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ROLE OF HEPATIC AND RENAL CYTOCHROME P-450 IVA1 IN THE METABOLISM OF LIPID SUBSTRATES

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Abstract—The role of clofibrate-inducible cytochrome P-450 IVA1 in the metabolism of endogenous lipids in both rat liver and kidney microsomal fractions has been investigated. 20(ω)-hydroxyarachidonic acid has been identified as a major metabolite after incubation with both tissue fractions and the structure confirmed by mass spectrometry. The arachidonic acid 20-hydroxylase activity is inducible by clofibrate in both liver and kidney, indicating that cytochrome P-450 IVA1 is probably the enzyme responsible for this activity. In addition, the kidney exhibited higher rates of arachidonate 20-hydroxylase activity than the liver (in both control and induced states). Although leukotriene B_4 was also hydroxylated in the 20-position in both liver and kidney, clofibrate induction resulted in a decrease (approximately 50%) in hydroxylase activity. In addition, the absolute level of leukotriene B_4 20-hydroxylase activity in both tissue homogenates and by purified cytochrome P-450 IVA1 in a reconstituted system, was 2–3 orders of magnitude lower than the corresponding activity for lauric acid and arachidonic acid as substrates, indicating that the leukotriene was not the preferred substrate for this enzyme. Computer modelling of the conformational geometries of the above three potential cytochrome P-450 IVA1 substrates have shown that both lauric and arachidonic acids adopt a compact, 'hairpin' structure that are almost superimposed on each other, thereby rationalizing why they are relatively good substrates for this isoenzyme. By contrast, leukotriene B_4 adopts a more bulky geometry than the two fatty acids, thereby providing a coherent structural reason why it is a poorer substrate for the cytochrome P-450 IVA1 isoenzyme.

The fatty acid hydroxylating cytochrome P-450s are constitutive enzymes that participate in the metabolism of endogenous substances. Medium chain length (C_6–C_{12}) fatty acids are oxidized by either of two microsomal pathways: ω- or ($\omega - 1$) oxidation [1–3]. Both of these fatty acid oxidation pathways have been shown to function in liver, kidney, lung, colon, intestinal mucosa and polymorphonuclear leukocytes [4–8].

It has been proposed that the ($\omega - 1$)-hydroxylating activity is catalysed by several cytochrome P-450 isoenzymes and is therefore non-specific. By contrast the ω-hydroxylase is highly specific for fatty acids [9]. Furthermore, parallel changes in renal ω- and $\omega - 1$ hydroxylation of lauric acid brought about by inducers and inhibitors suggests that a single cytochrome P-450 enzyme mediates both reactions in the kidney [10, 11]. In contrast, preferential elevation of hepatic laurate ($\omega - 1$) hydroxylation by phenobarbital treatment [12, 13] and preferential inhibition of this same activity by CO, SKF-525A, metyrapone, aminopyrine and α-naphthoflavone [10, 13] suggest that different cytochrome P-450 isozymes catalyse laurate ω- and ($\omega - 1$) hydroxylations in liver [13].

Early studies in our laboratory reported the isolation of a specific clofibrate-induced hepatic lauric acid ω-hydroxylase (termed cytochrome P-450 IVA1) [14]. Subsequent studies demonstrated that pretreatment of experimental animals with several hypolipidaemic agents resulted in a marked elevation in the hepatic microsomal hydroxylation of fatty acids in rat liver and kidney [15–17], with concomitant induction of cytochrome P-450 IVA1.

Recent studies have demonstrated that the liver microsomal cytochrome P-450 system metabolizes arachidonic acid to a variety of oxygenated products such as hydroxyeicosatetraenoic acids (HETEs), epoxyeicosatetraenoic acids (EETs) and ω- and ($\omega - 1$) hydroxylated acids [18]. Furthermore, our laboratory has reported that arachidonic acid serves as an excellent substrate for the highly-purified hypolipidaemic-induced hepatic cytochrome P-450 IVA1 in a reconstituted enzyme system [15]. Similarly, it has previously been reported that renal cytochrome P-450 is active in the ω- and ($\omega - 1$) oxidation of fatty acids [1–3], and various groups have investigated the regulation of arachidonic acid metabolism by cytochrome P-450 in the rabbit kidney [19–21].

A puzzling feature of the high specificity of lauric acid for cytochrome P-450 IVA1 is the fact that only trace amounts of both free and esterified laurate are found in the membrane of the hepatic and renal endoplasmic reticulum and it would therefore appear likely that lauric acid serves only as a model substrate for a physiologically more relevant endogenous substrate(s). Accordingly, it is our purpose to compare

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and contrast the ability of both control and clofibrate-induced rat liver and kidney microsomal fractions to hydroxylate lauric acid, arachidonic acid and leukotriene B_4.

