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IN VIVO AND *IN VITRO* PEROXISOME PROLIFERATION PROPERTIES OF SELECTED CLOFIBRATE ANALOGUES IN THE RAT

STRUCTURE–ACTIVITY RELATIONSHIPS

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Abstract—We have examined, relative to clofibric acid (CPIB), the effects of a chemical series of phenoxyacetic acids and of two asymmetric CPIB analogues, the *R*(+)- and *S*(−)-enantiomers of 2-(4-chlorophenoxy)propionic acid (4-CPPA) and 2-(4-chlorophenoxy)butyric acid (4-CPBA), on hepatic peroxisome proliferation both *in vivo* and *in vitro* utilizing cholesterol-fed rats and primary cultured rat hepatocytes respectively. Peroxisome proliferation was assessed by measuring changes in peroxisomal fatty acyl-CoA oxidase (FACO) and microsomal laurate hydroxylase (LH) activities as well as by electron microscopic examination of 3,3′-diaminobenzidine-stained liver slices. CPIB and enantiomers of 4-CPPA and 4-CPBA (0.6 mmol/kg/day for 7 days) produced hepatomegaly, lowered serum cholesterol levels, and caused 4.7- to 12.9-fold and 2.9- to 6.1-fold increases in hepatic FACO and LH activities, respectively, in cholesterol-fed rats. Electron micrographs of liver cells showed an increased number of peroxisomes from cholesterol-fed rats given *S*(−)-4-CPBA and CPIB. Likewise, these compounds (0.03 to 1.0 mM) induced FACO and LH in primary rat hepatocyte cultures after 72 hr. *R*(+)- and *S*(−)-Enantiomers of 4-CPPA produced similar concentration-dependent and maximal increases in both FACO and LH activities, whereas enantiomeric selectivity [*S*(−) > *R*(+)] for the induction of these two enzymes was observed with the isomers of 4-CPBA. The increases in the activities of FACO and LH caused by *S*(−)-4-CPBA were similar to those elicited by 1.0 mM CPIB (58.6- and 9.8-fold respectively). These results show that the enantiomers of 4-CPPA and 4-CPBA induce the peroxisome proliferation-associated enzymes FACO and LH *in vivo* and *in vitro*, and that the *S*(−)-isomer of 4-CPBA causes a greater induction of FACO and LH *in vitro* than its corresponding *R*(+)-isomer, indicating that these two enzymes are induced in an enantioselective manner. Optimal induction of the peroxisome proliferation-associated enzymes FACO and LH in rat hepatocyte cultures was produced by phenoxyacetic acids possessing (1) a chlorine atom at the 4-position of the phenyl ring, (2) a dimethyl or mono-ethyl substitution at the α-carbon atom of the carboxylic acid side chain; and (3) an *S*(−)-orientation for chiral analogues possessing a mono-ethyl group at the α-carbon atom of the carboxylic acid side chain. These results indicate that changes in the chemical and stereoisomeric structures of phenoxyacetic acids alter peroxisome proliferation and are consistent with a hypothesized receptor-mediated mechanism, and by employing the proper enantiomeric form, a greater dissociation of beneficial lipid lowering actions from adverse carcinogenic effects of peroxisome proliferators may be obtained.

Clofibric acid [2-(4-chlorophenoxy)-2-methylpropionic acid; CPIB] (Fig. 1), the active form of clofibrate, is a hypolipidemic drug used for the treatment of hyperlipoproteinemias [1]. In addition, this compound and structurally related (e.g. ciprofibrate and nafenopin) and unrelated (e.g. Wy-14,643 and BR-931) hypolipidemic drugs, plasticizers [e.g. di(2-ethylhexyl)phthalate] and herbicides (e.g. 2,4,5-trichlorophenoxyacetic acid) comprise a class of chemicals referred to as peroxisome proliferating agents [2, 3]. Chronic treatment of rodents with these compounds produces a lowering of serum triglyceride levels, hepatomegaly, an increase in the size and number of hepatic peroxisomes, and the induction of a number of peroxisomal and non-peroxisomal enzymes [2–4]. Furthermore, peroxisome proliferators cause an increased incidence of hepatocarcinomas in rodents which has been related to an increase in reactive oxygen species produced by induction of peroxisomal enzymes [2, 5–7].

Several studies have examined the structural requirements of hepatic peroxisome proliferation with most emphasizing an *in vivo* evaluation of phenoxyacetic acids (PAA) chemically related to clofibrate or to phenoxy acid herbicides [8–11]. In recent years, we [12, 13] and others [14–16] have demonstrated that primary cultures of rat hepatocytes are a useful model in which to investigate the effects of peroxisome proliferators. This *in vitro* hepatocyte model has shown morphological and biochemical responses to peroxisome proliferators that are in excellent agreement with *in vivo* studies, thereby making it possible to study structure–activity relationships of compounds that induce peroxisome proliferation. To date, little is known how structural and/or stereochemical changes of PAA affect the abilities of these compounds to induce hepatic

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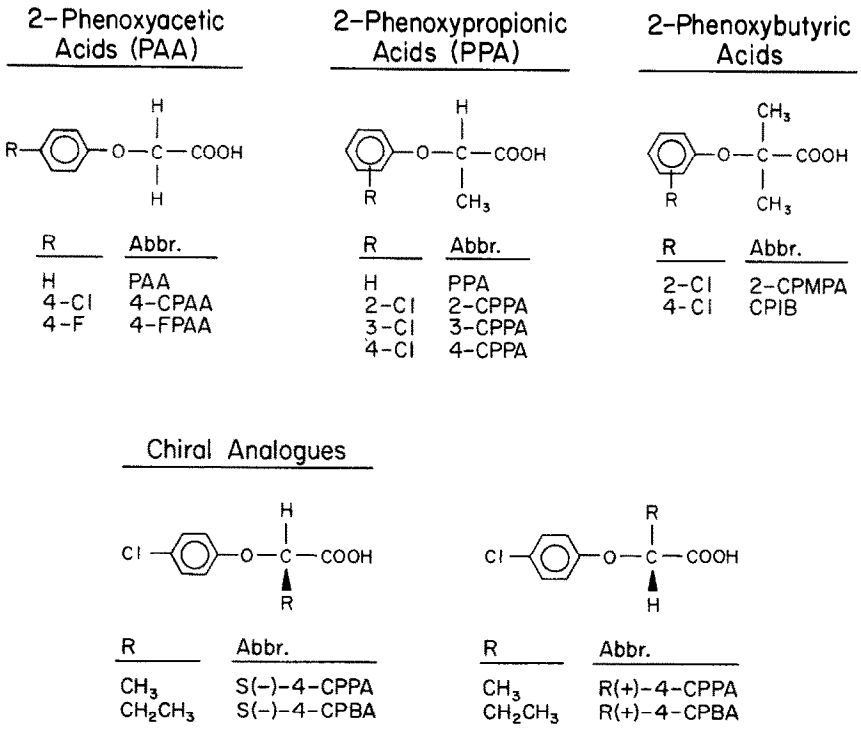

Fig. 1. Chemical structures and abbreviations of phenoxyacetic acid (PAA) analogues used.

peroxisome proliferation. However, one common feature that most peroxisome proliferators possess is an acidic function, the most common of which is a carboxylic acid moiety [2–7].

The existence of a specific cytosolic receptor capable of binding peroxisome proliferating compounds has been reported [17, 18] and has been proposed to mediate the phenomenon of peroxisome proliferation [2, 7, 17, 18]. One important property often demonstrated by many of the enzymes and receptors which modulate physiologic processes is enantiomeric selectivity for the bioactive agents with which they interact [19, 20]. The stereoselective induction of several liver enzyme systems, including lauric acid 12-hydroxylation, has been demonstrated [21]; therefore, it was of interest to determine if the induction of enzymes associated with hepatic peroxisome proliferation could be elicited in an enantioselective manner by chiral analogues of CPIB. The *R*(+)- and *S*(−)-enantiomers of 2-(4-chlorophenoxy)propionic acid (4-CPPA) and 2-(4-chlorophenoxy)butyric acid (4-CPBA) are structurally very similar to clofibric acid (Fig. 1) but possess a chiral center at the α-carbon atom of the carboxylic acid moiety allowing for the assessment of the enantioselective induction of peroxisome proliferation. The isomers of 4-CPPA and 4-CPBA possess hypolipidemic and enantioselective [*R*(+)- > *S*(−)-isomer] platelet antiaggregatory properties [22]. In contrast, the *S*(−)-isomers of 4-CPPA and 4-CPBA were found to possess highly specific inhibitory effects on chloride conductance in rat extensor digitorum longus muscle fibers, a model for the adverse myotonic activity demonstrated by this class of compounds [23]. Therefore, by employing the appropriate enantiomeric forms of these compounds, the beneficial hypolipidemic and platelet antiaggregatory effects may be dissociated from adverse actions such as peroxisome proliferation-associated carcinogenesis.

