Participation of peroxisomes in lipid biosynthesis in the harderian gland of guinea pig

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Peroxisomal enzyme activities in the guinea-pig harderian gland, which has a unique lipid composition, were studied. Activities of catalase, acyl-CoA oxidase and the cyanide-insensitive acyl-CoA β -oxidation system in this tissue were comparable with those in rat liver. The activities of dihydroxyacetone phosphate acyltransferase (DHAPAT, EC 2.3.1.42) and alkyl-DHAP synthase (EC 2.5.1.26) were appreciable, and the distributions of both activities were consistent with that of sedimentable catalase activity. Glycerol-3-phosphate acyltransferase (GPAT, EC 2.3.1.15), which is localized in both microsomes (microsomal fractions) and mitochondria in the rat liver, was a peroxisomal enzyme in the harderian gland, though the activity was only about one-tenth of the DHAPAT activity. These enzymes had different pH profiles and substrate specificity. The existence of high activities of enzymes of the acyl-DHAP pathway in peroxisomes suggests the physiological significance of peroxisomes in the biosynthesis of glycerol ether phospholipid and 1-alkyl-2,3-diacylglycerol in the guinea-pig harderian gland.

INTRODUCTION

Generally glycerol phospholipid and triacylglycerol are biosynthesized via phosphatidic acid, which is synthesized by direct acylation of glycerol 3-phosphate (GP) and subsequent acylation of the 1-acyl-GP [1–3]. On the other hand, dihydroxyacetonephosphate acyltransferase (DHAPAT, EC 2.3.1.42) and alkyl-DHAP synthase (EC 2.5.1.26) participate in the production of glycerol ether lipids [4–8]. Both enzymes are localized mainly in peroxisomes of guinea-pig liver, though the ultimate glycerol lipids are biosynthesized in the endoplasmic reticulum [9–11]. Rock *et al.* [12] reported that the pink portion of rabbit harderian gland contained a preponderance of alkyl glycerolipids. It is known that these ether lipids are actively secreted from this organ [13]. Yamazaki *et al.* [13] showed that guinea-pig harderian gland contains a unique lipid which consists of 1-alkyl-2,3-diacylglycerol as the main lipid instead of triacylglycerol.

There have been many investigations on peroxisomal lipid metabolism, but few data are available concerning the peroxisomal enzymes in the harderian gland [12]. In the present study we have compared the peroxisomal enzyme activities of harderian gland with those of rat liver and investigated whether or not peroxisomes participate in the biosynthesis of the alkyl-type glycerol lipids in this tissue.

EXPERIMENTAL

Animals and preparation of samples

Male Hartley guinea pigs, weighing about 300–400 g were used. Animals were anaesthetized with diethyl ether,

and the harderian glands were removed immediately after respiration ceased. Tissues were minced, then homogenized in 0.25 M-Tris/HCl, pH 7.4. The homogenates were centrifuged by the method of De Duve *et al.* [14] with a slight modification. The nuclear fraction was rehomogenized and spun at 600 g for 10 min and the resulting supernatant was combined with the previous supernatant. Sucrose-density-gradient centrifugation was done using the light mitochondrial (LM) fraction (from 33 000 to 21 000 g) of the harderian gland. A linear gradient of 28–54% (w/w) sucrose solution containing 10 mM-Tris/HCl (pH 7.4), 1 mM-EDTA and 0.1% ethanol was used. The LM fraction was layered on the sucrose gradient and centrifuged at 40 000 rev./min. for 3 h in a Hitachi RPV-50T rotor.

Enzyme assays

Activities of catalase (EC 1.11.1.6), D-amino-acid oxidase (EC 1.4.3.3), urate oxidase (EC 1.7.3.3), carnitine acyltransferases (EC 2.3.1.-), cyanide-insensitive acyl-CoA β -oxidation system, acyl-CoA oxidase, cytochrome *c* oxidase (EC 1.9.3.1), NADPH:cytochrome *c* reductase (EC 1.6.2.4) and acid phosphatase (EC 3.1.3.2) were measured as described in [15,16]. DHAPAT, GPAT, alkyl-DHAP synthase and acyl/alkyl-DHAP reductase (EC 1.1.1.101) were determined by the methods described in [17–19]. Protein contents were determined by the method of Lowry *et al.* [20].