MATERIALS AND METHODS

Chemicals. Clofibrate was a gift from I.C.I. plc, Pharmaceuticals Division (Macclesfield, U.K.), leukotriene B_4 and 20-hydroxy leukotriene B_4 were gifts from Merck Frost Canada Inc. (Quebec, Canada). 1-[^{14}C]arachidonic acid was purchased from the Radiochemical Centre (Amersham, U.K.), and NADPH was purchased from the Sigma Chemical Co. (Poole, U.K.). All other chemicals were obtained from commercial sources and were of the highest purity available.

Animals and drug pretreatment. Male Wistar albino rats (150–200 g body wt, University of Surrey Breeders, U.K.) were pretreated with clofibrate (250 mg/kg) by gastric intubation once daily for 3 days. Clofibrate was administered with peanut oil as the vehicle, control animals being given peanut oil at 5 ml/kg and all animals were killed at the start of the fourth day after commencement of treatment (i.e. 24 hr after the last dose). Male Wistar rats were also induced with phenobarbital [0.1% (w/v)] in their drinking water for 5 days and killed on day 6. Four animals were used per group.

The livers, following excision, were rinsed and perfused with 0.9% (w/v) sodium chloride to remove contaminating haemoglobin, blotted dry and weighed. The kidneys from animals of the same group were pooled and decapsulated. All subsequent steps were performed at 5°. The livers and kidneys were scissor-minced and homogenized in 0.24 M sucrose using a Potter Elvehjem glass Teflon homogenizer. The liver homogenate was adjusted to 25–33% (w/v) and kidney homogenate to 15% (w/v) by the addition of 0.25 M sucrose. Microsomes were prepared by a standard, rapid calcium precipitation and centrifugation method [22].

Enzyme assays. The products of arachidonic acid metabolism were isolated from an incubation mixture containing rat liver microsomes (1 nmol total cytochrome P-450/ml), 10 mM magnesium chloride, 50 mM Tris buffer (pH 7.5) and 0.1 mM [^{14}C]arachidonic acid (0.3 mCi/mmol). The [^{14}C]arachidonic acid was obtained in 10 mg sealed vials. The vial was broken open and 2 ml of 0.1 M Tris HCl (pH 7.5) was added. The pH was then adjusted to between 8.5 and 9.2 by the addition of 10 μl aliquots of 2 M sodium hydroxide. The total volume was then adjusted to 2.63 ml by the addition of distilled water. As the pH was adjusted the suspension cleared as the sodium salt was formed.

Following a 5 min pre-incubation period at room temperature, with gentle (magnetic bar) stirring, NADPH (1 mM final concentration) was added to initiate the reaction. The reaction mixture (final vol., 2 ml) was then transferred to a 37° shaking water bath. The reaction was terminated after 30 min by the addition of 2 ml ethyl acetate plus 0.5 ml of 1 M HCl and 0.01% (w/v) butylated hydroxytoluene as anti-oxidant. The samples were extracted three times with 2 ml of ethyl acetate, the combined extracts being dried over anhydrous sodium sulphate, filtered and evaporated to dryness under a stream of nitrogen. The dried ethyl acetate extracts were reconstituted in 150 μl of starting solvent (water/acetonitrile, 60:40, containing 0.1% acetic acid). The metabolites were separated by reverse-phase HPLC using a C_{18} Microbondapak (4.6 mm × 150 mm) MCH-10 column, using a shallow elution gradient ranging from water/acetonitrile (60:40 containing 0.1% acetic acid) to 100% acetonitrile, at a flow rate of 1 ml/min for 50 min. The elution profile of the radioactive products was monitored using a Berthold LB503 HPLC radioactivity monitor (Lab-Impex, Twickenham, U.K.) interfaced with a Commodore PET (Series 4000) thus allowing quantitation of the radioactive metabolites. Rates of metabolite formation were calculated from the fractional conversion of the radio-labelled substrate, making the estimation of recovery unnecessary. Replicate samples did not vary by greater than 10%.

The main metabolic peak resulting from arachidonic acid metabolism eluted consistently at approximately 43.8 min, was collected, evaporated to dryness, and dissolved in hexane. The metabolites were resolved by normal-phase HPLC on a Spherisorb 5 μm silica column (4.6 mm × 300 mm), utilizing a linear gradient of 1% isopropanol, 0.1% acetic acid and 96.9% hexane in 30 min at a flow rate of 3 ml/min. The metabolites from normal phase chromatography were collected and dried under nitrogen. The sample was then resuspended in 70 μl of methanol and mixed with 700 μl of ethereal diazomethane and left at room temperature for 10 min. The ethereal diazomethane was removed under nitrogen and the methylated sample was resuspended in 50 μl of bis(trimethylsilyl)-trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS) (Pierce, U.K.) and heated at 70° for 60 min.

