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THE INCORPORATION OF 3-PHENOXYBENZOIC ACID AND OTHER XENOBIOTIC ACIDS INTO XENOBIOTIC LIPIDS BY ENZYMES OF THE MONOACYLGLYCEROL PATHWAY IN MICROSOMES FROM ADULT AND NEONATAL TISSUES

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Abstract—The incorporation of 3-phenoxybenzoic acid (3PBA) into xenobiotic lipids by enzymes of the monoacylglycerol (MG) pathway was measured using microsomes prepared from rat liver as an enzyme source. The mean activities of the three enzymes involved were: acyl-CoA synthetase, 1.1 nmol/min/mg protein; MG acyltransferase, 75 pmol/min/mg protein; and diacylglycerol acyltransferase, 11.4 pmol/min/mg protein. MG and DG acyltransferases also showed activity with benzoyl-CoA or 1-naphthylacetyl-CoA as acyl donor but none with clofibryl-CoA or 2,4-dichlorophenoxyacetyl-CoA. MG acyltransferase activity, using 3PBA-CoA, was higher in microsomes from rat intestinal mucosa and pig liver, and lower in rat adipose tissue, rat liver and mouse liver. This ranking of activities corresponds to published activities using natural substrates. There was a large increase in MG acyltransferase, using either 3PBA-CoA or palmitoyl-CoA as substrate, in microsomes from the livers of rats 16–18 days old. Lysophosphatidic acid (lyso-PA) and lysophosphatidylethanolamine (lyso-PE), but not other phospholipids or detergents, stimulated MG acyltransferase activity more than two-fold. Lyso-PA (5 μM) increased the V_{max} but had little effect on the K_m for 2-hexadecylglycerol, whereas 100 μM lyso-PE decreased the K_m and had a smaller effect on the V_{max}. These results illustrate that the incorporation of xenobiotic acids into diacyl- and triacylglycerol by enzymes of the MG pathway may be a more general phenomenon than was previously suspected and that it may be subject to a variety of developmental and physiological controls.

It is now well-established that a number of xenobiotic compounds, in addition to forming classical water-soluble conjugates, may enter pathways of lipid metabolism and form lipophilic conjugates [1, 2]. The formation of xenobiotic analogues of triacylglycerol (TG§) and diacylglycerol (DG), in which one or more of the natural fatty acids is replaced with a xenobiotic carboxylic acid, has been observed both *in vivo* [3] and using liver slices incubated with the compound *in vitro* [4]. In a previous publication [5] we reported the presence in rat liver microsomes of a monoacylglycerol (MG) acyltransferase (acyl CoA:monoacylglycerol *O*-acyltransferase, EC 2.3.1.22) that was capable of incorporating 3-phenoxybenzoic acid (3PBA) from 3PBA-CoA into glycerolipid products. In this paper we report the extension of the study to the other enzymes of the MG pathway and present evidence that this mechanism may be of more general importance in the formation of xenobiotic acylglycerols.

MATERIALS AND METHODS

Materials. Chemical and biochemical reagents were obtained as described previously [5] or from the following indicated suppliers. Sigma Chemical Co. (Poole, U.K.): ATP, CoA (lithium salt), bovine serum albumin (essentially fatty acid free), *sn*-1-monoacyl phospholipids [lysophosphatidic acid, oleoyl (lyso-PA), lysophosphatidyl choline, oleoyl (lyso-PC), lysophosphatidyl ethanolamine, oleoyl (lyso-PE) and lysophosphatidyl serine (lyso-PS), from bovine brain], diacylphospholipids (PA, dioleoyl and PC, dioleoyl) and lipid standards for TLC; Aldrich Chemical Co. (Gillingham, U.K.): 3-phenoxybenzoic acid; BDH (Poole, U.K.): Merck silica gel $60F_{254}$ TLC plates, basic alumina (Brockmann grade I) and scintillation fluids (Cocktail O and Cocktail T); Amersham International (Amersham, U.K.): radiochemical precursors.

[^{3}H]2-Hexadecylglycerol ([^{3}H]2HDG) was a gift from Dr D. N. Brindley; labile ^{3}H was removed and the compound repurified as described previously [6]. 1-Naphthyl-[carboxyl-^{14}C]acetyl-CoA and [carboxyl-^{14}C]benzoyl-CoA were gifts from Dr P. Millburn [7].

Radiochemical synthesis. [*ring*-^{14}C]Clofibryl-CoA

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§ Abbreviations used: TG, triacylglycerol; DG, diacylglycerol; MG, monoacylglycerol; 3PBA, 3-phenoxybenzoic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; 2HDG, 2-hexadecylglycerol; V_{max}, maximum velocity; K_m, Michaelis constant; SDS, sodium dodecyl sulphate; c.m.c., critical micellar concentration.

was prepared from the cinnarizine salt of the correspondingly labelled clofibric acid by the mixed anhydride method of Huckle *et al.* [8]. The identity of the product was confirmed from its UV spectrum and by mass spectral analysis.

