Absence of monoacylglycerol pathway for triacylglycerol synthesis in goat mammary gland

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Both subcellular fractions of lactating goat mammary gland and dispersed lactating goat mammary-gland cells were able to incorporate [U-³H]2-*O*-hexadecylglycerol into monoalkyl-monoacylglycerol. However, the incorporation of [U-³H]2-*O*-hexadecylglycerol into monoalkyl-monoacylglycerol by dispersed cells was not accompanied by incorporation of fatty acid synthesized *de novo* or added radiolabelled fatty acid. The result therefore shows that an active monoacylglycerol pathway does not exist in goat mammary gland.

INTRODUCTION

Both positive (McBride & Korn, 1964; Bickerstaffe & Annison, 1971; Kinsella & Gross, 1973) and negative (Dils & Clark, 1962; Phynadath & Kumar, 1964) evidence has been presented for the presence of an active monoacylglycerol pathway for triacylglycerol synthesis in mammary gland in experiments with monoacylglycerols as substrate. However, these experiments are susceptible to criticism, because the monoacylglycerol substrate is likely to be hydrolysed by monoacylglycerol lipases (Bickerstaffe & Annison, 1971; McBride & Korn, 1964), and the 2-monoacylglycerol used in some experiments may readily isomerize to the 1(3)-acyl derivative.

To circumvent these problems, 2-*O*-monoalkylglycerols have been used as acyl acceptor instead of monoacylglycerols. Goat mammary subcellular preparations (Bickerstaffe & Annison, 1971) and freshly secreted milk (Christie, 1974) have been shown to be able to use 2-monoglycerol ethers as acyl acceptor. Further evidence for the existence of the monoacylglycerol pathway was obtained from incorporation of 2-monoglycerol ethers in infusion experiments with lactating goats (Bickerstaffe *et al.*, 1970).

The experimental evidence that can be obtained from the literature for the existence of an active operating monoacylglycerol pathway in mammary gland is therefore conflicting and confusing. Against this background, we decided to re-investigate this problem, using subcellular fractions of lactating goat mammary gland and dispersed goat mammary epithelial cells. We interpret the results as conclusive evidence that an operative monoacylglycerol pathway does not exist in goat mammary gland.

MATERIALS AND METHODS

Materials

Bovine serum albumin, dithiothreitol and DL-glycerol 3-phosphate were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. [2-³H]Glycerol, sodium [1-¹⁴C]acetate and [1-¹⁴C]palmitic acid were from Amersham International, Amersham, Bucks., U.K. [U-³H]2-*O*-Hexadecylglycerol was a gift from Professor David Brindley, Medical School, University of Nottingham, U.K. 2-*O*-Octadecylglycerol was a gift from Dr. Per Belfrage, Lund Universitet, Sweden. Glycerolipid and fatty acid standards for t.l.c. were obtained from

Nu-Chek-Prep., Elysian, MI, U.S.A. Monoalkyl-monoacylglycerol and monoalkyl-diacylglycerol standards were synthesized by partial acylation of 2-*O*-octadecylglycerol with palmitic acid as described by Bourne *et al.* (1949). CoA and ATP were supplied by Boehringer, Mannheim, West Germany. Fetal-calf serum, horse serum and powdered medium F12 were obtained from Grand Island Biological Co., Grand Island, NY, U.S.A. Medium 199 (powder; with Hanks' salts) was from Serva, Heidelberg, West Germany. All other chemicals were of analytical grade from E. Merck, Darmstadt, West Germany. Hydrocount for liquid-scintillation counting was obtained from J. T. Baker Chemicals B.V., Deventer, Holland.

Methods

Lactating goats 2–3 weeks *post partum* were used. Tissue preparation, homogenization and subcellular fractionation were carried out as described by Knudsen (1972). The incubations (0.5 ml) contained 25 mM-Tris/HCl, pH 7.0, 10 mM-MgCl₂, 8 mM-ATP, 4 mM-dithiothreitol, 50 μM-CoA, 1.0 mM-[1-¹⁴C]palmitic acid (sp. radioactivity 0.5 Ci/mol), 7.5 mg of bovine serum albumin/ml, 2.0 mM-[U-³H]2-*O*-hexadecylglycerol (sp. radioactivity 2.0 Ci/mol) and 1.0 mg of microsomal protein. Blanks without added enzyme were run in all experiments.

The incubation was divided into two equal portions, and the reaction was terminated by addition of 3.25 ml of methanol/chloroform/heptane (28:25:20, by vol.) and 1.05 ml of 0.05 M-K₂CO₃ buffer, pH 10.0, to each portion. After mixing and separation by centrifugation, the lower phases were carefully removed and evaporated to dryness, and the dry residue was taken up in a small volume of chloroform for t.l.c. analysis.