The primary objectives of this study were to determine the abilities of the enantiomers of 4-CPPA and 4-CPBA to induce peroxisome proliferation *in vivo* by utilizing cholesterol-fed rats and *in vitro* using primary cultured rat hepatocytes and to examine the effects of a number of chemically related phenoxyacetic acids in primary hepatocyte cultures in order to define structural features necessary for peroxisome proliferation. Peroxisome proliferation was assessed by electron microscopic examination of liver slices from animals treated with these compounds and by measurement of the activities of two enzymes known to be induced by peroxisome proliferating agents. The measured enzymes were fatty acyl-CoA oxidase (FACO), the first and rate-limiting enzyme of the peroxisomal fatty acid β-oxidation system [24, 25], and microsomal laurate hydroxylase (LH), a cytochrome P450 (P452) isozyme(s) which catalyzes the ω and ω-1 hydroxylation of lauric acid [26] and is induced by peroxisome proliferating agents [27]. A preliminary report of this work has appeared [28]. The chemical structures and abbreviations of all compounds are given in Fig. 1.

MATERIALS AND METHODS

Materials. Biochemicals and their sources were: *R*(+)- and *S*(−)-2-(4-chlorophenoxy)propionic acid

and 2-(4-chlorophenoxy)butyric acid (gifts from Dr. V. Tortorella, Department of Medicinal Chemistry, University of Bari, Bari, Italy); 5-aminolevulinic acid, antibiotic/antimycotic solution, bovine serum albumin (fraction V), 2-(2-chlorophenoxy)-2-methylpropionic acid, clofibric acid, deoxyribonuclease I, dexamethasone, FAD, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, hydrocortisone 21-hemisuccinate, 12-hydroxydodecanoic acid, insulin, lauric acid, NADH, NADP, palmitoyl-CoA, peroxidase, phenoxyacetic acid, pyruvic acid, scopoletin and trypan blue (Sigma Chemical Co., St. Louis, MO); 4-chlorophenoxyacetic acid, 2-(2-chlorophenoxy)propionic acid, 2-(3-chlorophenoxy)propionic acid, 2-(4-chlorophenoxy)propionic acid, 4-fluorophenoxyacetic acid, 2-phenoxypropionic acid and 3,3′-diaminobenzidine tetrahydrochloride dihydrate (Aldrich Chemical Co., Inc., Milwaukee, WI); [1-^{14}C]lauric acid (26 mCi/mmol) (Amersham, Arlington Heights, IL); Vitrogen (The Collagen Corp., Palo Alto, CA); collagenase type IV (Cooper Biomedical, Malvern, PA); Nu-Serum (Collaborative Research Inc., Lexington, MA); Williams Medium E (Gibco, Grand Island, NY); and Formula 963 (New England Nuclear, Boston, MA). Other reagents and organic solvents were of the highest purity available. Other materials used and their sources were: Anasil G-250 thin-layer chromatography plates (The Foxboro Co., North Haven, CT); Corning tissue culture dishes (100 × 20 mm) and Spectra Mesh (111 and 202 μm) (Fisher Scientific, Cincinnati, OH) and Millex-GV filters (0.22 μm) (Millipore Corp., Medford, MA). Male Sprague–Dawley rats (150–300 g) were obtained from Harlan Sprague–Dawley, Inc. (Indianapolis, IN) and housed in an American Association for the Advancement of Laboratory Animal Care accredited vivarium maintained at 25–26° with an alternating 12-hr light and dark cycle and with free access to Purina rat chow and water.

In vivo *treatment.* Adult male Sprague–Dawley rats (230–310 g) were maintained on a high cholesterol (1%) semisynthetic diet as described by Witiak *et al.* [29]. One group of six animals received only the semisynthetic diet with no cholesterol. Following 1 week of this high cholesterol diet, the rats were divided into six groups of six rats each and were treated twice daily with 0.3 mmol/kg body weight of the appropriate compound in a 0.25% methylcellulose vehicle via intragastric administration for 7 days while being maintained on the high cholesterol diet. Control animals were administered only the vehicle for 1 week. The body weights of the treatment groups did not change significantly during the course of the treatment. After this treatment period, blood was withdrawn for serum cholesterol determinations [22], and the animals were then killed, the livers were removed, cleaned and weighed, and liver slices were taken for electron microscopic examination. The remainder of the liver was stored at −70° until biochemical assays could be performed.

Liver slices were stained for the presence of peroxisomes with 3,3′-diaminobenzidine as described by Novikoff *et al.* [30] and subsequently prepared for electron microscopic examination. A 3- to 4-g section of liver from each animal was thawed and homogenized using a Teflon–glass homogenizer and diluted 1:4 with ice-cold 0.154 M KCl/20 mM Tris–HCl, pH 7.4 (Tris–KCl) buffer. The homogenate was then centrifuged at 600 *g* for 10 min, the pellet was discarded, and the supernatant fraction was centrifuged at 9000 *g* for 20 min. The resultant pellet from this spin (peroxisome-containing mitochondrial fraction) was then resuspended and centrifuged again at 9000 *g* for 20 min while the supernatant fraction was centrifuged at 105,000 *g* for 1 hr. The pellet from the second 9000 *g* spin was resuspended in Tris–KCl and homogenized by sonication. Aliquots of the homogenate were then taken for (1) a 1:50 dilution in distilled water and refrigerated until assayed for protein content, and (2) a 1:100 dilution in Tris–KCl and frozen at −20° until assayed for FACO activity. The pellet from the 105,000 *g* spin (microsomal fraction) was recentrifuged at 105,000 *g* for 1 hr, and the pellet was resuspended in Tris–KCl and homogenized by sonication. Aliquots of the homogenates were taken for (1) a 1:50 dilution in distilled water and refrigerated until assayed for protein content, and (2) a 1:20 dilution in Tris–KCl and frozen at −70° until assayed for LH activity.

Primary cultures of adult rat hepatocytes. Hepatocytes were isolated from male Sprague–Dawley rats by the recirculating collagenase perfusion method described by Seglen [31] with slight modifications. Immediately following a 10–15 min perfusion of the liver with collagenase, the cells were placed in approximately 25 mL of Williams Medium E containing 1% bovine serum albumin and filtered through 202 μm and 111 μm polyethylene Spectra/Mesh. This crude suspension of cells was then purified by centrifuging three times for 3 min at 50 *g* with the first two spins containing a few milligrams of deoxyribonuclease I. The trypan blue exclusion cell counting method was used to determine cell viability (usually greater than 90%) and cell yield. The hepatocyte suspension was then diluted to 1 million cells/2 mL with Williams Medium E supplemented with 100 μM 5-aminolevulinic acid, 5 μM dexamethasone, 10 μM hydrocortisone 21-hemisuccinate, 20 mI.U./mL insulin, 10% Nu-Serum, 50 units/mL penicillin, 50 μg/mL streptomycin and 0.125 μg/mL amphotericin B. The cells were plated onto collagen coated (Vitrogen) 100 mm petri dishes at a density of 2.5 million cells/5 mL medium and placed into a humidified 37°, 95% air/5% CO_2 incubator and allowed an attachment period of 3 hr.

The compounds were dissolved directly into the medium by sonication and the resulting solutions were sterilized by filtration through 0.22 μm Millex-GV filters. Following the attachment period, freshly prepared medium containing the compounds was added. Medium and compounds were then changed every 24 hr. By using protein content and lactate dehydrogenase (LDH) activity as a criteria for cultured hepatocyte viability, all compounds tested were found to be essentially noncytotoxic to the cultured cells at the highest concentration used (1.0 mM).

After a 72-hr incubation period, the dishes of hepatocytes were washed twice with ice-cold 0.154 M

KCl/50 mM Tris–HCl buffer, pH 7.4, and the hepatocytes were harvested by scraping into 1.25 mL of the buffer. The hepatocytes were homogenized by sonication, and aliquots of each homogenate were (1) taken for determination of LDH activity, (2) diluted 1:2 with 0.1 N NaOH and refrigerated until assayed for protein content, and (3) diluted 1:2 with Tris–KCl buffer and frozen at −20° until assayed for FACO activity. The remaining cell homogenates were stored at −70° until assayed for LH activity.

Biochemical assays of cell homogenates. Protein concentrations of the cell homogenates were determined by the method of Lowry *et al.* [32] utilizing bovine serum albumin as the standard. Activities of lactate dehydrogenase (LDH) in medium and cell homogenate were determined by measuring the pyruvate-dependent oxidation of NADH at 340 nm for 1–3 min at 30°. FACO activity was assessed by the fluorometric measurement of H_2O_2 production from palmitoyl-CoA as described by Walusimbi-Kisitu and Harrison [33].

