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ScienceDirect

Journal of Nutritional Biochemistry 25 (2014) 377–387

Journal of Nutritional Biochemistry

RESEARCH ARTICLES

Copper and lactational hormones influence the CTR1 copper transporter in PMC42-LA mammary epithelial cell culture models

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Received 20 June 2013; received in revised form 23 October 2013; accepted 22 November 2013

Abstract

Adequate amounts of copper in milk are critical for normal neonatal development, however the mechanisms regulating copper supply to milk have not been clearly defined. PMC42-LA cell cultures representative of resting, lactating and suckled mammary epithelia were used to investigate the regulation of the copper uptake protein, CTR1. Both the degree of mammary epithelial differentiation (functionality) and extracellular copper concentration greatly impacted upon CTR1 expression and its plasma membrane association. In all three models (resting, lactating and suckling) there was an inverse correlation between extracellular copper concentration and the level of CTR1. Cell surface biotinylation studies demonstrated that as extracellular copper concentration increased membrane associated CTR1 was reduced. There was a significant increase in CTR1 expression (total and membrane associated) in the suckled gland model in comparison to the resting gland model, across all copper concentrations investigated (0–50 μM). Regulation of CTR1 expression was entirely post-translational, as quantitative real-time PCR analyses showed no change to *CTR1* mRNA between all models and culture conditions. X-ray fluorescence microscopy on the differentiated PMC42-LA models revealed that organoid structures distinctively accumulated copper. Furthermore, as PMC42-LA cell cultures became progressively more specialised, successively more copper accumulated in organoids (resting<lactating<suckling), indicating a link between function and copper requirement. Based on previous data showing a function for CTR1 in copper uptake, we have concluded that under the influence of hormones and increased extracellular copper levels, CTR1 participates in uptake of copper by mammary epithelial cells, as a prerequisite for secretion of copper into milk.

Keywords: Copper; CTR1; Lactation; Human mammary gland; X-ray fluorescence microscopy; Metallothionein

1. Introduction

Copper in breast milk is critical for the postnatal growth and development of mammalian infants. The importance of copper at this stage is clearly illustrated by the toxic milk (*tx*) mouse model, where a defective mAtp7b (P_{1B}-type ATPase) protein impedes supply of copper into the dam's milk causing the suckling pups to become copper deficient; leading to their death after approximately 2 weeks of age [1,2]. During lactation copper levels fluctuate greatly in the mother's blood independent of dietary copper uptake [3]. Concurrently, the rate of copper absorbed by the mammary gland can increase up to 20-fold during lactation [4]. Mammalian milk copper concentrations are highest the first week after birth (humans; 0.4–0.6 mg/L, rodents; 3.2–6.4 mg/L) then decrease substantially (~50%) as lactation progresses (humans; 0.2–0.3 mg/L, rodents; 0.9–2.1 mg/L) [5–7], with copper secretion into milk from the mammary gland stimulated by suckling [8]. Copper secretion into milk is regulated to not expose the suckling infant to excess or deficient levels, and thus allowing for optimal growth and development.

Until pregnancy, mammary epithelial cells remain dormant. Hormonal changes in pregnancy play an important role in modification of the mammary gland, initiating the growth and proliferation of both ducts and alveoli [9]. Estrogen and progesterone regulate the differentiation and development of mammary epithelial cells into secretory alveoli. Postpartum, estrogen and progesterone levels then reduce to pre-pregnancy levels, initiating the secretion of milk [10,11]. Other hormones including dexamethasone, insulin and prolactin are crucial for the differentiation of mammary glands and in the induction of lactation [10,12]. Interestingly, circulating prolactin concentrations in maternal blood positively correlate with mammary gland copper transport and prolactin has been shown to modulate the three main copper-transport proteins, mCtr1 (copper transporter 1), mAtp7a (P_{1B}-type ATPase) and mAtp7b in mouse mammary epithelial cells [8]. However, the molecular mechanisms that regulate mammary gland copper uptake and secretion into milk, including interplay between the copper-transport proteins, are not well understood. In the mammary gland, the high affinity copper uptake protein, CTR1, localises along the basolateral membrane,

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0955-2863/$ - see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jnutbio.2013.11.011

positioned to mediate copper uptake from maternal blood directly into mammary epithelial cells [8,13,14]. CTR1 is a 190-amino-acid glycosylated protein that is approximately 35 kDa and has three transmembrane domains with an exofacial N-terminus and a cytosolic C-terminus [15]. Electron crystallography revealed that three CTR1 proteins can bind together to form a radially-symmetrical conical-shaped channel that is widest on the cytosolic side [16]. The exofacial N-termini coordinate Cu(II) from the extracellular environment. Subsequently, Cu(II) is reduced to Cu(I) and passively transported through the channel to the C-termini, where it becomes available to copper chaperones like ATOX1 [17,18]. In some mammalian cells (e.g., HEK293) elevated extracellular copper (1–100 μM) stimulates rapid internalisation of CTR1 from the plasma membrane [19], while in other cells (e.g., HeLa and Caco-2) CTR1 localisation remains unreceptive to copper [20]. Internalisation of CTR1 could provide a quick and reversible mechanism for the regulation of cellular copper uptake.

