Molecular and Cellular Endocrinology 440 (2017) 16–24

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

# Distinct roles of prolactin, epidermal growth factor, and glucocorticoids in β-casein secretion pathway in lactating mammary epithelial cells

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ARTICLE INFO

*Article history:*
Received 29 August 2016
Received in revised form
20 October 2016
Accepted 6 November 2016
Available online 9 November 2016

*Keywords:*
Prolactin
Epidermal growth factor
Glucocorticoid
Dexamethasone
β-casein
Mammary epithelial cell

ABSTRACT

Beta-casein is a secretory protein contained in milk. Mammary epithelial cells (MECs) synthesize and secrete β-casein during lactation. However, it remains unclear how the β-casein secretion pathway is developed after parturition. In this study, we focused on prolactin (PRL), epidermal growth factor (EGF), and glucocorticoids, which increase in blood plasma and milk around parturition. MECs cultured with PRL, EGF and dexamethasone (DEX: glucocorticoid analog) developed the β-casein secretion pathway. In the absence of PRL, MECs hardly expressed β-casein. EGF enhanced the expression and secretion of β-casein in the presence of PRL and DEX. DEX treatment rapidly increased secreted β-casein concurrent with enhancing β-casein expression. DEX also up-regulated the expression of SNARE proteins, such as SNAP-23, VAMP-8 and Syntaxin-12. Furthermore, PRL and DEX regulated the expression ratio of $\alpha_{s1}$-, β- and κ-casein. These results indicate that PRL, EGF and glucocorticoids have distinct roles in the establishment of β-casein secretion pathway.



## 1. Introduction

The mammary gland is a highly specialized organ that produces milk during lactation. Milk contains sufficient doses of proteins, carbohydrates, and lipids as nutrition for suckling infants. Most of these components are synthesized by alveolar mammary epithelial cells (MECs) and are then secreted into the alveolar lumen in lactating mammary glands. Alveolar MECs proliferate during pregnancy and differentiate into mature milk-secreting cells to produce adequate quantities of milk after parturition (Neville et al., 2002). The milk production ability of MECs is regulated by several factors including systemic hormones and local growth factors (Weaver and Hernandez, 2016). Prolactin (PRL) and glucocorticoids, which are known as lactogenic hormones and induce milk production, increase in blood plasma around parturition (Edgerton and Hafs, 1973; Nguyen et al., 2001). EGF regulates alveolar development and the expression of milk proteins together with PRL in *ex vivo* mammary gland cultures (Plaut, 1993). MECs are exposed to epidermal growth factor (EGF) in milk around parturition and during lactation (Xiao et al., 2002). In particular, colostrum, which is secreted immediately after parturition, contains high concentrations of EGF at 300 ng/ml in humans (Read et al., 1984). Thus, PRL, glucocorticoids and EGF are abundant in mammary glands when MECs initiate milk production.

Alveolar MECs synthesize and secrete various milk-specific proteins around parturition. Casein is the most major milk-specific protein and consists of multiple subtypes with some species-specific differences (Miller et al., 1990). For example, mouse milk contains $\alpha_{S1}$-, β-, γ-, δ-, and κ-casein, while bovine milk contains $\alpha_{S1}$-, $\alpha_{S2}$-, β-, and κ-casein. In addition, human milk contains α-, $\alpha_{S2}$-like-, β-, and κ-casein. Beta-casein is commonly found in milk across mammalian species and shows typical expression patterns as a milk-specific protein in accordance with the lactation

*Abbreviations:* DEX, dexamethasone; EGF, epidermal growth factor; EGFr, EGF receptor; FBS, fetal bovine serum; GR, glucocorticoid receptor; JAK2, Janus kinase 2; MEC, mammary epithelial cell; PBST, PBS containing 0.05% Tween 20; PRL, prolactin; qPCR, quantitative PCR; RER, rough endoplasmic reticulum; RT, reverse transcription; SNARE, Soluble N-ethylmaleimide-sensitive factor activating protein receptor; STAT5, signal transducer and activator of transcription 5.

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http://dx.doi.org/10.1016/j.mce.2016.11.006
0303-7207/

stage after parturition (Rijnkels, 2002; Edery et al., 1984; Rezaei et al., 2016). Beta-casein is also the most-investigated casein regarding gene expression regulatory mechanisms. PRL and glucocorticoids synergistically induce β-casein gene expression (Doppler et al., 1989; Kabotyanski et al., 2009). EGF enhances casein expression with PRL in cultured rat MECs (Sigurdson and Ip, 1993). These reports indicate that β-casein expression levels are regulated by PRL, glucocorticoids and EGF during parturition and lactation. However, it remains unclear how these factors are involved in the secretion of β-casein from MECs into the alveolar lumen.

Caseins are secretory proteins that form micelle structures in MECs. Caseins are synthesized in the rough endoplasmic reticulum (RER) and are then transported to the Golgi apparatus. Casen serine residues are phosphorylated in the Golgi apparatus and casein micelles are formed by calcium phosphate-mediated aggregation between phosphorylated casein serines (Clermont et al., 1993). Kappa-casein is glycosylated in the Golgi apparatus. Glycosylated κ-casein is predominantly located on the outer layer of the micelles due to its hydrophilic, phosphorylated and glycosylated C-terminal region (Day et al., 2015). Casein micelles are packaged into secretory vesicles and then transported into apical plasma membranes in alveolar MECs. Soluble N-ethylmaleimide-sensitive factor activating protein receptor (SNARE) proteins regulate the intracellular trafficking and exocytosis of casein micelles (Truchet et al., 2014). SNARE proteins are categorized as vesicular- and target-SNAREs. The vesicular-SNARE proteins (VAMPs) are localized on the vesicle membrane, while target-SNARE proteins (Syntaxins) are on the apical or basolateral membranes. The association of vesicular- and target-SANRE proteins leads to the SNARE complex formation for exocytosis. SNAP-23 interacts with Syntaxins and VAMPs as a central player for casein micelle secretion (Chat et al., 2011). Casein micelles are finally secreted into the alveolar lumen together with other milk components such as lactose and water.