LH activity was determined by measuring the amount of conversion of [1-^{14}C]lauric acid to combined 11- and 12-hydroxylauric acids as described by the radiometric and thin-layer chromatographic method of Parker and Orton [34] as modified by Lake *et al.* [27] for use with cultured rat hepatocytes. Incubation mixtures included 1–2 mg cellular protein, an NADPH-generating system (10 mM glucose-6-phosphate, 0.5 units/mL glucose-6-phosphate dehydrogenase, 6.25 mM $MgCl_2$ and 0.5 mM NADP) and 60 μM (0.11 μCi) [1-^{14}C]lauric acid in 50 mM Tris–HCl, pH 7.5, for a total volume of 2.0 mL. Lauric acid was added to the incubation mixtures in 2 μL of 95% ethanol (0.1% of total volume) in order to initiate the reaction.

Analysis of data. For the *in vivo* analysis, the number of samples for each treatment group was five livers. FACO activity was expressed as nanomoles H_2O_2 produced per minute per milligram mitochondrial fraction protein, while LH activity was expressed as nanomoles lauric acid hydroxylated per hour per milligram microsomal protein. For the *in vitro* evaluation of the effects of the compounds, all dishes of hepatocytes receiving a given treatment of the same compound and its respective concentration were grouped together over all of the experiments for the calculation of the results (N = 4–12 dishes). FACO and LH activities were expressed as nanomoles H_2O_2 produced per minute per milligram cellular protein and nanomoles lauric acid hydroxylated per hour per milligram cellular protein respectively. For comparative studies of PAA analogues in cultured hepatocytes, concentration–response data for induction of FACO and LH activities were normalized by percentage conversion relative to the effect obtained with 1.0 mM CPIB. This concentration of CPIB was found to give a maximal inductive response for these enzyme activities, and was included in all experiments for the normalization of data. Serum cholesterol levels of the drug treatment groups were compared at $P < 0.01$ using a one-way analysis of variance. Comparisons among means of the FACO and LH activities were made at $P < 0.05$ using one-way analysis of variance followed by the Student–Newman–Keuls test.

RESULTS

Effects of CPIB and the enantiomers of 4-CPPA and 4-CPBA on liver weights, liver-to-body weight ratios, serum cholesterol concentrations and hepatic FACO and LH activities in cholesterol-fed rats. The liver weight of the cholesterol-fed control group was 9.79 g, and the liver weights of animals treated with *R*(+)-4-CPBA, *S*(−)-4-CPBA and CPIB for 7 days were increased significantly ($P < 0.05$) relative to this control group by 1.5-, 1.3- and 1.3-fold respectively (Table 1). Both enantiomers of 4-CPPA caused 1.2-fold increses in liver weight but these were not significant. All compounds significantly ($P < 0.05$) increased the liver-to-body weight ratios relative to the control value of 3.63%. Treatment of the animals with *R*(+)- and *S*(−)-4-CPPA resulted in 1.2-fold increases in the liver-to-body weight ratios for both enantiomers, whereas *R*(+)- and *S*(−)-4-CPBA caused 1.5- and 1.4-fold increases, respectively, and CPIB treatment increased the liver-to-body weight ratio 1.3-fold. After 7 days of treatment, all compounds significantly ($P < 0.01$) decreased serum cholesterol concentrations from the control level of 267 mg/dL (Table 1). The magnitude of these reductions ranged from 28% for *S*(−)-4-CPBA to 44% for CPIB.

The hepatic peroxisomal FACO activity in the cholesterol-fed control animals was 3.89 nmol H_2O_2 produced/min/mg mitochondrial protein (Table 2). Treatment with CPIB, *R*(+)-4-CPBA and *S*(−)-4-CPBA produced significant ($P < 0.05$) increases in hepatic FACO activity that were 10.3-, 9.3- and 12.9-fold greater, respectively, than that seen in the control animals. The *R*(+)- and *S*(−)-enantiomers of 4-CPPA caused 5.5- and 4.7-fold increases, respectively, in FACO activity but these increases were not statistically significant. All of the compounds increased hepatic microsomal LH activities to significantly ($P < 0.05$) greater levels than the cholesterol-fed control LH activity of 51.51 nmol lauric acid hydroxylated/hr/mg microsomal protein (Table 2). Treatment with CPIB, *R*(+)-4-CPBA and *S*(−)-4-CPBA increased LH activity 5.6-, 6.1- and 5.2-fold, respectively, while the *R*(+)- and *S*(−)-enantiomers of 4-CPPA produced 4.6- and 2.9-fold increases in LH activity, respectively, relative to control. Electron micrographs of liver cells from cholesterol-fed rats administered *S*(−)-4-CPBA and CPIB show an increase in the number and size of peroxisomes relative to the cholesterol-fed control rat liver cells (Fig. 2).

Effects of CPIB and the enantiomers of 4-CPPA and 4-CPBA on FACO and LH activities in primary cultured rat hepatocytes. CPIB and the enantiomers of 4-CPPA and 4-CPBA each produced concentration-dependent increases in FACO and LH activities when incubated with cultured rat hepatocytes for 72 hr (Figs. 3 and 4). The control FACO activity was 0.159 nmol H_2O_2/min/mg cellular protein at this time, and all compounds produced significant ($P < 0.05$) increases in activity at concentrations of 0.3 and 1.0 mM. CPIB increased FACO activity 58.6-fold above control activity at 1.0 mM. The *R*(+)- and *S*(−)-isomers of 4-CPPA produced similar concentration–response curves for

Table 1. Effects of *R*(+)-4-CPPA, *S*(−)-4-CPPA, *R*(+)-4-CPBA, *S*(−)-4-CPBA and CPIB on the liver weights, liver-to-body weight ratios and serum cholesterol levels of cholesterol-fed rats

Treatment*	Liver weight† (g)	Liver-to-body weight ratio† (%)	Serum-cholesterol‡ (mg/dL)
Cholesterol-fed	9.79 ± 0.42	3.63 ± 0.10	267 ± 21.1
R(+)-4-CPPA	11.91 ± 0.41	4.31 ± 0.19§	191 ± 14.1‖
S(−)-4-CPPA	11.63 ± 0.52	4.23 ± 0.11§	184 ± 27.6‖
R(+)-4-CPBA	14.26 ± 0.91§	5.42 ± 0.18§	184 ± 17.2‖
S(−)-4-CPBA	13.18 ± 0.27§	5.02 ± 0.04§	193 ± 13.7‖
CPIB	13.00 ± 0.89§	4.58 ± 0.27§	149 ± 13.6‖

* Compounds were administered via a gastric tube at a dose of 0.6 mmol/kg/day in two divided doses for 7 days to cholesterol-fed (1% diet) rats.

† Values are means ± SE; N = 5–6 livers.

‡ Values are means ± SE of serum cholesterol concentrations of animals after 7 days of compound treatment; N = 6 rats.

§ Significantly different from cholesterol-fed treatment group ($P < 0.05$).

‖ Significantly different from cholesterol-fed treatment group ($P < 0.01$).

Table 2. Effects of *R*(+)-4-CPPA, *S*(−)-4-CPPA, *R*(+)-4-CPBA, *S*(−)-4-CPBA and CPIB on the *in vivo* induction of hepatic peroxisomal FACO and microsomal LH activities of cholesterol-fed rats

Treatment*	FACO activity† (nmol H_2O_2/min/mg protein)	LH activity‡ (nmol/hr/mg protein)
Cholesterol-fed	3.89 ± 0.81	51.51 ± 1.14
R(+)-4-CPPA	21.26 ± 2.71	234.40 ± 22.96§
S(−)-4-CPPA	18.20 ± 6.53	151.46 ± 15.99§
R(+)-4-CPBA	36.09 ± 5.74§	315.34 ± 18.42§
S(−)-4-CPBA	50.09 ± 7.30§	267.18 ± 31.41§
CPIB	40.40 ± 7.77§	290.83 ± 22.02§

* Compounds were administered via a gastric tube at a dose of 0.6 mmol/kg/day in two divided doses for 7 days to cholesterol-fed (1% diet) rats.

† Values are the means ± SE of N = 5 livers. Activity is expressed as nmol H_2O_2 produced/min/mg mitochondrial protein.

‡ Values are the means ± SE of N = 5 livers. Activity is expressed as nmol lauric acid hydroxylated/hr/mg microsomal protein.

