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Secretion of three enzymes for fatty acid synthesis into mouse milk in association with fat globules, and rapid decrease of the secreted enzymes by treatment with rapamycin

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ABSTRACT

The mammary epithelium produces numerous lipid droplets during lactation and secretes them in plasma membrane-enclosed vesicles known as milk fat globules. The biogenesis of such fat globules is considered to provide a model for clarifying the mechanisms of lipogenesis in mammals. In the present study, we identified acetyl coenzyme A carboxylase, ATP citrate lyase, and fatty acid synthase in mouse milk. Fractionation of milk showed that these three enzymes were located predominantly in milk fat globules. The three enzymes were resistant to trypsin digestion without Triton X-100, indicating that they were not located on the outer surface of the globules and thus associated with the precursors of the globules before secretion. When a low dose of rapamycin, an inhibitor of the mammalian target of rapamycin (mTOR), was injected into lactating mice, the levels of the three enzymes in milk were decreased within 3 h after injection. Since the protein levels of the three enzymes in tissues were not obviously altered by this short-term treatment, known transcriptional control by mTOR signaling was unlikely to account for this decrease in their levels in milk. Our findings suggest a new, putatively mTOR-dependent localization of the three enzymes for de novo lipogenesis.

Introduction

Milk is essential for the rearing of mammalian pups. For sufficient nutritional supply, milk contains various components including milk fat, more than 95% of which is triglycerides [1]. In the mammary epithelium, triglycerides have been reported to be initially synthesized within the endoplasmic reticulum (ER)[2] membrane [2], and they are then released from the ER with a lipid monolayer covering. The lipid droplets grow in size in the cytosol and are finally secreted through a characteristic mechanism, whereby they are coated with apical plasma membrane. These secreted droplets are known as milk fat globules (MFGs). Despite many detailed studies of the formation, growth, and secretion of MFGs, which have also been reviewed [2–5], details of the processes involved remain unclear. Analysis of lipid bodies in various cells is believed to be helpful for understanding the evolutionarily conserved and divergent regulation of lipogenesis, thus contributing to various aspects of human health such as the prevention of metabolic diseases [6,7]. Lipogenesis is increased in developing cancers, and proteins in this pathway are expected to become novel therapeutic targets [8].

After pups have been weaned, milk and lactating epithelial cells are unnecessary, and the mammary gland shifts to a regressive phase known as involution [9–11], in which most epithelial cells undergo apoptosis, and dying cells and accumulated milk are absorbed by surrounding tissues. Apoptosis reaches a peak around four days after weaning in mice, as estimated by fragmentation of nuclear DNA. Subsequent proteinase-dependent remodeling at the whole-tissue level ensures the tissue returns to a pre-pregnant state. To identify the genes involved in the initiation of involution, we have performed surveys using the mammary gland and milk of mice [12,13]. A proteomic survey identified acetyl-CoA carboxylase (ACC, EC 6.4.1.2), ATP citrate lyase (ACL, EC 2.3.3.8, former EC 4.1.3.8), and fatty acid synthase (FASN, EC 2.3.1.85) in milk, and demonstrated that they are included in MFGs.

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that plays an important role in cell growth [14]. As one of the mechanisms involved in promotion of cell growth, mTOR has been found to regulate lipogenesis, for example, by activating the transcription of lipogenetic genes, including those of ACC, ACL, and FASN [3,15]. Studies involving in vivo administration

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[2] *Abbreviations used:* ACC, acetyl-CoA carboxylase; ACL, ATP citrate lyase; ER, endoplasmic reticulum; FASN, fatty acid synthase; In2, involution of day 2; L10, day 10 of lactation; MFG, milk fat globule; mTOR, mammalian target of rapamycin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TOF-MS, time-of-flight mass spectrometry.

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of the mTOR inhibitor, rapamycin, have suggested that the association of the three enzymes with MFGs is controlled by mTOR activity. However, this control by mTOR does not seem to be fully explained by the known transcriptional effect of the three enzymes.

Materials and methods

Mouse experiments

Details of the collection of milk and mammary gland tissue from mice have been described previously [12]. Briefly, BALB/c mice (8–9 weeks old) in mid-lactation, including day 10 of lactation (L10), were used, and the number of pups per dam was adjusted to 6 or 7. Milk was collected from the fourth mammary gland of BALB/c mice by hand after intraperitoneal injection of oxytocin. All pups were removed for 3 h before milking to collect milk from the lactating mice, as without this step, insufficient amounts of milk were collectable by hand, due to vigorous and continuous suckling by pups in mid-lactation. Since irreversible execution of mammary involution has been reported to begin around two days after weaning [9–11], it was considered that the effects of this short-term removal of pups would be negligible. Tissue was collected from the inguinal mammary gland, and involution was induced at L10 by removing the pups.

For rapamycin (LC Laboratories, Woburn, MA, USA) treatment, the drug was dissolved in dimethyl sulfoxide, kept at −20 °C, and diluted in sterile phosphate-buffered saline just before injection. BALB/c mice at L10 were injected intraperitoneally once with 0.2 mg/kg body weight rapamycin. Control mice were injected in the same way with the same volume of dimethyl sulfoxide diluted in saline. Milk was collected from each mother at 3 h after injection, and then the mammary tissue was collected. The mothers were kept separated from the pups after the injection, in order to collect a sufficient amount of milk, as described above.