One half of the lipid extract was used for determination of substrate incorporation into monoalkyl-diacylglycerol and triacylglycerol. These compounds were separated from all other lipid classes on a t.l.c. plate coated with 0.5 mm-thick silica gel 60 HR (Merck 7744). The plate was developed twice in light petroleum (b.p. 60–80 °C)/diethyl ether/acetic acid (88:12:1, by vol.) followed by two developments with light petroleum (b.p. 60–80 °C)/diethyl ether/acetic acid (70:30:1, by vol.) to 5 cm above the application spot.

The second half of the lipid extract was used for determination of substrate incorporation into monoalkyl-

Fig. 1. Incorporation of $[U-^3H]2-O$ -hexadecylglycerol and $[1-^{14}C]$ palmitate into monoalkyl-diacylglycerol (a) and monoacylglycerol (b) by goat mammary-gland microsomal fraction

Microsomal fraction (2 mg/0.5 ml) was incubated for 50 min as described in the Materials and methods section, except that the $[1-^{14}C]$ palmitate concentration (sp. radioactivity 0.5 Ci/mol) was varied as indicated. Values are means of duplicates $\pm$ half the difference (bars). $\circ$, $\bullet$,

monoacylglycerol and diacylglycerol. These compounds were separated on a similar t.l.c. plate impregnated with 5% (w/w) boric acid. The plates were developed in chloroform/acetone (24:1, v/v), followed by two developments in chloroform/acetone (1:1, v/v) to 5 cm above the application spot.

The appropriate spots were scraped off and the radioactivity was determined by liquid-scintillation counting. The recovery of radioactivity applied to the plate by scraping off all radioactive spots was 88–98%.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), after acid precipitation as described previously. Bovine serum albumin was used as standard.

Purification of $[U-^3H]2-O$ -hexadecylglycerol

$[U-^3H]2-O$ -Hexadecylglycerol (approx. 500 μ Ci) was purified on a 20 cm $\times$ 20 cm t.l.c. plate coated with 0.5 mm-thick silica gel HR 60 (Merck 7744). The plate was developed in chloroform/methanol (10:1, v/v). The $[U-^3H]2-O$ -hexadecylglycerol band was scraped off and eluted from the gel with ethanol. Unlabelled 2-*O*-hexadecylglycerol was added to give a specific radioactivity of 2 Ci/mol. A 20 mM emulsion of $[U-^3H]2-O$ -hexadecylglycerol in water containing 0.6 mg of bovine serum albumin/ml was made by sonication.

Preparation and incubation of dispersed lactating goat mammary-gland cells

Cells were prepared and incubated as described by Hansen *et al.* (1986)

RESULTS AND DISCUSSION

The pattern of incorporation of $[U-^3H]$ -*O*-hexadecylglycerol into monoalkyl-monoacylglycerol and monoalkyl-diacylglycerol at different fatty acid concentrations with and without ATP is shown in Fig. 1. The incorporation into monoalkyl-monoacylglycerol without ATP was about 6 nmol/50 min per mg of protein (Fig. 1b). This incorporation was doubled when the fatty acid concentration was increased to 10 mM still without added ATP. The basic incorporation of $[U-^3H]2-O$ -hexadecylglycerol into monoalkyl-monoacylglycerol was increased to 10 nmol/50 min per mg of protein when ATP was added to the incubation and was further increased to about 18 nmol/50 min per mg of protein when palmitic acid was increased to 1.0 mM in the presence of ATP. The ATP-dependent incorporation of $[U-^3H]2-O$ -hexadecylglycerol was calculated by subtracting the values obtained without added ATP from those obtained with added ATP at the individual fatty acid concentrations. These values are plotted in Fig. 1(b), which shows that the ATP-dependent incorporation is always smaller than the ATP-independent incorporation. Fig. 1(b) also shows that the fatty acid-stimulated increase in ATP-independent incorporation of $[U-^3H]2-O$ -hexadecylglycerol is an indirect effect, because the $[1-^{14}C]$ palmitate is not incorporated. No incorporation of $[U-^3H]2-O$ -hexadecyl-

$[U-^3H]2-O$ -hexadecylglycerol incorporation without and with ATP respectively; $\square$, $\blacksquare$, $[1-^{14}C]$ palmitate incorporation without and with ATP respectively; $\triangle$, ATP-dependent incorporation of $[U-^3H]2-O$ -hexadecylglycerol calculated by subtracting $\circ$ from $\bullet$.

Table 1. Incorporation of [1-¹⁴C]acetate, [1-¹⁴C]palmitate, [2-³H]glycerol and [U-³H]2-*O*-hexadecylglycerol into the cellular diacylglycerol and triacylglycerol fractions by lactating goat mammary-gland cells

Cells (10⁶/ml) were incubated in medium 199/F12 (1:1, v/v) containing 20 mg of bovine serum albumin/ml in a total volume of 0.4 ml. The concentrations of substrates were 1 mM-[1-¹⁴C]acetate (4.25 Ci/mol), 125 μM-[1-¹⁴C]palmitate (0.5 Ci/mol), 2 mM-[2-³H]glycerol (2.85 Ci/mol) and 0.2 mM-[U-³H]2-*O*-hexadecylglycerol (2.0 Ci/mol). The incubation was stopped after 2 h, and the incorporation into various lipid classes was analysed as described in the Materials and methods section. The results are means of duplicates ± half the difference. Similar results have been obtained with four different cell preparations.