In this study we investigated the regulation of CTR1 in cellular models of the resting, lactating and suckling mammary gland. A disadvantage of many mammary epithelial cell lines utilised for in *vitro studies* is the lack of capacity to differentiate. Previous studies demonstrated that mammary epithelial PMC42-LA cells grown on plastic retain breast-specific features including lipid granules, swollen endoplasmic reticulum and large secretory vacuoles [21,22]. Additionally, when grown on specialised extracellular matrixes (e.g., derived from Engelbreth Holm-Swarm mouse sarcoma) PMC42-LA cells can form organoid structures similar to alveoli of the mammary gland, where secretory cells are arranged around a central lumen [23]. These cells develop a phenotype more representative of differentiated luminal epithelial cells, including the presence of tight junctions and microvilli, basally located nuclei and the presence of lipid granules [23]. Furthermore, differentiated PMC42-LA cells also express proteins found in milk and upon stimulation with lactational hormones express β-casein; a marker often used to identify fully differentiated lactating mammary epithelial cells [23–25]. Using PMC42-LA–based models representing resting, lactating and suckling mammary glands, we have demonstrated that both the degree of mammary epithelial differentiation and the level of extracellular copper greatly impact upon CTR1 expression and plasma membrane association. Furthermore, copper resides predominantly in organoid structures with the level rising as PMC42-LA cells become progressively more specialised towards suckled mammary gland. How CTR1 may aid the movement of copper from maternal blood into milk to sustain the rapid growth of the neonate is discussed.

2. Materials and methods

2.1. Cell culture

Breast adenocarcinoma PMC42-LA cells, a variant of the PMC42 line, were originally derived from a pleural effusion [21] and were cultured at 37ºC (5% CO_2) in RPMI 1640 medium (Thermo Fisher Scientific; Melbourne, Australia) supplemented with 10% FBS (Bovogen; Melbourne, Australia). PMC42-LA cells were differentiated on porous Transwell filters (BD Falcon; Sydney, Australia) coated thinly with undiluted extracellular matrix gel from Engelbreth Holm-Swarm mouse sarcoma (EHS matrix) (Sigma-Aldrich; Sydney, Australia). Cells were treated with lactogenic hormones to obtain a model of lactating epithelia as previously described [23–25]. Briefly, cells were grown for seven days on EHS matrix and then treated with 2.7 ng/ml β-estradiol (Sigma-Aldrich) and 157 ng/ml progesterone (Sigma-Aldrich) for three days. This was followed by another three-day treatment with 1 μg/ml dexamethasone (Sigma-Aldrich) and 0.6 μg/ml insulin (Sigma-Aldrich), in combination with either 200 ng/ml prolactin (Jomar Diagnostic; Melbourne, Australia) to simulate lactation, or with 800 ng/ml prolactin to simulate suckling. No hormones were added to the model that recapitulates the resting breast.

2.2. Immunocytochemistry

PMC42-LA cells were grown on either coverslips (undifferentiated), or on porous Transwell cell culture inserts coated with EHS matrix (differentiated). Cells were treated as outlined in Section 2.1, then prepared for immunocytochemistry as previously described [25]. Briefly, cells grown on coverslips were fixed in 4% (w/v) paraformaldehyde (Sigma-Aldrich) for 10 min, permeabilised with 0.1% (v/v) Triton X-100 (Sigma-Aldrich) for 10 min and blocked with 1% (w/v) BSA (bovine serum albumin) (Sigma-Aldrich) for 10 min [all in PBS (Amresco; Solon, OH, USA)]. Cells grown on Transwell inserts with EHS matrix were fixed in 4% (w/v) paraformaldehyde for 10 min, permeabilised with 3% (v/v) Triton X-100 for 10 min and blocked with 3% (w/v) BSA for 1 h. The CTR1 antibody was raised in rabbits against the first 98 amino acids of the human CTR1 protein [14,26,27]. Rabbit anti-CTR1 was diluted (1/1000) in 1% BSA before being applied to cells overnight at 4°C. Chicken anti-rabbit AlexaFluor 488 (Molecular Probes; Melbourne, Australia) diluted (1/5000) in 1% BSA was used for detection of primary antibodies. Cells were also counterstained with ethidium bromide (1 μg/mL) for visualisation of nuclei. Images were collected using a Leica confocal microscope system TCS SP2 (Leica; Melbourne, Australia).

