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QUANTITATIVE ASSESSMENT OF ENZYME INDUCTION BY PEROXISOME PROLIFERATORS AND APPLICATION TO DETERMINATION OF EFFECTS ON TRIGLYCERIDE BIOSYNTHESIS IN PRIMARY CULTURES OF RAT HEPATOCYTES

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Abstract—Potencies for the induction of peroxisomal fatty acyl-CoA oxidase (FACO) and microsomal laurate hydroxylase (LH) were determined for clofibric acid (CPIB), ciprofibrate (Cipro) and gemfibrozil (Gem) in primary cultures of rat hepatocytes based on complete concentration–response analysis and determination of theoretical maximum inductive responses for Cipro. CPIB and Cipro each induced FACO and LH in a concentration-dependent manner. Scatchard analysis of the data allowed calculation of EC_{50} values (mM) of 0.82 and 0.028 (for FACO) and 0.22 and 0.0081 (for LH) for CPIB and Cipro respectively. The EC_{50} ratios (CPIB/Cipro) were identical (29-fold) for induction of FACO and LH, supporting the concept that these enzymes are induced by CPIB and Cipro through a common mechanism. By comparison, Gem was relatively ineffective as an inducer of FACO and LH. Furthermore, Gem did not antagonize Cipro-mediated enzyme inductions, suggesting that Gem is a peroxisome proliferator of low potency rather than a partial agonist. Based on the potency and time–course profiles observed for induction of FACO and LH, the effects of CPIB, Cipro and Gem on triglyceride (TG) biosynthesis were determined in the cultured rat hepatocytes. Conditions of maximal FACO and LH induction by the drugs did not result in inhibition of TG biosynthesis in the cells. These results support the *in vivo* evidence which indicates that FACO and LH induction are not causally linked to the hypotriglyceridemic actions of peroxisome proliferating drugs.

Certain hypolipidemic drugs, including clofibrate and its structural analogs, are members of the class of chemicals known as peroxisome proliferators [1]. Peroxisome proliferators are distinguished by their abilities to stimulate growth of peroxisomes in size and number along with induction of certain peroxisomal and nonperoxisomal enzymes in the hepatocytes of several species [1]. These peroxisome proliferation-associated enzymes include peroxisomal fatty acyl-CoA oxidase (FACO), the first and rate-limiting enzyme of the peroxisomal fatty acid β-oxidation system, and microsomal laurate hydroxylase (LH), the form(s) of cytochrome P-450 which catalyzes the ω and ω-1 hydroxylations of lauric acid [2, 3].

An additional effect exerted by all peroxisome proliferators is hypotriglyceridemia [4]. The fact that FACO and LH are fatty acid-catabolizing enzymes suggests that there may be an association between the induction of these enzymes and hypotriglyceridemia. Specifically, induction of FACO and LH can be envisioned to result in (1) increased catabolism of cellular fatty acids, (2) decreased fatty acid availability for triglyceride (TG) biosynthesis, and (3) decreased very low density lipoprotein (VLDL) production and release into the blood. However, results of several *in vivo* studies designed to address the relationship between peroxisome proliferation-associated enzyme induction and hypotriglyceridemia have not supported such an association [5–10]. Thus, FACO and/or LH induction does not appear to be the primary hypotriglyceridemic mechanism for peroxisome proliferating hypolipidemic drugs. This is especially likely in view of the other effects exerted by these compounds, such as activation of endothelial lipoprotein lipase activity, which can also provide hypotriglyceridemic mechanisms [11].

It is possible, however, that induction of FACO and/or LH may play a secondary role in peroxisome proliferator-mediated hypotriglyceridemia. This possibility is not easily addressed *in vivo* where the major extrahepatic hypotriglyceridemic actions of these compounds can obscure any minor effects that may be attributable to peroxisome proliferation-associated fatty acid catabolism. As primary cultures of rat hepatocytes respond to the peroxisome proliferating and hypotriglyceridemic effects of drugs [12–18], this system would appear to provide a reasonable method with which to examine the potential relationship between these phenomena in isolation from the rest of the organism. Little such *in vitro* information, however, currently exists [19].

Before it is feasible to study the relationship between peroxisome proliferation-associated

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enzyme induction and hypotriglyceridemia in primary cultured hepatocytes, it is necessary to determine accurately the enzyme inducing potencies of the peroxisome proliferating agents. This is best done by construction of complete concentration–response relationships for FACO and LH induction. Such analysis can serve not only as a necessary prerequisite to further experiments, but can also provide some of the fundamental mechanistic information regarding the phenomenon of peroxisome proliferation-associated enzyme induction which is currently lacking.

