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CHARACTERIZATION OF THE HEPATIC RESPONSES TO THE SHORT-TERM ADMINISTRATION OF CIPROFIBRATE IN SEVERAL RAT STRAINS

CO-INDUCTION OF MICROSOMAL CYTOCHROME P-450 IVA1 AND PEROXISOME PROLIFERATION

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Abstract—The influence of ciprofibrate, a potent oxyisobutyrate derivative, on several hepatic enzyme parameters was studied in five rat strains following a 14-day treatment period. Ciprofibrate-dependent hepatomegaly was observed at two dose levels (2 and 20 mg/kg) in all rat strains examined. A 10- to 15-fold induction in the 12-hydroxylation of lauric acid with a less marked 1.5- to 5-fold induction of 11-hydroxylation was observed in treated animals. This dose-dependent increase in fatty acid hydroxylase activity was associated with a maximal 10-fold increase in the specific content of cytochrome P-450 IVA1 isoenzyme apoprotein, as assessed immunochemically using an ELISA technique. The activities of the cytochrome P-450 I (IA1 and IA2) and II (IIB1 and IIB2) families, as measured by ethoxyresorufin-*O*-deethylase and benzphetamine-*N*-demethylase activities respectively, were decreased on treatment. In the mitochondria, monoamine oxidase activity was significantly decreased at the higher dose level whereas α-glycerophosphate dehydrogenase activity was elevated. Total carnitine acetyltransferase activity (mitochondrial and peroxisomal) and peroxisomal β-oxidation were markedly increased at both dose levels in all strains examined. Cytosolic glutathione peroxidase activity, measured using both *t*-butylhydroperoxide and hydrogen peroxide as substrates, was decreased on treatment to approximately 50% of the control value. In treated animals, a marked increase in mRNA levels coding for cytochrome P-450 IVA1 and the peroxisomal bifunctional protein of the fatty acid β-oxidation spiral was observed. However, mRNA levels coding for glutathione peroxidase appeared unchanged following ciprofibrate administration, in contrast to the above-noted decrease of glutathione peroxidase enzyme activity. Taken collectively, our results have further substantiated a close association between the induction of microsomal cytochrome P-450 IVA1, peroxisomal β-oxidation and total carnitine acetyltransferase activity in rat liver, and have formed a conceptual basis for the rationalization of the chronic toxicity of peroxisome proliferators in this species.

Ciprofibrate, 2-(4-(2,2-dichlorocyclopropyl)-2-methyl-propanoic acid, is a recently developed and clinically effective phenoxyisobutyrate hypolipidaemic drug which effectively decreases serum cholesterol and triglyceride levels in normal laboratory animals and in animals rendered hyperlipidaemic by dietary manipulation [1]. In addition, ciprofibrate is a potent peroxisome proliferator in certain rodents (particularly the rat and mouse), a biological response that is in common with other drugs of this therapeutic class [2].

The administration of peroxisome proliferators to rodents results in a significant proliferation of cellular organelles including peroxisomes and smooth endoplasmic reticulum [2, 3]. Treatment is also associated with significant liver enlargement [4] and chronic exposure to these compounds results in the development of liver tumours [5–7]. A close association has been observed between the induction of microsomal cytochrome P-450 IVA1 (and its associated fatty acid hydroxylase activity) and peroxisomal, fatty acid β-oxidation enzymes [8], and the induction of microsomal and peroxisomal fatty acid metabolizing systems may represent an adaptive cellular response to acute changes in lipid metabolism resulting from treatment [4]. These early subcellular liver changes are sustained throughout acute and chronic treatment with these xenobiotics and may be associated with the development of hepatocellular carcinoma [2, 7], although the precise relationship between these acute hepatic enzyme changes and hepatocarcinogenesis has yet to be firmly established.

The hepatic effects of several structurally diverse peroxisome proliferators, including clofibrate, Wy-14,643, aspirin and the phthalate ester plasticisers, DEHP and MEHP, on hepatic peroxisomal and microsomal parameters has been established in the Wistar rat [4]. In addition, it has been reported that ciprofibrate administration causes hepatic peroxisome proliferation and induction of peroxisomal enzymes in the Fischer rat [9], although the influence of ciprofibrate on microsomal cytochrome P-450 IVA1 fatty acid hydroxylase activity has not been reported.

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Accordingly, the present study was designed to compare the hepatic responses in peroxisomal, mitochondrial, microsomal and cytosolic sub-cellular fractions in Gunn, Fischer, Sprague–Dawley, Wistar and Long Evans rat strains following short-term ciprofibrate administration to determine whether the sub-cellular hepatic enzymne changes are related in these strains, and whether we could identify a non-susceptible strain that may further shed light on the mechanistic aspects of the induced hepatic changes and the associated hepatocarcinogenesis.

MATERIALS AND METHODS

Chemicals. Ciprofibrate was donated by Sterling-Winthrop (Alnwick, Northumberland, U.K.). Lauric acid and NADPH were purchased from the Sigma Chemical Co. (Poole, Dorset, U.K.). 1-[^{14}C]Lauric acid was supplied by the Radiochemical Centre (Amersham, Bucks, U.K.). Ethoxyresorufin and resorufin were obtained from Molecular Probes Inc. (Junction City, OR, U.S.A.) and benzphetamine from the Upjohn Company (Kalamazoo, MI, U.S.A.).

All other chemicals were obtained from commercial sources and were of the highest purity available.

Animals and drug pretreatment. Male Wistar albino rats (University of Surrey breeders), male Gunn rats (University of Dundee breeders), male Sprague–Dawley, Fischer albino rats and Long Evans rats (Charles River U.K., Margate, Kent) of 140–160 g starting weight, were treated with ciprofibrate (2 and 20 mg/kg body weight) by gavage, once a day for 14 days. Ciprofibrate was administered as a suspension in 0.25% (w/v) gum tragacanth, at a volume of 5 mL/kg body weight. Control animals were given the vehicle alone, similarly at 5 mL/kg by gavage. All animals were killed on the 15th day after commencement of the treatment (i.e. 24 hr after the last dose). The livers were removed and perfused with 0.9% (w/v) saline prior to homogenization in 0.25 M sucrose. Samples of whole liver homogenate were taken for the determination of peroxisomal and mitochondrial marker enzymes. The microsomal and cytosolic fractions were prepared by ultracentrifugation [4] and the microsomal pellet was resuspended in 50 mM phosphate buffer, pH 7.25 containing 20% (v/v) glycerol.

Enzyme assays. Total, carbon monoxide-discernible cytochrome P-450 was determined according to Omura and Sato [10], using a difference absorption coefficient (450–490 nm) of 91 mM^{-1} cm^{-1} for the sodium dithionite-reduced carbon monoxide adduct. The specific hypolipidaemic-induced cytochrome P-450 IVA1 protein was determined immunochemically using an ELISA method as described by Sharma *et al.* [4]. A polyclonal antibody raised in sheep to a highly purified, electrophoretically homogenous preparation of rat cytochrome P-450 IVA1 was used as the immunogen. The antibody was relatively specific for cytochrome P-450 IVA1 as it recognised the homologous antigen in a Western blot as the major reaction product. In addition, a minor band (less than 10% of total reactive protein) was also identified with this antibody, this minor cross-reactivity probably corresponding to another related member of the cytochrome P450IV sub-family. Electrophoretically homogenous cytochrome P-450 IVA1 was used to construct a calibration curve which was shown to be linear up to 0.02 pmol cytochrome P-450 IVA1 per assay.