§ Significantly different from cholesterol-fed treatment group ($P < 0.05$).

the induction of FACO activity with maximal increases at 1.0 mM that were 33.8- and 37.9-fold greater than control activity respectively. However, the *R*(+)- and *S*(−)-enantiomers of 4-CPBA produced dissimilar profiles for the induction of FACO with the concentration–response curve of *S*(−)-4-CPBA located to the left of that for *R*(+)-4-CPBA. Additionally, 1.0 mM *S*(−)-4-CPBA produced a maximal increase in FACO activity that was 60.7-fold greater than control activity, whereas 1.0 mM *R*(+)-4-CPBA caused only a 33.1-fold increase. After 72 hr, control LH activity was 4.906 nmol lauric acid hydroxylated/hr/mg cellular protein and all compounds produced concentration-dependent increases in LH activity with activities significantly ($P < 0.05$) greater than control seen for the 0.3 and 1.0 mM treatment groups. A 9.8-fold increase in activity was seen with 1.0 mM CPIB, and the *R*(+)- and *S*(−)-enantiomers of 4-CPPA produced 8.4- and 7.1-fold increases, respectively, at 1.0 mM. In a manner similar to the induction profile for FACO activity, the concentration–response curve for the induction of LH activity by *S*(−)-4-CPBA was to the left of that for *R*(+)-4-CPBA with 6.3- and 8.3-fold increases in LH activity relative to control seen for 1.0 mM *R*(+)-4-CPBA and 1.0 mM *S*(−)-4-CPBA respectively.

Effect of the type of halogen substitution on the phenyl ring of phenoxyacetic acids (PAA). In these studies, clofibric acid (CPIB), the active metabolite of clofibrate [1], was used to determine whether primary cultures of hepatocytes respond to peroxisome proliferators and for the purpose of comparing the relative inductive effects of CPIB with PAA analogues. PAA, 4-chlorophenoxyacetic acid (4-CPAA) and 4-fluorophenoxyacetic acid (4-FPAA) represented the analogues which were available to examine the role of halogen substitution at the 4-phenyl position (see Fig. 1). Each compound produced a concentration-dependent increase in FACO and LH activities after 72 hr of incubation with primary cultures of rat hepatocytes (see Fig. 5A and Table 3). Incubation of the cultured hepatocytes with 1.0 mM 4-CPAA produced an increase in FACO

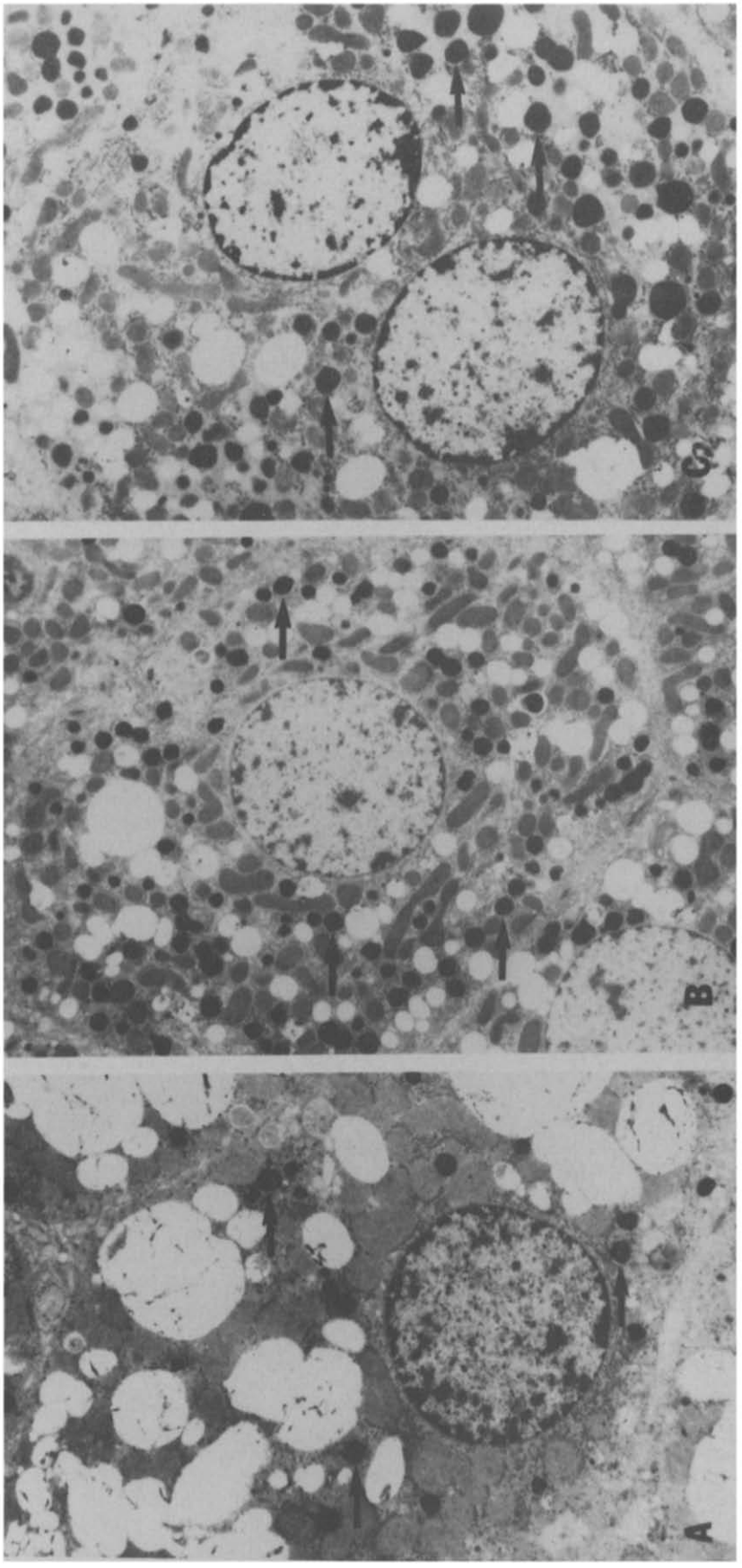

Fig. 2. Electron micrographs of hepatocytes from liver of control and drug-treated cholesterol-fed rats. The rats were maintained on a high cholesterol (1%) diet for 7 days and then treated with vehicle or drug (0.6 mmol/kg/day) for 7 days. Key: (A) control cholesterol-fed rat hepatocytes (magnification = 8000); (B) CPIB treatment (magnification = 6800); (C) *S*(−)-4-CPBA treatment (magnification = 8000). The peroxisomes are identified as the dense, darkly stained organelles (see arrows).

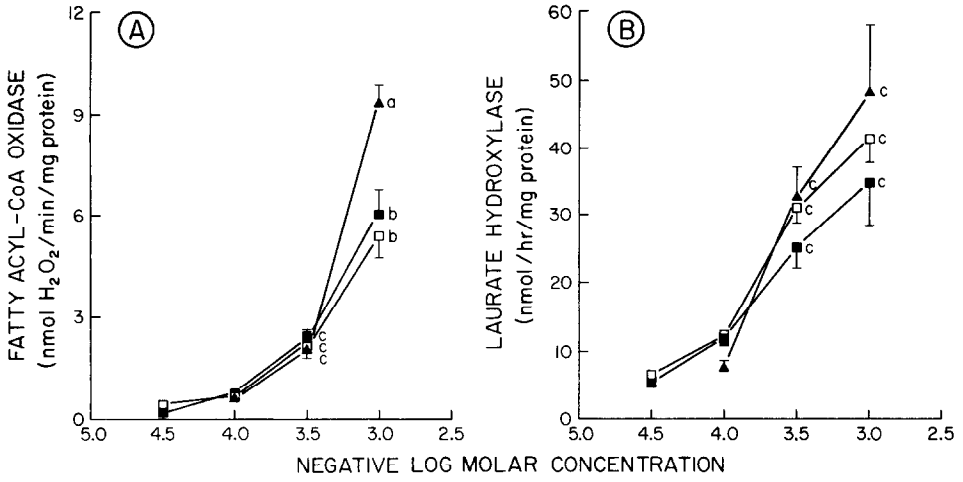

Fig. 3. Concentration–response curves for the induction of FACO (panel A) and LH (panel B) by *R*(+)-4-CPPA, *S*(−)-4-CPPA and CPIB. Cultured hepatocytes were incubated for 72 hr in the presence or absence of compound. For FACO activity, results are expressed as nmol H_2O_2 produced/min/mg cellular protein, and each point is the mean ± SE of determinations from 7–12 dishes of cells. For LH activity, results are expressed as nmol lauric acid hydroxylated/hr/mg cellular protein, and each point is the mean ± SE of determinations from 4–8 dishes of cells. Inductions of enzyme activity greater than control ($P < 0.05$) are indicated by letters, and points with different letters are significantly different ($P < 0.05$) from each other. Key: (□) *R*(+)-4-CPPA; (■) *S*(−)-4-CPPA; and (▲) CPIB. Control FACO activity was 0.159 nmol/min/mg protein and control LH activity was 4.906 nmol/hr/mg protein.