Samples were run on a Hewlett Packard 5890 gas chromatograph directly coupled to a TSQ-70 (Finnigan-MAT) mass spectrometer. The GC was carried out on a 30 m × 0.25 mm i.d. DB5 (WCOT) fused silica capillary column (Jones Chromatography) using a splitless injection technique and helium carrier gas with a head pressure of 15 psi. The GC conditions were 130° for 1 min then temperature programmed at 25°/min to 250° for 20 min with the injection port and GC–MS interface at 260°. Electron impact mass spectrometry was carried out at 70 eV.

The products of LTB_4 metabolism were isolated from reaction vessels containing liver or kidney microsomes (0.6–0.8 mg protein/ml, equivalent to either 1 nmol or 0.1 nmol cytochrome P-450/ml for liver or kidney homogenates respectively, unless otherwise indicated), unlabelled LTB_4 (5,12-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid, 10 μM) and magnesium chloride (5 mM) in a final volume of 0.8 ml of 50 mM Tris buffer (pH 7.5). After 1 min of preincubation, the reaction was initiated by the addition of NADPH (1 mM final concentration). Samples were incubated in a shaking water bath at 37°. The reaction was terminated 15 min later by the addition of 0.4 ml of 10% acetic acid and the samples immediately placed on ice. The incubation samples were extracted three times with

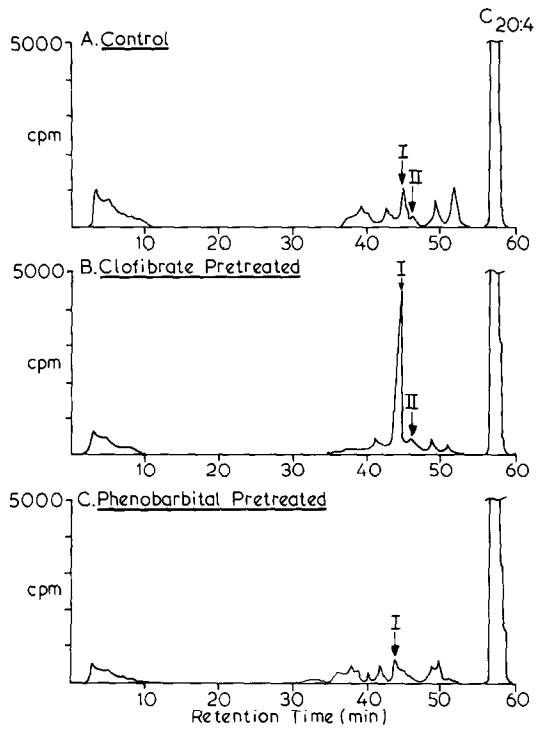

Fig. 1. Metabolism of arachidonic acid by rat liver microsomes. Microsomes from rats pretreated with either clofibrate or phenobarbital were incubated with arachidonic acid, separated by reverse phase HPLC with subsequent radiochemical detection as described in Materials and Methods. (A) Control; (B) clofibrate-pretreated; (C) phenobarbital-pretreated. $C_{20:4}$: arachidonic acid.

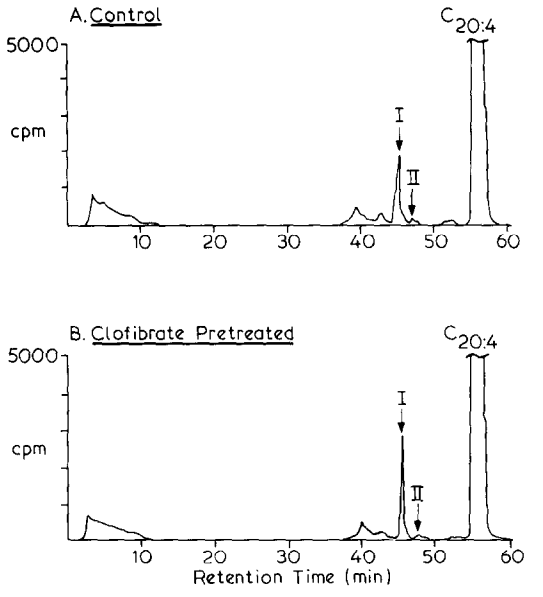

Fig. 2. Metabolism of arachidonic acid by rat kidney microsomes. *In vitro* incubations, metabolite separation by reverse phase HPLC and radiochemical detection were performed as described in Materials and Methods. Kidney microsomes were derived from either control (A) or clofibrate-treated rats (B). $C_{20:4}$: arachidonic acid.

ether (3 ml) and the ether evaporated under nitrogen.

The dried ether extracts were re-suspended in 200 μl of HPLC starting solvent (water: acetonitrile, 80:20, containing 0.1% acetic acid) and an aliquot taken for injection. LTB_4 and its metabolites were separated on an HPLC system consisting of two model 510 solvent delivery systems with automated gradient controller, a model 481 variable wavelength UV detector set at 280 nm, a UK6 manual injector (Waters Associates Inc., Milford, MA) and a C_{18} Ultrasphere ODS reverse phase column (4.6 mm × 150 mm). The percentage of acetonitrile was increased exponentially from 20 to 90% in 25 min, then held at 90% for an additional 5 min. The flow rate was maintained at 1 ml/min. Percentage recovery and calibration curves were estimated by the addition of known quantities of 20-OH LTB_4 standard to a blank run (i.e. minus NADPH). This was determined by peak heights at 280 nm corrected for recovery (80–90%) and replicate samples did not vary by greater than 10%.