The objectives of these experiments were: (1) to determine quantitatively the potencies for induction of FACO and LH of clofibric acid (CPIB), ciprofibrate (Cipro) and gemfibrozil (Gem), three clinically used hypolipidemic drugs [11], in primary cultures of rat hepatocytes, and (2) based on this information, to examine the effects of CPIB, Cipro and Gem on TG biosynthesis in the cultured rat hepatocyte system.

MATERIALS AND METHODS

Materials. Biochemicals and their sources were: collagenase type IV (Cooper Biochemical, Malvern, PA), [1,3-^{14}C]glycerol (48.6 mCi/mmol) (New England Nuclear, Boston, MA), [1-^{14}C]lauric acid (26 mCi/mmol) (Amersham, Arlington Heights, IL), Nu-Serum (Collaborative Research Inc., Lexington, MA), Vitrogen (The Collagen Corp., Palo Alto, CA) and Williams Medium E (Gibco, Grand Island, NY). Other biochemicals were obtained from Sigma Chemical Co. (St Louis, MO). Male Sprague–Dawley rats (150–275 g) were obtained from Harlan Sprague–Dawley, Inc. (Indianapolis, IN), housed in an animal facility accredited by the American Association for the Advancement of Laboratory Animal Care, and given food and water *ad lib.*

Primary culture of adult rat hepatocytes. Hepatocytes were isolated from male Sprague–Dawley rats by the recirculating collagenase perfusion method of Seglen [20] and plated onto collagen-coated petri dishes in supplemented Williams Medium E as described previously [21]. Cell viability was usually greater than 90% as determined by trypan blue exclusion cell counting. The dishes were placed in a humidified 37°, 95% air/5% CO_2 incubator, and cells were allowed 3 hr for attachment to the dishes.

Drugs were dissolved directly in medium by sonication, and the resulting solutions were sterilized by filtration. After the attachment period, medium and unattached cells were aspirated, and fresh medium containing drugs were added. Medium and drugs were subsequently renewed at 24-hr intervals. Aliquots of medium were routinely collected before medium changes to monitor cell viability by determination of lactate dehydrogenase (LDH) leakage.

After the desired time of incubation, dishes were washed twice with ice-cold 0.154 M KCl/50 mM Tris–HCl buffer, pH 7.4 (Tris–KCl), and hepatocytes were harvested by scraping them into 1.25 ml Tris–KCl. The cells were homogenized by sonication, and aliquots of each homogenate were (1) taken for assay of LDH activity, (2) diluted 2:1 with Tris–KCl and frozen at −20° until assayed for FACO activity, and (3) diluted 2:1 with 0.1 N NaOH and refrigerated until assayed for protein content. The remaining quantity of each homogenate was frozen at −80° until assayed for LH activity.

Biochemical assays. Protein was determined by the method of Lowry *et al.* [22]. LDH activity was determined by following the pyruvate-dependent oxidation of NADH at 340 nm. FACO activity was assayed by the fluorometric method of Walusimbi-Kisitu and Harrison [23].

LH activity was assayed by measuring the conversion of [1-^{14}C]lauric acid to combined 11- and 12-hydroxylauric acids according to the thin-layer chromatographic method described by Lake *et al.* [14] with minor modifications. Incubations included 1–2 mg cellular protein and an NADPH-generating system consisting of 0.5 mM NADP, 10 mM glucose-6-phosphate, 0.5 units/ml glucose-6-phosphate dehydrogenase and 6.25 mM $MgCl_2$. Lauric acid was introduced to the samples in 2 μl of 95% ethanol (0.1% of total volume).

TG biosynthesis was determined essentially as described by Lamb *et al.* [18]. After 24 or 72 hr of incubation, cells were washed once with fresh medium. Medium was then added to the dishes which contained 1.5 mM [^{14}C]glycerol (160 μCi/mmol), 1.0 mM palmitate and 1% fatty acid free bovine serum albumin (BSA). After 90 min of incubation, medium was removed from the dishes. The cells were washed twice with Dulbecco's phosphate-buffered saline (DPBS) and then were scraped into 1.25 ml DPBS. The cells were homogenized by sonication, and aliquots were saved as described above. The remaining quantity of each homogenate was frozen at −80° until processed for [^{14}C]triglyceride content. Lipids were extracted from the cell homogenates by the method described by Kates [24] for microorganisms. Neutral lipids were separated by thin-layer chromatography using isopropyl ether–acetic acid (96:4) followed by petroleum ether (b.p. 60–90°)–ethyl ether–acetic acid (90:10:1) in the same direction [24]. TG spots were visualized with iodine and identified by comparing their migrations with those of authentic samples of tripalmitin. The TG spots were scraped into 17 ml plastic scintillation vials, and the silica scrapings were dissolved by addition of 0.5 ml of 95% ethanol and 0.25 ml of hydrofluoric acid. Following the addition of 10 ml of New England Nuclear Formula 963, the samples were counted for ^{14}C content by liquid scintillation spectrometry.