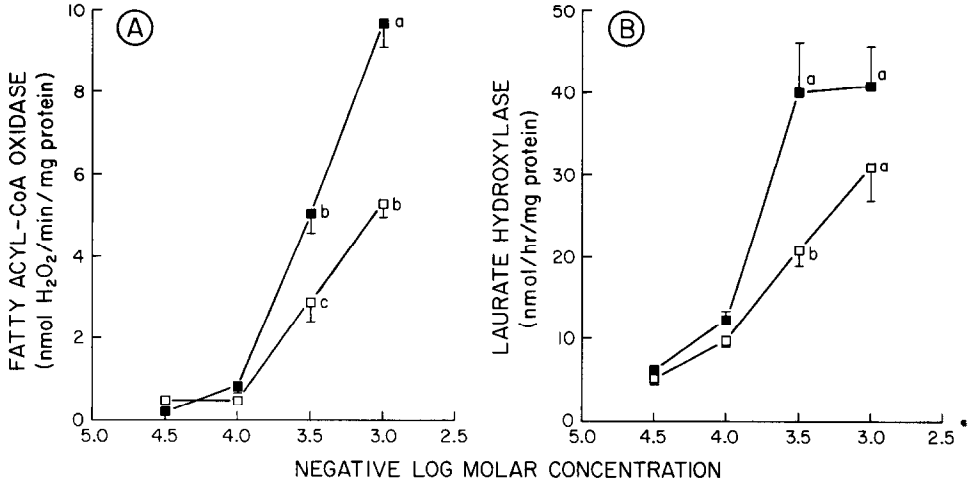

Fig. 4. Concentration–response curves for the induction of FACO (panel A) and LH (panel B) by *R*(+)-4-CPBA and *S*(−)-4-CPBA. Cultured hepatocytes were incubated for 72 hr in the presence or absence of compound. For FACO activity, results are expressed as nmol H_2O_2 produced/min/mg cellular protein, and each point is the mean ± SE of determinations from 4–8 dishes of cells. For LH activity, results are expressed as nmol lauric acid hydroxylated/hr/mg cellular protein, and each point is the mean ± SE of determinations from 4–8 dishes of cells. Inductions of enzyme activity greater than control ($P < 0.05$) are indicated by letters, and points with different letters are significantly different ($P < 0.05$) from each other. Key: (□) *R*(+)-4-CPBA; and (■) *S*(−)-4-CPBA. Control FACO activity was 0.159 nmol/min/mg protein and control LH activity was 4.906 nmol/hr/mg protein.

activity that was 51% of the CPIB 1.0 mM response and also statistically greater than the increases by 1.0 mM PAA and 4-FPAA (35 and 34% of CPIB 1.0 mM response respectively). In addition, PAA and 4-CPAA caused concentration-dependent increases in LH activity with 1.0 mM responses that were 24 and 23% of the CPIB 1.0 mM response, respectively, whereas 4-FPAA failed to elicit a significant increase in LH activity.

Effect of the location of the halogen substituent on the phenyl ring of phenoxyacetic acids. A series of racemic 2-phenoxypropionic acids (PPA, 2-CPPA, 3-CPPA and 4-CPPA) as well as a pair of 2-phenoxy-2-methylpropionic acids (2-CPMPA and CPIB) were

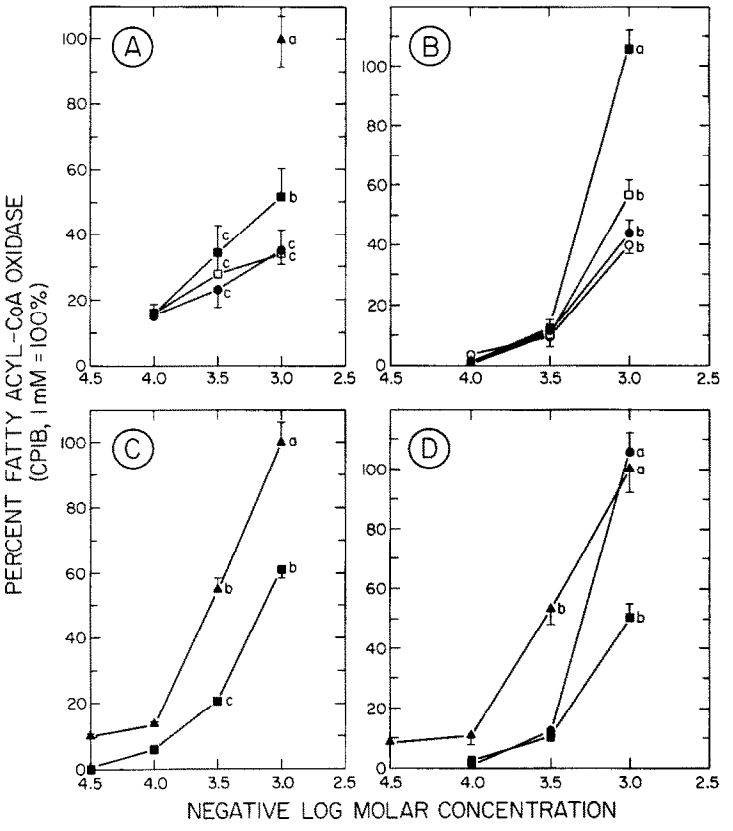

Fig. 5. Concentration–response curves for the induction of FACO by PAA (●), 4-CPAA (■), 4-FPAA (□), and CPIB (▲) (panel A); PPA (○), 2-CPPA (●), 3-CPPA (□), and 4-CPPA (■) (panel B); 2-CPMPA (■) and CPIB (▲) (panel C); and 4-CPPA (■), 4-CPPA (●), and CPIB (▲) (panel D). Cultured hepatocytes were incubated for 72 hr in the presence or absence of compound. The activities are expressed as a percent of the CPIB 1.0 mM response, and each point is the mean ± SE of determinations from 8–24 dishes of cells. The mean control activity was 9.0% of the CPIB 1.0 mM response (8.63 nmol H_2O_2/min/mg cellular protein). Inductions of enzyme activity greater than control ($P < 0.05$) are indicated by letters, and points with different letters are significantly different ($P < 0.05$) from each other.

used to evaluate the effect of the position of the halogen substituent on the phenyl ring of phenoxyacetic acids for induction of FACO and LH activities (see Fig. 1). Increases in FACO activities of each of the 2-phenoxypropionic acid compounds were observed in a concentration-dependent manner (see Fig. 5B). All of the compounds tested in this series of experiments at a 1.0 mM concentration significantly ($P < 0.05$) increased FACO activity in the treated hepatocytes over that observed in the control cultured cells. When exposed to a 1.0 mM concentration of each compound, increases in FACO activity that were 34, 44, 57 and 106% of the CPIB 1.0 mM response were observed in primary cultured hepatocytes treated with PPA, 2-CPPA, 3-CPPA and 4-CPPA respectively. The increase in FACO activity caused by 1.0 mM 4-CPPA was significantly ($P < 0.05$) greater than those caused by the other 2-phenoxypropionic acids. As with the induction of FACO activity, these compounds also induced LH activity in a concentration-dependent manner (see Table 3). Treatment of the cultured hepatocytes with 1.0 mM concentrations of PPA, 2-CPPA, 3-CPPA and 4-CPPA produced increases in LH activity that were 66, 68, 46 and 66% of the CPIB 1.0 mM response respectively. However, all of these increases were significantly ($P < 0.05$) less than the induction caused by 1.0 mM CPIB.

Concentration-dependent increases in FACO and LH activities were also seen with 2-CPMPA and CPIB (see Fig. 5C and Table 3). FACO activity was increased to 61% of the CPIB 1.0 mM response by 1.0 mM 2-CPMPA; however, the induction caused by 2-CPMPA was significantly ($P < 0.05$) less than that caused by CPIB. Additionally, cultured rat hepatocytes treated with 1.0 mM 2-CPMPA produced an increase in LH activity that was 104% of that for the CPIB 1.0 mM response.

