Molecular and Cellular Endocrinology 439 (2017) 35–45

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

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

In vitro characterization and endocrine regulation of cholesterol and phospholipid transport in the mammary gland

Corneille Edgar Ontsouka [a, b], Xiao Huang [a, b], Eldar Aliyev [a], Christiane Albrecht [a, b, *]

[a] Institute of Biochemistry and Molecular Medicine, Faculty of Medicine, University of Bern, Buehlstrasse 28, 3012, Bern, Switzerland
[b] Swiss National Center of Competence in Research, NCCR TransCure, University of Bern, Bern, Switzerland

ARTICLE INFO

Article history:
Received 22 July 2016
Received in revised form
25 September 2016
Accepted 15 October 2016
Available online 17 October 2016

Keywords:
Cholesterol
Phospholipid
Transport
Regulation
Hormone
Efflux
Apolipoprotein A-1

ABSTRACT

Cell-based studies previously showed that the ATP-binding cassette transporter A1 (ABCA1) transfers cholesterol across mammary epithelial cells (MEC). Data for phospholipid transport are lacking, and it is unclear from which cellular source the transported cholesterol stems, whether this transport activates signaling pathways, and how lactogenic hormones regulate it. To clarify these aspects, lipid transport and expressional analyses were performed in bovine primary (bMEC) and/or immortalized (MAC-T) MEC cultures. Lipid efflux and ABCA1, ABCG1 and liver X receptorα mRNA levels were higher in MAC-T than bMEC. In MAC-T, the transported cholesterol originated mainly from the plasma membrane. ABCA1 dependent cholesterol efflux was higher than phosphatidylcholine efflux, was suppressed by probucol (ABCA1 inhibitor), AG490 (janus kinase-2 inhibitor), PD98059 (mitogen activated protein kinase kinase inhibitor) and pretreatment with β-cyclodextrin (lowering membrane cholesterol). Insulin was the only hormone significantly increasing cholesterol efflux. In conclusion, this study gives novel mechanistic and regulatory insights into the transport of cholesterol and phospholipids in MEC.

1. Introduction

The mammary gland (MG) principally serves to metabolically assemble subconstituents and secrete milk – a complex biological fluid enriched in diverse important nutrients (e.g. lipids, vitamins) – for nourishment of the offspring. Given the varying nutrient demands of the growing offspring, it is crucial to better understand the molecular aspects regulating the appropriate nutrient profile of colostrum and milk.

Milk synthesis occurs within mammary alveolar secretory epithelial cells (MEC), which are functional units of the MG. Hormones and growth factors, whose levels vary along the pregnancy-lactation cycle, play important roles in this process (Neville et al., 2002). Prolactin and hydrocortisone, together with insulin, mediate for instance MEC differentiation and lactogenesis (Neville et al., 2002; Hovey et al., 2002). The biological effects of endocrine factors (e.g. prolactin) require their interaction with specific receptors, which leads to downstream activation of intracellular signaling molecules such as janus kinase (JAK)-2 and signal transducer and activator of transcription (STAT)-5 (Liu et al., 2012; Wyszomierski et al., 1999).

The composition of milk ultimately depends on the transport of nutrients across MEC and their accumulation inside the MG alveolar lumen. These transport processes are likely associated with cell surface expression and activity of membrane nutrient transporters. Regarding the milk lipid content, major advances in understanding the lipid transport processes were gained with the identification of the lipid transport proteins ATP-binding cassette (ABC) A1 (ABCA1) and ABCG1 in MG tissues (Ontsouka and Albrecht, 2014; Mani et al., 2010, 2011). Indeed, the expression of ABCA1 and ABCG1 was localized in the alveolar epithelium (Ontsouka and Albrecht, 2014), and apolipoprotein (apo)A-1, a major constituent of high density lipoprotein (HDL), was found in milk fat globule membranes isolated from alveolar milk (Reinhardt and Lippolis, 2006; Fong et al., 2007). These findings led to the establishment of the first, yet preliminary lipid transport models in MG involving ABC transporters (Mani et al., 2010).

In general, the transport activities of ABCA1 and ABCG1 depend on both extracellular and cellular factors. With respect to extracellular factors, ABCA1 activity requires its interaction with extracellular apoA-1, whereas ABCG1 interacts with HDL, to allow cellular lipid transport (Wang et al., 2004; Vaughan and Oram,

* Corresponding author. Institute of Biochemistry and Molecular Medicine, University of Bern, Buehlstrasse 28, 3012, Bern, Switzerland.
E-mail address: christiane.albrecht@ibmm.unibe.ch (C. Albrecht).