Addition	Substrate incorporated (nmol/h per 10 ⁶ cells)			
	Diacylglycerol		Triacylglycerol	
	¹⁴ C	³ H	¹⁴ C	³ H
[1- ¹⁴ C]Acetate	0.1 ± 0.0	—	4.7 ± 0.6	—
[1- ¹⁴ C]Acetate + [2- ³ H]glycerol	0.2 ± 0.0	0.1 ± 0.0	5.3 ± 0.1	2.8 ± 0.1
[1- ¹⁴ C]Acetate + [U- ³ H]2- <i>O</i> -hexadecylglycerol	0.3 ± 0.0	5.1 ± 0.1	3.5 ± 0.1	0.1 ± 0.0
[1- ¹⁴ C]Palmitate + [U- ³ H]2- <i>O</i> -hexadecylglycerol	0.3 ± 0.0	4.9 ± 0.1	1.3 ± 0.1	0.1 ± 0.0

glycerol into monoalkyl-diacylglycerol was observed without addition of ATP, and the incorporation was independent of the addition of fatty acids (Fig. 1a).

The rate of incorporation of [2-³H]glycerol into triacylglycerols by the microsomal fractions was 25 times the rate of incorporation of [U-³H]2-*O*-hexadecylglycerol into monoalkyl-diacylglycerol (results not shown). When the experiments in Fig. 1 were repeated with a second microsomal fraction, the same pattern of incorporation was obtained, but the incorporation of [U-³H]2-*O*-hexadecylglycerol was 23% and 37% lower with and without ATP respectively. Glycerol 3-phosphate stimulated incorporation of [U-³H]2-*O*-hexadecylglycerol in the presence of ATP, whereas it was without effect or slightly inhibitory in the absence of ATP (results not shown).

The above results indicate that the monoacylglycerol pathway plays a minor role as a route to triacylglycerol synthesis compared with the glycerol 3-phosphate pathway in goat mammary gland. The fact that the major part of the [U-³H]2-*O*-hexadecylglycerol incorporation is ATP-independent indicates that the incorporation of this compound is catalysed by non-specific acyltransferases. The enzymes involved are most probably lipases. It has been shown that cellular lipases catalyse acyl transfer from one monoacylglycerol molecule to another, forming diacylglycerols and free glycerol (Waite & Sisson, 1973, 1974).

The observation that glycerol 3-phosphate only stimulated [U-³H]2-*O*-hexadecylglycerol incorporation in the presence of ATP further indicates that non-specific acyltransferases are involved. In the presence of ATP, glycerol 3-phosphate might give rise to formation of glycerolipids, which can serve as substrates for the non-specific acyltransferases.

Mammary epithelial cells also incorporated [2-³H]-glycerol at a high rate into the triacylglycerol fraction; however, the incorporation of [U-³H]2-*O*-hexadecylglycerol into this fraction, which contains the monoalkyl-diacylglycerols, was very small (Table 1).

The large incorporation of [U-³H]-*O*-hexadecylglycerol into the monoalkyl-monoacylglycerol fraction was surprisingly not followed by a similar (on a molar basis) incorporation of newly synthesized ¹⁴C-labelled fatty acids (Table 1). This lack of incorporation was not due

to the fact that these acids were not available for the glycerolipid-synthesizing system, because they were to a large extent incorporated into triacylglycerols (Table 1).

The presence of palmitic acid in the incubation medium did not affect the incorporation of [U-³H]2-*O*-hexadecylglycerol into the triacylglycerol fraction (Table 1). Furthermore, [1-¹⁴C]palmitic acid was only incorporated into the diacylglycerol fraction containing the monoalkyl-monoacylglycerols at a rate which was 6% of the rate of [U-³H]2-*O*-hexadecylglycerol incorporation into this fraction. The lack of incorporation of [1-¹⁴C]palmitic acid into the monoalkyl-monoacylglycerols was not caused by an inability of the cell to take up this fatty acid and activate it, because it was incorporated to a great extent in the triacylglycerol fraction.

The above results show that neither ¹⁴C-labelled fatty acids synthesized *de novo* nor added [1-¹⁴C]palmitic acid were incorporated into the monoalkyl-monoacylglycerols formed from [U-³H]hexadecylglycerol. The fatty acids used for monoalkyl-monoacylglycerol synthesis can therefore only come from the pool of esterified fatty acids.

We therefore conclude that the monoacylglycerol pathway for triacylglycerol synthesis does not exist in goat mammary-gland epithelial cells.

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