Analysis of data. Each treatment (drug and concentration) was performed in quadruplicate (four dishes) in each experiment (cell preparation). Enzyme activities were expressed per milligram of cellular protein as the mean ± SE. TG biosynthesis data were expressed as nanomoles [^{14}C]glycerol incorporated per milligram of cellular protein ± SE. Means were compared at $P < 0.05$ by one-way ANOVA followed by either *t*-tests (for planned comparisons) or the Student–Newman–Keuls test (for unplanned comparisons).

The EC_{50} values for the induction of FACO and LH were determined in the following manner. Three concentration–response experiments were

performed each of which included Cipro, CPIB and Gem. For each experiment, the Cipro concntration–response data (points between 0.001 and 0.3 mM for FACO; points between 0.001 and 0.1 mM for LH) were subjected to Scatchard analysis, and E_{max} values were determined as the X-intercepts of the least squares lines of the Scatchard plots. Within each experiment, data for each drug were expressed as percentages of the corresponding calculated E_{max}, the percentages subjected to the arcsine transformation, and least squares lines fit to the log concentration-arcsine curves. The EC_{50} values for each experiment were calculated as the abscissa coordinates of the least squares lines corresponding to a 50% response. Mean EC_{50} values and 95% confidence intervals were calculated for each drug from the EC_{50} values determined in the three separate experiments.

RESULTS

Concentration–response effects of CPIB, Cipro and Gem on FACO and LH activities. CPIB and Cipro each caused concentration-dependent increases in FACO activity (Fig. 1). The maximal increase produced by Cipro was 67% higher than that produced by CPIB. The CPIB and Cipro inductive responses decreased markedly at 3.0 and 1.0 mM respectively. Decreases in cellular LDH activity were also observed at these concentrations (data not shown), indicating that the changes in FACO were probably manifestations of drug toxicity. In contrast, Gem produced a much smaller effect on FACO as an increase of only 1.4-fold was seen at the highest nontoxic concentration used (0.3 mM). Scatchard analysis of the Cipro concentration–FACO activity data in these experiments gave least squares lines with correlation coefficients ranging from 0.790 to 0.965. As Fig. 1 shows, the E_{max} values determined from the Scatchard plots agreed well with the responses produced by 0.3 mM Cipro. Also, the EC_{50} values calculated from the Scatchard plots agreed with those estimated directly from the Cipro concentration–response curves. Scatchard analysis of the CPIB concentration–response data did not produce the clear linear relationships obtained for Cipro. From the analysis of the Cipro data, EC_{50} (95% C.I.) values (mM) of 0.82 (0.44–1.5) and 0.028 (0.013–0.060) were determined for CPIB and Cipro respectively.

CPIB and Cipro also each induced LH in a concentration-dependent manner (Fig. 2). Similar maximal responses were produced by both drugs. Again Gem was much less effective, producing only a 1.8-fold induction of LH at 0.3 mM. The CPIB and Cipro responses decreased at 3 and 0.3 mM, respectively, again indicating cytotoxicity at these concentrations. Scatchard analysis of the Cipro concentration–LH activity data in these experiments gave least squares lines with correlation coefficients ranging from 0.907 to 0.976. The E_{max} values obtained from the Scatchard plots agreed well with the responses produced by maximally effective concentrations of Cipro (0.1 mM) and CPIB (1 mM). Also, the EC_{50} values determined from the Scatchard plots agreed with