Materials

[³²P]DHAP and [³²P]GP were prepared as described [17] and [¹⁴C]hexadecanol was synthesized from [¹⁴C]palmitic acid [18]. Palmitoyl-DHAP and 1-*O*-hexadecyl-DHAP were prepared by the method of Hajra

Abbreviations used: DHAPAT, dihydroxyacetonephosphate acyltransferase; GPAT, glycerol-3-phosphate acyltransferase; CAT, carnitine acetyltransferase; COT, carnitine octanoyltransferase; CPT, carnitine palmitoyltransferase; M, mitochondrial; LM, light mitochondrial; P, microsomal.

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et al. [21]. Radioactive compounds were purchased from Amersham Japan. CoA derivatives and nicotinamide nucleotides were obtained from Sigma, and L-carnitine was donated by the Otsuka Pharmaceutical Factory.

RESULTS AND DISCUSSION

The lipid composition of the harderian gland of guinea pig is very different from those of other tissues, inasmuch as it contains alkyl- and branched-chain-fatty-acid moieties and small contents of unsaturated fatty acids [13]. Yamazaki *et al.* [13] reported that more than 90% of total lipid in the harderian gland of guinea pig was 1-alkyl-2,3-diacylglycerol. On the other hand, it is known that peroxisomes contain DHAPAT and alkyl-DHAP synthase [9–11]. We therefore examined the distribution of enzymes participating in the formation of ether lipids in this tissue.

Table 1 shows the enzyme activities in the harderian gland of guinea pig. The catalase activity was approx. 0.5- and 0.8-fold of that of rat liver when the activity was expressed per g of tissue and per mg of protein respectively. The activities of D-amino-acid oxidase and urate oxidase were not detected under the same assay conditions employed for the rat liver enzymes. The activities of fatty acyl-CoA oxidase, the first enzyme of the peroxisomal β -oxidation system, and the system measured in terms of the reduction of NAD⁺, were also recognized in this organ. The activities of carnitine acetyltransferase (CAT) and carnitine octanoyltransferase (COT) measured by using acetyl-CoA and octanoyl-CoA as substrates respectively, were very high compared with those in rat liver, whereas carnitine palmitoyltransferase (CPT) activity was not high. On a per-gram-of-tissue basis, the activities of cytochrome *c*

oxidase, NADPH:cytochrome *c* reductase and acid phosphatase, which are representative of mitochondrial, microsomal and lysosomal enzymes respectively, were within the range of 0.3–0.4-fold of those of rat liver. The activities of enzymes relating to the acyl-DHAP pathway were relatively high. The activities in harderian gland and rat liver were 568 and 68 nmol/min per g of tissue respectively. On the other hand, the activity of GPAT, which is localized in both microsomes and mitochondria of all the tissues reported [22–24], was only 0.23-fold of that in rat liver.

Since high activities of CAT, COT and DHAPAT were recognized in the harderian gland, the subcellular distributions of these enzymes were investigated (Fig. 1). Catalase activity was localized in both the light mitochondrial (LM) and the cytosolic fractions, though the activity in the latter fraction might have been released from peroxisomes during the homogenization procedure. The activities of cytochrome *c* oxidase and NADPH:cytochrome *c* reductase were mainly distributed in the mitochondrial (M) and microsomal (P) fractions. The acyl-CoA oxidase and CPT activities were localized mainly in the LM and M fractions respectively, suggesting the presence of separate fatty acid β -oxidation systems in peroxisomes and mitochondria of the harderian gland. The activities of CAT and COT were recognized in both M and LM fractions. Since COT and CPT activities showed different distribution patterns, it was considered that, in the harderian gland of guinea pig, the low activity of CPT was not due to the contribution of COT, which has some activity towards palmitoyl-CoA as a substrate. However, at present we do not know whether CAT and COT activities are attributable to the same enzyme protein or not. The activities of DHAPAT, GPAT and alkyl-DHAP synthase were exclusively localized in the LM fraction, and the activity of alkyl-DHAP

Table 1. Enzyme activities and protein contents of guinea-pig harderian gland and rat liver.

