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IMMUNOCHEMICAL STUDY ON THE CONTRIBUTION OF HYPOLIPIDAEMIC-INDUCED CYTOCHROME P-452 TO THE METABOLISM OF LAURIC ACID AND ARACHIDONIC ACID

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Abstract—The influence of four hypolipidaemic drugs (clofibrate, WY-14,643, clobuzarit and bezafibrate) on hepatic cytochrome P-450 and fatty acid metabolism in male rat liver microsomes has been investigated. All of the hypolipidaemic drugs tested significantly induced the hydroxylation of lauric acid and, furthermore, this was accompanied by a concomitant 3-fold induction of a specific isoenzyme of cytochrome P-450 (termed cytochrome P-452) as determined by a single radial immunodiffusion technique. In addition, immunochemical quantitation of cytochrome P-452 in control, uninduced rat liver microsomes revealed that this particular isoenzyme constituted 22% of the total carbon monoxide-discernible cytochrome P-450 population. This has led us to the conclusion that cytochrome P-452 is a constitutive cytochrome P-450 isoenzyme and therefore that hypolipidaemic agents function as inducers of constitutive haemoprotein isoenzymes.

Cytochrome P-452 plays a significant role in the hydroxylation of lauric acid as evidenced by inhibition of hydroxylase activity in the presence of an anti-P-452 IgG fraction. In addition, this antibody preferentially inhibits the 12-hydroxylation of lauric acid in rat liver microsomes by comparison to the 11-hydroxylase activity. Our studies have also shown that arachidonic acid serves as an excellent substrate for hypolipidaemic-induced cytochrome P-452, resulting in the formation of several metabolites that have been separated by reverse phase HPLC. Furthermore, a specific metabolite (or group of metabolites) of arachidonic acid is induced by clofibrate pretreatment and that the formation of this metabolite(s) is inhibited by an antibody to cytochrome P-452. By comparison, other metabolites of arachidonic acid remain refractory to induction by clofibrate and are not inhibited by the presence of anti-P-452 IgG.

In addition, a reconstituted enzyme system containing highly purified cytochrome P-452 actively catalyses the above specific oxidation of arachidonic acid, a reaction that is significantly stimulated by the presence of cytochrome b_5.

Taken collectively, our data provide compelling evidence that hypolipidaemic agents induce a specific isoenzyme of hepatic microsomal P-450 that readily oxidizes fatty acids and that arachidonic acid may serve as an excellent endogenous substrate for this novel haemoprotein.

The administration to rats of a number of hypolipidaemic agents which are capable of lowering plasma triglycerides is uniformly associated with hepatomegaly and a marked proliferation of both peroxisomes and microsomal fractions [1–4]. The peroxisomal proliferation is almost always associated with an increase in the peroxisomal enzyme activities [3], including the fatty acid β-oxidation system that is considered to be partly responsible for the lipid-lowering effect of hypolipidaemic drugs [5].

In addition to their effect on peroxisomal enzyme activities, a number of hypolipidaemic agents have been shown to increase microsomal enzyme activities, including the mixed function oxidase system [6], certain forms of glucuronyl transferase [7] and epoxide hydrolase [8]. The increase of hepatic mixed function oxidase activity in response to hypolipidaemic drugs is characterized by an increase in the 12-hydroxylation of lauric acid and no change or decrease in the metabolism of model drug substrates [6], suggesting the induction of a specific isoenzyme of cytochrome P-450. In this respect, cytochrome P-450 has been isolated and highly purified from the livers of clofibrate-induced rats and has been described as a unique isoenzyme of the cytochrome P-450 class of haemoproteins in that it does not readily catalyse the hydroxylation of most model drug substrates, but rather exhibits a distinct substrate specificity for the 12-hydroxylation of lauric acid [10]. In addition, the clofibrate-induced cytochrome P-450 (termed cytochrome P-452 based on its spectral properties) is distinct from the major cytochrome P-450s induced by both phenobarbital and β-naphthoflavone as characterized by it physicochemical, biochemical and immunological properties [10].

In the present study we have investigated the ability of the four hypolipidaemic agents clofibrate, WY-14,643, clobuzarit and bezafibrate to induce the above cytochrome P-452 isoenzyme as assessed both immunologically and catalytically. Furthermore, in

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view of the ability of hepatic microsomal cytochrome P-450 to metabolize arachidonic acid [11, 12] and induction of this activity by the hypolipidaemic drug ciprofibrate (R. W. Estabrook, personal communication), we have utilized an antibody to cytochrome P-452 to determine the role played by this isoenzyme in the oxidation of arachidonic acid.

MATERIALS AND METHODS

Chemicals. The hypolipidaemic compounds (Fig. 1) were obtained from the following sources: WY-14,643 from Wyeth Laboratories (Radnor, PA); bezafibrate from Boehringer Ingelheim (F.R.G.), and both clofibrate and clobuzarit were gifts from ICI Pharmaceuticals Division (Macclesfield, U.K.). Lauric acid, arachidonic acid and NADPH were obtained from Sigma (Poole, Dorset, U.K.). 1-^{14}C Lauric acid and 1-^{14}C arachidonic acid were supplied by the Radiochemical Centre (Amersham, Bucks., U.K.). All other chemicals were obtained from commercial sources and were of the highest purity available.

