Effect of Exogenous Long-Chain Fatty Acids on Lipid Biosynthesis in Dispersed Ruminant Mammary Gland Epithelial Cells: Esterification of Long-Chain Exogenous Fatty Acids

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ABSTRACT
Dispersed epithelial cells from lactating bovine and goat mammary glands incorporated acetate into all fatty acids (C₄ to C₁₆) that were incorporated into mainly triacylglycerols. The cells secreted free fatty acids only into the incubation medium, and this secretion was dependent on the concentration of albumin and the type and amount of exogenous fatty acid added to the medium.

Addition of palmitic acid to the incubation medium stimulated synthesis and incorporation of fatty acids synthesized de novo into triacylglycerols, whereas stearic and linoleic acid were inhibitory.

INTRODUCTION
All the short-chain and medium-chain fatty acids in milk lipids are synthesized de novo in the mammary gland, whereas the main part of the long-chain fatty acids are absorbed from the blood (16). The synthesis of medium-chain fatty acids by ruminant mammary gland fatty acid synthetase is terminated by a transacylation reaction, and the synthesis of these acids is dependent upon simultaneous removal of the produced acyl-Coenzyme A (CoA) ester from the synthetase complex (12). This removal process can be carried out by the microsomal triacylglycerol synthesizing system (4, 6). However, short-chain and medium-chain fatty acids can only be esterified into the sn-2 and sn-3 position of the glycerol backbone (17). It can therefore be expected that the rate of fatty acid synthesis de novo in the mammary gland would be dependent upon simultaneous supply of exogenous fatty acids as previously suggested (6). In the present study we explore this hypothesis by investigating the effect of exogenous fatty acids on fatty acid synthesis de novo in isolated lactating ruminant cells.

MATERIALS AND METHODS
Materials
1-Carbon-14-Labeled lauric acid, stearic acid, linoleic acid, and 9, 10-³H labeled palmitic and oleic acid were obtained from the Radiochemical Centre, Amersham, Buckinghamshire, UK. Unlabelled fatty acids and standards for thin-layer chromatography were from Nu-Check-Prep., Inc., MI. Bovine serum albumin (BSA) was purchased from Boehringer, Mannheim, West Germany. All other materials were obtained as described (7).

Methods
Preparation and incubations with goat and bovine mammary gland epithelial cells were as described (7). Bovine serum albumin was treated with charcoal to remove residual fatty acids (3). Radiolabeled fatty acids were mixed with carrier fatty acids to obtain a specific activity of 5 Ci/mol. Fatty acid solutions were prepared by slow addition of warm solution of the sodium salt of the fatty acid to medium 199/medium F12 (M199/F12) containing 40 mg/ml fatty acid-free BSA. The final solution was adjusted to pH 7.4 with .1 N HCl and the concentration of the radioactive fatty acids was determined by liquid scintillation counting. Incorporation of radioactivity into various lipid classes and determination of fatty acid composition were as described by Hansen et al. (5).
TABLE 1. Composition of fatty acids synthesized de novo and incorporated into various lipid classes by goat mammary gland cells.  

<table>
<thead>
<tr>
<th>[1-14C] Acetate incorporation (nmol/10^6 cells × 2 h)</th>
<th>mol %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4</td>
</tr>
<tr>
<td>PL^2</td>
<td>2.08 ± .01</td>
</tr>
<tr>
<td>DG</td>
<td>1.50 ± .04</td>
</tr>
<tr>
<td>FA</td>
<td>.69 ± .15</td>
</tr>
<tr>
<td>TG</td>
<td>15.45 ± .24</td>
</tr>
</tbody>
</table>

1 The values shown are means ± half the difference between duplicates and representative for 10 separate experiments.
2 PL = Polar lipids; DG = diacylglycerol; FA = fatty acids; TG = triacylglycerol.

RESULTS

Dispersed mammary gland cells from lactating goat and cow incorporated [1-14C]-acetate into all lipid classes; however, the main part of the incorporated radioactivity was recovered from the triacylglycerol fraction (Tables 1 and 2). Analysis of the fatty acid composition of the various lipid classes revealed that fatty acids with a chain length from 4 to 8 carbon atoms was only incorporated in the triacylglycerol fraction and that decanoic and lauric acid were esterified to a greater extent in the triacylglycerol fraction than in the other lipid fractions (Tables 1 and 2).