Results are expressed as means $\pm$ S.D. for five samples. One unit of activity of catalase, urate oxidase and cytochrome *c* oxidase was defined as the amount giving a *k* value of 1, where *k* is the decrease in the absorbance/min. One unit of other enzyme activities was expressed as nmol/min. Abbreviation: N.D., not detected.

Enzyme	Activity (units/g of tissue)		
	Guinea-pig harderian gland (a)	Rat liver (b)	Ratio (a/b)
Catalase	1086 $\pm$ 192	2214 $\pm$ 186	0.49
D-Amino-acid oxidase	N.D.	1850 $\pm$ 409	—
Urate oxidase	N.D.	2.87 $\pm$ 0.40	—
Acyl-CoA β -oxidation system	296 $\pm$ 34	765 $\pm$ 181	0.39
Acyl-CoA oxidase	318 $\pm$ 29	779 $\pm$ 127	0.41
Carnitine acetyltransferase	15010 $\pm$ 1206	1531 $\pm$ 305	9.80
Carnitine octanoyltransferase	16913 $\pm$ 3501	1840 $\pm$ 224	9.19
Carnitine palmitoyltransferase	1254 $\pm$ 68	2450 $\pm$ 234	0.51
Cytochrome <i>c</i> oxidase	588 $\pm$ 138	1336 $\pm$ 102	0.44
Glutamate dehydrogenase	N.D.	9141 $\pm$ 1108	—
NADPH:cytochrome <i>c</i> reductase	964 $\pm$ 114	2424 $\pm$ 190	0.40
Acid phosphatase	635 $\pm$ 11	1799 $\pm$ 69	0.35
DHAPAT	568 $\pm$ 79	67.8 $\pm$ 8.8	8.38
GPAT	35.0 $\pm$ 6.4	157.2 $\pm$ 11.9	0.22
Alkyl-DHAP synthase	122.8 $\pm$ 19.4	69.9 $\pm$ 17.7	1.76
Acyl/alkyl-DHAP reductase	8563 $\pm$ 1425	8190 $\pm$ 1015	1.05
Protein (mg/g of tissue)...	137.9 $\pm$ 24.4	224.7 $\pm$ 18.0	0.61

Fig. 1. Cell fractionation of homogenate of guinea-pig harderian gland

The homogenate was centrifuged as described in the Experimental section. The columns represent, from left to right, the nuclear, mitochondrial (M), light mitochondrial (LM), microsomal (P) and soluble fractions. (a) Catalase (recovery, 103%); (b) cytochrome *c* oxidase (87%); (c) NADPH:cytochrome *c* reductase (106%); (d) acyl-CoA oxidase (95%); (e) CPT (89%); (f) acid phosphatase (97%); (g) CAT (95%); (h) COT (86%); (i) alkyl DHAP synthase (112%); (j) DHAPAT (91%); (k) GPAT (103%); (l) alkyl-DHAP reductase (91%).

Fig. 2. Effect of pH on the activities of DHAPAT and GPAT in the peroxisomal fraction

Peroxisomal fraction was obtained by sucrose-density-gradient centrifugation. Preparation of peroxisomes and the assay conditions are described in the text. (a) DHAPAT (maximum activity 27.2 units/ng of protein); (b) GPAT (maximum activity 3.8 units/mg of protein): ●, Mes; ▲, Tris/HCl.

Fig. 3. Effects of substrate concentration on the DHAPAT and GPAT activities of harderian-gland peroxisomes

Assay conditions are given in the text. The palmitoyl-CoA concentration was 60 μM . GPAT activity was measured at pH 8.0. The assay mixture contained 3.1 μg of peroxisomal protein. (a) DHAPAT activity with (○) and without (●) 2.5 mM-GP. The V_{max} for DHAPAT was 29.1 nmol/min per mg of protein; the K_m for DHAPAT was 67 μM , and the K_i for GP is 80 μM . (b) GPAT activity with (○) and without (●) 2.5 mM-DHAP. The V_{max} for GPAT was 4.1 nmol/min per mg of protein, the $V_{\text{max, inhibited}}$ was 1.2 nmol/min per mg of protein, and the K_m for GPAT was 95 μM .

reductase showed bimodal distributions in the LM and P fractions. When palmitoyl-DHAP instead of hexadecyl-DHAP was used as a substrate in the reaction mixture for the reductase, the same distribution pattern was obtained. Furthermore, the intracellular distributions of these enzyme activities were confirmed by the result of sucrose-density-gradient centrifugation of the post-nuclear supernatant from the harderian gland (S. Horie & T. Suga, unpublished work). These results indicate that peroxisomes of guinea-pig harderian gland have a high potency in the biosynthesis of alkyl glycerolipids.