Table 1. The effect of ciprofibrate on liver size and hepatic cytochrome P-450/P450 IVA1 content in different rat strains

Rat strain	Dose level	Liver/body weight ratio (%)	Total cytochrome P-450 specific content (nmol/mg)	Specific cyt. P-450 IVA1 quantitation	
				Specific cyt. P-450 IVA1 (nmol/mg)	% of total cyt. P-450
Gunn	Control	5.16 ± 0.38	0.93 ± 0.35	0.04 ± 0.01	4.48 ± 1.44
	2 mg/kg	6.36 ± 0.09†	0.94 ± 0.09	0.22 ± 0.02‡	23.71 ± 2.15
	20 mg/kg	8.47 ± 0.60†	0.75 ± 0.16	0.20 ± 0.02‡	27.09 ± 4.60
Sprague–Dawley	Control	5.21 ± 0.14	0.78 ± 0.16	0.03 ± 0.01	4.29 ± 0.76
	2 mg/kg	6.76 ± 0.51†	1.28 ± 0.06‡	0.24 ± 0.03‡	18.53 ± 2.44
	20 mg/kg	9.78 ± 0.42‡	0.91 ± 0.22	0.23 ± 0.09†	24.67 ± 4.32
Fischer	Control	5.23 ± 0.30	0.56 ± 0.12	0.03 ± 0.01	5.73 ± 1.28
	2 mg/kg	7.15 ± 0.35‡	1.00 ± 0.09‡	0.24 ± 0.04‡	23.98 ± 1.53
	20 mg/kg	10.18 ± 0.28‡	0.72 ± 0.11*	0.26 ± 0.03‡	35.99 ± 3.99
Wistar	Control	5.18 ± 0.25	0.66 ± 0.11	0.02 ± 0.01	3.31 ± 0.68
	2 mg/kg	6.76 ± 0.29‡	0.86 ± 0.06*	0.22 ± 0.04‡	25.19 ± 2.83
	20 mg/kg	9.85 ± 0.48‡	0.72 ± 0.15	0.21 ± 0.04‡	29.08 ± 6.20
Long Evans	Control	5.93 ± 0.47	0.63 ± 0.16	0.03 ± 0.01	4.71 ± 2.46
	2 mg/kg	7.94 ± 0.28‡	0.86 ± 0.06*	0.30 ± 0.03‡	34.53 ± 2.46
	20 mg/kg	11.33 ± 0.59‡	0.95 ± 0.11*	0.46 ± 0.12‡	48.13 ± 8.44

Ciprofibrate was administered by gavage for 14 days at the dose levels indicated. Values are means ± SD from 3–5 individual animals. P values for results significantly different (Student's *t*-test) from control data: * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$.

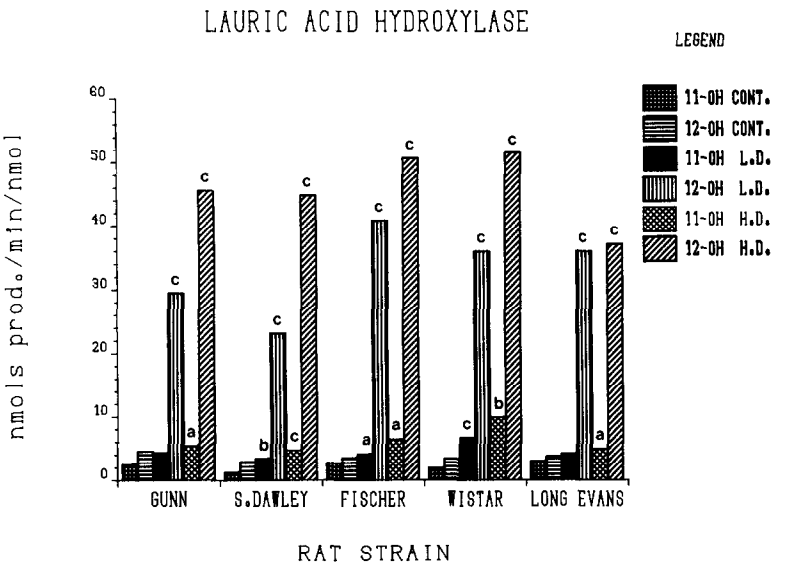

Fig. 1. Influence of ciprofibrate on the 11- and 12-hydroxylation of lauric acid by rat liver microsomes. Animals were administered ciprofibrate by gavage for 14 days at a dose level of either 2 mg/kg/day or 20 mg/kg/day. P values at: [a] $P < 0.05$, [b] $P < 0.01$ and [c] $P < 0.001$. Results are expressed as nmol metabolite produced/min/nmol P-450. Key: 11-OH, 11-hydroxylaurate; 12-OH, 12-hydroxylaurate; CONT., Control; L.D., Low Dose (2 mg/kg); H.D., High Dose (20 mg/kg).

Table 2. The effect of ciprofibrate on hepatic drug-metabolizing activity in different rat strains

Rat strain	Dose level	Benzphetamine-*N*-demethylase (nmol HCHO formed/min/nmol P-450)	Ethoxyresorufin-*O*-deethylase (nmol resorufin formed/min/nmol P-450)
Gunn	Control	10.52 ± 3.38 (100)	0.10 ± 0.04 (100)
	2 mg/kg	8.64 ± 0.66 (82)	0.05 ± 0.01 (50)
	20 mg/kg	6.63 ± 1.02 (63)	0.04 ± 0.01 (40)
Sprague–Dawley	Control	11.83 ± 0.76 (100)	0.04 ± 0.01 (100)
	2 mg/kg	6.75 ± 0.84† (57)	0.02 ± 0.01† (50)
	20 mg/kg	5.69 ± 0.77† (48)	0.01 ± 0.01* (25)
Fischer	Control	28.17 ± 8.69 (100)	0.05 ± 0.01 (100)
	2 mg/kg	9.31 ± 0.56* (33)	0.02 ± 0.01† (40)
	20 mg/kg	5.14 ± 0.85† (18)	0.01 ± 0.01† (20)
Wistar	Control	18.84 ± 3.63 (100	0.04 ± 0.01 (100)
	2 mg/kg	10.38 ± 2.06* (55)	0.01 ± 0.01* (25)
	20 mg/kg	9.19 ± 1.86* (49)	0.01 ± 0.01* (25)
Long Evans	Control	22.93 ± 4.38 (100)	0.03 ± 0.01 (100)
	2 mg/kg	12.04 ± 2.42* (53)	0.01 ± 0.01* (33)
	20 mg/kg	6.57 ± 2.20† (29)	0.01 ± 0.01* (33)

Ciprofibrate was administered by gavage for 14 days at the dose levels indicated. Values are means ± SD from 3–5 individual animals. Figures in brackets are values expressed as percentage of corresponding control. P values for results significantly different (Student's *t*-test) from control data: * $P < 0.01$, † $P < 0.001$.

The 11- and 12-hydroxy metabolites of lauric acid [11] were separated by reverse-phase HPLC using an ultrasphere ODS column (15 × 0.4 cm). The metabolites were resolved using a linear gradient of water (containing 0.1% (v/v) acetic acid): methanol (45:55) to 100% methanol over a 35-min period at a flow rate of 1.5 mL/min. The HPLC eluate containing the radioactive metabolites, was monitored with a Berthold LB 503 Radiodetector flow-cell (Lab-Impex, Twickenham, U.K.), interfaced with a Commodore PET (series 4000), enabling quantitative analysis of lauric acid metabolites. The analytical variation in this assay was approximately 5%.

Benzphetamine-*N*-demethylase activity was determined by the colorimetric procedure of Nash [12] and ethoxyresorufin O-deethylation was measured spectrofluorimetrically according to Burke *et al.* [13].