2,4-Dichlorophenoxy-[2-^{14}C]acetyl-CoA ([^{14}C]-2,4-D-CoA) was prepared from [^{14}C]2,4-D using a modification to the mixed anhydride method of Huckle *et al.* [8]. In this modification the mixed anhydride residue was redissolved in tetrahydrofuran/water (1:1, v/v) in which CoA had already been dissolved. The solution was maintained at pH 8.0 with 2,4,6-trimethylpyridine. Even using these modifications, the method gave a low yield of 2,4-D-CoA.

Animals. Male rats of between 200–250 g, timed-mated pregnant rats, both of Wistar strain and male mice of CFI strain were obtained from Charles River UK Ltd (Manston, Ramsgate, U.K.) and were allowed free access to water and 41B pellets obtained from Grain Harvesters Ltd (Wingham, U.K.). Neonatal rats were kept with their mother and the litter size was not manipulated. The litter size varied from 9 to 11. Rats were weaned at 25 days *post partum*, then caged separately and allowed free access to food and water. Pig liver was obtained from Landrace pigs (*Sus scofra*), reared at Wye College Farm (Pig Unit), Wye, Ashford, Kent, U.K.

Preparation of sub-cellular fractions. Adult rats were killed by a blow on the head, and the livers removed. Livers were homogenized in 2 volumes of "homogenization buffer" (0.25 M sucrose/1 mM Hepes pH 7.4 purged with N_2) at 4°. Cell nuclei and debris were sedimented at 2000 *g* for 10 min and the supernatant was centrifuged at 33,000 *g* for 20 min to sediment mitochondria, peroxisomes and lysosomes. The latter centrifugation was repeated, then microsomes were collected by sedimenting from the supernatant at 100,000 *g* for 70 min. The microsomal pellet was resuspended in a minimum volume of homogenization buffer. Further washing of the microsomes was achieved by diluting them in 0.154 M NaCl/1 mM Hepes pH 7.4 and layering the resulting suspension over homogenization buffer in a centrifuge tube. The microsomes were re-sedimented as before and resuspended in homogenization buffer.

Neonatal rats were killed between 9 a.m. and 10 a.m. by cervical dislocation. An entire litter of suckling rats was used on each occasion. It was necessary to pool the livers from up to four of the younger neonatal rats. Washed microsomes were prepared by the method used with the adults.

Adult rat small intestine was washed *in situ* with 0.154 M NaCl at 4°. The small intestine was excised, everted and the mucosa removed by scraping. Microsomes were prepared as described for adult rat liver except that two centrifugation stages at 19,000 *g* for 10 min replaced those at 33,000 *g*. Fat-free homogenate of the epididymal fat pads of adult rats was prepared as previously described [6]. Pig liver microsomes were prepared as described for adult rat liver microsomes.

Microsomal suspensions and homogenates were stored until required in small aliquots at −20°, for up to 4 months without any apparent loss of MG acyltransferase.

Enzyme assays. Fatty acyl-CoA synthetase (EC 6.2.1.3) was assayed by the method of Lloyd-Davies and Brindley [9] with minor modifications. Each assay contained, in a final volume of 250 μL, 25 mM Tris–HCl buffer pH 7.4, 1.5 mg of bovine serum albumin, 2.5 mM ATP, 2.5 mM $MgCl_2$, 55 μM CoA lithium salt, 0.6 mM [9, 10-^{3}H]potassium palmitate (1.5 μCi/μmol) or 0.6 mM [^{14}C]3-PBA (10 μCi/μmol) and 0.5 mg of microsomal protein. Solutions were purged with N_2 gas prior to use. Incubations at 37° were started by the addition of microsomes and stopped after 3 min by the addition of Dole reagent [10]. The CoA ester was extracted by the method of Bar-Tana *et al.* [11]. Aliquots of the aqueous phase were either subjected to liquid scintillation counting or further purified on silica TLC. These plates were developed for 10 cm with chloroform:methanol:acetone:acetic acid:water (7:5:4:2:1, by vol.) then twice in the same direction with hexane:diethyl ether:acetic acid (60:40:1, by vol.) for the full length of the plate. Areas corresponding to genuine acyl-CoA were scraped from the plate and subjected to liquid scintillation counting.