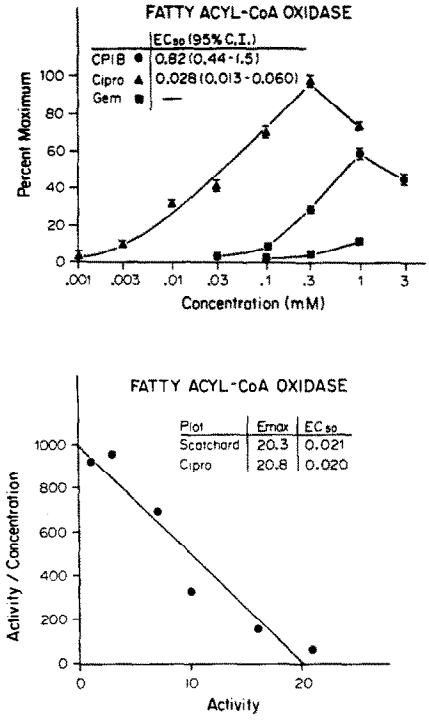

Fig. 1. Upper panel: Concentration–response curves for the induction of fatty acyl-CoA oxidase (FACO) activity by CPIB, Cipro and Gem. Hepatocytes were incubated with the drugs for 72 hr. Results are expressed as a percentage of the maximum response. Each point represents the mean ± SE of determinations from twelve dishes of cells (three separate experiments with four dishes/experiment). The inset shows the EC_{50} values with 95% confidence intervals for CPIB and Cipro. Lower panel: Scatchard plot with least squares line of Cipro concentration–response data from one experiment for FACO induction. Each point represents the mean of determinations from four dishes of cells. The inset shows (1) the E_{max} value determined from the Scatchard plot versus the response produced by 0.3 mM Cipro, and (2) the EC_{50} value determined from the Scatchard plot versus the EC_{50} value estimated from the corresponding Cipro concentration–response curve.

those estimated directly from the Cipro concentration–response curves. EC_{50} (95% C.I.) values (mM) of 0.22 (0.18–0.28) and 0.0081 (0.0052–0.013) were calculated for CPIB and Cipro respectively.

It is apparent from Figs 1 and 2 that Cipro is much more potent than CPIB as an inducer of FACO and LH. Furthermore, the EC_{50} values determined for LH induction by Cipro and CPIB were lower than those obtained for FACO induction. However, the EC_{50} ratios (CPIB/Cipro; mean ± SE, N = 3) for FACO and LH were 29.2 ± 1.0 and 28.5 ± 4.1, respectively, and therefore the inductions of the two enzymes by these drugs were not different.

Time–courses for induction of FACO and LH activities by CPIB, Cipro and Gem and drug combination effects. CPIB (1 mM) and Cipro (0.1 mM) each produced time-dependent increases in FACO and LH activities in the cultured hepatocytes. In the presence of these drugs, FACO activity increased throughout a 96-hr incubation whereas LH activity

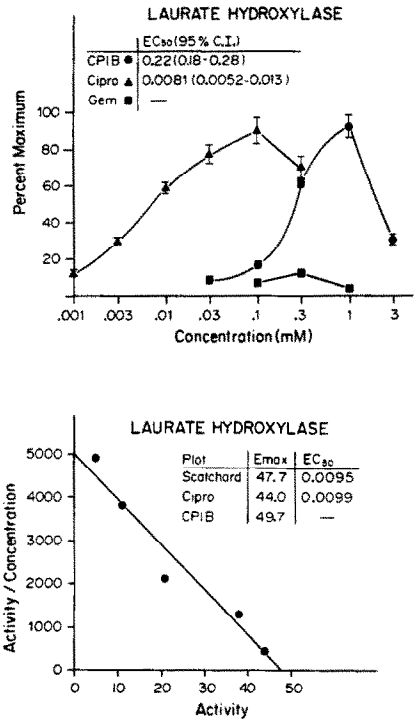

Fig. 2. Upper panel: Concentration–response curves for the induction of laurate hydroxylase (LH) activity by CPIB, Cipro and Gem. Hepatocytes were incubated with the drugs for 72 hr. Results are expressed as a percentage of the maximum response. Each point represents the mean ± SE of determinations from twelve dishes of cells (three separate experiments with four dishes/experiment). The inset shows the EC_{50} values with 95% confidence intervals for CPIB and Cipro. Lower panel: Scatchard plot with least squares line of Cipro concentration–response data from one experiment for LH induction. Each point represents the mean of determinations from four dishes of cells. The inset shows (1) the E_{max} value determined from the Scatchard plot versus the responses produced by 0.3 mM Cipro and 1 mM CPIB, and (2) the EC_{50} value determined from the Scatchard plot versus the EC_{50} value estimated from the corresponding Cipro concentration–response curve.