Whole tissue homogenates were used to determine peroxisomal and mitochondrial marker enzyme

Table 3. The effect of ciprofibrate on hepatic mitochondrial enzyme activities in different rat strains

Rat strain	Dose level	Monoamine oxidase activity (nmol product/min/mg protein)	Succinate dehydrogenase activity (nmol product/min/mg protein)	α-Glycerophosphate dehydrogenase activity (change in absorbance/min/mg protein)
Gunn	Control	4.85 ± 0.86	19.72 ± 2.01	0.02 ± 0.01
	2 mg/kg	5.26 ± 0.64	18.16 ± 1.12	0.03 ± 0.01†
	20 mg/kg	3.07 ± 0.38*	18.11 ± 1.35	0.03 ± 0.01*
Sprague–Dawley	Control	4.67 ± 0.84	13.69 ± 1.72	0.01 ± 0.01
	2 mg/kg	4.70 ± 1.18	13.89 ± 1.64	0.02 ± 0.01†
	20 mg/kg	3.43 ± 0.62*	12.60 ± 1.19	0.03 ± 0.01†
Fischer	Control	5.45 ± 1.32	11.79 ± 1.00	0.02 ± 0.01
	2 mg/kg	4.64 ± 0.31	11.35 ± 1.50	0.02 ± 0.01
	20 mg/kg	3.51 ± 0.29*	11.57 ± 0.47	0.03 ± 0.01*
Wistar	Control	5.37 ± 0.93	13.16 ± 1.11	0.01 ± 0.01
	2 mg/kg	5.57 ± 1.43	13.00 ± 1.28	0.02 ± 0.01*
	20 mg/kg	2.91 ± 0.58†	11.48 ± 1.14	0.04 ± 0.01‡
Long Evans	Control	7.27 ± 0.88	14.86 ± 1.14	0.04 ± 0.01
	2 mg/kg	6.21 ± 0.34	15.57 ± 2.81	0.10 ± 0.01‡
	20 mg/kg	3.27 ± 0.51‡	13.45 ± 0.65	0.18 ± 0.01‡

Ciprofibrate was administered by gavage for 14 days at the dose levels indicated. Values are means ± SD from 3–5 individual animals. P values for results significantly different (Student's *t*-test) from control data: * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$.

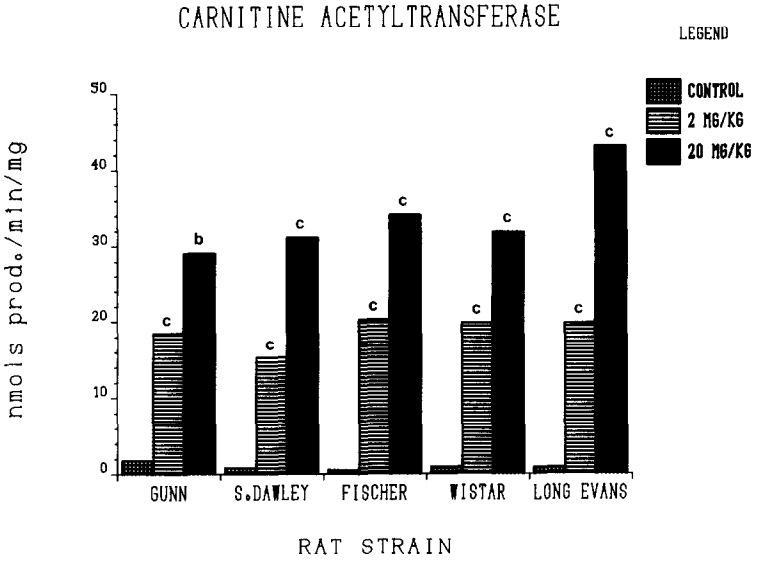

Fig. 2. Influence of ciprofibrate on hepatic mitochondrial and peroxisomal carnitine acetyltransferase activity in different rat strains. Animals were administered ciprofibrate by gavage for 14 days at a dose level of either 2 mg/kg/day or 20 mg/kg/day. P values at: [a] $P < 0.05$, [b] $P < 0.01$ and [c] $P < 0.001$. Results are expressed as nmol CoA formed/min/mg protein.

activities. Catalase was determined as described by Bock *et al.* [14]. The disappearance of the substrate, hydrogen peroxide, was monitored at 240 nm for 30–50 sec and the rate constant, *K*, was derived from the absorbance change over this period. Cyanide-insensitive palmitoyl-CoA oxidation was measured by the procedure of Bronfman *et al.* [15], and uricase activity by the method of Beaufay *et al.* [16]. Succinate dehydrogenase was determined by the method of Prospero [17], and α-glycerophosphate dehydrogenase activity according to Lee and Lardy [18]. Carnitine acetyl-CoA transferase activity was measured by the method of Bieber *et al.* [19] and Bock *et al.* [14]. Monoamine oxidase activity was determined using the modified fluorimetric method of Krajl [20] using kynuramine as the substrate which undergoes cyclization to 4-hydroxyquinoline, the reaction product.

Table 4. The effect of ciprofibrate on hepatic peroxisomal enzyme activities in different rat strains

Rat strain	Dose level	Catalase activity (K/mg protein)	Uricase activity (nmol metabolized/min/mg protein)
Gunn	Control	1.86 ± 0.12 (100)	14.23 ± 2.62 (100)
	2 mg/kg	2.02 ± 0.23 (109)	15.50 ± 1.77 (109)
	20 mg/kg	2.01 ± 0.11 (108)	18.17 ± 1.77 (128)
Sprague–Dawley	Control	1.55 ± 0.19 (100)	11.55 ± 2.32 (100)
	2 mg/kg	1.58 ± 0.34 (102)	9.87 ± 0.57 (85)
	20 mg/kg	2.07 ± 0.23† (133)	11.16 ± 1.90 (97)
Fischer	Control	2.00 ± 0.50 (100)	13.16 ± 2.23 (100)
	2 mg/kg	1.77 ± 0.19 (88)	10.71 ± 1.16 (81)
	20 mg/kg	1.83 ± 0.09 (91)	11.85 ± 1.58 (90)
Wistar	Control	1.95 ± 0.04 (100)	19.75 ± 1.94 (100)
	2 mg/kg	1.92 ± 0.16 (98)	15.39 ± 2.49* (78)
	20 mg/kg	2.41 ± 0.29† (124)	18.51 ± 0.62 (94)
Long Evans	Control	2.30 ± 0.31 (100)	14.32 ± 1.82 (100)
	2 mg/kg	2.52 ± 0.35 (110)	11.33 ± 1.59* (79)
	20 mg/kg	2.56 ± 0.36 (111)	15.60 ± 3.08 (109)

Ciprofibrate was administered by gavage for 14 days at the dose levels indicated. Values are means ± SD from 3–5 individual animals. Figures in brackets are values expressed as percentage of corresponding control. P values for results significantly different (Student's t-test) from control data: * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$.