Patterns of fatty acids synthesized de novo found in phospholipids, diacylglycerols, and free fatty acids were similar in goat and cow mammary gland cells (Tables 1 and 2). There was a minor difference in the free fatty acid fraction; palmitic acid was the dominant fatty acid in the goat and myristic acid was the species dominant in the cow.

Under the assumption that polar lipids and triacylglycerols have a common biosynthetic pathway to the level of phosphatidic acid or even to the level of diacylglycerol, the data presented clearly indicate that short-chain fatty acids are exclusively esterified into the sn-3 position of the glycerol backbone and that medium-chain fatty acids may be esterified in sn-2 position as well as in the sn-3 position, although the latter appears to be the preferred position.

The dispersed cells did not actively secrete any glycerolipids during the incubation, and only radioactively labeled free fatty acids could

TABLE 2. Composition of fatty acids synthesized de novo and incorporated into various lipid classes by cow mammary gland cells.  

<table>
<thead>
<tr>
<th>[1-14C] Acetate incorporation (nmol/10^6 cells × 2 h)</th>
<th>mol %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4</td>
</tr>
<tr>
<td>PL^2</td>
<td>2.48 ± .05</td>
</tr>
<tr>
<td>DG</td>
<td>.98 ± .02</td>
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<tr>
<td>FA</td>
<td>1.72 ± .05</td>
</tr>
<tr>
<td>TG</td>
<td>14.5 ± .3</td>
</tr>
</tbody>
</table>

1 The values shown are means ± half the difference between duplicates and representative for 3 independent experiments.
2 PL = Polar lipids; DG = diacylglycerol; FA = fatty acids; TG = triacylglycerol.
Figure 1. Effect of bovine serum albumin (BSA) on triacylglycerol synthesis (a) and free fatty acid secretion (b) by goat mammary gland cells. The cells (25 × 10⁶/ml) were incubated as described in the Materials and Methods section with 2 mM sodium [1-14C] acetate (specific radioactivity 5 Ci/mol) and BSA as indicated in a total volume of 400 µl. After 2 h of incubation in a shaking waterbath at 37°C under air:CO₂ (19:1), tubes were centrifuged at 5000 rpm, and the lipid content of the pellet and supernatant was analyzed separately as described in the Materials and Methods. Results are means ± half the difference between duplicates and representative for at least four separate goats. a: cells (pellet); b: medium (supernatant); triacylglycerol (%); free fatty acids (o).

be recovered from the incubation medium (Figure 1). Total lipid synthesis was stimulated by fatty acid free BSA, which also stimulated free fatty acid secretion to the medium. In order to obtain fatty acid binding properties similar to that found in plasma, a BSA concentration of 20 mg/ml was chosen for the following experiments.

The dispersed cells readily incorporated added long-chain fatty acids into triacylglycerols and phospholipids (Figure 2). There were only minor differences in the esterification efficiency into triacylglycerols of various fatty acids, except for palmitic acid, which was a clearly better substrate than the other fatty acids tested. In the phospholipid fraction palmitic acid was by far the most efficient substrate.

In vivo, the mammary gland simultaneously takes up fatty acids from the blood and synthesizes fatty acids de novo. In order to investigate the direct effect on the mammary gland of dietary fatty acids, we incubated dispersed lactating goat mammary gland cells with a number of different fatty acids, as shown in Figure 3. The effect of various long-chain fatty acids in the incubation medium on the esterification of fatty acids derived from [1-14C]acetate into triacylglycerol was very different. Palmitic acid clearly stimulated esterification of fatty acids synthesized de novo, despite the fact that this fatty acid itself was incorporated with high efficiency. Lauric acid had a neutral effect, whereas oleic acid stimulated triacylglycerol synthesis slightly at low concentration and became inhibitory at higher concentrations. Stearic and linoleic acid inhibited triacylglycerol synthesis from newly synthesized fatty acids at all concentrations.
We also monitored the effect of long-chain fatty acids on the secretion of free fatty acids synthesized de novo (Figure 4) and found that palmitic acid as well as lauric acid stimulated secretion of fatty acids from the cells, whereas stearic acid was inhibitory. Oleic and linoleic acid had no effect on fatty acid release from the cells. The fact that stearic acid decreases triacylglycerol as well as fatty acid release indicates that this fatty acid inhibits fatty acid synthesis in the cells.