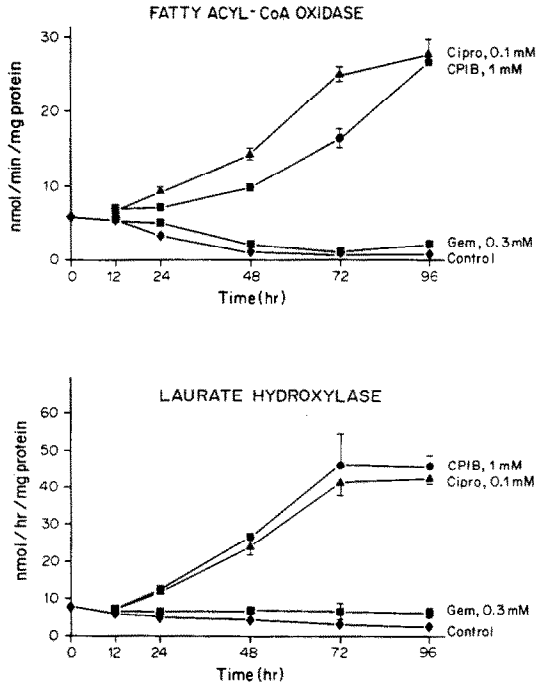

Fig. 3. Time courses for the induction of fatty acyl-CoA oxidase (FACO, upper panel) and laurate hydroxylase (LH, lower panel) activities by 1 mM CPIB, 0.1 mM Cipro and 0.3 mM Gem and for the enzyme activities in untreated cells. Results from one experiment are expressed as activity per milligram of cellular protein, and each point represents the mean ± SE of determinations from four dishes of cells. Similar results were obtained in two separate experiments.

was clearly maximal by 72 hr (Fig. 3). Control FACO and LH activities decreased throughout the incubation period and were 11 and 28% of the 0 hr value at 96 hr respectively. In contrast to CPIB and Cipro, Gem (0.3 mM) was ineffective as an inducer of FACO and LH activities at all time points.

Combinations of Cipro (0.1 mM) and Gem (0.1 and 0.3 mM) and Cipro (0.1 mM) and CPIB (0.3 mM) were tested for any possible inhibition or potentiation of drug effects on FACO and LH activities. FACO induction was significantly lower in cells coincubated with both 0.1 mM Cipro and Gem (0.1 and 0.3 mM) than in those incubated with 0.1 mM Cipro alone (Fig. 4). However, this reduction in response was not concentration dependent. Also, the combination of 0.1 mM Cipro and 0.3 mM CPIB produced a similarly reduced FACO induction response. Similar results were observed for LH induction, except that the reductions observed for the various drug combinations were not significant relative to the response produced by 0.1 mM Cipro alone.

Effects of drugs on glycerolipid biosynthesis and secretion. Experiments were designed to test the effects of CPIB, Cipro and Gem on TG biosynthesis (using incorporation of [^{14}C]glycerol as an index) under conditions in which FACO and LH induction should or should not occur. Accordingly, cells were incubated with CPIB and Cipro for early (24 hr) or late (72 hr) time points on the enzyme induction time–course curves, and were incubated in the presence of low or high drug concentrations. Since Gem did not produce substantial peroxisome proliferation-associated enzyme induction, only the highest nontoxic concentration (0.3 mM) was tested.

Preliminary experiments were performed to optimize the length of the assay incubation and the components of the assay medium for TG biosynthesis (data not shown). The hypothesis under examination is that induction of FACO and LH reduces TG biosynthesis by decreasing the availability of fatty acids. Therefore, conditions for the subsequent experiments were chosen to (1) give high levels of TG biosynthesis, (2) be saturating with respect to glycerol, and (3) be linear with respect to palmitate. Under this design, effects on cellular fatty acid concentration should translate to effects on TG biosynthesis. Assay incubations were conducted, therefore for 90 min and included 1.5 mM [^{14}C]glycerol and

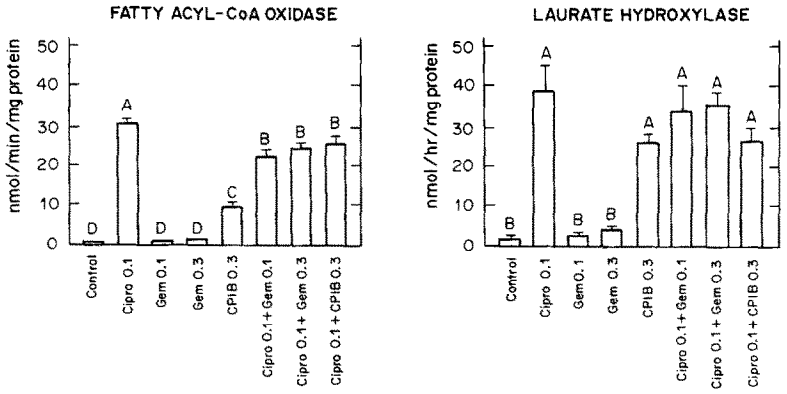

Fig. 4. Effects of drug combinations on the induction of fatty acyl-CoA oxidase (FACO, left panel) and laurate hydroxylase (LH, right panel) activities. Cells were incubated for 96 hr in the presence of the indicated drugs. All drug concentrations are millimolar. Results from one experiment are expressed as activity per milligram of cellular protein, and each bar represents the mean ± SE of determinations from four dishes of cells. Bars with different letters are significantly different at $P < 0.05$. Similar results were obtained in two separate experiments.