Effect of alkyl substitution at the α-carbon atom of phenoxyacetic acids. The compounds used to evaluate the effect of the alkyl substitution at the α-carbon atom of phenoxyacetic acids were 4-CPAA, 4-CPPA and CPIB (see Fig. 1). As seen in Fig. 5D, FACO activity was increased in a concentration-dependent manner in primary cultured rat hepatocytes exposed to these compounds. In cells treated with 1.0 mM 4-CPPA, FACO activity was increased to 106% of the 1.0 mM CPIB response which was significantly ($P < 0.05$) greater than the 51% relative increase produced by 1.0 mM 4-CPAA. Likewise, these com-

Table 3. Effects of a series of phenoxyacetic acids (PAA) on LH activities in primary cultured rat hepatocytes

Treatment	Concentration (mM)	Percent CPIB response*
Control		8.89 ± 2.62
PAA	0.1	10.42 ± 1.63
	1.0	24.05 ± 0.01
4-FPAA	0.1	5.49 ± 1.35
	1.0	7.80 ± 1.02
4-CPAA	0.1	6.92 ± 1.44
	1.0	22.51 ± 1.61
PPA	0.1	25.19 ± 0.96
	1.0	66.46 ± 2.86†
2-CPPA	0.1	20.27 ± 1.06
	1.0	67.93 ± 4.36†
3-CPPA	0.1	48.13 ± 0.95†
	1.0	45.68 ± 1.55†
4-CPPA	0.1	42.38 ± 3.56†
	1.0	66.34 ± 5.44†
2-CPMPA	0.1	6.20 ± 0.58
	1.0	104.17 ± 16.26†

* Results are the means ± SE of LH activity expressed as a percent of the LH response in CPIB 1.0 mM treated primary cultured rat hepatocytes, N = 3–12 dishes. The mean control and CPIB (1.0 mM)-induced LH activities were 1.54 ± 0.33 and 25.12 ± 2.73 nmol lauric acid hydroxylated/hr/mg cellular protein respectively; N = 12 dishes.

† Significantly greater than control treatment ($P < 0.05$).

pounds also induced LH activity in a concentration-dependent manner with 1.0 mM 4-CPPA producing an increase that was 66% of the 1.0 mM CPIB response and also was significantly ($P < 0.05$) greater than the 22% relative increase produced by 1.0 mM 4-CPAA (Table 3).

DISCUSSION

A number of studies on the hepatic peroxisome proliferating effects of clofibrate and its active metabolite, CPIB, have been reported [10, 12, 14, 35, 36]; however, no studies of the role of stereochemistry on peroxisome proliferation have appeared. We have examined the peroxisome proliferating activities of two chiral analogues of CPIB both *in vivo* and *in vitro* and have demonstrated that the enantiomers of 4-CPPA and 4-CPBA reduce serum cholesterol concentrations in cholesterol-fed rats to a degree similar to that elicited by CPIB. The enantiomers of 4-CPBA and 4-CPPA, like CPIB, caused hepatomegaly in rats and also induced the peroxisome proliferation-associated enzymes FACO and LH to varying degrees. No enantiomeric selectivity was observed for the induction of FACO and LH by the isomers of 4-CPPA. Moreover, the decreased abilities of both enantiomers of 4-CPPA to induce these enzyme activities relative to CPIB *in vivo* and *in vitro* suggests that the loss of one methyl group from the α-carbon atom results in a decrease in the ability to induce peroxisome proliferation. In contrast, enantioselectivity was observed for the *in vitro* induction of FACO and LH by the isomers of 4-CPBA. *S*(−)-4-CPBA induced both enzymes to an extent similar to that of CPIB and to a greater extent than *R*(+)-4-CPBA which may indicate that the induction of peroxisome proliferation-associated enzymes is dependent upon a specific receptor-mediated mechanism responsible for these events. The loss of enantiomeric selectivity for the induction of FACO and LH *in vivo* may perhaps be explained by pharmacokinetic factors such as absorption, distribution, metabolism and/or excretion but further research into the disposition of these enantiomers *in vivo* is required to determine if any of these factors contributes to the observed results.

Our work shows that the presence, type and position of the halogen substituent on the phenyl ring, and the presence of mono- or di-methyl substitution at the α-carbon atom of the carboxylic acid side chain of PAA influence the peroxisome proliferating activity in primary cultured rat hepatocytes. From these studies, we found that: (1) placement of a chlorine atom at the 4-position of the phenyl ring of PAA results in a compound (4-CPAA) that causes greater induction of peroxisomal FACO activity than the corresponding nonhalogenated (PAA) or fluorinated (4-FPAA) analogues, both of which produced approximately equal inductions of FACO. However, this order of potency was not seen with the induction of another peroxisome proliferation-associated enzyme, LH; (2) the position of the chlorine atom on the phenyl ring also affects the abilities of these analogues to induce FACO activity as demonstrated by the greater induction evoked by the 4-chloro-substituted agents relative to the inductions caused by the 2- or 3-chloro-substituted compounds, whereas the inductions of LH by these analogues did not show the same degree of dependency on the ring position of the chlorine atom; (3) the methylation of the α-carbon atom of the carboxylic acid side chain also affects the abilities of the PAA compounds to induce peroxisome proliferation-associated enzymes. Addition of one methyl group to the α-carbon atom of 4-CPAA results in a large increase in the ability of the resultant compound (4-CPPA) to induce FACO and LH activities, and (4) the presence of an asymmetric center at the α-carbon atom of the carboxylic acid side chain influences the induction of FACO and LH activities. In this regard, we have shown that monosubstitution of the α-carbon atom with an ethyl group, but not a methyl group produced an enantioselective [*S*(−)-isomer > *R*(+)-isomer] induction of these peroxisome proliferation-associated enzymes.

Previously reported *in vivo* studies generally complement our findings using primary cultures for assessing the peroxisome proliferating capabilities of PAA analogues. The observed induction of FACO by 4-CPAA is similar to that shown by Lundgren *et al.* [9] who demonstrated that 4-CPAA causes only a small increase in hepatic palmitoyl-CoA oxidase activity in mice exposed to this compound. Further, we have shown that the type of halogen present at the 4-position of the phenyl ring appears to affect the ability of these compounds to induce peroxisomal FACO activity *in vitro*. In this regard, the 4-chloro-substituted compound (4-CPAA) caused a greater induction in this enzyme activity than the corresponding 4-fluoro-substituted agent (4-FPAA) or

the nonhalogenated analogue (PAA). These findings follow the same trend that was shown in an *in vivo* study in rats by Azarnoff *et al.* [8] which showed that clofibrate, a 4-chloro-substituted compound, produces substantial peroxisome proliferation (as determined by electron microscopy) whereas its unhalogenated (ethyl 2-methyl-2-phenoxypropionate) and 4-fluorinated [ethyl 2-(4-fluorophenoxy)-2-methylpropionate] analogues do not cause peroxisome proliferation in rats treated with the compounds. Accordingly, the addition of a chlorine atom at the 4-position of the phenyl ring increases the size and lipophilicity of 4-CPAA relative to the unsubstituted or fluorinated compounds, and would be expected to have a greater influence on the activity.

The position of the halogen substituent on the phenyl ring of these PAA compounds also appears to affect the ability of these compounds to induce peroxisome proliferation-associated enzyme activities as demonstrated by the differences in induction of FACO activity caused by a series of racemic 2-(2-, 3- and 4-chlorophenoxy)-2-methylpropionic acids. Azarnoff and coworkers [8] examined the peroxisome-proliferating effects of the 2- and 3-chloro-substituted analogues [ethyl 2-(2-chlorophenoxy)-2-methylpropionate and ethyl 2-(3-chlorophenoxy)-2-methylpropionate respectively] of clofibrate in rats and showed that neither of these compounds produced peroxisome proliferation. In contrast, we demonstrated that 2-CPMPA [the hydrolysis product of ethyl 2-(2-chlorophenoxy)-2-methylpropionate] increased peroxisomal FACO activity but not to the same degree as CPIB. The difference between these results may perhaps be explained by the high concentration (up to 1.0 mM) of 2-CPMPA exposed to the primary cultured rat hepatocytes as opposed to the dosage of these agents [0.25% (w/w) in the diet of the rats] used in the study by Azarnoff *et al.* [8]. This high concentration of compound could allow for the induction of FACO and expression of peroxisome proliferation in cultured rat hepatocytes.

In addition to the induction of FACO activity, 2-PPA, 2-CPPA, 3-CPPA and 4-CPPA induced microsomal LH activity in primary cultured hepatocytes in a concentration-dependent manner. However, unlike the induction of peroxisomal FACO activity, the position of the chlorine atom on the phenyl ring did not appear to influence the efficacy and/or potency of these compounds in inducing LH activity since each of these compounds produced similar degrees of induction. Raza and Levine [37] also demonstrated that 3-CPPA, 4-CPPA and CPIB induced *in vivo* hepatic microsomal LH activity in rats to similar extents. CPIB and 2-CPMPA produced similar large increases in LH activity in cultured rat hepatocytes which agrees with the results seen with the 2-phenoxypropionic acids in that the position of the chlorine atom does not appear to affect the degree of LH induction caused by these compounds. Additionally, the *in vitro* inductions of LH by CPIB and 2-CPMPA agrees with the findings of the previously described study by Raza and Levine [37] which showed that these two compounds produce similar inductions of hepatic microsomal LH activities in rats. Therefore, the induction of LH activity by these agents does not appear to show the same strict dependency on the physico-chemical property of phenyl ring chlorine placement as the induction of FACO activity demonstrates.