Animals. Male Wistar Albino rats (80–100 g body weight, University of Surrey breeders) were used throughout the study. The hypolipidaemic compounds were administered in a standard powdered laboratory diet, for 14 days, at the concentrations indicated (w/w): Wy-14,643, 0.25%; clofibrate, 0.4%; bezafibrate, 0.2%; clobuzarit, 0.05%. Control animals were fed drug-free diet. The animals were starved overnight prior to sacrifice. Liver microsomes were prepared by the calcium precipitation method as described by Cinti *et al.* [13].

Antibodies to clofibrate-induced cytochrome P-452 were raised in sheep. A female sheep was immunized with 0.6 mg of highly purified, electrophoretically homogenous preparation of cytochrome P-452 in Freund's complete adjuvant. Four weeks later the sheep was boosted with 0.3 mg of purified antigen and seven days later, blood was collected from the jugular vein. IgG-enriched fractions were prepared from the sera by a combination of ammonium sulphate and DEAE-chromatography [14] and the final IgG fractions were concentrated to approximately 70–100 mg protein/ml using an Amicon PM-30 membrane. Control sera and the respective IgG were prepared from the blood of pre-immunized sheep.

Enzyme assays. Cytochrome P-450 content was determined according to the method of Omura and Sato [15]. The specific cytochrome P-452 isoenzyme was quantitated in solubilized microsomes using the single radial immunodiffusion assay of Thomas *et al.* [16] with the following modifications: 3% (w/v) polyethylene glycol was incorporated into the agarose and 40 μg of purified anti-P-452 IgG was used per plate. Under these conditions, a calibration curve

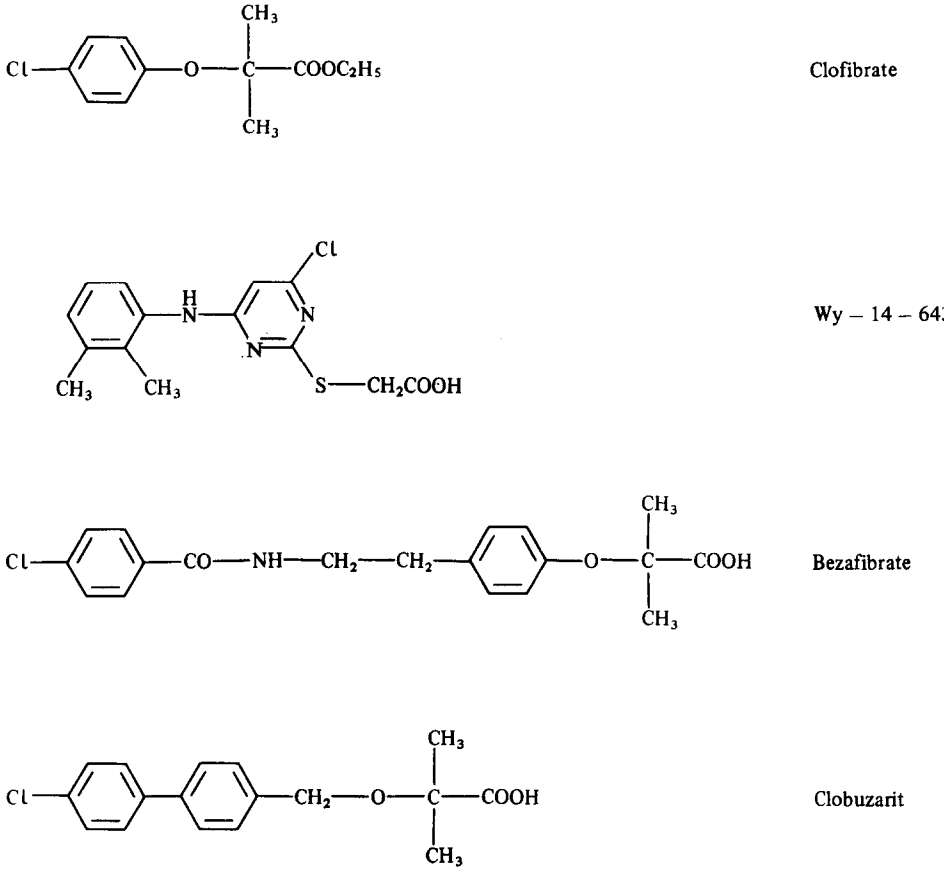

Fig. 1. Chemical structures of hypolipidaemic agent

for electrophoretically homogeneous cytochrome P-452 was linear up to 25 pmoles haemoprotein per assay. The combined 11- and 12-hydroxylation of lauric acid was routinely determined by the thin layer autoradiography assay of Parker and Orton [17]. The 11- and 12-hydroxy derivatives of lauric acid were separated by HPLC using a Micropak MCH-10 column (30 × 0.4 cm, Varian Associates Ltd., Walton-on-Thames, Surrey, U.K.). The products of the reaction were resolved using a linear gradient of water : methanol (45 : 55 containing 0.5% acetic acid) to 100% methanol over a 30-min period at a flow rate of 1.5 ml/min. The radioactive metabolites were quantitated by a liquid scintillation technique.