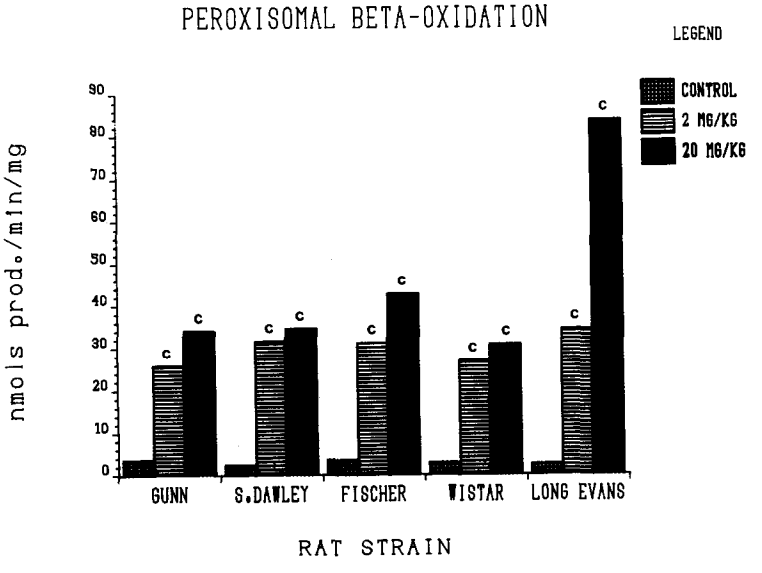

Fig. 3. Influence of ciprofibrate on hepatic cyanide-insensitive peroxisomal β-oxidation in different rat strains. Animals were administered ciprofibrate by gavage for 14 days at a dose level of either 2 mg/kg/day or 20 mg/kg/day. P values at: [a] $P < 0.05$, [b] $P < 0.01$ and [c] $P < 0.001$. Results are expressed as nmol NAD^+ reduced/min/mg protein.

Cytosolic glutathione peroxidase activity was determined using the method described by Awasthi *et al.* [21] and Lawrence and Burk [22]. Hydrogen peroxide, cumene hydroperoxide and t-butylhydroperoxide were used as substrates. Glutathione S-transferase activity was determined as the difference between the total activity using cumene hydroperoxide and the activity using hydrogen peroxide as the substrate as described by Lawrence *et al.* [23] and Mohandas *et al.* [24]. Protein concentrations were determined in whole homogenate, microsomal and cytosolic fractions by the method of Lowry *et al.* [25], using bovine serum albumin as the standard.

RNA isolation and dot blot hybridization. Total cellular RNA was isolated from whole liver homogenates from control and treated rats using a modification of the procedures of Chirgwin *et al.* [26]. RNA dot blots were prepared using nylon filters [27]

Table 5. The effect of ciprofibrate on hepatic cytosolic glutathione peroxidase and glutathione S-transferase activities in different rat strains

Rat strain	Dose level	Glutathione peroxidase activity (μmol metabolized/min/mg protein) Substrates: t-Butyl hydroperoxide	Hydrogen peroxide	Cumene hydroperoxide	Glutathione S-transferase activity (μmol metabolized/min/mg protein)
Gunn	Control	0.47 (100)	1.00 (100)	1.28 (100)	0.28 (100)
	2 mg/kg	0.47 (100)	0.95 (95)	1.18 (92)	0.23 (82)
	20 mg/kg	0.29 (62)	0.66 (66)	0.76 (59)	0.10 (36)
Sprague–Dawley	Control	0.60 (100)	1.25 (100)	1.51 (100)	0.26 (100)
	2 mg/kg	0.32 (53)	0.70 (56)	0.85 (56)	0.16 (61)
	20 mg/kg	0.27 (45)	0.59 (47)	0.72 (48)	0.13 (50)
Fischer	Control	0.51 (100)	0.92 (100)	—	—
	2 mg/kg	0.34 (67)	0.82 (89)		
	20 mg/kg	0.29 (57)	0.58 (63)		
Wistar	Control	0.65 (100)	1.37 (100)	—	—
	2 mg/kg	0.44 (68)	0.94 (69)		
	20 mg/kg	0.25 (38)	0.60 (44)		
Long Evans	Control	0.42 (100)	0.86 (100)	—	—
	2 mg/kg	0.34 (81)	0.66 (77)		
	20 mg/kg	0.22 (52)	0.45 (52)		

Ciprofibrate was administered by gavage for 14 days at the dose levels indicated. Values are expressed as an average of two experimental results as cytosolic fractions from animals of each dose group were pooled to give one pool per dose group. Figures in brackets are values expressed as percentage of corresponding control.

and nick translation of the cDNA/genomic probes and filter hybridization was accomplished as described by Earnshaw *et al.*[28].

RESULTS

The effect of ciprofibrate on liver/body weight ratio, total cytochrome P-450 content and the specific cytochrome P-450 IVA1 isoenzyme apoprotein levels in different rat strains is shown in Table 1. Significant hepatomegaly was observed in all test groups, approximately 2-fold at the high dose level in all rat strains, with the exception of the Gunn rat which displayed less extensive liver enlargement. Variable changes in the specific content of total cytochrome P-450 were observed in treated animals, with a significant increase at both dose levels in the Fischer and Long Evans rats. Cytochrome P-450 content was significantly elevated in the low dose groups in Sprague–Dawley and Wistar strains.

Cytochrome P-450 IVA1 isoenzyme levels, as determined immunochemically using an ELISA technique, were found to constitute between 3% and 6% of the total cytochrome P-450 population in untreated animals. Ciprofibrate was found to significantly increase cytochrome P-450 IVA1 content (nmol/mg protein) in all test groups, representing a 10-fold induction at the high dose level in the Long Evans rat and an increase between 5 to 8 in the remaining rat strains.

Lauric acid is considered to be a suitable marker substrate for the hypolipidaemic-inducible cytochrome P-450 IVA1 omega(12)-hydroxylase activity [29]. Reverse phase HPLC analysis showed that the 12-hydroxylation of lauric acid was preferentially induced over the 11-hydroxylation in rat liver microsomes of ciprofibrate treated animals (Fig. 1). The ratio of 12/11 hydroxy metabolites in untreated animals is in the range of 1.3 to 2.3 in the rat strains used in these experiments. However, in treated animals the 12/11 metabolite ratio was approximately 5 to 9. In treated Sprague–Dawley, Wistar, Fischer and Long Evans rats, the marked increase in 12-hydroxylation of lauric acid was associated with a marked decrease in the dealkylation of benzphetamine and ethoxyresorufin, marker substrates for cytochromes P-450 IIB1/IIB2 and IA1/IA2 respectively (Table 2). However it must be noted that the latter decreases in the dealkylation specific activities are partially offset by the noted inducer-dependent increases in liver weight (Table 1).

The activity of monoamine oxidase, an outer mitochondrial membrane enzyme, was significantly decreased at the high dose level in all rat strains (Table 3). The activity of the inner mitochondrial membrane enzyme, succinate dehydrogenase, was not significantly altered with ciprofibrate administration (Table 3). Mitochondrial α-glycerophosphate dehydrogenase activity was elevated at both dose levels in the Gunn, Sprague–Dawley, Wistar and Long Evans rats (Table 3). Enzyme activity in the Fischer rat was induced only in the high dose group.

Total carnitine acetyl-CoA transferase activity (localized in the mitochondrial and peroxisomal fractions) was significantly induced in all test groups (Fig. 2). The extent of induction was approximately 16-fold in the Gunn rat and between 36- and 70-fold in the other rat strains. Catalase activity was significantly increased at the high dose level in Sprague–Dawley and Wistar rat strains (Table 4), whereas activity in the Fischer rat was decreased on treatment, although this was not significant. The activity of uricase, a peroxisomal enzyme, was not altered by ciprofibrate treatment in Gunn, Fischer and Sprague–Dawley rats yet was significantly decreased at the 2 mg/kg dose level in the Wistar and Long Evans rats (Table 4). Ciprofibrate treatment resulted in a significant dose-dependent increase in peroxisomal fatty acid β-oxidation (Fig. 3). Induction was 9- to 15-fold in the Gunn, Sprague–Dawley, Wistar and Fischer strains. In the Long Evans rat, a 34-fold increase was observed.