We also observed a step-wise increase in the induction of LH activity upon the addition of one or two methyl groups to the α-carbon of the acetic acid side chain of 4-CPAA, as in 4-CPPA and CPIB. Fournel *et al.* [21] demonstrated that the *in vivo* induction of rat hepatic laurate hydroxylase was similarly dependent upon the number of methyl groups located at the α-carbon of 4-CPAA. These results suggest that the addition of methyl groups to the α-carbon atom of PPAs increases the ability of these compounds to induce microsomal LH activity.

Based upon our studies, substitution of the chlorine atom at the 4-position of PAA creates compounds which possess the greatest ability to induce peroxisomal FACO activity. This is expected since a chlorine atom placed at this position would cause the greatest increase in lipophilicity and electronic effects in the PAA compounds. It has also been demonstrated that the placement of additional chlorine atoms (to form di- and trichlorophenoxyacetic acids), which would further increase the lipid solubility and electronic effects of these compounds, results in an even greater induction of peroxisome proliferation-associated enzymes both *in vivo* and *in vitro* [9, 15, 16]. Studies of structure–activity requirements for peroxisome proliferation in primary rat hepatocyte cultures by chemically similar and dissimilar compounds have demonstrated a good correlation between biological activity and electronic structural parameters derived from molecular orbital calculations [15, 16]. Taken collectively, these results suggest that peroxisome proliferators may act at a common receptor site in liver cells, either by the cytosolic peroxisome proliferator receptor mechanism of Reddy and coworkers [2, 7, 17, 18], or at other intracellular enzyme sites of substrate overload-perturbation of lipid metabolism [4]. Our results show that differences exist in the induction of the peroxisome proliferation-associated enzymes FACO and LH caused by structural and stereoisomeric alterations in the chemical structure of the PAA which may be explained by a receptor-mediated mechanism as proposed by Reddy and Rao [7]. Accordingly, a cytosolic peroxisome proliferator binding protein has been identified and partially characterized [17, 18], although recent reports [4, 38] have challenged this proposal. Therefore, it appears that further studies are necessary in order to determine whether the binding of a peroxisome proliferator to a receptor protein is responsible for the initiation of peroxisome proliferation or whether other events such as perturbations of lipid metabolism [4] may elicit this response. Based upon our results, the availability of enantiomerically specific inducers of peroxisome proliferation should be valuable in characterizing potential sites of interaction for this unique class of compounds.

The effect of structural changes on the hypolipidemic properties of compounds closely related to clofibrate has been examined by Azarnoff *et al.* [8] but the effect of structural changes of the compounds examined in this study on the hypolipidemic and

carcinogenic properties of these agents remains to be elucidated. Although the hypolipidemic action attributed to these compounds has been suggested to be associated with the induction of peroxisomal fatty acid β-oxidizing enzymes [24, 39] and microsomal fatty acid ω and ω-1 hydroxylase enzymes [26, 40], recent evidence does not support this association [41–44]. Further research is necessary in order to clarify the role of these induced fatty acid oxidizing enzyme systems in the hypolipidemic actions of these compounds. The occurrence of hepatocarcinomas in rodents administered peroxisome proliferators has also been proposed to be the result of the proliferation of peroxisomes and the subsequent production of reactive oxygen species which initiate the carcinogenic event [2]. The evidence to date [2–7, 45, 46] supports the contention that peroxisome proliferators constitute a unique class of carcinogens. Along these lines, the use of primary cultured rat hepatocytes for the evaluation of the peroxisome proliferating effects of PAA, and of other chemically unrelated classes provides an inexpensive and relatively easy screening system for determining the structural features required for the expression of this response. This information, in turn, may then be useful in the development of safer hypolipidemic drugs which exhibit a diminished carcinogenic potential that is associated with hepatic peroxisome proliferation.

Our *in vivo* results show that CPIB and the enantiomers of 4-CPPA and 4-CPBA all significantly lowered serum cholesterol concentrations of cholesterol-fed rats after 7 days of treatment. However, no enantioselectivity was observed at this dose in the *in vivo* model. It has also been shown that none of these compounds alters liver cholesterol concentrations and that only CPIB significantly reduces serum triglyceride levels [22]. Enantiomeric selectivity has been demonstrated for the inhibition of human platelet aggregation with a relative potency of $R(+)$-4-CPPA $>$ $R(+)$-4-CPBA $\geq$ $S(-)$-4-CPPA $>$ $S(-)$-4-CPBA $\geq$ CPIB reported [22]. Another enantioselective effect of these compounds is inhibition of chloride conductance which has been implicated in the detrimental side effect of myotonia associated with the use of clofibrate-like hypolipidemic agents [1]. The $S(-)$-isomers of both 4-CPPA and 4-CPBA greatly inhibit chloride conductance, whereas the $R(+)$-enantiomers and CPIB are virtually inactive as inhibitors of chloride conductance [23]. Thus, by taking advantage of the stereochemical features of these compounds, a greater dissociation of the beneficial effects such as serum cholesterol lowering and platelet antiaggregatory activities from the adverse properties involving inhibition of membrane chloride conductance in skeletal muscle fibers and hepatic peroxisome proliferation may be realized. Since hyperlipidemia and platelet hyperactivity are both involved in the atherosclerotic process, the $R(+)$-enantiomers of 4-CPPA and 4-CPBA may serve as leads for the development of safer and more efficacious drugs for the treatment of coronary artery disease which possess less potential for carcinogenesis associated with hepatic peroxisome proliferation.

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REFERENCES

1. Feller DR, Hagerman LM, Newman HA and Witiak DT, Antilipidemic drugs. In: *Principles of Medicinal Chemistry* (Ed. Foye WO), pp. 481–501. Lea & Febiger, Philadelphia, PA, 1989.
2. Reddy JK and Lalwani ND, Carcinogenesis by hepatic peroxisome proliferators: Evaluation of the risk of hypolipidemic drugs and industrial plasticizers to humans. *CRC Crit Rev Toxicol* **12**: 1–58, 1983.
3. Feller DR and Kocarek TA, Chemical carcinogenicity of peroxisome proliferating agents. In: *Environmental Toxicity and Carcinogenesis* (Eds. Ruchirawat M and Shank RC), pp. 105–118. Text & Journal Corp, Bangkok, Thailand, 1986.
4. Lock EA, Mitchell AM and Elcombe CR, Biochemical mechanisms of induction of hepatic peroxisome proliferation. *Annu Rev Pharmacol Toxicol* **29**: 145–163, 1989.
5. Reddy JK, Azarnoff DC and Hignite CE, Hypolipidemic hepatic peroxisome proliferators form a novel class of chemical carcinogens. *Nature* **283**: 397–398, 1980.
6. Fahl WE, Lalwani ND, Watanabe T, Goel SK and Reddy JK, DNA damage related to increased hydrogen peroxide generation by hypolipidemic drug-induced liver peroxisomes. *Proc Natl Acad Sci USA* **81**: 7827–7830, 1984.
7. Reddy JK and Rao MS, Peroxisome proliferation and cancer: Mechanisms and implications. *Trends Pharmacol Sci* **7**: 438–443, 1986.
8. Azarnoff DL, Reddy J, Hignite C and Fitzgerald T, Structure activity relationships of clofibrate-like compounds on lipid metabolism. In: *Proceedings of the Sixth International Congress of Pharmacology* (Ed. Vapaatalo H), Vol. 4, pp. 137–147. Pergamon Press, New York, 1975.
9. Lundgren B, Meijer J and DePierre JW, Induction of cytosolic and microsomal hydrolases and proliferation of peroxisomes and mitochondria in mouse liver after dietary exposure to *p*-chlorophenoxyacetic acid, 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid. *Biochem Pharmacol* **36**: 815–821, 1987.
10. Lalwani ND, Reddy MK, Qureshi SA, Sirtori CR, Abiko Y and Reddy JK, Evaluation of selected hypolipidemic agents for the induction of peroxisomal enzymes and peroxisome proliferation in the rat liver. *Hum Toxicol* **2**: 27–48, 1983.
11. Lundgren B, Meijer J and DePierre JW, Examination of the structural requirements for proliferation of peroxisomes and mitochondria in mouse liver by hypolipidemic agents, with special emphasis on structural analogues of 2-ethylhexanoic acid. *Eur J Biochem* **163**: 423–431, 1987.
12. Feller DR, Singh Y, Shirhatti VR, Kocarek TA, Liu C and Krishna G, Characterization of ciprofibrate and clofibric acid as peroxisomal proliferators in primary cultures of rat hepatocytes. *Hepatology* **7**: 508–516, 1987.
13. Kocarek TA and Feller DR, Induction of peroxisomal fatty acyl-CoA oxidase and microsomal laurate hydroxylase activities by beclobric acid and two metabolites in primary cultures of rat hepatocytes. *Biochem Pharmacol* **36**: 3027–3032, 1987.
14. Gray TJB, Lake BG, Beamand JA and Gangolli SD, Use of primary hepatocyte cultures for studies on the toxicological significance of peroxisome proliferation. In: *Alternative Methods in Toxicology* (Ed. Goldberg

