **265**: 11880–11884, 1990.
45. Wilcox HG and Heimberg M, Effects of hyperthyroidism on synthesis, secretion and metabolism of the VLDL apoproteins by the perfused liver. *Biochim Biophys Acta* **1081**: 246–252, 1991.
46. Cianflone KM, Yasruel Z, Rodriguez MA, Vas D and Sniderman AD, Regulation of apo B secretion from Hep G2 cells: Evidence for a critical role of cholesteryl ester synthesis in the response to a fatty acid challenge. *J Lipid Res* **31**: 2045–2055, 1990.
47. Bronfman M, Amigo L and Morales MN, Activation of hypolipidemic drugs to acyl-coenzyme A thioesters. *Biochem J* **239**: 781–784, 1986.
48. Dawson JR, Adams DJ and Wolf CR, Induction of drug metabolizing enzymes in human liver cell line Hep G2. *FEBS Lett* **183**: 219–222, 1985.