1.0 mM palmitate. As [^{14}C]glycerolipid in the medium was found to constitute only approximately 5% of that in the cells, incorporation of [^{14}C]glycerol into cellular TG was considered to represent total TG biosynthesis.

Peroxisome proliferating agents are known to exert a variety of biological effects, including direct inhibitions of lipid biosynthetic enzymes [25] as well as the enzyme inductions which occur following chronic drug treatment. Experiments were designed, therefore, to discriminate between drug effects on TG biosynthesis attributable to acute inhibitory actions on lipid biosynthetic enzymes and those due to the FACO and LH inductions resulting from chronic drug treatment. Thus, drugs were included (1) only during the 24- or 72-hr incubations with the standard supplemented Williams Medium E (referred to as the "induction incubation") to determine the changes in TG biosynthesis that resulted from the chronic effects of the drugs on the system (this is referred to as a "Drug/No Drug" incubation); (2) only during the 90-min incubation with the medium containing [^{14}C]glycerol and palmitate ("assay incubation") to determine the changes in TG biosynthesis that resulted from the acute effects of the drugs upon the lipid-metabolizing enzymes ("No Drug/Drug" incubation); and (3) during both the induction and assay incubations to most closely mimic the *in vivo* state ("Drug/Drug" incubation).

In 24-hr cultured hepatocytes, CPIB and Cipro produced either no changes or small increases in TG biosynthesis when present during the induction incubation (Drug/No Drug or Drug/Drug) (Fig. 5). The decrease seen when Cipro was present during both the induction and assay incubations (Drug/Drug) is probably attributable to a toxic effect produced by this treatment as LDH leakage was increased significantly in this group (data not shown). No changes in TG synthesis were seen when the drugs were present only during the assay incubation (No Drug/Drug). In 72-hr cultured cells, CPIB, Cipro and Gem produced either no changes or concentration-dependent increases in TG biosynthesis

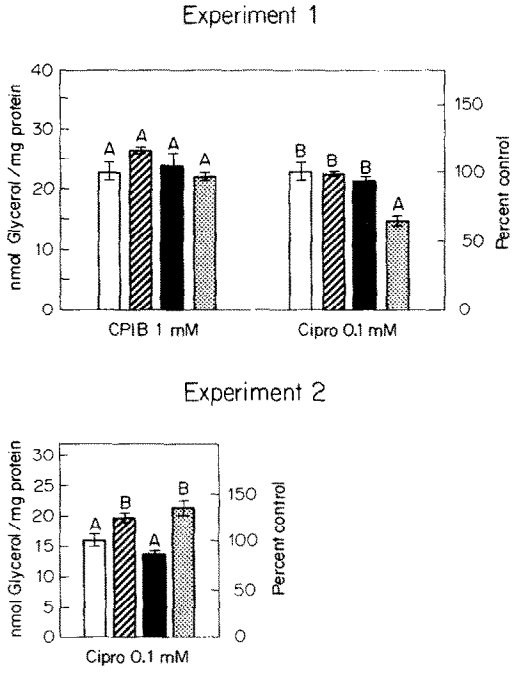

Fig. 5. Effects of CPIB and Cipro on the incorporation of [^{14}C]glycerol into cellular triglycerides (TG) in 24-hr cultured hepatocytes. Results from two separate experiments are shown. Induction and assay incubation conditions are defined in the text. Key: (□) No Drug/No Drug; (▨) Drug/No Drug; (■) No Drug/Drug and (▩) Drug/Drug. Values are expressed as nanomoles [^{14}C]glycerol incorporated per milligram of cellular protein and as a percent of control (no drug/no drug incubation), and each bar represents the mean ± SE of determinations from four dishes of cells. Bars with different letters (within one drug) are significantly different at $P < 0.05$.

when present during the induction incubation (Drug/No Drug and Drug/Drug) and no changes when present only during the assay incubation (No Drug/Drug) (Fig. 6).