The activity of glutathione peroxidase was measured using hydrogen peroxide and *t*-butyl hydroperoxide as substrates. A dose-dependent decrease in enzyme activity was observed with both substrates (Table 5). It should be noted that the enzyme activity expressed as a percentage of control, was similar with both substrates. At the high dose level, activity was 40–65% of the control value. Glutathione *S*-transferase activity was determined in the Sprague–Dawley and Gunn rats and was attributed to the difference in activity between cumene hydroperoxide and hydrogen peroxide as substrates. Glutathione *S*-transferase activity was decreased in a dose-dependent manner. Glutathione peroxidase activity, expressed as percentage of control, was not significantly different between the three substrates.

A positive hybridization of all liver RNA samples with the cytochrome P-450 IVA1 and peroxisomal bifunctional protein cDNA probes was observed and both mRNA levels were clearly increased in all treated animals as compared to their corresponding controls (Figs 4 and 5). Duplicate filters probed with an actin cDNA probe demonstrated low signal intensities of equal magnitude from hepatic RNA derived from all control and pretreated rat strains (data not shown), confirming that similar amounts of RNA were indeed applied to the filters and that the above ciprofibrate-dependent elevation of cytochrome P-450 IVA1 and peroxisomal bifunctional protein mRNA levels did not arise as a result of a general gene activation phenomenon.

The influence of ciprofibrate on glutathione peroxidase mRNA levels in the rat strains was also examined, and unlike the elevation of cytochrome P-450 IVA1 and peroxisomal bifunctional protein mRNAs, ciprofibrate has no effect on the mRNA coding for this cytosolic enzyme in all rat strains examined (Fig. 6).

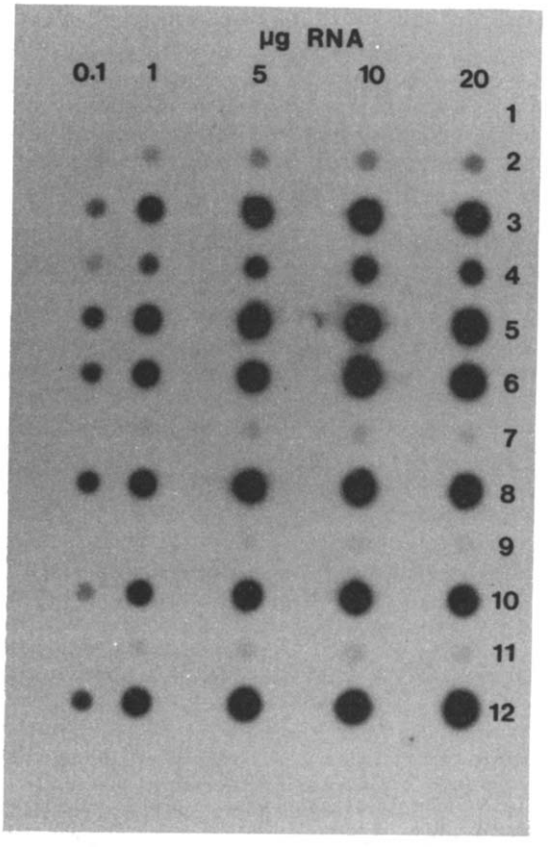

Fig. 4. Influence of ciprofibrate on hepatic cytochrome P-450 IVA1 mRNA in several rat strains. RNA was applied to the nylon filters in the amounts indicated, and the filter probed with a ^{32}P-labelled cDNA for cytochrome P-450 IVA1 as described in Materials and Methods. RNA was derived from: 1. tRNA (negative control); 2. Gunn rat, control; 3. Gunn rat, ciprofibrate-pretreated, 20 mg/kg; 4. Sprague–Dawley rat, control; 5. Sprague–Dawley rat, ciprofibrate-pretreated, 20 mg/kg; 6. Duplicate of 5, demonstrating reproducibility; 7. Fischer rat, control; 8. Fischer rat, ciprofibrate-pretreated, 20 mg/kg; 9. Wistar rat, control; 10. Wistar rat, ciprofibrate-pretreated, 20 mg/kg; 11. Long Evans rat, control; 12. Long Evans rat, ciprofibrate-pretreated 20 mg/kg.

DISCUSSION

The data presented on this paper demonstrates that the short-term effects of ciprofibrate on rat liver are qualitatively similar in each strain and additionally dose dependent. A 1.5- to 2-fold increase in liver/body weight ratio was observed and the response in the Fischer rat was consistent with published data [9, 30]. Cytochrome P-450 IVA1, a constitutive isoenzyme, was found to constitute between 3–6% of the total CO-discernible cytochrome P-450 population in uninduced rat liver microsomes. Immunodetectable cytochrome P-450 IVA1 protein was increased 6- to 10-fold with treatment with a minimal increase in 11-hydroxylation but with a marked induction in the formation of 12-hydroxylaurate (10- to 16-fold). Similar changes have been demonstrated in the Wistar rat following treatment with a variety of peroxisome proliferators [4]. This increase in catalytic activity is consistent with the marked increase in both the apoprotein and mRNA coding for cytochrome P-450 IVA1 in treated animals observed in this study. However, induction with ciprofibrate was more marked in the current

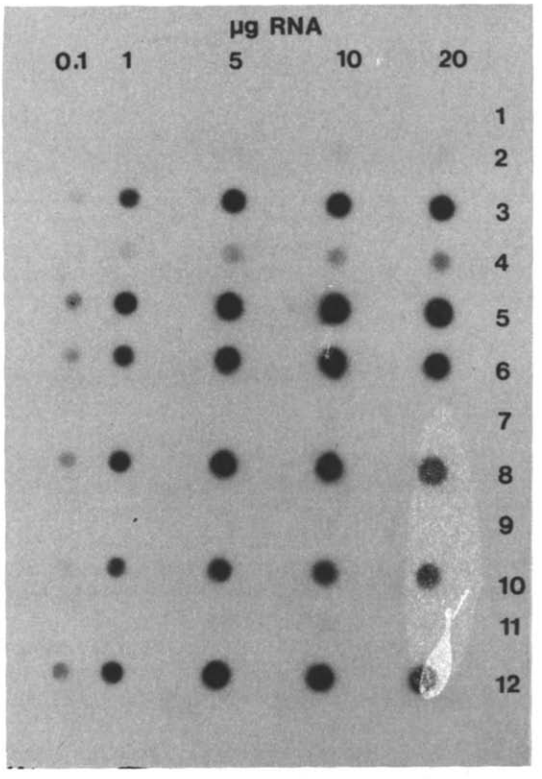

Fig. 5. Influence of ciprofibrate on hepatic bifunctional protein mRNA in several rat strains. RNA was applied to the nylon filters in the amounts indicated and the filter probed with a ^{32}P-labelled cDNA probe to the peroxisomal bifunctional protein, as described in Materials and Methods. RNA was derived from: 1. tRNA (negative control); 2. Gunn rat, control; 3. Gunn rat, ciprofibrate-pretreated, 20 mg/kg; 4. Sprague–Dawley rat, control; 5. Sprague–Dawley rat, ciprofibrate-pretreated, 20 mg/kg; 6. Duplicate of 5, demonstrating reproducibility; 7. Fischer rat, control; 8. Fischer rat, ciprofibrate-pretreated, 20 mg/kg; 9. Wistar rat, control; 10. Wistar rat, ciprofibrate-pretreated, 20 mg/kg; 11. Long Evans rat, control; 12. Long Evans rat, ciprofibrate-pretreated, 20 mg/kg.

