Biosynthesis of medium-chain fatty acids by mammary epithelial cells from virgin rats

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Epithelial cells were isolated from the undifferentiated mammary glands of mature virgin female rats, and their lipogenic characteristics were studied. These cells synthesized predominantly medium-chain fatty acids, albeit at a low rate. In contrast, whole tissue from mammary glands of virgin rats synthesized predominantly long-chain fatty acids at a relatively higher rate, indicating that the lipogenic activity is dominated by the adipocyte component of the gland. Enzyme assays revealed that thioesterase II, the enzyme which regulates production of medium-chain fatty acids by the fatty acid synthetase, was present at a high activity in the undifferentiated mammary epithelial cells of virgin rats. Immunohistochemical studies confirmed this observation and showed that the regulatory enzyme was present exclusively in the epithelial cells lining the alveolar and ductal elements of the undifferentiated gland. This study demonstrates that the potential to elaborate tissue-specific medium-chain fatty acids is already expressed in the undifferentiated tissue of virgin rats and is not acquired as a result of the differentiation associated with the lactogenic phase of development. In this species mammary epithelial cells apparently synthesize predominantly medium-chain fatty acids at all stages of development, and only the overall rate of synthesis is increased on induction of the fatty acid synthetase during lactogenesis.

During pregnancy, the mammary epithelium responds to hormonal influences by undergoing cell replication (Topper & Freeman, 1980). There is a dramatic increase in the number of lobuloalveolar structures, which subsequently undergo a series of morphological and biochemical changes resulting in the formation of mammary secretory cells. These fully differentiated cells are capable of synthesizing and secreting vast quantities of tissue-specific products, notably casein and α -lactalbumin, lactose and milk fat. A unique feature of the milk fat of many species is the presence of significant amounts of medium-chain (C_8 – C_{14}) fatty acids (Smith, 1980). In non-ruminants, the key enzyme responsible for the synthesis of medium-chain fatty acids is a thioesterase which interacts with the fatty acid synthetase causing hydrolysis of medium-chain acyl moieties from the thioester linkage to the synthetase

(Knudsen *et al.*, 1976; Libertini *et al.*, 1976; Libertini & Smith, 1978). This thioesterase is, in mammals, a truly tissue-specific enzyme and cannot be detected in tissues other than the mammary epithelium (Smith, 1980; Nolin *et al.*, 1982; Pasco *et al.*, 1982).

It is clear that, on a quantitative basis, development of the ability of the gland to synthesize fatty acids is associated with the lactogenic phase of differentiation, during which the fatty acid synthetase is induced (Strong & Dils, 1972; Dils *et al.*, 1976; Smith & Ryan, 1979; Smith & Stern, 1981). However, earlier work in our laboratory indicated that the increase in thioesterase content of the rat mammary gland paralleled closely the proliferative or mammogenic phase rather than the differentiation or lactogenic phase of development (Smith & Ryan, 1979). The experiments described in the present paper were performed in an attempt to discover whether the thioesterase was already at a high activity in the undifferentiated mammary epithelial cells of the virgin rat.

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Experimental

Preparation of mammary epithelial cells

Mammary glands were excised from adult virgin female rats of the Fisher strain, minced and then digested at 37°C by incubation for 3 h with 0.4% (w/v) collagenase (Worthington, type III) in medium 199 (Gibco, Santa Clara, CA, U.S.A.) supplemented with 5% foetal bovine serum (Sterile Systems, Logan, UT, U.S.A.). The digest was filtered through Nitex cloth (150 μ m mesh size) and the cells were washed twice with medium 199 and finally with Krebs–Henseleit bicarbonate buffer, pH 7.4 (Krebs & Henseleit, 1932).