Molecular modelling of substrates. Molecular structures were generated from first principles using the COSMIC software package (written by J. G. Vinter, A. Davis and M. R. Saunders, Smith Kline and French Ltd, Welwyn Garden City, U.K.) where a combination of Simplex and Newton–Raphson minimization procedures was employed. In essence these procedures utilize bond lengths and bond angles for each substrate as the raw data and the routines identify a minimum energy (i.e. most thermodynamically stable) conformation. The minimum energy conformers were then computer-fitted and superimposed by utilization of routines present in the COSMIC package. Some reorientation of the molecular structures occurs during the fitting process and, therefore, the view of each structure may change depending on the particular second substrate chosen. All calculations were performed on a Sigmex S6130 graphics workstation, coupled to a MicroVax II computer.

RESULTS

Characterization of hydroxylated arachidonic acid metabolites reverse phase HPLC

Figure 1 shows the profile of metabolites formed during the NADPH-dependent hydroxylation of arachidonic acid by different microsomal preparations. Pretreatment with the hypolipidaemic drug, clofibrate, results in a dramatic change in the metabolite profile catalysed by the microsomal fractions compared to control and phenobarbitone-pretreated microsomes. The formation of peak I (retention time 44 min) is the predominant feature of the reaction catalysed by clofibrate pretreated microsomal fractions (Fig. 1B). From previous studies on hypolipidaemic-induced arachidonic acid metabolism [15, 18] we can tentatively postulate that peak I represents the 20-hydroxyarachidonic acid metabolite, and that peak II (retention time 46 min) represents the 19-hydroxy metabolite.

Capdevila *et al.* [18] reported that treatment of rats with ciprofibrate results in a clear stimulation of ω- and (ω − 1) oxidation concomitant with a marked

Table 1. Effect of clofibrate pretreatment on the rates of 20- and 19-hydroxylation of arachidonic acid catalysed by liver and kidney microsomal fractions*

	Kidney		Liver	
Product	Control	Clofibrate pretreated	Control	Clofibrate pretreated
19-Hydroxy	0.06 ± 0.01	0.11 ± 0.03†	0.03 ± 0.01	0.04 ± 0.01
20-Hydroxy	4.97 ± 0.66	7.28 ± 1.20‡	0.22 ± 0.05	1.16 ± 0.05†

* The values given are the averages ± SD of four determinations and values are expressed as nmol of product formed per min per nmol of total cytochrome P-450, and are derived from the reverse phase column.

P values for results significantly different (Student's *t*-test) from control data at: † $P < 0.001$ and ‡ $P < 0.01$.

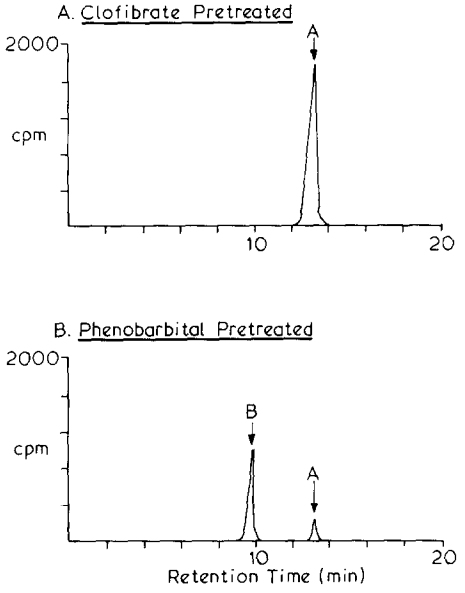

Fig. 3. Resolution of arachidonic acid metabolites by normal phase HPLC. Liver microsomes from clofibrate (A) or phenobarbital-pretreated rats (B) were initially separated by HPLC as described in Fig. 1. Peak I (of retention time 44 min initially separated by reverse phase HPLC (Fig. 1)) was rechromatographed by normal phase HPLC as described in Materials and Methods.

decrease in the ability of the liver microsomal suspensions to catalyse both olefin epoxidation and allylic oxidations. Although the formation and identification of EETs and HETEs was not directly addressed in this study Fig. 1B clearly demonstrates that the induction of ω- and $(\omega - 1)$ oxidation following clofibrate pretreatment is concomitant with a marked decrease in the formation of all other metabolites. The polar material which elutes between 2 and 10 min (Figs 1 and 2) represents either an impurity or non-cytochrome P-450-dependent metabolism, as demonstrated by its presence in a blank run minus the co-factor, NADPH. Oliw *et al.* [23] demonstrated that these metabolites co-chromatographed with prostaglandins, the products of cyclooxygenase metabolism, on reverse phase HPLC.