The products of arachidonic acid metabolism were isolated from an incubation mixture containing rat liver microsomes (1 mg protein/ml), 10 mM $MgCl_2$, 50 mM Tris buffer (pH 7.5) and 0.1 mM [^{14}C]arachidonic acid (0.3 mCi/mmole). After 5-min equilibration at 25°, NADPH (1 mM) was added to initiate the reaction. The reaction was terminated after 10 min by the addition of 2 ml ethyl acetate containing 0.05 ml of 1 M HCl and 0.01% butylated hydroxytoluene as antioxidant. The samples were extracted twice with 2 ml of ethylacetate, the combined extracts dried over anhydrous sodium sulphate, filtered and evaporated to dryness under a stream of nitrogen. Samples were dissolved in a minimal volume of ethanol and the products were separated by HPLC using a Micropak MCH-10 column. The arachidonic acid metabolites were separated using a linear solvent gradient programme ranging from water/acetonitrile (50 : 50 containing 0.1% acetic acid) to 100% acetonitrile, at a flow rate of 1 ml/min for 35 min. The elution profile of the radioactive products was monitored using a Berthold LB 503 HPLC radioactivity monitor (Laboratory Impex Ltd., Middlesex, U.K.) and radioactive metabolites were quantitated using a liquid scintillation technique.

When antibody inhibition of fatty acid metabolism was studied, rat liver microsomes were preincubated with IgG for 10 min at 25° in 0.4 ml of 10 mM potassium phosphate buffer, pH 7.4 before the addition of cofactors and substrates.

The reconstitution of the cytochrome P-452 containing monooxygenase reaction system was carried out as follows: sonicated L-α-dilauroylglycerophosphocholine (100 μg) was incubated with 0.5 nmole purified cytochrome P-452, 1 nmole of purified NADPH-cytochrome P-450 reductase and, where included, 1 nmole purified cytochrome b_5. The mixture was incubated for 2 min at 25° and then diluted to 1 ml using a buffer mixture containing 50 mM Tris–Cl (pH 7.5) and 10 mM $MgCl_2$. [^{14}C] Arachidonic acid was added to give a 0.1 mM final concentration. The mixture was pre-incubated for 2 min and NADPH (1 mM final concentration) was added to initiate the reaction. The reaction was stopped after 5 min by the addition of an equal volume of a mixture of 1 M HCl and ethylacetate, resulting in pH 3 in the aqueous phase. The metabolic products were extracted and analysed as described above.

Electrophoretically homogeneous preparations of cytochrome P-452, NADPH-cytochrome P-450 reductase and cytochrome b_5 were isolated from rat liver microsomes as previously described [10] and exhibited specific contents of 13.0, 11.8 and 31.3 nmoles/mg protein respectively.

Protein determination. Protein concentration was determined by the method of Lowry *et al.* [18] using crystalline bovine serum albumin as the standard.

RESULTS

Table 1 shows the effects of the administration of clofibrate, bezafibrate, clobuzarit and WY-14,643 on the relative liver weights, total cytochrome P-450 content and lauric acid hydroxylation. Marked hepatomegaly was produced following the dietary administration of hypolipidaemics to rats, in good agreement with earlier reports [3]. The mean liver weights increased markedly compared to that of control rats following pretreatment of animals with WY-14,643 and clobuzarit. Significant hepatomegaly was also produced with clofibrate and bezafibrate, although the effect was less marked than that observed with WY-14,643 and clobuzarit. There was also an increase in the total cytochrome P-450 levels above control levels, with maximum induction observed with both clobuzarit and WY-14,463. In agreement with previous findings [6, 17], lauric acid hydroxy-

Table 1. Effect of hypolipidaemic agents on the rat hepatic microsomal drug metabolizing enzyme system

Parameter	Animal treatment				
	Control	WY-14,643 (0.25%)	Clofibrate (0.4%)	Bezafibrate (0.20%)	Clobuzarit (0.05%)
Liver/body weight (%)	5.10 ± 0.60	†7.50 ± 0.60	6.30 ± 0.30	6.60 ± 0.50	†8.30 ± 0.80
Cytochrome P-450 (nmoles/mg)	1.09 ± 0.03	*1.37 ± 0.16	1.27 ± 0.11	1.17 ± 0.11	*1.42 ± 0.24
Lauric acid hydroxylase (total 11- and 12-hydroxy metabolites formed, nmoles/min per mg protein)	2.2 ± 1.20	‡8.30 ± 0.50	‡6.90 ± 0.80	‡7.90 ± 0.70	‡7.5 ± 0.30

Compounds were administered in the diet for 14 days at the percentages indicated. Values are means ± S.D. from four individual animals.

* Significantly different from control at $P < 0.05$.

† Significantly different from control at $P < 0.01$.

‡ Significantly different from control at $P < 0.001$.