MG acyltransferase was assayed, and products extracted and isolated essentially as described by Imhof *et al.* [5]. The standard assay contained, in a final volume of 250 μL, 25 mM Hepes buffer pH 7.4, 1.5 mg of bovine serum albumin, 6 mM $MgCl_2$, 2 mM 2HDG, rat liver microsomes (up to 0.3 mg of protein) with either 250 μM [benzoyl-^{14}C]3PBA-CoA (5.7–9.2 μCi/μmol) or 250 μM [1-^{14}C]palmitoyl-CoA (0.5–1.1 μCi/μmol). Other [^{14}C]acyl-CoAs were occasionally used as the acyl donor as indicated in the results. When 1-naphthylacetyl-CoA was the acyl donor, silica gel TLC plates were developed with hexane:diethyl ether:ammonia (sp.g. = 0.88) (50:50:1, by vol.) to retain at the origin an unidentified acidic radioactive contaminant which had contributed to high background readings. Control incubations from which 2HDG was omitted were also carried out and the values thus obtained were subtracted from values obtained from incubations which included 2HDG.

Diacylglycerol acyltransferase (acyl CoA: 1,2-diacylglycerol *O*-acyltransferase, EC 2.3.1.20) was assayed by a method based on that of Bell and Miller [12]. In a final volume of 200 μL each assay contained 175 mM Tris–HCl buffer pH 7.4, 200 μg of bovine serum albumin, 6 mM $MgCl_2$, 0.1 mM 1,2-dipalmitoyl-*sn*-glycerol (added as 10 μL of 2 mM solution in ethanol), 250 μM [^{14}C]3PBA-CoA (9.2 μCi/μmol) and 0.3 mg of rat liver microsomal protein. [^{14}C]3PBA-CoA was replaced, at times, by other [^{14}C]acyl-CoA compounds as indicated in the results section or by a [^{3}H]palmitoyl-CoA generating system [5]. Solutions were purged with N_2 before use. Reactions were stopped by the addition of 1.88 mL of chloroform:methanol (1:2, v/v) followed by 0.3 mL of H_2O and extracted by the method of Bligh and Dyer [13]. Acidic precursors were removed by column chromatography on basic alumina [5] and the eluate subjected to TLC in order to separate the TG product from the ethyl ester which forms as a result of an ethanol acyltransferase [14, 15]. TLC was performed on silica gel plates using light petroleum (b.p. 60–80°):diethyl ether:acetic acid

(90:10:1, by vol.) or, in the case when 1-naphthylacetyl-CoA was used as acyl donor, hexane:diethyl ether:ammonia (sp.g. = 0.88) (90:10:1, by vol.).

Determination of protein and radioactivity. Protein was determined by a biuret method [16] or a microbiuret method [17].

Radioactivity was determined by liquid scintillation counting using an external standard channels ratio method to correct for quench. Samples were prepared for scintillation counting as follows. Samples in solution in chloroform were reduced to dryness and then mixed with 5 mL of Cocktail O. Water (1 mL) was added to samples scraped from silica TLC plates followed by 10 mL of Cocktail T and the whole mixture thoroughly dispersed using a vortex mixer.

RESULTS

Enzyme activities

Preliminary experiments with [^{14}C]3PBA-CoA revealed a rapid hydrolysis to [^{14}C]3PBA, which appeared not to be enzyme-dependent but to be due entirely to the presence of dithiothreitol. For this reason the dithiothreitol was excluded from all enzyme assays and purging with N_2 gas was used instead to reduce the oxidation of thiol groups.

3PBA, as well as palmitate, was shown to be a substrate for acyl-CoA synthetase from rat liver microsomes. The activity with 3PBA as substrate was first order with respect to time of incubation for more than 5 min, and with respect to protein concentration up to 100 μg of microsomal protein per 250 μL assay. The activities recorded were 1.1 ± 0.2 nmol of 3PBA-CoA formed/min/mg protein (mean ± SE from five determinations) and 27.4 ± 3.4 nmol of palmitoyl-CoA formed/min/mg protein (from 13 determinations). These figures, at optimum substrate concentrations, may give a misleading impression of the relative activities with the two substrates. Accordingly the ability of 3PBA and oleic acid to inhibit palmitoyl-CoA synthetase activity was compared. Figure 1 shows that while 0.25 mM oleic acid was required to cause 50% inhibition, 14 mM 3PBA was necessary to achieve the same effect.

We have previously shown that rat liver microsomal MG acyltransferase will incorporate 3PBA into diacylglycerol analogues. This activity was dependent upon the presence of the CoA ester, a 2-acylglycerol or 2-alkylglycerol acyl-acceptor and active enzyme. The reaction was characterized in terms of its dependence on time of incubation, protein concentration and substrate concentration. Products were isolated and identified. All these observations were consistent with the presence of a genuine MG acyltransferase enzyme [5].