Figure 2 represents the reverse phase separation of metabolites formed by control and clofibrate-pretreated renal microsomes. Peak I had the same retention time (44 min) as that observed in liver, but unlike control liver, represented a major metabolite in control kidney microsomes. Furthermore, in the kidney, peak I was only induced 1–2-fold following clofibrate pretreatment, whereas in the liver a 5–6-fold induction of this metabolite(s) was observed.

Quantitative analysis of peaks I and II from reverse phase HPLC, thought to represent the ω- and $(\omega$-1) hydroxylation products of arachidonic acid is shown in Table 1. In renal microsomes, clofibrate pretreatment results in a 1.5-fold and a 1.8-fold induction of the ω- and $(\omega - 1)$ hydroxy metabolites, respectively. The elevation of the ω-hydroxy metabolite of arachidonic acid is much more marked in clofibrate pretreated liver. A 5–6-fold induction of the ω-hydroxy metabolite is observed with no induction of the $(\omega - 1)$ metabolite being observed (Table 1).

Another feature of renal arachidonic acid metabolism is the comparative rate of conversion to the ω-hydroxy metabolite. This is particularly evident in the control situation where control kidney microsomes exhibit a 22-fold higher rate of conversion to the ω-hydroxy metabolite when compared to control liver microsomes. By comparison, following clofibrate pretreatment, a 6-fold higher rate in the ω-hydroxylation of arachidonic acid was observed in the kidney as compared to the liver.

Normal phase HPLC

To further purify and identify peak I (Figs 1 and 2), subsequent normal phase HPLC was performed on peak I, the resulting eluate being subjected to gas chromatography followed by mass spectrometry.

Peak I (Figs 1 and 2) was separately collected from the reverse phase separation of hepatic and renal, control and clofibrate incubations and dried under nitrogen. The dried extracts were reconstituted in 200 μl hexane and subjected to normal phase HPLC, as described in Materials and Methods. Figure 3 shows the results of the chromatography of peak I on normal phase HPLC. Figure 3A shows the chromatogram of peak I derived from clofibrate pretreated liver microsomes. A single peak with a retention time of 13.5 min (peak A) was observed. This suggests that peak I from clofibrate-pretreated hepatic microsomes represented only one metabolite.

Table 2. Recovery of radioactive metabolites from normal phase HPLC

	Counts from reverse phase HPLC	Counts from normal phase HPLC	% Recovery	Arachidonic acid 20-hydroxylase activity (normal phase)*
Control kidney	950 ± 28	675 ± 20	71	3.53 ± 0.47
Clofibrate kidney	1180 ± 46	885 ± 35	75	5.46 ± 0.90
Control liver	526 ± 30	400 ± 23	76	0.16 ± 0.04
Clofibrate liver	2075 ± 57	1660 ± 46	80	0.93 ± 0.04

The values given are the means ± SD of four determinations.
* nmol of 20-hydroxy arachidonic acid formed per min per nmol of total cytochrome P-450 and are corrected for recovery for the normal phase HPLC step.

Although the size of this peak varied, with respect to the microsomal incubation (Table 2), only the one peak was observed and the retention time was consistent. The quantitative differences in the formation of peak I by control and clofibrate-induced microsomes (Table 1) are clearly reflected in the counts in the metabolite peaks and turnover rate observed following normal phase HPLC (Table 2).

Figure 3B represents the separation of peak I from phenobarbital pretreated microsomes (Fig. 1C). Two peaks are observed with retention times of 10 min (peak B) and 13.5 min (peak A). The ratio of peaks B:A is 3:1 with peak A corresponding to peak A seen in Fig. 3A. Capdevila *et al.* [24] have identified peaks B and A as the 19- and 20-hydroxyarachidonic metabolites, respectively. These observations with phenobarbital-pretreated microsomes also suggest that peak A obtained from control and clofibrate-induced hepatic and renal microsomal incubations (Fig. 3A) represents the 20-hydroxyarachidonic acid metabolite.

Gas chromatography–mass spectrometry (GC–MS)

The 70 eV electron impact mass spectra of the trimethylsilyl derivative of the methyl ester of peak A, is shown in Fig. 4A. This was obtained from a peak with a retention time equivalent to a C-value of 22.6 when compared to a standard mixture of C_{16}, C_{18}, C_{20}, C_{22} and C_{24} fatty acid methyl esters. The m^+ of 406 indicated a mono-hydroxylated fatty acid while the C-value and fragmentation pattern indicated that hydroxylation had occurred at the C-20 position. Major ions in the upper mass range were m/z 406 (m^+), 391 $(m - 15)^+$, 316 $(m - 90)^+$. The mass spectrum and C-value almost certainly confirm the structure as 20-hydroxyarachidonic acid and is in full agreement with the structural data presented by Oliw *et al.* [23]. In addition, the absence of a major peak at m/z 117 (characteristic of the 19-hydroxy metabolite, Ref. 23) again indicates that hydroxylation had taken place exclusively at the 20-position. In addition, although an in-chain hydroxylated metabolite would give the same molecular ion as the 20-hydroxy metabolite, the fragmentation pattern noted is not consistent with this possibility.