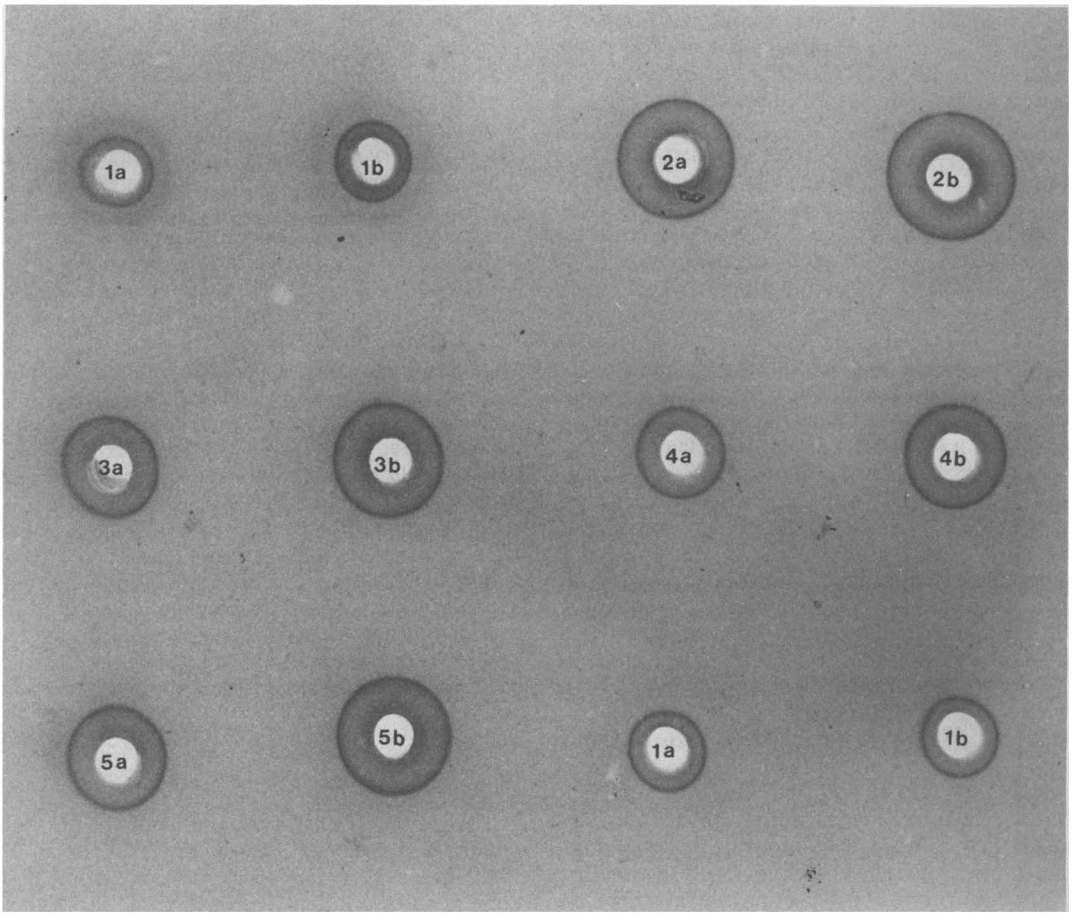

Fig. 2. Single radial immunodiffusion analysis of cytochrome P-452 in control and hypolipidaemic-induced rat liver microsomes. The following amounts of total CO-discernible cytochrome P-450 were loaded on the immunodiffusion plates: 1a and 1b, control microsomes, 16 and 20 pmoles; 2a and 2b; clobuzarit-induced microsomes, 16 and 20 pmoles; 3a and 3b, WY-14,643-induced microsomes, 11 and 15 pmoles; 4a and 4b, clofibrate-induced microsomes, 9 and 14 pmoles; 5a and 5b, bezafibrate-induced microsomes, 9 and 13 pmoles.

lation (total 11 + 12) was significantly increased following pretreatment with hypolipidaemics.

Single radial immunodiffusion analyses in the presence of anti-P-452 IgG of control and hypolipidaemic-induced liver microsomes are shown in Fig. 2. The data indicated an increase in the amount of cytochrome P-452 isoenzymes in the drug-treated animals when similar amounts of total carbon monoxide-discernible cytochrome P-450 were loaded on the diffusion plates. Quantitative analysis of this immunoreactive material with reference to a standard curve revealed that approximately 22% of the total cytochrome P-450 in control rat liver microsomes was cytochrome P-452 isoenzyme and furthermore, pretreatment of rats with hypolipidaemics resulted in a three-fold increase in cytochrome P-452 levels (Table 2). With the four hypolipidaemics, approximately 54–67% of the total cytochrome P-450 was immunochemically identical to cytochrome P-452 isoenzyme.