DG acyltransferase was assayed using an ethanol-dispersed preparation of dipalmitoylglycerol as acyl acceptor. This required separation of the two possible products namely the TG and the ethyl ester. Using 3PBA-CoA as an acyl donor both ethyl-3PBA and a 3PBA-containing TG analogue were identified as products. The rate of the TG formation was first order with time of incubation for up to 30 min and

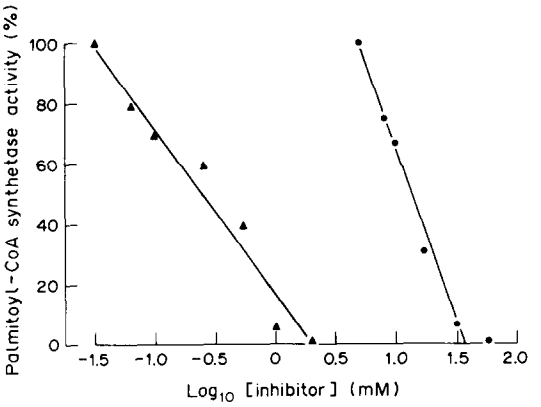

Fig. 1. Inhibition of palmitoyl-CoA synthetase by oleate and 3PBA. Palmitoyl-CoA synthetase was assayed in rat liver microsomes as described in Materials and Methods but using only 0.1 mM [^{3}H]potassium palmitate and in the presence of the indicated concentration of potassium oleate (▲) or potassium 3PBA (●). The activities are expressed as a percentage of the activity measured in the absence of oleate and 3PBA which was 15.9 nmol palmitoyl-CoA formed/min/mg protein.

with concentration of microsomal protein up to 750 μg protein/200 μL assay. The activity with [^{14}C]3PBA-CoA as the acyl donor was found to be 11.3 ± 3.4 pmol 3PBA incorporated into TG/min/mg microsomal protein compared to 2.9 ± 1.1 nmol palmitate incorporated into TG/min/mg microsomal protein (means ± SE from three determinations). When [^{14}C]3PBA-CoA was replaced with [^{14}C]3PBA at various concentrations no incorporation of radiolabel into TG was detected.

Monoacylglycerol acyltransferase in adult and neonatal tissues

Table 1 shows the results of experiments to measure the 3PBA-CoA-dependent MG acyltransferase activity in a variety of tissues. Some concomitant determinations of the palmitoyl-CoA dependent activity are also shown. It can be seen that highest activities were obtained with rat intestinal mucosal microsomes followed by pig liver microsomes. It is also of interest that a DG acyltransferase activity of 350 pmol/min/mg protein was measured in pig liver microsomes with 3PBA-CoA as the acyl-donor. With the mucosal microsomes only, MG acyltransferase activity was measured as the 3PBA-CoA-dependent incorporation of [^{3}H]2HDG into TLC bands corresponding to DG and TG. This was because the high concentration of endogenous MG in the preparation made it difficult to measure incorporation of [^{14}C]3PBA-CoA dependent upon added 2HDG. Dilution of the [^{3}H]2HDG by endogenous MG will have resulted in underestimation of the true MG acyltransferase activity. A similar problem occurred when defatted rat adipose tissue homogenate was used: 2HDG-dependent activity represented only one third of the total incorporation of [^{14}C]3PBA-CoA into DG and TG. Lower activities were also detected in microsomes from rat liver and mouse liver but not from rat lung or kidney microsomes.

Table 1. Tissue variation in MG acyltransferase activity

Species	Tissue	Preparation	Activity of MG acyltransferase using 3PBA-CoA	Activity of MG acyltransferase using Palmitoyl-CoA
Rat	Liver	Microsomes	75 ± 42 (40)	177 ± 64 (3)
	Small intestinal mucosa	Microsomes	2550	
	Epididymal adipose	Defatted homogenate	115 ± 38 (3)	120 ± 14 (2)
	Kidney	Microsomes	ND	
	Lung	Microsomes	ND	80
Mouse	Liver	Microsomes	17	
Pig	Liver	Microsomes	480	12100

MG acyltransferase was assayed in the indicated tissue preparation as described in the text. Activities, expressed as pmol/min/mg protein, are given as mean ± SE with the number of observations in parentheses.
ND, not detected.