It is known that GPAT is the first enzyme required for the production of glycerolipid by direct acylation of GP, and the activity contributes to the formation of phosphatidic acid in mammalian cells [1–3]. In our present study, however, it was confirmed that the activity of GPAT was extremely low compared with that of DHAPAT, and the distribution of the former enzyme activity was consistent with that of the latter enzyme activity. Then we examined some properties of both enzymes. Fig. 2 shows the pH profiles of DHAPAT and GPAT. The pH optimum for DHAPAT activity was situated in the range pH 6.6–7.5, and no significant activity was recognized at pH 5.5–5.7, which is the environmental pH for the peroxisomal DHAPAT in the livers of guinea pig and rat [24,25]. When microsomal samples from rat brain and rabbit lung were examined, low pH optima for DHAPAT activities were also reported [17,26]. The GPAT activity tended to be active over a higher range of pH (about pH 8.5). Thus the two activities have very different pH optima. Rock *et al.* [12] reported that the activity of DHAPAT in the pink portion of rabbit harderian gland was stimulated by treatment with cholate and deoxycholate. When we

determined the activities of DHAPAT and GPAT in the presence of deoxycholate in the assay system (in the range from 0 to the critical micellar concentration), however, both activities were decreased to almost the same extent. It would be of interest to investigate in a future study the distribution of these enzymes in rabbit harderian gland.

Fig. 3 shows the effects of GP on DHAPAT activity and of DHAP on GPAT activity. DHAPAT activity was slightly inhibited by 2.5 mM-GP in the reaction mixture. When Lineweaver-Burk plots were constructed in the presence or absence of GP, a slight competitive-type inhibition was observed (Fig. 3a). On the other hand, GPAT activity was strongly inhibited by the addition of DHAP, in a non-competitive manner (Fig. 3b). Since the value of K_i (80 μ M) for GP in the DHAPAT reaction is close to the K_m value (67 μ M) of DHAP, it is reasonable to consider that the inhibition is due to the competition between common substrates for the same active centre of DHAPAT. The inhibition of GPAT by DHAP did not cause to any change in apparent K_m value, though the V_{max} value was markedly reduced. Accordingly it is possible that DHAP associates with the active centre or an adjacent site of GPAT. These results indicate that DHAPAT and GPAT in the harderian gland are different from those observed in other tissues such as liver [27], extrahepatic tissues [28] and fibroblasts [29]. However, we cannot exclude the possibility that DHAPAT and GPAT activities are manifested by a single protein having dual catalytic sites, i.e. a bifunctional enzyme.

Generally speaking, GPAT is found in microsomes and mitochondria, whereas DHAPAT is mainly localized in peroxisomes and microsomes. In studies on rat liver, Hajra *et al.* [10,11] demonstrated that the peroxisomal DHAPAT was responsible for most of the hepatic DHAP acylation, but that the DHAP pathway contributed to only a minor extent to the overall hepatic glycerolipid synthesis. On the other hand, the present data indicated that peroxisomes play a major role in overall glycerolipid biosynthesis in the harderian gland. We conclude that peroxisomes of harderian gland have a very important role in glycerolipid metabolism. Yamazaki *et al.* [13] showed that the aliphatic chains of 1-alkyl-2,3-diacylglycerol in guinea-pig harderian gland were exclusively saturated, and so it appears that the fatty acids of glycerolipid in this tissue were produced endogenously rather than by uptake from the surroundings. Further studies are necessary on the properties of 1-alkyl-2-lysophosphatidic acid acyltransferase and on further metabolism in order to establish the physiological significance of the high activities of CAT and COT in the guinea-pig harderian gland.

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