Metabolic experiments

Cell suspensions were incubated for 2 h at 37°C under an atmosphere of O₂/CO₂ (19:1) in plastic tubes containing 1.0 ml of Krebs–Henseleit bicarbonate buffer, pH 7.4, supplemented with 10 mM-glucose and 10 mM-sodium [1-¹⁴C]acetate (100 μ Ci). Reactions were stopped by centrifuging the reaction tubes for 5 min at 1000 g, to sediment the cells. The supernatant was discarded and the cell pellet was disrupted in 1.5 ml of hexane/propan-2-ol (3:2, v/v) in a Polytron homogenizer. Lipids were extracted and saponified, and the fatty acids were isolated (Radin, 1981). Radioactivity in a portion of the fatty acid extract was determined by liquid-scintillation spectrometry (Smith & Ryan, 1979) and the remainder of the sample was subjected to gas-liquid radiochromatography (Agradi & Smith, 1976).

Enzyme assays

Cells were homogenized in 0.5 ml of 0.25 M-sucrose/1 mM-EDTA/1 mM-dithiothreitol, pH 7.0, in a small Potter–Elvehjem homogenizer. Thioesterase was assayed by a radiochemical adaptation of the previously described spectrophotometric procedure (Smith, 1981b). Incubation systems contained, in a final volume of 100 μ l: 0.1 M-potassium phosphate buffer (pH 8.0), 50 μ M-acetyl-CoA, 100 μ M-malonyl-CoA (45 nCi), 0.25 mM-NADPH, 50 μ g of trypsin-treated fatty acid synthetase (Smith, 1981b), and cytosol [which had been previously titrated with rabbit anti-(rat fatty acid synthetase) serum to remove all endogenous fatty acid synthetase activity]. Reactions were allowed to continue for 15 min at 37°C and were then stopped by the addition of 0.4 ml of 7% (w/v) HClO₄, followed by 1.5 ml of hexane/propan-2-ol (3:2, v/v) and 2 mg of carrier fatty acid mixture (C₄–C₂₀). The two phases were mixed by vigorous vortex mixing and then separated by centrifugation at 1000 g for 2 min. The upper phase was transferred to a small vial and the lower phase mixed twice more with 1.0 ml of hexane. The combined hexane extracts were evaporated in a

stream of air, 3 ml of scintillation fluid (33% 2-ethoxyethanol/67% toluene, containing 5 mg of Omnifluor/ml) was added and radioactivity was determined. Activity measured in this assay system, with cytosol derived from mammary epithelial cells of virgin rats as the source of enzyme, was completely inhibited by antibodies raised against the medium-chain-terminating thioesterase, confirming the specificity of the procedure.

Fatty acid synthetase was assayed spectrophotometrically (Smith, 1981a).

In both assay procedures, enzyme activity was related to amount of enzyme present by the addition of a known amount of homogeneous thioesterase or fatty acid synthetase to each cytosol, as an internal standard. The increment in activity on addition of the purified enzyme was always equal to that predicted on the basis of the amount of enzyme added; thus there was no evidence of any inhibitory or stimulatory factors present in the cytosol.

Immunochemical procedures

Thioesterase II was isolated from lactating rat mammary gland (Smith, 1981b). Purity of the enzyme preparations is routinely better than 95% (Libertini & Smith, 1978). Other proteins which exhibit thioesterase activity are completely removed during the purification procedure. For example, fatty acid synthetase, which exhibits palmitoyl-CoA thioester hydrolase activity, and a non-specific decanoylpantetheine thioester hydrolase are resolved from thioesterase II during the DEAE-cellulose chromatography step. Rabbit anti-(thioesterase II) antiserum was raised (Smith, 1973) and antibodies were partially purified by (NH₄)₂SO₄ precipitation and DEAE-cellulose chromatography (Fahey & Horbett, 1959). Ouchterlony double-diffusion analyses were carried out as described previously (Smith & Stern, 1979).