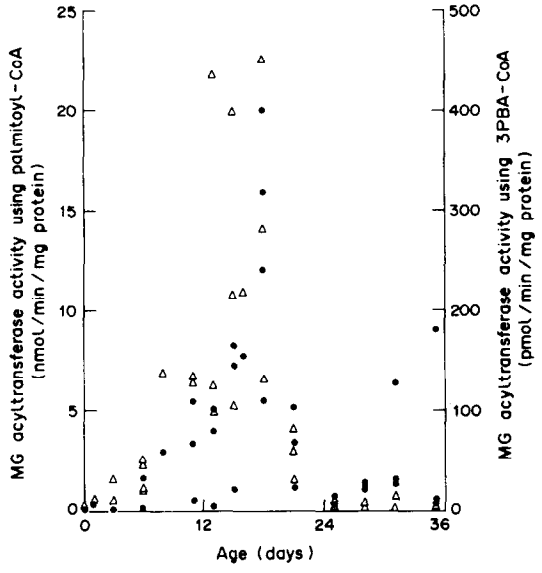

Fig. 2. The effect of age on hepatic MG acyltransferase activity in the rat. Microsomes were prepared from neonatal rat livers and MG acyltransferase activity assayed using palmitoyl-CoA (△) or 3PBA-CoA (●) as described in Materials and Methods. Individual points represent the activity measured from a single preparation at three different protein concentrations.

Coleman and Haynes [18] have reported the presence of a very active MG acyltransferase in microsomes prepared from the livers of suckling rats, 6–8 days old. Figure 2 shows the results of similar experiments using [^{14}C]3PBA-CoA or [^{14}C]palmitoyl-CoA as the precursor. It can be seen that a large peak in both activity curves occurred between 15 and 18 days of age, after which both activities decreased rapidly. After weaning at 25 days the 3PBA-CoA-dependent activity, increased to approach the activity observed in adults.

Alternative xenobiotic substrates for MG and DG acyltransferase

Table 2 summarizes the activities of the MG and DG acyltransferases in rat liver microsomes using 3PBA-CoA and four other aromatic acyl-CoAs as acyl donors. After 3PBA-CoA the highest MG acyltransferase activity was achieved with benzoyl-CoA. Figure 3a shows that the sigmoid benzoyl-CoA requirement curve reached a plateau at about 2 mM. The rate was first order with respect to time for 30 min and to protein concentration up to 0.6 mg microsomal protein/250 μL assay. Analysis of the radiolabelled products by TLC showed that 83% co-chromatographed with a DG standard and the remainder co-chromatographed with a TG standard. Figure 3b shows that, up to 1 mM 1-naphthylacetyl-CoA, the activity of MG acyltransferase had not reached a plateau. A limited supply of the substrate precluded extending this curve to higher concentrations. Using 250 μM 1-naphthylacetyl-CoA, MG acyltransferase activity was first order with respect to time for 20 min and to a protein concentration of 1 mg microsomal protein/250 μL assay. The majority of radiolabelled product co-chromatographed with DG standards. No activity of MG acyltransferase was detected with clofibryl-CoA or 2,4-D-CoA as acyl donors.

The rates of DG acyltransferase with benzoyl-CoA or 1-naphthylacetyl-CoA were similar to that obtained with 3PBA-CoA (Table 2). First order rates using 500 μM benzoyl-CoA were obtained up to 10 min incubation time and 1 mg microsomal protein/200 μL assay, and with 250 μM 1-naphthylacetyl-CoA for 20 min incubation time and up to 1.4 mg microsomal protein/200 μL assay. In both cases the rate of ethyl ester formation was less than 1 pmol/min/mg protein.

Effects of the addition of monoacyl- and diacylphospholipids on MG acyltransferase activity

The effects of adding monoacyl- and diacylphospholipids to the [^{14}C]3PBA-CoA dependent assay for MG acyltransferase were investigated. The standard assay conditions [5] were used except for the inclusion of the phospholipid and 8 mM NaF. The latter was included to inhibit the activities of phospholipases and phosphatidate phosphohydrolase (EC 3.1.3.4) which might otherwise have contributed to the formation of product. The effect of the NaF upon the incorporation of [^{14}C]3PBA

Table 2. Activity of MG and DG acyltransferase with different acyl-CoA substrates

Substrate	Concentration	Activity (pmol/min/mg protein) MG acyltransferase	DG acyltransferase
3PBA-CoA	250 μM	75 ± 42 (40)	11.4 ± 3.4 (3)
Benzoyl-CoA	250 μM	21 ± 10 (6)	
	1 mM	41	10.9 ± 3.6 (4)
	2 mM	52	
1-Naphthylacetyl-CoA	250 μM	4.4 ± 3.0 (3)	
	1 mM	7.7	9.1 ± 3.9 (2)
Clofibryl-CoA	25 μM–2 mM	ND	
2,4-D-CoA	250 μM	ND	

MG and DG acyltransferase were assayed in microsomes from adult rat liver as described in Materials and Methods except that the indicated concentration of the different acyl-CoA substrates replaced the standard substrates. Results are expressed as mean ± SE with the number of observations given in parentheses.

ND, not detected.