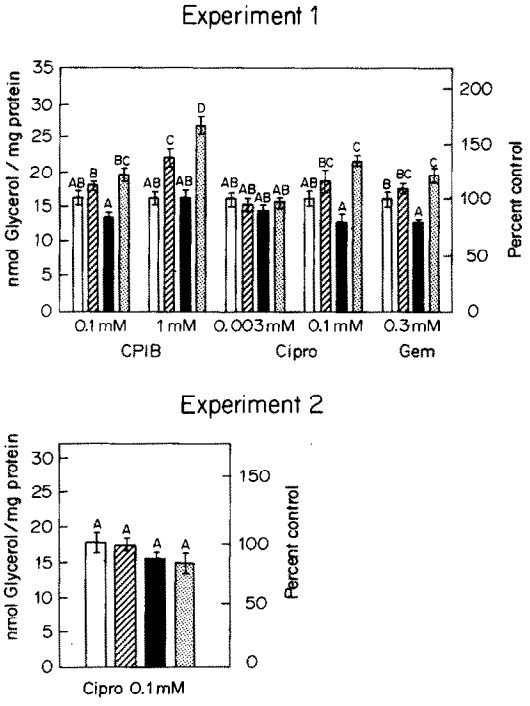

Fig. 6. Effects of CPIB, Cipro and Gem on the incorporation of [^{14}C]glycerol into cellular triglycerides (TG) in 72-hr cultured hepatocytes. Results from two separate experiments are shown. Induction and assay incubation conditions are defined in the text. Key: (□) No Drug/No Drug; (▨) Drug/No Drug; (■) No Drug/Drug and (▩) Drug/Drug. Values are expressed as nanomoles [^{14}C]glycerol incorporated per milligram of cellular protein and as a percent of control (no drug/no drug incubation), and each bar represents the mean ± SE of determinations from four dishes of cells. Bars with different letters (within one drug) are significantly different at $P < 0.05$.

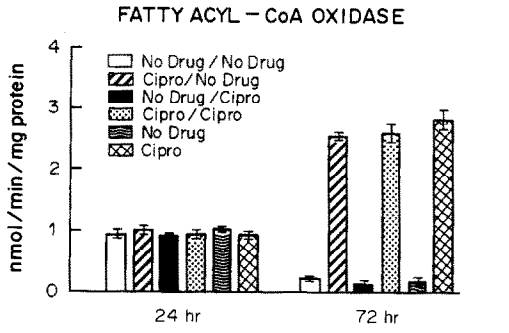

Fig. 7. Effects of 0.1 mM Cipro on fatty acyl-CoA oxidase (FACO) activity in 24- and 72-hr cultured hepatocytes. Incubation conditions are given in the inset and are as defined in the text. The last two groups (No Drug and Cipro) were not exposed to the assay medium. Results from one experiment are shown and values are expressed as activity per milligram of cellular protein; each bar represents the mean ± SE of determinations from four dishes of cells.

Figure 7 shows that FACO activity was appropriately induced by 0.1 mM Cipro under the various incubation conditions. Thus, Cipro produced 13-fold increases in FACO activity after 72 hr of exposure when present during the induction incubation (Drug/No Drug and Drug/Drug). The induced FACO activity was approximately the same as that produced in cells not exposed to the assay medium, indicating that the cells were not affected adversely by the high concentrations of palmitate and glycerol present in the assay medium.

DISCUSSION

Our studies demonstrate that primary cultures of rat hepatocytes provide a practical system for performing concentration–response analysis of the effects of peroxisome proliferators. Previously, Lewis *et al.* [26] examined the concentration–response effects of thirteen compounds on the induction of peroxisomal fatty acid β-oxidation in primary cultures of rat hepatocytes. They expressed the inductions as a percentage of control activity (after 70 hr in culture) and estimated the potencies of the compounds as the concentrations which produced 3-fold inductions over control. However, we have found that after 72 hr in culture (1) control enzyme activities are usually very low and (2) these low control activities may fluctuate substantially from one experiment to another. Expressing potencies as multiples of small values that fluctuate considerably between experiments may result in estimates which represent different parts of the concentration–response curve in different experiments. To overcome this problem, we found that theoretical maximum induction responses could be predicted from Scatchard plots of the Cipro concentration–response data, and that these theoretical maxima agreed well with the observed maximum responses produced by Cipro (for FACO and LH) and CPIB (for LH). Therefore, we were able to express our concentration–response data as percentages of the maximum drug response and estimate EC_{50} values as the concentrations of the compounds that produced 50% of the maximum response. Treatment of the data in this way should result in greater accuracy of EC_{50} estimations and in greater consistency of drug potency determinations between experiments.

Reddy and co-workers have proposed that peroxisome proliferators exert their effects by acting through one or more specific peroxisome proliferator receptors [1, 27]. Although a cytosolic peroxisome proliferator binding protein has been detected and isolated [28, 29], there is no convincing evidence to demonstrate that this protein is an actual receptor. In fact, there is little available evidence to demonstrate that peroxisome proliferation-associated phenomena satisfy any of the requirements for a pharmacological receptor-mediated activity. As stated above, Scatchard analysis lincarized our Cipro concentration–response data for the induction of FACO and LH. While this does not by any means prove the existence of a receptor, it does provide evidence that, consistent with a receptor-mediated event, the induction of FACO and LH occurs through a saturable mechanism in hepatocyte culture.