Further confirmation of the hydroxy metabolite (mol wt 406) was obtained by chemical ionization mass spectrometry using ammonia as the reagent gas. This technique yielded the data shown in Fig. 4B showing intense m/z ions, 424 $(m + NH_4)^+$ and 407 $(m + 1)^+$.

Peak A fractions from control and clofibrate induced rat liver and kidney microsomes all gave similar results on mass spectral analysis (data not shown).

Effects of clofibrate pretreatment on hepatic and renal leukotriene B_4 (LTB_4) metabolism

LTB_4 and its metabolites were quantitated by a reverse phase HPLC system as described in Materials and Methods. Table 3 shows that control kidney and liver microsomes metabolize LTB_4 preferentially to the 20-hydroxy LTB_4. The ratio of 19:20 hydroxy metabolite formation is 1:4 in control kidney microsomes and 1:9 in control liver microsomes. This ratio remains unaltered in both tissues following clofibrate pretreatment. However, a 50% reduction in the formation of both the ω- and $(\omega - 1)$ metabolites in both tissues was observed. Clearly LTB_4 is not a preferred substrate for the clofibrate-induced cytochromes P-450 IVA1 of rat liver and kidney.

Electrophoretically homogeneous hepatic cytochrome P-450 IVA1 [14] was also used as an enzyme source in a reconstituted enzyme system with the following rates of 20- and 19-hydroxylation of LTB_4 being observed:

19-OH LTB_4 = 3.50 ± 0.31 pmol/min/nmol cytochrome P-450 IVA1
20-OH LTB_4 = 24.80 ± 6.34 pmol/min/nmol cytochrome P-45 IVA1

This low turnover rate coupled with the results of microsomal incubations additionally suggests that LTB_4 hydroxylases may be distinct from the laurate hydroxylase (i.e. cytochrome P-450 IVA1-dependent) and furthermore are not induced by hypolipidaemic agents.

Molecular modelling of cytochrome P-450 IVA1 substrates

Figure 5A shows the overlaid structures of arachidonic acid and lauric acid and indicates a close similarity in the structural conformation of these two fatty acids. However, in the case of LTB_4, the effect of the sequence of *trans* carbon–carbon double bonds is to bring about a displacement in the positions of the two alkyl chains that form the arms of the 'hairpin' structure, relative to those of lauric and arachidonic acids (Fig. 5B and C). Such variabilities in the structural conformation of LTB_4 compared to arachidonic acid and lauric acid may therefore affect its binding

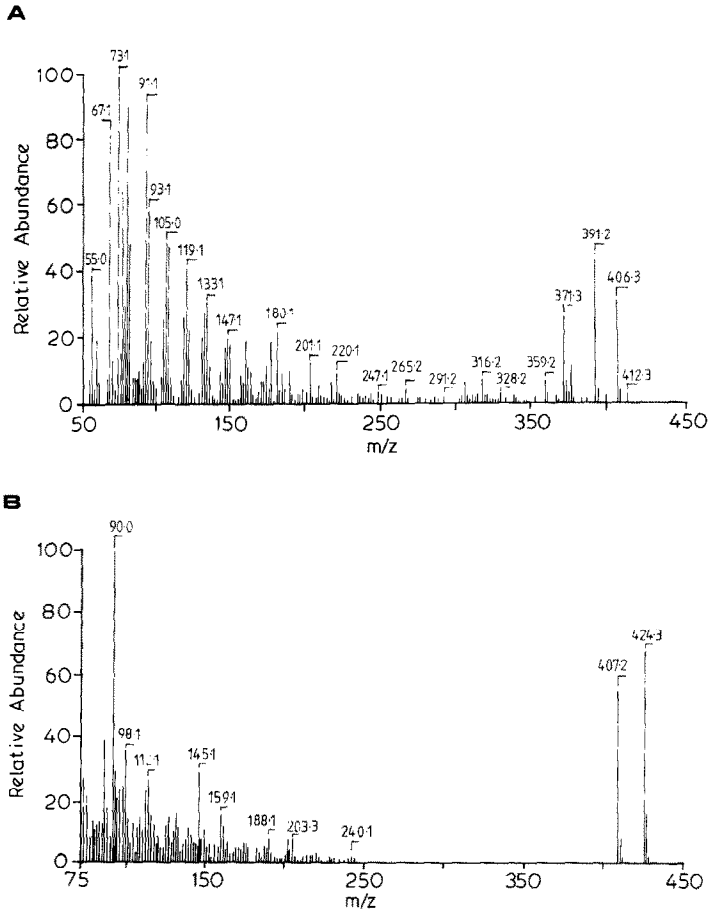

Fig. 4. Electron impact mass spectrum of arachidonic acid metabolite. (A) Metabolite peak A (Fig. 3) was methylated and the trimethylsilyl derivative prepared and analysed by gas chromatography–electron impact mass spectrometry as described in Materials and Methods. (B) Metabolite peak A (Fig. 3) was subjected to chemical ionization mass spectrometry, using ammonia as the reagent gas.