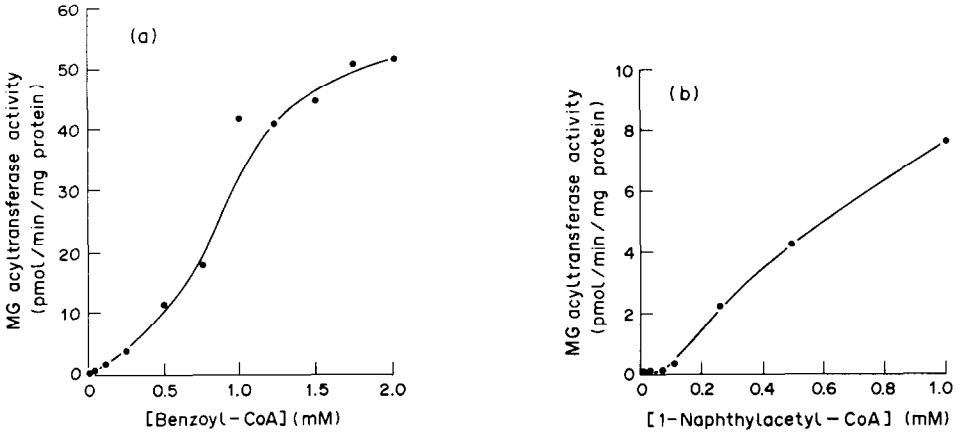

Fig. 3. Benzoyl-CoA and 1-naphthylacetyl-CoA substrates for MG acyltransferase. The standard MG acyltransferase assay in rat liver microsomes was carried out except that (a) benzoyl-CoA or (b) 1-naphthylacetyl-CoA replaced the usual acyl-CoA as substrate.

into neutral lipid products was a small inhibition of about 5%.

Figure 4 shows that MG acyltransferase activity was increased by about 80% in the presence of 5–200 μM lyso-PA and by about 140% in the presence of 150–300 μM lyso-PE. In contrast, in the range 0 to 100 μM, PA, PC and lyso-PC all failed to produce a significant stimulation of MG acyltransferase. Lyso-PS (100 μM) produced a stimulation of 46 ± 4% (mean ± range from two determinations). Figure 5 shows the effect of varying 2HDG concentration in the presence of a fixed concentration of lyso-PA or PE. The activity of MG acyltransferase (using [^{14}C]3PBA-CoA) was increased at all concentrations of 2HDG, in the range 0.25 to 4 mM, in the presence of 5 μM lyso-PA or 100 μM lyso-PE. The apparent V_{max} rose by 84% to 115 pmol/min/mg protein in the presence of 5 μM lyso-PA and by 29% in the presence of 100 μM lyso-PE. The apparent K_m for 2HDG fell from 1.9 mM to 0.6 mM in the presence of 100 μM lyso-PE but was virtually unchanged at 2.1 mM in the presence of 5 μM lyso-PA.

These large stimulations, typically two-fold, observed using rat liver microsomes prepared from adult rats were not seen in one experiment using the more active microsomes prepared from 18-day-old rats. In the latter case a rate of 242 pmol/min/mg protein, measured in the absence of phospholipid, became 227 pmol/min/mg protein in the presence of 10 μM lyso PA and 307 pmol/min/mg protein in the presence of 100 μM lyso-PE.

MG acyltransferase assays were also carried out in the presence of sodium dodecylsulphate (SDS) or sodium deoxycholate to determine whether the effects of lyso-PA could be mimicked by other anionic detergents. It was found that SDS had no effect in the concentration range 0 to 100 μM and that 0 to 10 mM deoxycholate progressively inhibited MG acyltransferase by as much as 70% at 10 mM.

DISCUSSION

A prior communication [5] reported that a MG acyltransferase activity in rat liver microsomes was responsible for the incorporation of [^{14}C]3PBA from

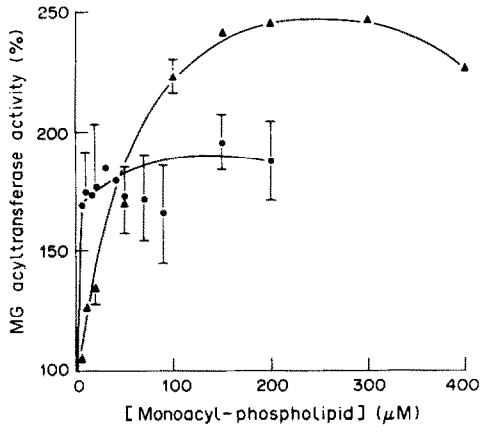

Fig. 4. Effect of lysophospholipids upon the MG acyltransferase incorporation of 3PBA into neutral lipids. The standard MG acyltransferase assay using [^{14}C]3PBA-CoA as substrate was carried out, as described in the Materials and Methods section, in the presence of the indicated concentrations of lyso-PA (●) or lyso-PE (▲). Error bars represent mean ± SD from three determinations or mean ± range from two determinations. The results are expressed as a percentage of the activity measured in the absence of lysophospholipids which was 45.6 ± 6.07 pmol/min/mg protein (mean ± SE from three determinations).