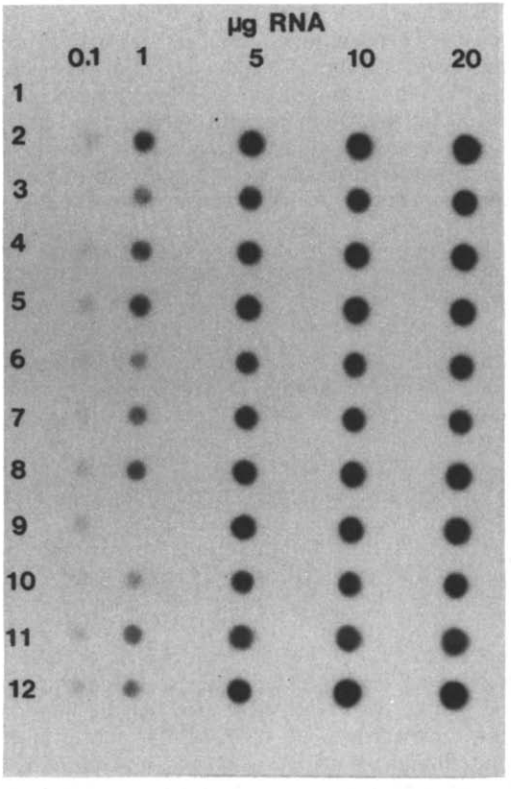

Fig. 6. Influence of ciprofibrate on hepatic glutathione peroxidase mRMA in several rat strains. RNA was applied to the nylon filters in the amounts indicated and the filter probed with a ^{32}-P-labelled genomic probe to glutathione peroxidase, as described in Materials and Methods. RNA was derived from: 1. tRNA (negative control); 2. Gunn rat, control; 3. Gunn rat, ciprofibrate-pretreated, 20 mg/kg; 4. Sprague–Dawley rat, control; 5. Sprague–Dawley rat, ciprofibrate-pretreated, 20 mg/kg; 6. Duplicate of 5, demonstrating reproducibility; 7. Fischer rat, control; 8. Fischer rat, ciprofibrate-pretreated, 20 mg/kg; 9. Wistar rat, control; 10. Wistar rat, ciprofibrate-pretreated, 20 mg/kg; 11. Long Evans rat, control; 12. Long Evans rat, ciprofibrate-pretreated 20 mg/kg.

study (as compared to several other peroxisomal proliferators investigated previously [4]) reflecting its greater potency as a peroxisome proliferator. The induction of lauric acid 12-hydroxylase in ciprofibrate-treated animals was associated with a marked decrease in the metabolism of model drug substrates (benzphetamine and ethoxyresorufin, Table 2), indicating that ciprofibrate is not a general inducer of all cytochrome P450s.

Peroxisomal fatty acid β-oxidation was increased 9- to 34-fold in the rat with a concomitant increase in mRNA levels coding for the peroxisomal bifunctional protein, an enzyme involved in the β-oxidation spiral, in agreement with previously published data [9, 31]. Unlike the similar responses in rat strains noted herein, the induction of peroxisomal acyl-CoA oxidase has been shown to be strain dependent in the mouse [32] in that the C57 and C3H inbred strains differ significantly from one another and from their F_1 hybrid in both their basal and nafenopin-induced acyl-CoA oxidase activity. The hepatic inducing effect of ciprofibrate has also been investigated in nine mouse strains to ascertain whether all strains show similar peroxisome proliferation or if any strains are refractory to the induction of peroxisome proliferation [33]. The least responsive mouse strain was CBA/Ca and B6C3F1 was the most responsive, however these latter results indicate no significant interstrain differences in ciprofibrate-induced hepatic pleiotropic responses [33], and the reason for this inducer-dependent differences in mouse strains is at present unclear.

The specific activity of catalase, the peroxisomal marker enzyme, was only minimally increased at the high dose level in the Sprague–Dawley and Wistar rats, in contrast to the more extensive, clobuzarit-dependent 2-fold increase in catalase activity [34], and a similar increase with ciprofibrate in the Fischer rat [9, 30]. Although the specific activity of catalase was largely unchanged in this study the total liver

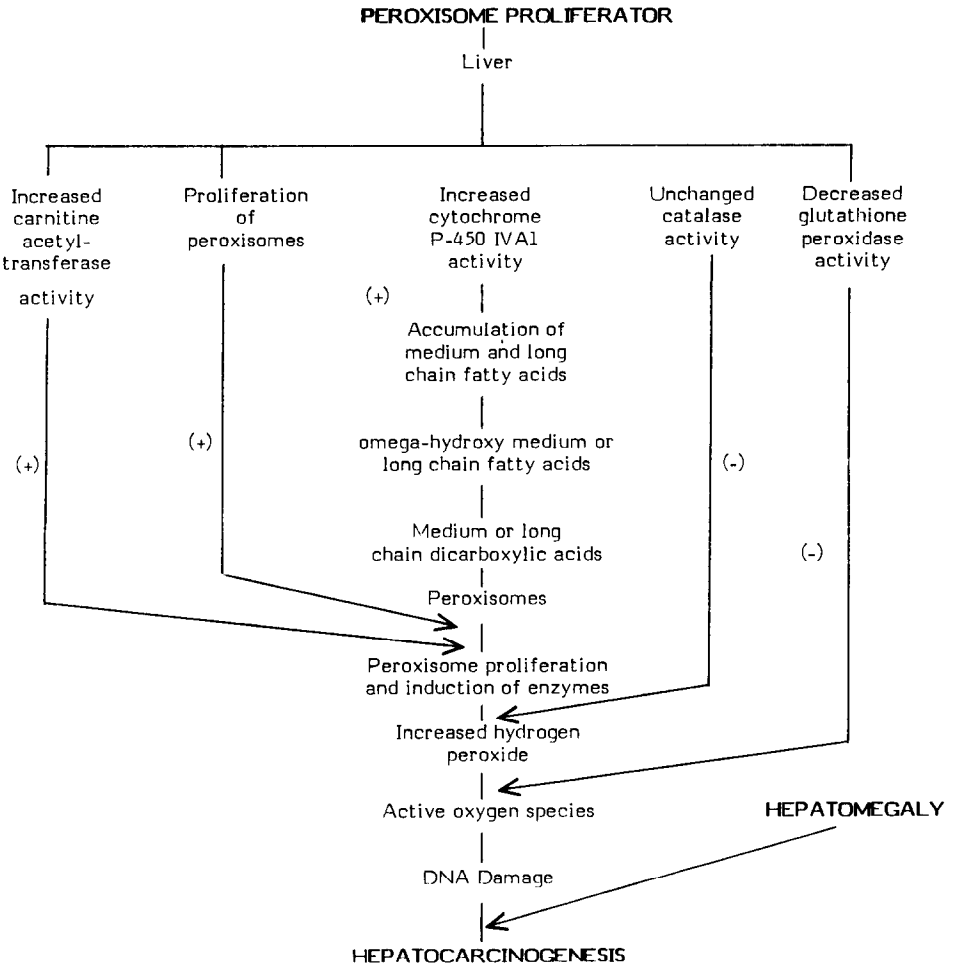

Fig. 7. Scheme relating ciprofibrate-dependent liver enzyme changes to hepatocarcinogenesis. (+), induction of enzyme activity; (−), inhibition of enzyme activity.

catalase activity was increased (approximately 2-fold) as a result of the observed hepatomegaly. Similarly, the specific activity of the peroxisomal enzyme uricase remained unchanged with ciprofibrate treatment indicating that uricase synthesis is increased in line with the cellular proliferation of peroxisomes. However, other studies have demonstrated uricase specific activity was decreased in male rats treated with di-(2-ethylhexyl)phthalate for 28 days and this was attributed to proliferation of hepatic peroxisomes lacking the uricase rich core [35].