Table 3. Effect of clofibrate treatment on hepatic and renal microsomal hydroxylation of leukotriene B_4*

Hydroxy metabolite	Kidney microsomes		Liver microsomes	
	Control	Clofibrate pretreated	Control	Clofibrate pretreated
19-OH ($\omega - 1$)	11.38 ± 2.02	5.25 ± 0.96†	9.38 ± 1.80	4.88 ± 1.11†
20-OH (ω)	47.76 ± 3.20	21.46 ± 2.36†	85.45 ± 4.68	43.34 ± 2.45†

* The values are the averages ± SD for four determinations and are expressed as pmol of product formed per min per nmol total cytochrome P-450.

† P values for results significantly different (Student's t-test) from control data at $P < 0.001$.

to the active site of cytochrome P-450 IVA1 and its subsequent metabolism to hydroxylated products.

DISCUSSION

The results of this study have demonstrated that arachidonic acid serves as an excellent endogenous substrate for cytochrome P-450 IVA1 in rat liver and kidney microsomes (Table 2). Mass spectral studies showed that the major metabolite in both hepatic and renal arachidonate metabolism was the 20-hydroxyarachidonic acid. Interestingly, the 20-hydroxylation of this fatty acid represented 60% of the total metabolism observed in control kidney microsomes, whereas in control liver, it represented only 5%. This suggests that in rat kidney, cytochrome P-450 IVA1 represents a major constitutive form, or that a structurally-related isoenzyme exists with a higher specificity towards the 20-hydroxylation of arachidonic acid. Other workers have previously

Table 4. Comparison of lauric acid and arachidonic acid as substrates for cytochrome P-450 IVA1

	Arachidonic acid* ω-hydroxylase activity	Lauric acid*† ω-hydroxylase activity
Control kidney	4.97	34.62
Clofibrate kidney	7.28	91.88
Control liver	0.22	2.55
Clofibrate liver	1.16	14.90

* The values are expressed as nmol of product formed per min per nmol total cytochrome P-450.
† Data derived from Ref. 16.

reported [23, 25–27] that the NADPH-dependent arachidonate oxygenase of rabbit kidney cortex catalyses arachidonic acid oxidation to form the 20- and 19-hydroxyarachidonic acids as major reaction products (85% of total) while other metabolites (EETs plus DHETs) account for about 8% of the total reaction products [28].

Clofibrate pretreatment results in a differential induction of hepatic and renal cytochrome P-450 IVA1-dependent metabolism of arachidonic acid. A 5–6-fold stimulation of the 20-hydroxylation is observed in the liver, whereas only a 1–2-fold induction is seen in the kidney. However, in both control and clofibrate-induced states, the kidney remains more efficient at metabolizing arachidonic acid to the 20-hydroxy metabolite. Previous studies in this laboratory using lauric acid as a substrate [16, 17] have also demonstrated a much higher rate of formation of the ω-hydroxy metabolite in control and clofibrate-induced kidney microsomes (Table 4). It is also interesting to note that the rate of hepatic and renal arachidonic acid metabolism is several-fold lower than the rate observed for lauric acid (Table 4). Ellin *et al.* [1, 2] have provided a hypothesis regarding the nature of the active site of a kidney microsomal cytochrome P-450 active in the ω- and (ω − 1) oxidation of saturated fatty acids. Their studies demonstrated an effect of fatty acid chain length on the rate of ω- and (ω − 1) oxidation. Maximal activity was obtained for straight chain fatty acids with about 12 carbons, i.e. oxidation rates decreased progressively as the carbon chain was increased or decreased. Of interest also is the decrease in the ratio of ω/ω − 1 oxidation with increasing chain length. Ellin *et al.* [2] suggested a 'binding pocket' for this cytochrome P-450 which best accommodates those fatty acids that conform to the geometry of its active site.