Immunohistochemical localization of medium-chain-terminating thioesterase

Mammary tissue from mature virgin female rats was fixed in Bouin's fluid, dehydrated in ethanol, cleared in methyl benzoate and benzene, embedded in paraffin and sectioned. Indirect staining of the thioesterase was performed by the avidin–biotin–peroxidase procedure, essentially as described by Hsu *et al.* (1981). Reagents, with the exception of the anti-thioesterase immunoglobulin G, were purchased in the form of Vectastain ABC kit, from Vector Laboratories, Burlingame, CA, U.S.A. The procedure involved initial treatment of paraffin sections with 0.3% H₂O₂ in methanol followed by 10% normal goat serum, to block endogenous peroxidase activity and decrease non-specific staining respectively. The tissue was then exposed

Table 1. *Lipogenic activity of epithelial cells and whole tissue derived from mammary glands of mature virgin rats*
Values are means $\pm$ s.d. for four individual preparations of cells. For further details see the text.

	Fatty acids synthesized (mol %)							Lipogenic rate (fmol of acetate incorporated/h per cell)
	C ₆	C ₈	C ₁₀	C ₁₂	C ₁₄	C ₁₆	C ₁₈	
Epithelial cells	6.7 $\pm$ 5.0	25.9 $\pm$ 3.2	37.8 $\pm$ 5.4	10.0 $\pm$ 3.6	6.5 $\pm$ 3.6	8.6 $\pm$ 2.3	4.5 $\pm$ 3.0	0.37 $\pm$ 0.29
Whole tissue	0	1.7 $\pm$ 1.9	1.3 $\pm$ 1.4	1.2 $\pm$ 0.5	12.0 $\pm$ 1.3	72.5 $\pm$ 24	11.3 $\pm$ 6.4	5.5 $\pm$ 4.8

successively to anti-thioesterase immunoglobulin G, biotinylated goat anti-(rabbit immunoglobulin G) and a mixture of avidin and biotinylated horseradish peroxidase. The chromogen, 3-amino-9-ethyl-carbazole, gave a distinctive red product when coupled with the horseradish peroxidase reactions. Finally, the tissue was counterstained with haematoxylin and mounted in Kaiser's glycerine.

Estimation of cell number

The content of DNA in tissue samples was estimated fluorimetrically (Hinegardner, 1971), by using a suspension of mammary epithelial cells, counted with a haemocytometer, as a standard.

Radiochemicals

Both sodium [1-¹⁴C]acetate and [2-¹⁴C]malonyl-CoA were obtained from New England Nuclear, Boston, MA, U.S.A.

Results and discussion

Epithelial cells derived from the mammary glands of mature virgin rats synthesized predominantly medium-chain fatty acids (Table 1). However, the lipogenic rate of the epithelial cells was considerably lower than that of the gland as a whole. Since the proportion of epithelial components in the undifferentiated gland is small (Munford, 1963), it is clear that the lipogenic activity of the gland is dominated by the contribution of the adipocytes. It was not surprising therefore that the products of lipogenesis in the whole tissue were mainly long-chain fatty acids, despite the fact that the epithelial cells contributed predominantly medium-chain acids (Table 1).

Enzyme assays revealed that the content of medium-chain thioesterase, on a per-cell basis, was more than 12-fold higher in the epithelial cells than in the whole tissue (Table 2). This observation is consistent with the localization of the enzyme in the epithelial cells of the gland. In contrast, assays for fatty acid synthetase showed no evidence of enrichment of this enzyme in the epithelial cells, indicating that the synthetase is present in both adipocytes and epithelial cells.

Table 2. *Lipogenic enzyme content of epithelial cells and whole tissue derived from mammary glands of mature virgin rats*

Values are means $\pm$ s.d. for four separate experiments. For further details see the text.