Total carnitine acetyltransferase activity (mitochondrial and peroxisomal) was consistently induced 16- to 70-fold on ciprofibrate administration in all strains tested and at both dose levels examined in our study. Similarly, in the Fischer rat, a 58-fold increase in enzyme activity has been reported following the dietary administration of ciprofibrate at a concentration of 0.1% (w/w) [9], in agreement with the 70-fold, ciprofibrate-dependent noted herein. Carnitine is not involved in the entry of fatty acids into peroxisomes but may play a role in the removal of end products which could be shuttled to the mitochondria for further oxidation or conversion to ketone bodies [36]. It has been reported that clofibrate administration elevates carnitine acetyltransferase in both subcellular fractions [37], and a similar response is likely in this study with ciprofibrate.

Monoamine oxidase activity was decreased in all strains which is comparable to enzyme changes observed with clofibrate administration [38]. The activity of succinate dehydrogenase, a mitochondrial marker enzyme, was unchanged on ciprofibrate treatment. Mitochondrial α-glycerophosphate dehydrogenase acts as a specific marker for the metabolic activity of thyroxine in the liver [39], and the elevation of enzyme activity observed in all rat strains is probably due to the synthesis of new protein [39].

The ciprofibrate-dependent decrease in glutathione peroxidase activity and the lack of effect on the corresponding mRNA levels is worthy of comment. Quite clearly, ciprofibrate does not influence the transcription of the glutathione peroxidase gene and the decrease in enzyme activity using three different substrates in all rat strains examined may be explained by a ciprofibrate-dependent inhibition of glutathione peroxidase mRNA translation,

enhanced enzyme degradation or a direct inhibition of enzyme activity, possibilities that require further experimentation.

Gunn rats are unable to glucuronidate bilirubin as this rat strain lacks hepatic bilirubin UDP-glucuronyltransferase activity [40]. These animals were used in the current study to examine whether the absence of this phase II enzyme would influence the hepatic responses to ciprofibrate. Clofibrate and its structural analogues, are hydrolysed to the free acid (i.e. the pharmacologically active compound) prior to conjugation giving rise to the acylglucuronide which is rapidly eliminated in the urine [41]. Impairment of this metabolic conjugation pathway could result in an *in vivo* enhanced response to ciprofibrate as this compound would be present only as the pharmacologically active parent acid. However, the Gunn rat was the least responsive rat strain to peroxisome proliferators although the variation in the enzyme parameters tested was minimal and the differences were not significant using analysis of variation. This would indicate that the enzymic capacity for hepatic glucuronidation of phenoxyisobutyrates is unchanged in the Gunn rat. This has been substantiated by recent studies demonstrating that Gunn rats conjugate clofibric acid [40] and that clofibrate specifically induced bilirubin glucuronidation [42] but not the conjugation of the drug itself [43].

Taken collectively, the results presented here indicate a close relationship between induction of microsomal and peroxisomal fatty acid metabolising systems following ciprofibrate challenge in all rat strains studied, and are consistent with recently published data with other peroxisome proliferators in the Wistar rat [4, 8]. It was therefore suggested that induction in these two subcellular fractions is separated temporally, with the early biological response to treatment being the induction of cytochrome P-450 IVA1 and the corresponding increase in the production of omega-hydroxy fatty acids (Fig. 7). Subsequent oxidation to dicarboxylic acids occurs [44], thereby providing the postulated proximal stimulus for peroxisome proliferation. These enzyme changes and others described in this study are not associated with liver toxicity *per se* and may be used as an index of toxicity to predict the probability of chronic liver damage, ultimately hepatocarcinogenesis, as outlined in Fig. 7. Peroxisome proliferators are uniformly negative in a wide range of mutagenicity tests and have therefore been classified as non-genotoxic (epigenetic) carcinogens [2, 7]. It has been suggested that the marked increase in peroxisomal fatty acid β-oxidation leads to an excessive production of hydrogen peroxide by the induced peroxisomal fatty acyl-CoA oxidase. The total activity of catalase is only minimally increased in parallel with hepatomegaly and may be insufficient to remove the excessive hydrogen peroxide produced in stimulated peroxisomes. These proliferated peroxisomes may be 'leaky' [2] resulting in the diffusion of hydrogen peroxide into the cytoplasm. The decrease in hepatic glutathione peroxidase activity (to approximately 50% of the control value) observed with ciprofibrate administration in this study, may potentiate H_2O_2-dependent oxidative stress by the inadequate removal of cytosolic hydrogen peroxide (Fig. 7) and all these factors, in combination with sustained hepatomegaly of a hyperplastic nature, may predispose the animal to hepatocarcinogenesis. Although the precise relationship between the acute liver enzyme changes noted herein and hepatocarcinogenesis has not been fully elucidated, these early liver changes appear to be a prerequisite for the development of hepatocellular carcinomas by this class of compound.

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REFERENCES

1. Arnold A, McAuliff JP, Powers LG, Phillips DK and Beyler L, The results of animal studies with ciprofibrate, a new orally effective hypolipidaemic drug. *Atherosclerosis* **32**: 155–163, 1979.
2. Reddy JK and Lalwani ND, Carcinogenesis by hepatic peroxisome proliferators: evaluation of the risk of hypolipidaemic drugs and industrial plasticisers to humans. *CRC Crit Rev Toxicol* **12**: 1–58, 1983.
3. Hawkins JM, Jones WE, Bonner FW and Gibson GG, The effects of peroxisome proliferators on microsomal, peroxisomal and mitochondrial enzyme activities in the liver and kidney. *Drug Metab Rev* **18**: 441–515, 1987.
4. Sharma R, Lake BG, Foster J and Gibson GG, Microsomal cytochrome P-452 induction and peroxisome proliferation by hypolipidaemic agents in rat liver. A mechanistic inter-relationship. *Biochem Pharmacol* **37**: 1193–1201, 1988.
5. Kluwe WM, Haseman JK, Fielding D, Douglas J and Huff JE, The carcinogenicity of dietary di-(2-ethylhexyl)phthalate (DEHP) in Fischer 344 rats and B6C3F1 mice. *J Toxicol Environ Health* **10**: 797–815, 1982.
6. Lalwani ND, Reddy MK, Qureshi SA, Sirtori CR, Abiko Y and Reddy JK, Development of hepatocellular carcinomas and increased peroxisomal fatty acid β-oxidation in rats fed 4-chloro-6-(2,3-xylidino)-2-pyrimidinyl-thioacetic acid (Wy-14643) in the semipurified diet. *Carcinogenesis* **2**: 645–650, 1981.
7. Reddy JK, Azarnoff DL and Hignite CE, Hypolipidaemic hepatic peroxisome proliferators form a novel class of chemical carcinogens. *Nature* **283**: 397–398, 1980.
8. Sharma R, Lake BG and Gibson GG, Co-induction of microsomal cytochrome P-452 and the peroxisomal fatty acid β-oxidation pathway in the rat by clofibrate and di-(2-ethylhexyl)phthalate. Dose response studies. *Biochem Pharmacol* **37**: 1203–1206, 1988.
9. Lalwani ND, Reddy MK, Qureshi SA, Sirtori CR, Abiko Y and Reddy JK, Evaluation of selected hypolipidaemic agents for the induction of peroxisomal enzymes and peroxisome proliferation in the rat liver. *Human Toxicol* **2**: 27–48, 1983.
10. Omura T and Sato R, The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its haemoprotein nature. *J Biol Chem* **239**: 2370–2378, 1964.
11. 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 JA, Carlstedt-Duke J, Mode A and Rafter J), pp. 373–377. Elsevier Press, Amsterdam, 1980.
12. Nash T, Colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *J Biol Chem* **53**: 416–421, 1953.