AM), Vol. 3, pp. 169–187. Mary Ann Liebert Inc., New York, 1985.
15. Lewis DFV, Lake BG, Gray TJB and Gangolli SD, Structure activity requirements for induction of peroxisomal enzyme activities in primary rat hepatocyte cultures. *Arch Toxicol [Suppl]* **11**: 39–41, 1987.
16. Lake BG, Lewis DFV and Gray TJB, Structure activity relationships for hepatic peroxisome proliferation. *Arch Toxicol [Suppl]* **12**: 217–224, 1988.
17. Lalwani ND, Fahl WE and Reddy JK, Detection of a nafenopin-binding protein in rat liver cytosol associated with the induction of peroxisome proliferation by hypolipidemic compounds. *Biochem Biophys Res Commun* **116**: 388–393, 1983.
18. Lalwani ND, Alvares K, Reddy MK, Reddy MN, Parikh I and Reddy JK, Peroxisome proliferator-binding protein: Identification and partial characterization of nafenopin-, clofibric acid-, and ciprofibrate-binding proteins from rat liver. *Proc Natl Acad Sci USA* **84**: 5242–5246, 1987.
19. Ariens EJ, Stereochemistry, a source of problems in medicinal chemistry. *Med Res Rev* **6**: 451–466, 1986.
20. Patil PN, Miller DD and Trendelenburg U, Molecular geometry and adrenergic drug activity. *Pharmacol Rev* **26**: 323–392, 1974.
21. Fournel S, Caldwell J, Magdalou J and Siest G, Stereospecific induction of rat liver bilirubin UDP-glucuronosyltransferase and lauric acid 12-hydroxylation by arylcarboxylic acids chemically related to clofibrate. *Biochim Biophys Acta* **842**: 202–213, 1985.
22. Feller DR, Kamanna VS, Newman HAI, Romstedt KJ, Witiak DT, Bettoni G, Bryant SH, Conte-Camerino D, Loiodice F and Tortorella V, Dissociation of hypolipidemic and antiplatelet actions from adverse myotonic effects of clofibric acid related enantiomers. *J Med Chem* **30**: 1265–1267, 1987.
23. Bettoni G, Loiodice F, Tortorella V, Conte-Camerino D, Mambrini M, Ferrannini E and Bryant SH, Stereospecificity of the chloride ion channel: The action of chiral clofibric acid analogues. *J Med Chem* **30**: 1267–1270, 1987.
24. Lazarow PB and DeDuve C, A fatty acyl-CoA oxidizing system in rat liver peroxisomes: Enhancement by clofibrate, a hypolipidemic drug. *Proc Natl Acad Sci USA* **73**: 2043–2046, 1976.
25. Inestrosa NC, Bronfman M and Leighton F, Detection of peroxisomal fatty acyl-coenzyme A oxidase activity. *Biochem J* **182**: 779–788, 1979.
26. Tamburini PP, Masson HA, Bains SK, Makowski RJ, Morris B and Gibson GG, Multiple forms of hepatic cytochrome P-450: Purification, characterization and comparison of a novel clofibrate-induced isozyme with other major forms of cytochrome P-450. *Eur J Biochem* **139**: 235–246, 1984.
27. Lake BG, Gray TJB, Stubberfield CR, Beamand JA and Gangolli SD, Induction of lauric acid hydroxylation and maintenance of cytochrome P-450 content by clofibrate in primary cultures of rat hepatocytes. *Life Sci* **33**: 249–254, 1983.
28. Esbenshade TA, Kocarek TA, Kamanna VS, Loiodice F, Tortorella V, Newman HAI, Witiak DT and Feller DR, Hepatic proliferating actions of clofibric acid-related analogs *in vivo* and *in vitro*: Stereostructure activity relationships. *Fed Proc* **46**: 6867, 1987.
29. Witiak DT, Kokrady SS, Patel ST, Huzoor-Akbar, Feller DR and Newman HAI, Hypocholesterolemic and antiaggregatory properties of 2-hydroxytetronic acid redox analogues and their relationship to clofibric acid. *J Med Chem* **25**: 90–93, 1982.
30. Novikoff AB, Novikoff PM and Davis C, Studies on microperoxisomes II. A cytochemical method for light and electron microscopy. *J Histochem Cytochem* **20**: 1006–1023, 1972.
31. Seglen PO, Preparation of isolated rat liver cells. *Methods Cell Biol* **29**: 29–83, 1976.
32. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
33. Walusimbi-Kisitu M and Harrison EH, Fluorometric assay for rat liver peroxisomal fatty acyl-coenzyme A oxidase activity. *J Lipid Res* **24**: 1077–1084, 1983.
34. Parker GL and Orton TC, Induction by oxyisobutyrates of hepatic and kidney microsomal cytochrome P-450 with specificity towards hydroxylation of fatty acids. In: *Biochemistry, Biophysics and Regulation of Cytochrome P-450* (Eds. Gustafsson J, Duke JC, Mode A and Rafter J), pp. 373–377. Elsevier/North-Holland Biomedical Press, Amsterdam, 1980.
35. Lake BG, Gray TJB, Rijcken WRP, Beamand JA and Gangolli SD, The effect of hypolipidaemic agents on peroxisomal β-oxidation and mixed-function oxidase activities in primary cultures of rat hepatocytes. Relationship between induction of palmitoyl-CoA oxidation and lauric acid hydroxylation. *Xenobiotica* **14**: 269–276, 1984.
36. Moody DE and Reddy JK, The hepatic effects of hypolipidemic drugs (clofibrate, nafenopin, tibric acid and Wy-14,643) on hepatic peroxisomes and peroxisome-associated enzymes. *Am J Pathol* **90**: 435–446, 1978.
37. Raza H and Levine WG, Effect of hypolipidemic drugs on the metabolism of lauric acid and dimethylaminoazobenzene by rat liver microsomes. *Biochem Pharmacol* **36**: 774–778, 1987.
38. Milton MN, Elcombe CR, Kass GE and Gibson GG, Lack of evidence for a hepatic peroxisome proliferator receptor and an explanation for the binding of hypolipidemic drugs to liver homogenates. *Biochem Pharmacol* **37**: 793–798, 1988.
39. Lazarow PB, Rat liver peroxisomes catalyze the β-oxidation of fatty acids. *J Biol Chem* **253**: 1522–1528, 1978.
40. Bains SK, Gardiner SM, Mannweiler K, Gillet D and Gibson GG, Immunochemical study on the contribution of hypolipidaemic-induced cytochrome P-452 to the metabolism of lauric acid and arachidonic acid. *Biochem Pharmacol* **33**: 3077–3080, 1984.
41. Harrison EH, Action of clofibrate and its analogs in rats: Dissociation of hypolipidemic effects and the induction of peroxisomal β-oxidation. *Biochim Biophys Acta* **796**: 163–168, 1984.
42. Lazarow PB, Shio H and Leroy-Houyet MA, Specificity in the action of hypolipidemic drugs: Increase of peroxisomal β-oxidation largely dissociated from hepatomegaly and peroxisome proliferation in the rat. *J Lipid Res* **23**: 317–326, 1982.
43. Moody DE, Reddy JK and Azarnoff DL, Peroxisome-associated enzymes and serum lipids in tumor-bearing rats treated with peroxisome proliferating agents. *Biochem Pharmacol* **33**: 2591–2597, 1984.
44. Reich NO and Ortiz de Montellano PR, Dissociation of increased lauric acid ω-hydroxylase activity from the antilipidemic action of clofibrate. *Biochem Pharmacol* **35**: 1227–1233, 1986.
45. Lalwani ND, Reddy MK, Qureshi SA and Reddy JK, Development of hepatocellular carcinomas and increased peroxisomal fatty acid β-oxidation in rats fed [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]-acetic acid (Wy-14,643) in the semi-purified diet. *Carcinogenesis* **2**: 645–650, 1981.
46. Reddy JK, Lalwani ND, Reddy MK and Qureshi SA, Excessive accumulation of autofluorescent lipofuscin in the liver during hepatocarcinogenesis by methyl clofenapate and other hypolipidemic peroxisome proliferators. *Cancer Res* **42**: 259–266, 1982.