Alveolar MECs develop the above intracellular β-casein secretion pathway after initiating β-casein expression during parturition and maintain it during lactation (Anderson et al., 2007). However, it remains unclear whether PRL, glucocorticoids and EGF regulate β-casein secretion in MECs. We have previously reported a cell culture model using primary MECs where casein secretion is induced in the presence of PRL, EGF, and dexamethasone (DEX) (Kobayashi et al., 2016). In this study, we investigated the influences of these factors on the β-casein secretion pathway using an MEC culture model.

## 2. Materials and methods

### 2.1. Animals

Virgin female ICR mice were purchased from Japan SLC Inc. (Shizuoka, Japan) and were maintained under conventional conditions at 22–25 °C. The mice were decapitated, and the fourth mammary glands were excised for isolation of MECs. All of the experimental procedures in this study were approved by the Animal Resource Committee of Hokkaido University and were conducted in accordance with Hokkaido University guidelines for the care and use of laboratory animals.

### 2.2. Cell culture

MECs were isolated by a previously described procedure (Kobayashi et al., 2016). Briefly, the mammary glands were minced with a scalpel and incubated with RPMI-1640 medium containing type III collagenase (Worthington Biochemical Corporation, Lakewood, NJ) at 1.5 mg/ml for 2 h at 37 °C, followed by treatment with 0.2% trypsin in RPMI-1640 for 5 min at room temperature. After centrifugation, the pellet was resuspended in 60% fetal bovine serum (FBS, Gibco®/Thermo Fisher Scientific, Waltham, MA) containing RPMI-1640 medium and then centrifuged at 5×*g* for 5 min. The precipitants were used as the mammary epithelial fragments. The fragments were seeded on a culture dish with RPMI-1640 supplemented with 10% FBS, 10 μg/mL insulin (Sigma-Aldrich, St. Louis, MO), 10 ng/mL EGF (BD Biosciences, San Diego, CA), 100 U/mL penicillin, and 100 μg/mL streptomycin as the growth medium. For immunofluorescence staining, the fragments were seeded on a poly-L-lysine-coated glass coverslip. After the MECs spread outwards from the epithelial fragments and reached confluence, the culture medium was changed to the differentiation medium (RPMI-1640 supplemented with 1% FBS, 10 μg/mL insulin, 10 ng/mL EGF, 0.5 U/mL PRL from sheep pituitary (Sigma-Aldrich), and 1 μM DEX (Sigma-Aldrich). Incomplete differentiation medium, which lacks either of EGF, PRL, or DEX, was also prepared.

### 2.3. Immunofluorescence staining

MECs on glass coverslips were fixed with methanol for 10 min at −20 °C followed by 1% formaldehyde in PBS for 10 min at 4 °C. After treatment with 0.2% Triton X-100 in PBS for 5 min at room temperature, the fixed MECs were incubated with phosphate-buffered saline (PBS) containing 5% bovine serum albumin (BSA; Sigma-Aldrich) to block nonspecific interactions. They were then incubated with the primary antibody diluted in blocking solution overnight at 4 °C. The following antibodies served as primary antibodies: (1) rabbit polyclonal antibodies against GPR78 (Sigma-Aldrich, #G9043, 1:400) and GM130 (Abcam, Cambridge, UK, #ab7970, 1:1000); (2) a mouse monoclonal antibody against cytokeratin 18 (CK18; Progen Biotechnik, Heidelberg, Germany, #61028, 1:50) and SNAP-23 (Sigma-Aldrich, #WH0008773M1, 1:100) and (3) a goat polyclonal antibody against β-casein (Santa Cruz Biotechnology, #sc-17969, 1:200). After washing with PBS containing 0.05% Tween 20 (PBST), the glass coverslips were incubated with secondary antibodies diluted in the blocking solution for 1 h at room temperature. The following secondary antibodies were purchased from Life Technologies (Gaithersburg, MD): (1) Alexa Fluor 546-conjugated donkey anti-rabbit IgG antibody, (2) Alexa Fluor 546-conjugated donkey anti-mouse IgG antibody, and (3) Alexa Fluor 488-conjugated donkey anti-goat IgG antibody. Control samples were processed in the same manner, with the exception that the primary antibody was absent. Immunofluorescence staining images were obtained with a confocal laser-scanning microscope (TCS SP5; Leica, Mannheim, Germany).

### 2.4. Western blot

MECs were lysed in Laemmli SDS-sample buffer (62.5 mM Tris: pH 6.8, 5% β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol), incubated for 10 min at 70 °C, and stored at −20 °C as samples for western blotting. The MEC samples were electrophoresed using a 12.5% SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA). The membranes were immersed for 1 h in PBST containing 5% BSA and then incubated overnight at 4 °C with primary antibodies diluted in PBST containing 2.5% BSA. The following antibodies served as primary antibodies: (1) a mouse monoclonal antibody against β-actin (Sigma-Aldrich, #A5441, 1:10,000) and (2) a goat polyclonal antibody against β-casein (1:750). Subsequently, the membranes were washed in PBST and incubated for 45 min at room temperature with horseradish peroxidase -conjugated anti-mouse or anti-goat IgG antibodies diluted in PBST containing 2.5% BSA. The immunoreactive bands were detected using the Luminata Forte Western HRP substrate (Millipore, Billerica, MA). The images