The animal experiments in this study were performed using protocols approved by the Animal Research Committee of Nagoya University.

Fractionation and triglyceride content of milk

Fresh whole milk (about 0.2 ml) was centrifuged at 25 °C for 15 min at 2500*g*. The skim milk was then collected carefully using a pipette to prevent contamination of the cream on top of the milk. The cream containing milk fat was then washed once with isotonic buffer (10 mM Tris–HCl, pH 6.8, containing 0.25 M sucrose and 10 mM KCl). The skim milk was mixed with an equal volume of the isotonic buffer and subjected to centrifugation at 10,000 *g* for 15 min at 25 °C to separate the whey (supernatants) and micellar caseins (precipitates). The caseins were washed once with distilled water before use. Total triglyceride contents of whole milk samples were determined enzymatically using a commercial kit (L-type Wako TG-M, Wako Pure Chemicals, Osaka, Japan).

Preparation of protein samples from milk and mammary gland

Proteins in whole milk and milk fat fractions were suspended in a modified sample buffer for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), containing 10% SDS, and incubated at 60 °C for 6 h to solubilize MFG proteins effectively [16]. Proteins in the whey and casein fractions were dissolved in conventional SDS–PAGE sample buffer, containing 1% SDS, and boiled for 10 min. For protein extraction from the mammary gland, the tissue was minced with scissors, homogenized in 50 mM Tris–HCl, pH 7.5, containing 5 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 10 μm/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin A, and 50 μM proteasome inhibitor I, using a Potter-type homogenizer with a Teflon pestle, and centrifuged twice at 14,000 *g* for 10 min at 2 °C. The supernatants thus obtained were used as the protein samples of the tissue.

Protein electrophoresis and immunoblotting

SDS–PAGE, gel staining with Coomassie brilliant blue R-250, electroblotting, immunodetection, and measurement of the band intensities of the immunoblots were performed as described previously [17]. Rabbit polyclonal antibodies against mouse MFG-E8 and caseins were those described previously [18]. The following antibodies were purchased: rabbit polyclonal antibodies against ACC, ACL, ribosomal protein S6, and phospho-S6 ribosomal protein (Ser240/244) from Cell Signaling Technology (Danvers, MA, USA); mouse monoclonal anti-FASN antibody from BD Biosciences (San Jose, CA, USA); rabbit polyclonal anti-mouse transferrin antibody from Inter-Cell Technologies (Jupiter, FL, USA); mouse monoclonal antibody against α-tubulin from Sigma; goat polyclonal anti-xanthine oxidoreductase antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Mass spectrometry (MS) and protein identification

Details of MS, including in-gel digestion with MS-grade trypsin (Promega, Madison, WI, USA), desalting with ZipTip C_{18} resin (Millipore), matrix-assisted laser-desorption time-of-flight (TOF) MS using a 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA), and data analysis with Mascot (http://www.matrixscience.com), were performed as described previously [12,13,19]. The TOF-MS data were searched against NCBI-nr protein sequence databases using the Mascot peptide mass fingerprint search program. Protein identification was considered significant if the Mascot score was higher than that of a random match at $P < 0.05$.

Tryptic digestion and microscopic observation of MFGs

For tryptic digestion, the milk fat fractions containing MFGs were suspended in the isotonic buffer described above and divided equally into three. Triton X-100 and trypsin (Sigma, St Louis, MO, USA) were added to appropriate MFG samples at concentrations of 1.0% (v/v) and 1.0 mg/ml, respectively. The three MFG samples were then kept at 25 °C for 20 min. To terminate trypsin digestion, phenylmethanesulfonyl fluoride was added at 1.5 mM.

As the control experiments, pure ACC, ACL, and FASN were digested with trypsin in the absence of Triton X-100 as follows. Recombinant human ACC and ACL, which were produced in insect cells, affinity-purified, and enzymatically active, were purchased from BPS Bioscience (San Diego, CA, USA). Each enzyme (1 μg) was subjected to tryptic digestion without Triton X-100, under the same conditions as described above, and then to immunoblotting with antibody against the enzyme. For purification of cytosolic FASN, the mammary gland at L10 was minced, homogenized in the isotonic buffer described above using the Potter-type homogenizer, and centrifuged at 700*g* for 10 min at 4 °C. The post-nuclear supernatant was centrifuged at 20,000*g* for 10 min at 4 °C and then ultracentrifuged at 100,000*g* for 60 min at 4 °C. The cytosolic supernatant, containing 1.5 mg proteins, was pre-treated with 20 μl of protein G coupled to magnetic beads (New England BioLabs, Ipswich, MA, USA) at 4 °C for 15 h to remove material non-specifically bound to the beads. The pre-treated supernatant was incubated with the mouse anti-FASN antibody or control mouse IgG (Chemicon, Temecula, CA, USA) at 4 °C for 120 min. After the addition of 20 μl protein G-magnetic beads, each sample was incubated with rotation at 4 °C for 14 h. Protein G-bound immune com-