Table 2. Quantitation of cytochrome P-452 in rat liver microsomes by single radial immunodiffusion

Parameter	Animal treatment				
	Control	WY-14,643 (0.25%)	Clofibrate (0.4%)	Bezafibrate (0.20%)	Clobuzarit (0.05%)
Specific cytochrome P-452 isoenzyme (nmoles/mg)	0.24 ± 0.08	*0.82 ± 0.17	*0.73 ± 0.01	*0.78 ± 0.05	*0.76 ± 0.12
% Total cytochrome P-450	22.0	59.90	57.50	66.70	53.50

Values are means ± S.D. from four individual animals.
* Significantly different from control at $P < 0.001$.

Table 3. Inhibition of lauric acid hydroxylase activity (total 11 + 12 hydroxylation) in rat liver microsomes with anti-P-452 IgG

Anti-P-452 IgG (mg IgG/nmole P-450)	Control	WY-14,643 (0.25%)	Clofibrate (0.4%)	Bezafibrate (0.20%)	Clobuzarit (0.05%)
0	100	100	100	100	100
10	69.3 ± 0.2	87.0 ± 2.3	81.6 ± 0.7	87.5 ± 2.2	91.4 ± 5.3
20	59.4 ± 1.4	76.2 ± 3.1	50.6 ± 1.0	77.8 ± 5.9	65.3 ± 4.0
30	41.7 ± 0.8	50.5 ± 2.8	34.5 ± 1.2	50.9 ± 4.3	39.1 ± 4.0

Compounds were administered in the diet for 14 days at the percentages indicated. Preimmune IgG, added at the same concentrations as anti-P-452 IgG had no significant effect on the catalytic activity.
Results are expressed as % of original activity.

An antibody prepared against clofibrate-induced cytochrome P-452 isoenzyme was tested for its inhibitory effect on lauric acid hydroxylation, a preferred substrate for this isoenzyme. As shown in Table 3 lauric acid hydroxylation (total 11 + 12) was substantially inhibited following preincubation of microsomes with anti-P-452, in both control and hypolipidaemic pretreated rats. These findings indicate that the cross-reactivity of anti-P-452 with the cytochrome P-450 present in microsomes is sufficient to form an immune complex with the haemoprotein and thus inhibit catalytic activity.

Considerable efforts have been made in recent years in elucidating whether the terminal and penultimate oxidation of fatty acids is catalysed by different cytochrome P-450s (reviewed in ref. 19). Studies with the clofibrate-induced purified cytochrome P-452 isoenzyme have shown that the isoenzyme is relatively regio-selective in hydroxylation of lauric acid, yielding a 12/11 hydroxylation product ratio of up to 6 [9]. Preincubation of clofibrate-induced microsomes with anti-P-452, followed by separation of metabolites by HPLC reveals that both the 11- and 12-hydroxylation of lauric acid are inhibited (Fig. 3). However, the terminal and penultimate hydroxylation were differentially inhibited by anti-P-452 IgG, i.e. at the highest antibody concentration, 12-hydroxylation was inhibited to approximately 25% of the original activity, whereas 11-hydroxylation was less sensitive to anti-P-452 IgG with the catalytic activity being inhibited to only approximately 65% of the original activity.

The incubation of ^{14}C-arachidonic acid with rat liver microsomes, followed by extraction of metabolites and analysis by HPLC shows the formation of a number of radioactive metabolites (Fig. 4). These metabolites were absolutely dependent on the presence of both microsomal protein and NADPH (results not shown). Pretreatment of rats with clofibrate resulted in a marked change in both the qualitative and quantitative metabolic profile of cytochrome P-450-dependent oxidation of arachidonic acid (compare Figs. 4a and 4b). For example, in the presence of control microsomes, metabolite(s) B was the major product formed and this peak was essentially absent in the presence of clofibrate microsomes. In addition, metabolite(s) D was the major product after incubation with clofibrate microsomes