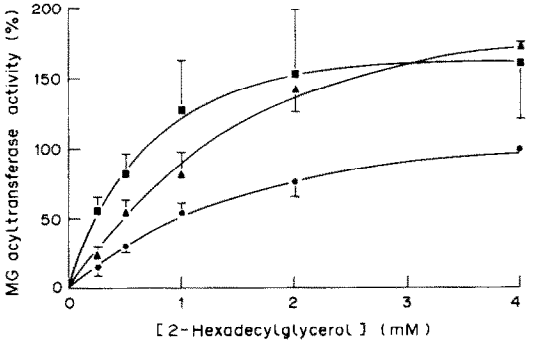

Fig. 5. The dependence of MG acyltransferase activity on 2HDG concentration in the presence and absence of lysophospholipids. MG acyltransferase was assayed in liver microsomes prepared from adult rats using [^{14}C]3PBA-CoA as substrate as described in Materials and Methods except that the indicated concentration of 2HDG replaced the standard concentration. The assays were carried out in the absence of added lysophospholipid (●) or in the presence of 5 μM lyso-PA (▲) or 100 μM lyso-PE (■). Error bars represent the mean ± SE from seven determinations for the unsupplemented incubations or from three determinations when lyso-phospholipid supplements were included. All activities are expressed as a percentage of the activity at 4 mM 2HDG in the absence of supplements which was 32.1 ± 7.4 pmol/min/mg protein (mean ± SE from seven determinations).

3PBA-CoA into DG. In this paper, evidence is presented that the other two enzymes of the "monoacylglycerol pathway" [19] are also operative with 3PBA or 3PBA-CoA as a substrate.

Previous experiments had provided indirect evidence for the activity of acyl-CoA synthetase with 3PBA because a low activity of MG acyltransferase could be demonstrated using [^{14}C]3PBA plus an acyl-CoA generating system [5]. The rate of 3PBA-CoA synthesis reported in this paper (1.1 nmol/min/mg protein) is an order of magnitude higher than MG acyltransferase activity. The high concentration of 3PBA, compared to oleic acid, required to inhibit palmitoyl-CoA synthesis could be an indication of a relatively low affinity of the enzyme for 3PBA or that different enzymes are responsible for the formation of the two thioesters [20]. These measurements would suggest that 3PBA-CoA formation is probably not rate limiting in the biosynthesis of 3PBA-containing lipids in the steady state conditions likely to occur *in vivo*. In this sense, the previous conclusion [5], that acyl-CoA synthetase was rate limiting in the coupled assay of [^{14}C]3PBA incorporation into neutral lipid products *in vitro*, is not supported.

The low activity of 3PBA-CoA utilization by DG acyltransferase compared to MG acyltransferase may be explained in part by the non-physiological presentation of the DG substrate. The activity of the enzyme with palmitoyl-CoA was very much higher than with 3PBA-CoA and this does suggest a strong discrimination against the xenobiotic acyl donor and is perhaps surprising in view of the lower acyl specificity often shown by DG acyltransferase [21, 22]. The participation of DG acyltransferase, demonstrated directly in these experiments, was also inferred from the product composition of MG acyltransferase assays [5] where approximately 30% of radiolabelled product was TG.

The limited survey of aromatic acyl-CoAs shows that 3PBA-CoA is not a unique xenobiotic substrate for either MG or DG acyltransferase. Both benzoyl-CoA and 1-naphthylacetyl-CoA were able to replace 3PBA-CoA in the DG acyltransferase assay, showing similar activities, and in the MG acyltransferase assay with reduced activity. Neither compound had previously been shown to be incorporated into lipids.

MG acyltransferase has been measured in only a very few animal tissues. High activities have been reported in intestinal mucosa from a number of species [19, 23–25] and there is one report of high activity in pig liver microsomes [26] which we now confirm. Lower activities have been reported in adipose tissue homogenates from hamster [27] and rat [6] and in rat liver [28]. MG acyltransferase measured using 3PBA-CoA as the acyl donor also exhibited high activity in rat intestinal mucosa and pig liver microsome preparations, and lower activities in rat adipose tissue homogenate and rat or mouse liver microsomes. The similarity of the tissue distribution provides further evidence that 3PBA-CoA is incorporated into the DG analogue by a genuine MG acyltransferase. It also demonstrates that the phenomenon is unique neither to the rat nor to the liver.