13. Burke MD, Prough RA and Mayer RT, Characteristics of a microsomal cytochrome P-448 mediated reaction. Ethoxyresorufin-*O*-deethylation. *Drug Metab Dispos* **5**: 1–8, 1977.
14. Bock PP, Kramer R and Pavelka M, Peroxisomes and related particles. In: *Cell Biology Monographs 7*, pp. 44–74. Springer, Berlin, 1980.
15. Bronfman M, Inestrosa NC and Leighton F, Fatty acid oxidation by human liver peroxisomes. *Biochem Biophys Res Commun* **88**: 1030–1036, 1979.
16. Beaufay H, Bendall DS, Baudhuin P, Wattiaux R and De Duve C, Tissue fractionation studies. Analysis of mitochondrial fractions from rat liver by density-gradient centrifugation. *Biochem J* **73**: 628–637, 1959.
17. Prospero TD, A simplified assay for succinate dehydrogenase. *Meth Dev Biochem* **4**: 411–412, 1974.
18. Lee YP and Lardy HA, Influence of thyroid hormones on L-α-glycerophosphate dehydrogenases and other dehydrogenases in various organs of the rat. *J Biol Chem* **240**: 1427–1436, 1965.
19. Bieber LL, Abraham T and Helmrath T, A rapid spectrophotometric assay for carnitine palmitoyltransferase. *Anal Biochem* **50**: 509–518, 1965.
20. Krajl M, A rapid microfluorimetric determination of monoamine oxidase. *Biochem Pharmacol* **14**: 1683–1685, 1965.
21. Awasthi YC, Beutler E and Srivastava SK, Purification and properties of human erythrocyte glutathione peroxidase. *J Biol Chem* **250**: 5144–5149, 1975.
22. Lawrence RA and Burk RF, Glutathione peroxidase activity in selenium-deficient rat liver. *Biochem Biophys Res Commun* **71**: 952–958, 1976.
23. Lawrence RA, Parkhill LK and Burk RF, Hepatic cytosolic non selenium-dependent glutathione peroxidase activity: its nature and the effect of selenium deficiency. *J Nutr* **108**: 981–987, 1978.
24. Mohandas J, Marshall JJ, Duggin GG, Horvath JS and Tiller DJ, Differential distribution of glutathione and glutathione-related enzymes in rabbit kidney. Possible implications in analgesic neuropathy. *Biochem Pharmacol* **33**: 1801–1807, 1984.
25. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
26. Chirgwin JM, Przybyla AE, MacDonald RJ and Rutter WJ, Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochem J* **18**: 5294–5299, 1979.
27. White BA and Bancroft FC, Cytoplasmic dot hybridisation. Simple analysis of relative mRNA levels in multiple small cell or tissue samples. *J Biol Chem* **257**: 8569–8572, 1982.
28. Earnshaw D, Dale JW, Goldfarb PS and Gibson GG, Differential splicing in the 3′ non-coding region of rat cytochrome P-452 (P-450IVA1) mRNA. *FEBS Lett* **236**: 357–361, 1988.
29. Tamburini PP, Masson HA, Bains SK, Makowski RJ, Morris B and Gibson GG, Multiple forms of hepatic cytochrome P-450. Purification, characterisation and comparison of a novel clofibrate-induced isoenzyme with other major forms of cytochrome P-450. *Eur J Biochem* **139**: 235–246, 1984.
30. Lalwani NE, Reddy MK, Qureshi SA, Moehle CM, Hayashi H and Reddy JK, Noninhibitory effect of antioxidants ethoxyquin, 2(3)-tert-butyl-4-hydroxyanisole and 3,5-di-tert-butyl-4-hydroxytoluene on hepatic peroxisome proliferation and peroxisomal fatty acid β-oxidation induced by a hypolipidaemic agent in rats. *Cancer Res* **43**: 1680–1687, 1983.
31. Gibson GG and Sharma R, The use of cytochrome P-450 isoenzymes as an index of endoplasmic reticulum changes. In: *Cells, Membranes and Disease, including Renal* (Eds. Reid E, Cook GMW and Luzio JP), pp. 79–88. Plenum Publishing Corporation, New York, 1987.
32. Butler EG, England PJ and Williams GM, Genetic differences in enzymes associated with peroxisome proliferation and hydrogen peroxide metabolism in inbred mouse strains. *Carcinogenesis* **9**: 1459–1463, 1988.
33. Dwivedi RS, Alvares K, Nemali MR, Subbarao V, Reddy MK, Usman MI, Rademaker AW, Reddy JK and Rao MS, Comparison of the peroxisome proliferator-induced pleiotropic response in the liver of nine strains of mice. *Toxicologic Pathology* **17**: 16–26, 1989.
34. Orton TC, Adam HK, Bentley M, Holloway B and Tucker MJ, Species differences in the morphological and biochemical response of the liver following chronic administration. *Toxicol Appl Pharmacol* **73**: 138–151, 1984.
35. Mitchell FE, Price SC, Hinton RH, Grasso P and Bridges JW, Time and dose-response study of the effects on rats of the plasticiser di-(2-ethylhexyl)phthalate. *Toxicol Appl Pharmacol* **81**: 371–392, 1985.
36. Lazarow PB, Compartmentation of β-oxidation of fatty acids in peroxisomes. In: *Metabolic Compartmentation* (Ed. Sies H), pp. 317–329. Academic Press, New York, 1982.
37. Goldenberg H, Huttinger M, Kampfer P, Kramer R and Pavelka M, Effect of clofibrate application on morphology and enzyme content of liver peroxisomes. *Histochemie* **46**: 189–196, 1976.
38. Gear ARL, Albert AD and Bednarek JM, The effect of the hypocholesterolemic drug clofibrate on liver mitochondrial biogenesis. A role for neutral mitochondrial proteases. *J Biol Chem* **249**: 6495–6504, 1974.
39. Lee YP, Takemori AE and Lardy H, Enhanced oxidation of α-glycerophosphate by mitochondria of thyroid fed rats. *J Biol Chem* **234**: 3051–3054, 1959.
40. Burchell B and Blanckaert N, Bilirubin mono- and diglucuronide formation by purified rat liver microsomal bilirubin UDP-glucuronyl-transferase. *Biochem J* **223**: 461–465, 1984.
41. Emudianughe TS, Caldwell J, Sinclair KA and Smith RL, Species differences in the metabolic conjugation of clofibric acid and clofibrate in laboratory animals and man. *Drug Metab Dispos* **11**: 97–102, 1983.
42. Fournel S, Caldwell J, Magdalou J and Siest G, Stereospecific induction of rat liver UDP-glucuronosyltransferase and lauric acid 12-hydroxylation by the isomers of 2-phenylpropionic acid. *Biochim Biophys Acta* **882**: 469–472, 1986.
43. Odum J and Orton TC, Hepatic microsomal glucuronidation of clofibric acid in the adult and neonate albino rat. *Biochem Pharmacol* **32**: 3565–3569, 1983.
44. Mitz MA and Heinrikson RL, Omega hydroxy fatty acid dehydrogenase. *Biochim Biophys Acta* **46**: 45–50, 1961.