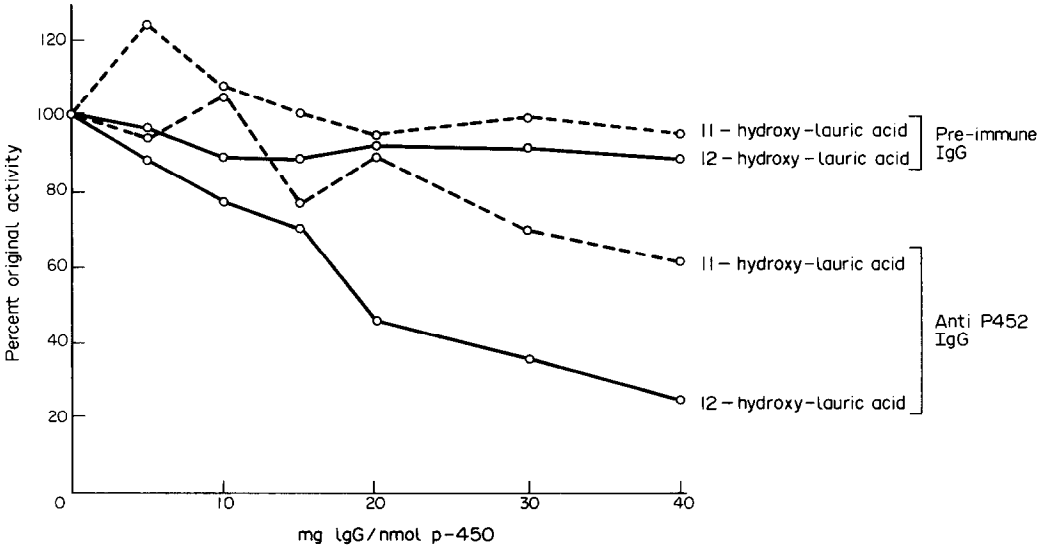

Fig. 3. Differential inhibition by anti-P-452 IgG of the 11- and 12-hydroxylation of lauric acid in liver microsomes derived from clofibrate-pretreated rats. Preincubation with IgG and HPLC metabolite analysis were carried out as described in Materials and Methods. The uninhibited activities were 0.93 and 7.71 nmole 11- and 12-hydroxylauric acid formed per min per mg protein respectively.

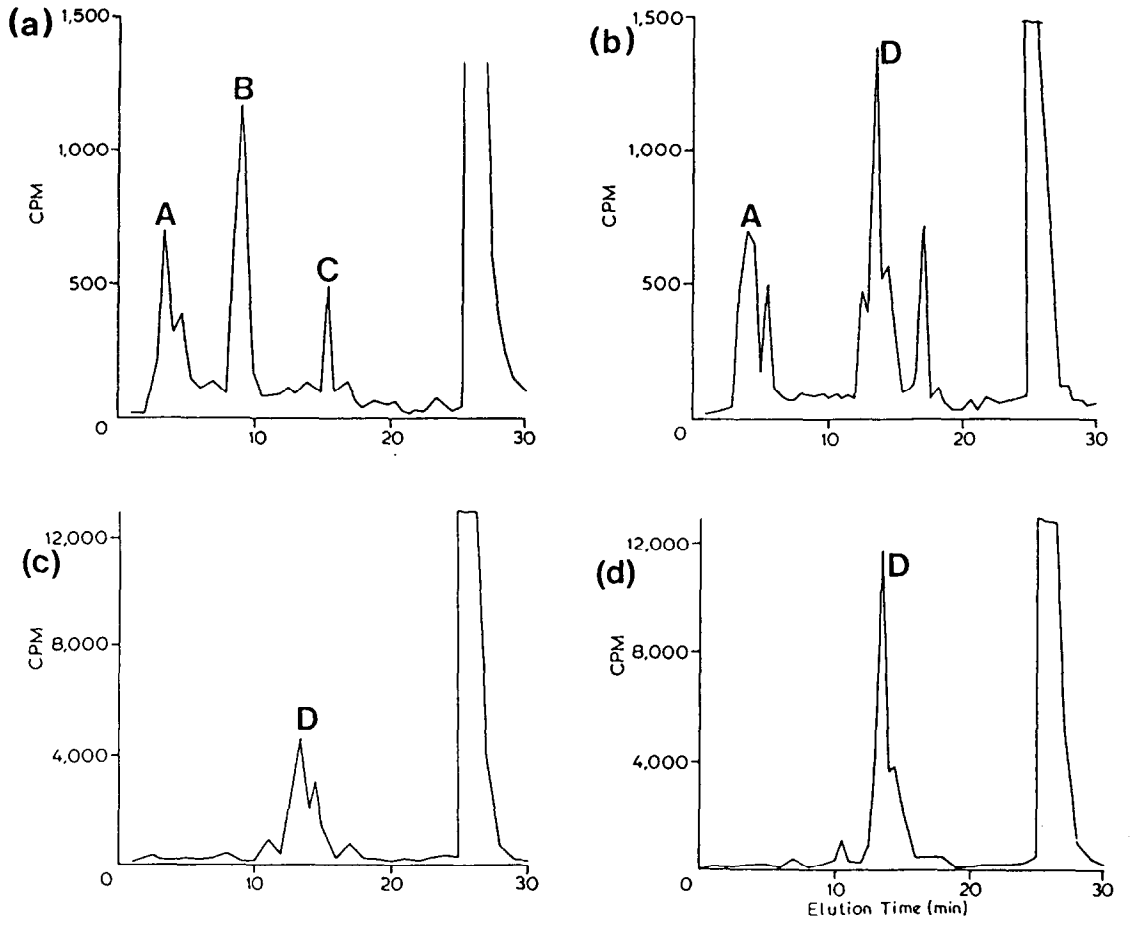

Fig. 4. Arachidonic acid metabolism by rat liver microsomes derived from control and clofibrate-pretreated rats. Incubation and HPLC conditions were as described in Materials and Methods, and typical HPLC profiles are shown for arachidonic acid metabolism as catalysed by (a) control rat liver microsomes, (b) clofibrate-induced rat liver microsomes, (c) reconstituted system containing cytochrome P-452 and (d) reconstituted system containing both cytochrome P-452 and cytochrome b_5. Cytochrome b_5 did not significantly change the yield of radioactivity during extraction and the yields were 80.5 ± 2.8 (in (c)) and 88.1 ± 4.5 (in d)).

(Fig. 4b) and was not present after incubation with control microsomes. Metabolite(s) A was produced in approximately similar amounts by both control and clofibrate-induced microsomes.