Our studies on the metabolism of LTB_4 suggest that it is a relatively poor substrate for the hepatic and renal cytochrome P-450 IVA1 enzyme system (Table 3). Induction of cytochrome P-450 IVA1 by clofibrate leads to a lowering of ω- and (ω − 1) LTB_4 hydroxylase activity in both liver and kidney. LTB_4 hydroxylase is present as a constitutive form of cytochrome P-450 in both liver and kidney, but appears to be distinct from the laurate hydroxylase enzyme system (cytochrome P-450 IVA1-dependent). The induction, or specific gene 'switch on' of laurate hydroxylase by clofibrate seems to result in a specific

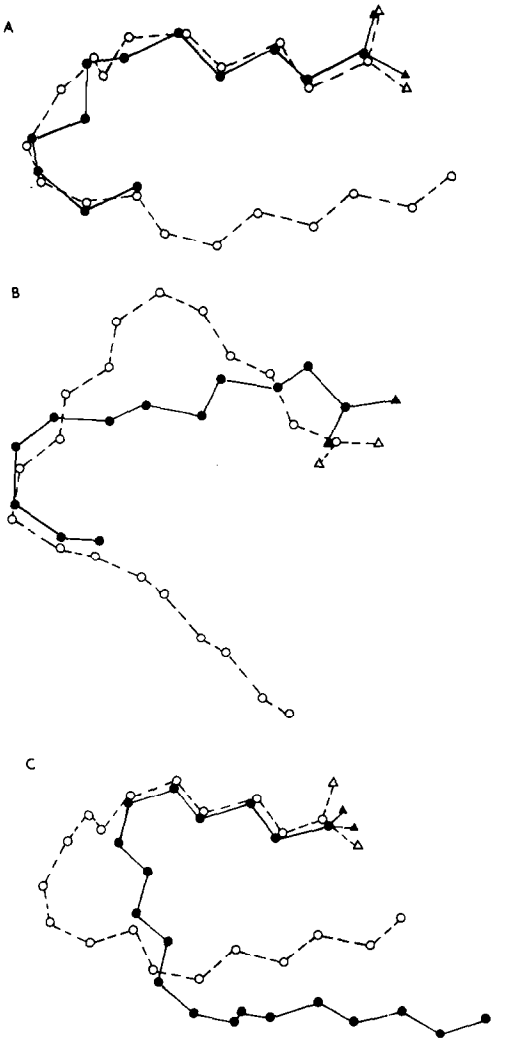

Fig. 5. Computer modelling of potential substrates for cytochrome P-450 IVA1. (A) Laurate (●—●) vs arachidonate (○—○); (B) laurate (●—●) vs leukotriene B_4 (○—○); (C) arachidonate (○—○) vs leukotriene B_4 (●—●). Substrate geometries were determined as described in Materials and Methods and only the carbon atoms (circles) and oxygen atoms (triangles) are shown for clarity. It is important to recognise that the absolute conformations of substrate are not shown, but rather *mutually* best-fit conformations for two substrates. Accordingly, the structure of a particular substrate will change according to what second substrate it is being compared against.

gene 'switch off' of LTB_4 hydroxylase. These observations are supported by Romano *et al.* [29] who have suggested that the isoenzymes of cytochrome P-450 responsible for LTB_4 hydroxylation are distinct from those which mediate the hydroxylation of laurate. Alternatively, it may be possible that clofibrate induces a pathway which reduces the concentration of LTB_4, such that subsequent metabolite concentration is reduced. However, it must be emphasized that this conclusion is only speculative and we have no evidence for this.

Inspection of molecular models reveals that arachidonic acid and lauric acid can adopt a more compact

'hairpin-like' conformation as compared to the bulkier LTB_4. The fact that lauric acid is almost superimposable on the arachidonic acid structure offers a rational explanation why these two fatty acids are relatively good substrates for cytochrome P-450 IVA1, whereas the bulkier molecular geometry of LTB_4 dictates that it is a poorer substrate. Although substrate conformation is clearly an important factor in determining rates of hydroxylation, it should also be borne in mind that physicochemical factors of these lipid substrates would also have a significant influence on the rate of metabolism.

Other lipids that may possibly be substrates of cytochrome P-450 IVA1 are the prostaglandins, particularly PGE_1 and PGE_2, as their metabolism is known to be cytochrome P-450-dependent [4, 9] and inspection of molecular models as described above shows a similar, but not identical conformation (data not shown). In this context, a prostaglandin ω-hydroxylase has recently been isolated from rabbit lung, the enzyme (termed cytochrome $P\text{-}450_{p-2}$ or P-450 IVA4) sharing 74% amino acid sequence homology with cytochrome P-450 IVA1 [30]. However, these latter workers also demonstrated that although cytochrome $P\text{-}450_{p-2}$ efficiently hydroxylates prostaglandins (including PGE_1, PGE_2 and PGA_2), cytochrome P-450 IVA1 showed no significant hydroxylase activity towards these substrates. In addition, cytochrome P-450 IVA1 isolated from the kidney cortex of rats was also incapable of prostaglandin hydroxylation [30].

In conclusion, the demonstration that arachidonic acid is 20- and 19-hydroxylated by hepatic and renal cytochrome P-450 IVA1 raises the possibility of whether exposure to hypolipidaemic agents such as clofibrate can perturb the *in vivo* steady-state levels of this fatty acid as well as its more physiologically potent metabolites. Furthermore, the high basal levels of ω-hydroxylase activity in kidney microsomes may suggest a role for cytochrome P-450 IVA1 in either the bioactivation or elimination of arachidonic acid and consequently in renal physiology.

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