Our results revealed some differences in the concentration–response and time–course induction profiles of FACO and LH by CPIB and Cipro. These differences included (1) different maximum responses for FACO induction by the two drugs, but identical maxima for LH induction; (2) lower EC_{50} values for the induction of LH by the drugs than for the induction of FACO; and (3) somewhat different time courses for the induction of the two enzymes. The available information suggests that the inductions of FACO and LH occur through a common mechanism. Thus, Lake *et al.* [13] reported a high degree of correlation between the inductions of peroxisomal fatty acid β-oxidation and LH produced by single concentrations of ten peroxisome proliferating agents in primary cultures of rat hepatocytes. Although our results illustrate some differences in the induction profiles of FACO and LH, they actually also argue in favor of a common induction mechanism. It seems unlikely that two completely different mechanisms would possess identical potency differences for two drugs. The EC_{50} ratios of CPIB/Cipro were identical for the induction of FACO and LH.

Our data indicated that control FACO and LH activities decreased over time in culture. This finding is consistent with the decreases in certain differentiated liver characteristics, including cytochrome P-450 content, which have been reported previously in primary hepatocyte cell culture [30]. Such phenotypic changes are no considered to represent diminished cell viability [30]. We began our drug treatments only 3 hr after plating when notable levels of FACO and LH activities remained. Therefore, the increases in these enzyme activities that occurred following drug treatment (relative to same time controls) may reflect some stabilization of the existing enzymes either by the drugs themselves or as a result of some effect of the drugs on the internal environment of the cells. However, the fact that the final induced enzyme activities were considerably higher than the 0 hr values (4.5- to 5.8-fold) suggests that substantial *de novo* synthesis of the enzymes occurred. This conclusion is bolstered by evidence which indicates that peroxisome proliferating compounds increase mRNA levels of several peroxisome proliferation-associated enzymes [31–33].

Our experiments showed that, unlike Cipro and CPIB, Gem was nearly ineffective as an inducer of FACO and LH in our cell culture system. This was somewhat unexpected since Gem has been shown to be a hepatic peroxisome proliferating agent by others [1] and has even been reported to cause peroxisome proliferation in monkeys [34]. However, in their study of the effects of ten compounds in rats, Lalwani *et al.* [4] found that Gem produces comparatively small inductions of hepatic carnitine acetyltransferase and peroxisomal fatty acid β-oxidase.

Although peroxisome proliferation has been proposed to occur through a receptor mechanism [1, 27], no compounds that could possibly be acting as receptor antagonists have yet been identified. Since Gem was a relatively poor inducer of FACO and LH, we speculated that it may function as a partial agonist with regard to these enzyme inductions which should then inhibit the induction produced by agonists such as Cipro and CPIB. Our results showed that, although Gem reduced the inductions of FACO and LH by Cipro, these reductions were unlikely to have been due to any specific inhibitory effect. First, the reductions in Cipro-induced enzyme activities by Gem were not concentration dependent, as they should have been if Gem was acting as a receptor antagonist. Second, similar reductions in the induction of FACO and LH by Cipro were produced by CPIB, another peroxisome proliferation agonist. The reductions were, therefore, more likely a reflection of the toxicity produced by these drug combinations.

Available *in vivo* data strongly suggest that FACO and/or LH induction is not the major hypotriglyceridemic mechanism of peroxisome proliferating drugs [5–10]. The primary cultured rat hepatocyte system allows examination of the effects of peroxisome proliferators on TG biosynthesis in isolation from the rest of the organism. Drug concentrations and incubation times can be selected to achieve either low or high levels of peroxisome proliferation-associated enzyme induction, and to discriminate between acute and chronic drug effects. In this regard, we found either no changes or increases, but never decreases, in TG biosynthesis when substantial FACO and LH induction should have occurred, i.e. following 72-hr incubation with 1 mM CPIB or 0.1 mM Cipro. Thus, in these experiments FACO and LH inductions were not associated with decreased TG biosynthesis. These findings provide further evidence which indicates that FACO and LH inductions are not causally linked to hypotriglyceridemia for this class of drugs.

In summary, we have (1) established, in a quantitative manner, concentration–response and time–course profiles for the induction of FACO and LH by CPIB, Cipro and Gem; (2) shown that Cipro and CPIB induced FACO and LH in a saturable manner; (3) provided evidence in support of the concept that the inductions of FACO and LH occur through a common mechanism; (4) shown that Gem was a FACO and LH inducer of low potency rather than a partial agonist; and (5) demonstrated that conditions of maximal FACO and LH induction did not result in inhibition of TG biosynthesis in a primary cultured rat hepatocyte system. These *in vitro* findings support the *in vivo* evidence that FACO and LH induction are not responsible for the hypotriglyceridemic actions of this class of drugs.

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