Thus it would appear that the formation of metabolite(s) D was induced by clofibrate pretreatment and that clofibrate-dependent induction of cytochrome P-452 may have a role to play in this metabolic pathway. This hypothesis was tested by preincubation of clofibrate-induced microsomes, arachidonic acid and anti-P-452 IgG and subsequent separation of metabolites by HPLC. As shown in Table

Table 4. Inhibition of arachidonic acid metabolism by anti-P-452 IgG in the presence of clofibrate-induced microsomes

Anti-P-452 IgG (mg IgG/nmole P-450)	Arachidonic acid metabolism	
	Metabolite(s) A	Metabolite(s) D
0	100	100
10	116.0	75.7
20	127.0	50.8
30	95.0	20.1

Results are expressed as % of original uninhibited activity, where the absolute 100% values were 0.14 and 0.32 nmole product formed/min per mg protein for metabolite(s) A and D respectively.

Preimmune IgG added at the same concentrations as anti-P-452 IgG, had no significant effect on the catalytic activity.

4, the formation of metabolite(s) D was inhibited by a range of anti-P-452 IgG concentrations, and at the highest antibody concentration tested, the formation of metabolite(s) D was inhibited to approximately 20% of the control activity. In contrast, metabolite(s) A was refractory to inhibition by this antibody, indicating the non-involvement of cytochrome P-452 in this particular metabolic pathway.

Further evidence for the involvement of cytochrome P-452 in the formation of metabolite(s) D from arachidonic acid, was provided by reconstitution studies using the highly purified cytochrome P-452 isoenzyme as described in Materials and Methods. As shown in Fig. 4c, incubation of arachidonic acid with purified cytochrome P-452 resulted in the exclusive formation of metabolite(s) D with a turnover number of 2.50 ± 0.28 nmoles product/nmole cytochrome P-452 per min. Furthermore as shown in Fig 4d, the addition of a twofold molar excess of highly purified liver cytochrome b_5 to the reconstituted cytochrome P-452 system, resulted in approximately a threefold stimulation in metabolite(s) D formation, exhibiting a turnover number of 6.90 ± 1.07 nmoles product/nmole cytochrome P-452 per min. It should be pointed out that cytochrome b_5 probably plays a synergistic and not an oligatory role in arachidonic acid metabolism, in view of the low fatty acid turnover in the reconstituted cytochrome b_5 system in the absence of cytochrome P-452 (0.36 ± 0.12 nmoles product/nmole cytochrome b_5 per min).

DISCUSSION

The results described in this study demonstrate that pretreatment of rats with hypolipidaemic agents results in induction of the cytochrome P-452 isoenzyme as assessed immunochemically. In addition, the induction of cytochrome P-452 is mirrored by a concomitant, parallel increase in lauric acid hydroxylase activity.

An important consideration in the immunochemical analysis of cytochrome P-452 is the specificity of the antibody used, as non-specific interactions would result in an over-estimation of this isoenzyme. We believe that our immunochemical method is specific for the following reasons. Firstly, as we have previously reported, the antibody to cytochrome P-452 does not cross-react with either the major haemoprotein isoenzymes isolated from the hepatic microsomes of rats pretreated with phenobarbital or β-naphthoflavone [10]. Secondly, as shown in Fig. 2, immunodiffusion of control and hypolipidaemic induced rat liver microsomes in the presence of anti-P-452 IgG reveals a single ring of precipitation. If the anti-P-452 IgG was grossly non-specific, the radial immunodiffusion analyses would have resulted in multiple concentric rings, and this was not observed. Thirdly, when control and induced microsomes are subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate and then analysed by Western blotting with anti-P-452 IgG, only one polypeptide band is observed (data not shown). This latter polypeptide has a monomeric molecular weight of approximately 51.5 kilodaltons, equivalent to the molecular weight of authentic cytochrome P-452. Accordingly, for the above reasons, we maintain that the anti-P-452 IgG is specific for its homologous antigen, does not cross-react with other microsomal proteins and therefore constitutes a sound analytical technique for the quantitation of this isoenzyme.

Using the above immunochemical method, we determined the amount of cytochrome P-452 to be 22% of the total cytochrome P-450 population in control liver microsomes. This relatively high level of cytochrome P-452 isoenzyme is in direct contrast to the amounts of other well-characterised cytochrome P-450 isoenzymes in control rat liver microsomes. For example, both cytochrome $P\text{-}450_b$ and cytochrome $P\text{-}450_c$ (major forms induced in the rat by phenobarbital and polycyclic aromatic hydrocarbons respectively) only occur to the extent of approximately 1–2% in control microsomes [20] and are essentially at the immunochemical minimum level of detection. After induction with phenobarbital or 3-methylcholanthrene, cytochromes $P\text{-}450_b$ and $P\text{-}450_c$ are elevated to 35% and 68% respectively of the total cytochrome population [20], approximately equal to the amounts of cytochrome P-452 isoenzyme in hypolipidaemic-induced microsomes observed in the present study. Although the $P\text{-}450_b$ and $P\text{-}450_c$ isoenzymes have been extensively studied and characterized, much less information is available on constitutive cytochrome P-450 isoenzymes. Accordingly, the use of hypolipidaemic drugs in inducing constitutive cytochrome P-452 concentrations may well prove to be a useful tool in the study of the properties of the constitutive haemoprotein(s).