2.3. Western blot analysis

PMC42-LA cell lysates were prepared using 1% (w/v) SDS (2% for cells on Transwell inserts) in 10 mM Tris–HCl (pH 7.5) with 1× EDTA-free protease inhibitor cocktail (Roche Diagnostics; Melbourne, Australia). Lysates were homogenised by repeated passing through a 21-gauge needle followed by sonication (15 pulses, 40% power output, 30% duty cycles) (Microson XL2000 Ultrasonic Cell Disruptor; Misonix, Farmingdale, NY, USA) on ice. Total protein content of cell lysates were measured using the BCA Protein Assay Kit (Pierce; Melbourne, Australia) calibrated against BSA standards. Protein samples (60 μg) were fractionated using the Mini-PROTEAN Tetra System (12% gels) (BioRad, Gladesville, Australia) and transferred to nitrocellulose (Whatman, Dassel, Germany) using the XCellII blot module and the Xcell Surelock mini-cell system (Invitrogen; Melbourne, Australia) according to the manufacturer's instructions. The nitrocellulose membrane was then blocked using 5% (w/v) milk powder in TBS (50 mM Tris–HCl, 150 mM NaCl, pH 7.6) (1 h at room temperature) before being incubated with primary antibody diluted in TBS-T (TBS with 0.1% (v/v) Tween 20) (overnight at 4°C). The following primary antibody dilutions were used: rabbit anti-CTR1 (diluted 1/3000); mouse anti-metallothionein (diluted 1/50); (Dako; Melbourne, Australia; #M0639); mouse anti-Na/K ATPase (diluted 1/1000); (Thermo Fisher Scientific; #sc-48345); mouse anti-β-actin (diluted 1/5000); (Sigma-Aldrich; #A5441). The following secondary antibody dilutions were used: HRP-conjugated goat anti-rabbit IgG (diluted 1/4000); (Millipore; Melbourne, Australia); HRP-conjugated goat anti-mouse IgG (diluted 1/4000); (Millipore). Proteins were detected by Immobilon enhanced chemiluminescence Substrate (Millipore) and imaged using a LAS-3000 FujiFilm Lumino-Image Analyser (Fuji Photo Film; Tokyo, Japan). Pixel intensities (arbitrary units) were quantified using Multi Gauge software [28] and levels were normalised against loading controls, being either β-actin or Na/K ATPase. At least three independent experiments were used for all comparisons.

2.4. Quantitative real time PCR (qRT-PCR)

PMC42-LA cells were harvested using trypsin solution (0.025% trypsin, 0.02% EDTA) for 5 min (incubated at 37°C). Alternatively, PMC42-LA cells grown on Transwell inserts with EHS matrix were harvested with 0.5% (w/v) dispase (Gibco; Melbourne, Australia) dissolved in PBS. Cells were incubated in dispase solution for 45 min until detached from the EHS matrix. Harvested cells were washed three-times in PBS and RNA was then isolated using the RNeasy Mini Kit (QIAGEN; Melbourne, Australia) following the manufacturer's instructions. The RNA preparation was further purified using the DNA-free kit (Ambion; Melbourne, Australia) and the concentration determined using a Nanodrop 100 spectrophotometer (Thermo Fisher Scientific). The high capacity cDNA reverse transcription kit (Applied Biosystems; Melbourne, Australia) was used to generate cDNA from the RNA preparation, following the manufacturer's instructions. Quantitative real-time PCR was performed using a 7500 real-time PCR system (Applied Biosystems) with 20 μl sample volumes containing the following: 8 μl SYBR green mastermix (Applied Biosystems), 0.3 μM of both forward (5′-ACTCCAACAGTACCATGCAACCT) and reverse (5-AGTTCCACATTCTTAAAGCCAAAG-TAG) CTR1 primers and 20 ng of cDNA. Reactions were run on a 7500 real-time PCR system (Applied Biosystems) on the following program: one cycle of 50°C for 2 min, one cycle of 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. Relative mRNA expression was determined by comparing target CTR1 gene amplification with a β-actin housekeeping control (primers; 5′-GACAGGATGCAGAAGGAGATTACT and 5′-TGATCCACATCTGCTGGAAGGT) using the equation $2^{-\Delta\Delta CT\pm SD}$, where ΔΔCT was the difference between the control ΔCT and the treatment ΔCT [29].

2.5. Surface biotinylation

PMC42-LA cells grown in 6-well plates (undifferentiated), or on Transwell inserts coated with EHS matrix (differentiated), were biotinylated as previously described [19]. Briefly, cells were cooled to 4°C and washed three times with PBS supplemented with 0.1 mM $CaCl_2$ and 1.0 mM $MgCl_2$. Cells were incubated in 0.77 mg/mL EZ-Link Sulfo-NHS-SS-biotin (Pierce) in a biotinylation buffer (10 mM triethanolamine (Sigma-Aldrich), 2 mM $CaCl_2$, 150 mM NaCl, pH 7.5) for 25 min on ice. The cells were washed twice with a quenching buffer (PBS with 0.1 mM $CaCl_2$, 1.0 mM $MgCl_2$, 100 mM Glycine) for 20 min on ice to remove excess biotin. The cells were then lysed using a