**DISCUSSION**

Incorporation of fatty acids synthesized de novo into individual lipid classes is similar to what has previously been reported (9, 10) for dispersed bovine mammary gland cells. However, the content of butyric and hexanoic acid in the triacylglycerol fraction is significantly higher in the present study and corresponds to what must be expected in vivo, if it is assumed that these fatty acids are synthesized exclusively in the cells, and that fatty acid synthesis de novo contributes to about 50% of the milk lipids. The low values found in the previous studies might be explained by loss of volatile short-chain fatty acids in the methylation procedure. The method used for methylation in the present study ensures more than 90% recovery of butyrate in methyl esters (13).

In a previous study (10), it was reported that dispersed lactating cow mammary cells actively secreted triacylglycerols, which is in contrast to our results, which show that only free fatty acids were secreted. However, Kinsella and McCarthy (10) used long incubation times, and the presence of 14C label in the medium triacylglycerols after 24 h of incubation might be ascribed to cell lysis. In our cells, trypan blue exclusion is lowered about 25% after incubation overnight (results not shown).

The relative esterification rate of various added free fatty acids by dispersed mammary cells in the present study is similar to the rates reported by Kinsella (8), who also observed that palmitic acid is the preferred substrate and that linoleic acid is esterified less efficiently.
Figure 3. Effect of exogenous fatty acids on triacylglycerol synthesis from fatty acids synthesized de novo by goat mammary gland cells. The incorporation of [1-14C] acetate into triacylglycerol with increasing amounts of added fatty acids is shown relative to the control (100%), which incorporated 8.2 ± 2.0 nmol/2 h × 10⁶ cells. The results are means ± SEM from five individual experiments. Incubation conditions were as described in the Materials and Methods section. C12:0 = ■, C16:0 = □, C18:0 = ○, C18:1 = ●, C18:2 = ▲.

Askew et al. (1) found the same order of esterification efficiencies using subcellular fractions of cow mammary gland.

Rate of fatty acid synthesis de novo and incorporation of these fatty acids into triacylglycerol is dependent upon the kind and amount of exogenous fatty acids supplied. Palmitic acid stimulated de novo synthesis and incorporation into triacylglycerols whereas other fatty acids were either neutral or inhibitory (Figure 4). The stimulating effect of palmitate is in agreement with previous observations by Kinsella and Gross (11), who reported that palmitic acid was the preferred fatty acid for esterification of the sn-1 position of glycerolipids. Palmitic acid presumably acts as a “primer” for triacylglycerol synthesis by acylation of the sn-1 position; thereby it allows medium-chain and short-chain fatty acids to be incorporated into the sn-2 and sn-3 positions. A similar effect of palmitic acid on the esterification of octanoic acid by hepatocytes has been observed by Mayorek and Bar-Tana (15).

Figure 4. Effect of exogenous fatty acids on secretion of fatty acids synthesized de novo from goat mammary gland cells. Incorporation of [1-14C] acetate into secreted fatty acids with increasing amounts of added fatty acids is shown relative to control (100%), which incorporated 1.46 ± .27 nmol/2 h × 10⁶ cells. Results are means ± SEM from five individual experiments. C12:0 = ■, C16:0 = □, C18:1 = ●, C18:2 = ▲.
The most likely explanation for the inhibitory effect of certain fatty acids on the rate of de novo synthesis is that they compete with newly synthesized medium-chain acyl-CoA for the sn-2 and sn-3 positions of the triacylglycerol backbone.

Long-chain acyl-CoA compete effectively with butyryl-CoA for the sn-3 position in ruminant mammary gland (14). However, the possibility remains that some long-chain acyl-CoA could inhibit fatty acid synthesis de novo by inhibition of acetyl-CoA carboxylase.

That long-chain fatty acids synthesized de novo are secreted by the cells indicates that endogenous as well as exogenous fatty acids belong to a common pool. Furthermore, this pool seems to be in equilibrium with the pool of free fatty acids in the extracellular medium. Whether this also is the case in vivo remains to be shown. The present results conclusively show that a number of the observed changes in milk lipid composition resulting from manipulation with dietary fat (2) can be explained by a direct action of dietary fatty acids on the mammary gland.

REFERENCES