	10 ⁻⁶ $\times$ Enzyme content (molecules/cell)	
	Thioesterase	Fatty acid synthetase
Epithelial cells	5.1 $\pm$ 1.0	0.3 $\pm$ 0.06
Whole tissue	0.4 $\pm$ 0.1	0.3 $\pm$ 0.05

Evidence that the antibodies used in the immunohistochemical studies were monospecific for thioesterase II was obtained by Ouchterlony double-diffusion analysis (Fig. 1). A single immunoprecipitin line was obtained when the antibodies were diffused against either purified thioesterase II or cytosol obtained from rat mammary gland; the precipitin lines fused, indicating a reaction of complete identity between purified thioesterase II and the cytosolic antigen. Other enzymes which exhibit thioesterase activity but which have physicochemical properties that distinguish them from thioesterase II (e.g. fatty acid synthetase and a non-specific decanoylpantetheine thioester hydrolase) were not recognized by the antibodies (Fig. 1). The specificity of the immunohistochemical staining procedure for thioesterase II was confirmed by control experiments in which the primary antibody was replaced by anti-thioesterase antibody that had been preabsorbed with pure antigen or by normal rabbit serum; no staining was observed in either control.

Immunohistochemical procedures demonstrated clearly the presence of thioesterase in the mammary glands of virgin rats. The enzyme is localized exclusively in the epithelial cells of both alveolar and ductal elements (Fig. 2). Staining was always heavier in the alveolar cells. No immunoreactive enzyme was detectable in adipose, stromal or myoepithelial components of the gland.

The amount of fatty acid synthetase present in the mammary epithelia is about 75-fold higher during

Fig. 1. Immunodiffusion studies with anti-thioesterase antibodies

Centre well contained rabbit anti-thioesterase II antibodies (34 mg/ml). Outer wells contained: 1 and 4, purified thioesterase II (0.15 mg/ml; sp. activity 1000 nmol of decanoylpantetheine hydrolysed/min per mg); 2, 0.15 M-NaCl/0.01 M-sodium phosphate buffer, pH 7.2; 3, cytosol prepared from rat mammary gland (12 mg of protein/ml; total decanoylpantetheine hydrolase activity 13 nmol/min per mg); 5, partially purified non-specific thioesterase from rat mammary gland (1.1 mg of protein/ml; sp. activity 380 nmol of decanoylpantetheine hydrolysed/min per mg); 6, purified fatty acid synthetase (0.4 mg/ml; sp. activity of associated thioesterase 990 nmol of palmitoyl-CoA hydrolysed/min per mg).

lactation [$25 (\pm 7) \times 10^6$ molecules per cell, $n = 5$] than in the virgin state. In contrast, the amount of thioesterase present in the epithelia is only about 3-fold higher during lactation [$15 (\pm 7) \times 10^6$ molecules per cell, $n = 5$] than in the virgin state. Thus, compared with the fatty acid synthetase, which increases dramatically during the lactogenic phase, the medium-chain thioesterase could almost be considered as constitutive to the rat mammary epithelium.

Both the medium-chain fatty acid synthetase and the lactose synthetase of rat mammary gland are unusual enzyme systems, since both are formed by the reversible association of two protein components. In both systems the product specificity of a ubiquitous enzyme, either fatty acid synthetase or UDP-D-galactose:*N*-acetyl-D-glucosamine β -4-galactosyltransferase, is modified by a protein unique to mammary gland, either the medium-chain thioesterase or α -lactalbumin. Nevertheless, the analogy between these two biosynthetic systems is imperfect. The regulatory protein of the lactose synthetase is induced during the lactogenic phase of mammary differentiation and switches the product specificity of the galactosyltransferase from *N*-acetyl-lactosamine to lactose (Brew, 1970; Nakhasi & Qasba, 1979). However, the regulatory protein of the medium-chain fatty acid synthetase is already expressed in the undifferentiated epithelium. Its presence preordains the ultimate product specificity of the fully differentiated gland, and full expression of this unique pathway awaits only induction of the universal lipogenic enzymes at lactogenesis.

Fig. 2. Immunohistochemical localization of medium-chain-terminating thioesterase in mammary gland of a virgin female rat

The thioesterase is localized exclusively in epithelial cells lining the lumina of alveolar (a) and ductal (b) structures. Magnification $\times 500$. The photographs illustrate sections representative of results from four separate experiments.

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