A number of recent papers by Coleman and coworkers have detailed the existence of a very active MG acyltransferase in neonatal rats [18, 29–31] and foetal guinea pig [32]. Our results confirm that, during their suckling period, rats possess liver MG acyltransferase activity about 50 times that found in adults, but the peak activity occurred at 16–18 days

post partum, 10 days later than in the previous studies [18]. This could only be accounted for by a strain difference (Coleman and Haynes used C-D rats; Wistar rats were used here). The induction and decay of 3PBA-CoA-dependent MG acyltransferase activity coincides with that dependent on palmitoyl-CoA, providing further evidence that the two activities are catalysed by the same enzyme. After weaning, the 3PBA-CoA-dependent activity recovers from the lowest point towards its normal adult activity. The palmitoyl-CoA activity, however, stabilizes at or below the 25 day activity which is very close to the adult activity. Thus the ratio of activities using the two acyl donors is different in the adult from the 16–18-day-old neonate. This change could be explained by the existence of neonatal and adult isoforms of the enzyme with different properties. Such an idea was suggested by Coleman *et al.* [31] to explain the different properties of MG acyltransferases from neonatal rat liver and adult rat intestinal mucosa. The observation that the enzyme in liver microsomes from 18-day-old rats was stimulated to a much lower extent than that from the adult by lyso-PA or lyso-PE would support this view, although an alternative explanation would be that the neonatal enzyme was already in an activated state and was therefore less responsive to added activator.

The observation that lyso-PA and lyso-PE can stimulate MG acyltransferase in adult rat liver is also of interest. Choy and Vance [33] have shown that lyso-PE and a number of other phospholipids can stimulate another lipid-synthesizing enzyme, CTP:cholinephosphate cytidylyltransferase (EC 2.7.7.15). There are three likely causes for the stimulation observed here: (i) an improved dispersal of the emulsified substrate 2HDG; (ii) a general detergent effect on the structure of the microsomal membrane carrying the MG acyltransferase activity; and (iii) a more specific, possibly physiological, effect on the enzyme itself or its immediate membrane environment. If the state of the 2HDG emulsion was a major factor it is probable that a large decrease in the apparent K_m for 2HDG, reflecting better substrate availability, would have been observed. With lyso-PE the apparent K_m for 2HDG was reduced by 50% in the presence of 5 μM activator (data not shown) and by 70% in the presence of 100 μM activator. These observations together with the small increase in V_{max} are consistent with, but not conclusive evidence for, an effect upon substrate dispersion. Lyso-PA at 5 μM, on the other hand, produced a large increase in V_{max} with no decrease in apparent K_m, effects which cannot readily be explained by the same mechanism.

It is more difficult to distinguish between the other two explanations. The sigmoid shape of both 3PBA-CoA and benzoyl-CoA requirement curves suggests a detergent-like activation of the enzyme mediated by an effect on the membrane. This is supported by a similar substrate dependence for palmitoyl-CoA in 16-day-old rats (Imhof and Dodds, unpublished data) but not by a report of an absence of a "lag phase" in the palmitoyl-CoA dependence curve in neonatal rat liver microsomes by Coleman and Haynes [30]. The failure of other acidic detergents, SDS and sodium deoxycholate, to effect similar stimulations also argues against this mechanism. If detergent action played an important role in the activation by lyso-PA and lyso-PE then it could be expected that the dependence on the concentrations of the two activators would be related to their critical micellar concentrations (c.m.c.). The c.m.c. of the two phospholipids (*sn*-1-monooleoyl forms) may be estimated, as described by Marsh and King [34] at 0.15 M NaCl, as being 1.9 μM for lyso-PE at pH 7.0 and between 5 and 30 μM for lyso-PA depending upon the degree of ionization but probably approximately 15 μM at pH 7.4. Therefore, while the lyso-PA activation curve does appear to reach a plateau in the region of its c.m.c. the lyso-PE activation curve does not reach its maximum until about 100 μM lyso-PE which is a concentration about 50 times higher than the c.m.c. Thus the activation properties of the two lyso-phospholipids are not as predicted by their c.m.c.: the action of lyso-PA could be explained by its detergent properties but this would not appear to be the case for lyso-PE. The activation phenomenon appears to be specific both for phospholipids in the monoacyl form and for a limited number of head groups. Such specificity suggests that a direct interaction with the enzyme may play a role in the activation. At this stage there is insufficient evidence to speculate about a possible physiological role.

In conclusion, this paper confirms that 3PBA may be incorporated into "xenobiotic-triacylglycerols" by the enzymes of the monoacylglycerol pathway. It presents evidence that this is not an isolated phenomenon and may be generally applicable to a number of xenobiotic acids in a number of tissues from a variety of species. More importantly, the finding that the xenobiotic-dependent MG acyltransferase activity may respond to a variety of developmental and physiological stimuli suggests that xenobiotic-lipid synthesis may be of greater significance in, say, neonatal or foetal animals than has hitherto been suspected from studies with adult animals.

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