The role of cytochrome P-450 isoenzymes in the oxidation of fatty acids at the terminal and penultimate carbon atoms has received considerable attention (reviewed in ref. 19). For example, phenobarbital pretreatment of rats results in an increase in the 11-hydroxylation of lauric acid and no change in the 12-hydroxylase activity, whereas pretreatment with β-naphthoflavone results in little change in either activity [21]. To our knowledge, hypolipidaemic-induced cytochrome P-452 is the only isoenzyme of cytochrome P-450 that exhibits a preferential, regio-selectivity for the 12-hydroxylation of lauric acid, as previously described by us [9]. This selectivity was further confirmed in the present study as evidenced by the preferential inhibition of the lauric acid 12-hydroxylase activity by anti-P-452 IgG.

A puzzling feature of the high specificity of lauric acid for cytochrome P-452 is the observation that only trace amounts of this fatty acid are found in hepatic microsomal membranes and it appears likely that lauric acid serves only as a model substrate for a physiologically more relevant endogenous substrate. In the current studies, we have shown that arachidonic acid may serve as a suitable endogenous substrate for cytochrome P-452 as evidenced both by the clofibrate-induced increase in metabolite(s) D formation and inhibition of formation of this metabolite(s) in microsomes by anti-P-452 IgG. Although we have shown that exogenous arachidonic acid serves as an excellent substrate for cytochrome P-452, the role of this isoenzyme in the metabolism of endogenous arachidonate was not directly addressed by our studies. However we have no reason to sus-

pect that endogenous and exogenous arachidonate are metabolized any differently in the liver. This conclusion is substantiated by the observation of Capdevila and co-workers, who have reported that arachidonate metabolites similar to those reported by us are found *in vivo*, and they tentatively concluded that the production of these metabolites were cytochrome P-450-dependent [22].

The ability of cytochrome P-452 to oxidatively metabolize arachidonic acid was further substantiated in our studies by the observation that highly purified cytochrome P-452 actively metabolized this fatty acid in a reconstituted enzyme system (Figs. 4c and 4d). Furthermore, the exclusive production of metabolite(s) D by purified cytochrome P-452 indicates that metabolites A, B and C (Fig. 4) are either metabolic products of other microsomal isoenzymes of cytochrome P-450 or are derived from a different oxidase enzyme system. The synergistic involvement of cytochrome b_5 in the cytochrome P-452-dependent oxidation of arachidonic acid was established in our studies, but the mechanism whereby cytochrome b_5 acts, clearly remains to be established.

Our conclusions regarding the role of cytochrome P-452 in the metabolism of fatty acids are in general agreement with other workers who have shown that arachidonic acid is metabolized by other cytochrome P-450 isoenzymes to a variety of metabolites including mono-, di- and tri-hydroxy and epoxides [23, 24]. In particular, pretreatment of rats with the hypolipidaemic drug ciprofibrate resulted in a substantial induction of the terminal ω-hydroxylation of arachidonic acid at carbon 20 (R. W. Estabrook, personal communication). Clearly, in our studies, the chemical nature of metabolite(s) D remains to be established, and is the subject of our current work. The observation that this metabolite(s) is substantially increased by clofibrate pretreatment and its relative elution position from the HPLC column is not inconsistent with the possibility that it is a mono-hydroxylated metabolite of arachidonic acid, possibly the 20-hydroxylated metabolite. In light of the above discussion, it is clear that induction of cytochrome P-452 by hypolipidaemic agents provides an opportunity for the metabolism of free arachidonic acid in addition to that derived from microsomal phospholipids. The profound changes in the metabolism of arachidonic acid may therefore have a pronounced effect on cellular homeostasis, as evidenced by the potent biological activities of the oxidized metabolites of arachidonic acid [25–27].

Although the mode of action of hypolipidaemic drugs is not fully understood, it has been suggested that the stimulation of the peroxisomal β-oxidation of fatty acids may play a central role [5]. The relative contribution of the ω-hydroxylation of fatty acids in the triglyceride-lowering pharmacology of hypolipidaemics remains to be established. Further studies are necessary to establish whether the stimulation of ω-hydroxylation is reflected in similar increases in ω-oxidation. It is possible that stimulation of ω-oxidation subsequent to hypolipidaemic exposure may lead to inhibition of triglyceride synthesis. This mechanism has been rationalized by Bjorkhelm [28], who showed that there is competition for fatty acid substrates for the two competing pathways of triglyceride biosynthesis and fatty acid catabolism via ω-oxidation. Further work is required to test this possibility and to establish whether or not the ω-hydroxylation of fatty acids in general is stimulated at therapeutic doses of hypolipidaemic drugs and whether such increases in ω-hydroxylation actually reflect similar increases in ω-oxidation.

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