http://dx.doi.org/10.1016/j.mce.2016.10.016
0303-7207/

2005; Nagao et al., 2012). Unlike these membrane protein-specific extracellular acceptors, cyclodextrin (CD), existing as α, β, and γ isoforms of different cavity sizes, has been reported to extract cholesterol with differential potencies (β-CD ≫ γ-CD > α-CD) or phospholipids (α-CD ≫ β-CD > γ-CD) from the plasma membrane (Ohtani et al., 1989; Christian et al., 1997; Haynes et al., 2000). Regarding the cellular factors interfering with cholesterol transport, studies in macrophages showed that the intracellular pools of not esterified cholesterol are the preferred substrate source for the ABCA1-dependent transport (Kojima et al., 2001; Chen et al., 2001). The size of the intracellular cholesterol pools can be modulated by the expression and activity of other genes involved in cellular lipid metabolism. They include trancriptional factors such as sterol regulatory element-binding proteins (SREBPs) or liver X receptors (LXR), and also enzymes such as acyl-coenzyme A:cholesterol acyltransferase (ACAT2) or intracellular transporters e.g. Niemann-Pick C1 (NPC1), which act as sensors of intracellular cholesterol levels (Ontsouka and Albrecht, 2014; Tamehiro et al., 2007). Other cellular factors influencing or modulating ABCA1/ABCG1 activities include regulatory proteins. In this context, studies in cells other than MEC indicated that cellular ABCA1 and ABCG1 expression is upregulated at the transcriptional level by LXRα (Hu et al., 2010). The effect of other molecules, including prolactin response element binding protein (PREB), a prolactin related transcriptional factor, on the transcriptional regulation of ABCA1 was also reported (Nishiuchi et al., 2010). At the protein level, ABCA1 expression is regulated through modulation of its stability and turnover rate (Arakawa and Yokoyama, 2002; Lu et al., 2008) under action of drugs such as probucol (Favari et al., 2004). Concerning ABCG1 protein, in vitro studies using *spodoptera frugiperda ovarian* (Sf9) cells reported an inhibitory effect of ABCG1 activity exerted by benzamil (Cserepes et al., 2004).

However, many aspects related to the molecular mechanisms and regulation of lipid transport by ABCA1 and/or ABCG1 in MEC are still unclear. Indeed, reports on phospholipid transport in MEC are lacking. Additionally, it is unclear from which intracellular compartment the cholesterol stems which is transported by ABCA1 and/or ABCG1, whether this process activates signaling pathways, and how lactogenic hormones regulate it. The current study was undertaken to answer these questions, and thereby contributes not only to the refinement of the current lipid transport model, but also to better understand the relevance of the mentioned transporters and regulatory aspects for the nutrition of neonates/infants. Since cell cultures are generally valuable tools for in vitro studies of MG biology (Ontsouka et al., 2013, 2016; Huynh et al., 1991), our objectives were to 1) determine an optimal cell culture model for mammary lipid transport studies by comparing the mRNA expression of lipid transporters (ABCA1, ABCG1, NPC1, NPC1-like1) and of lipid homeostasis regulators (LXRα, SREBP2, ACAT2, and PREB) in bovine primary MEC and immortalized mammary alveolar cells (MAC-T), 2) characterize the mechanisms of apoA-1 and HDL mediated ^{3}H-cholesterol and ^{14}C-phosphatidylcholine transport by treating MEC separately with probucol and benzamil as well as selective signaling pathway inhibitors (e.g. JAK-2 inhibitor; AG490), 3) identify the source of the apoA-1/ABCA1 transported cholesterol in MEC by carrying out transport studies in cells treated with β-CD, and 4) determine how selected hormones influence the lipid transport in MEC.

2. Materials and methods

2.1. Bovine MEC and cell culture

The suitability of primary bMEC cells or immortalized MEC line obtained by stable transfection with simian virus-40 (SV-40) large T-antigen (MAC-T) for in vitro studies on MG biology has been recently debated (Ontsouka et al., 2016). The MAC-T cell line, being described to maintain milk secretory characteristics (Huynh et al., 1991; Berry et al., 2003), was obtained from Dr. Laura Hernandez, University of Wisconsin at passage 3. Primary bMEC cells were isolated from a late lactating Swiss Holstein Friesian cow (Zbinden et al., 2014) and provided by Dr. Olga Wellnitz, University of Bern at passage 1.

MAC-T and bMEC were grown in DMEM-F12 medium (Gibco) supplemented with 10% fetal bovine serum (FBS), 1% (v/v) antibiotics/antimycotics solution containing penicillin, 100 units/ml; streptomycin, 100 μg/ml; and amphotericin B, 0.25 μg/ml (Gibco). In addition, cells were supplemented with 1x insulin-transferrin-selenite (ITS) mixture (Sigma) containing 10 ng/mL, 5.5 ng/mL and 6.7 pg/mL of each component, respectively. MAC-T cells required between 3 and 5 days of culture to reach confluence, while bMEC needed up to 7 days. Cells were passaged after treatment with 0.05% trypsin-EDTA (3–5 ml/75 cm^{2}flask) until cells detached.

2.2. Quantitative RT-PCR of selected genes of lipid homeostasis

The primer pairs used for amplification of ABCA1, ABCG1, NPC1, NPC1L1, LXRα, SREBP2, ACAT2, PREB, ubiquitin and β-actin are shown in Table 1.

For RNA extraction and the functional studies described below, confluent bMEC and/or MAC-T cells were used. The RNA isolation procedure using SV RNA isolation kit (Promega) as well as the SYBR-green based real-time PCR measurements on 384-well plates on the ViiA7 (Applied Biosystems) were previously reported (Huang et al., 2013).

2.3. Lipid efflux

The procedure used to study the transport of cholesterol and PC was adapted from a previous study (Ontsouka et al., 2013). In brief, cells maintained in complete growth medium were loaded with 0.5 μCi/ml of ^{3}H-cholesterol or 0.25 μCi/ml of ^{14}C-PC for 24 h. Thereafter, the medium was discarded and cells were provided with serum-free medium during a 18 h lasting equilibration phase. The lipid efflux was initiated by adding fresh serum-free medium containing either apoA-1 (5 μg/ml) or HDL (40 μg/ml) for 4 h. After removal of the efflux media, cells were frozen at −20 °C for 30 min. Then, 1 ml of dPBS per well was added to the cells, and the plates were shaken for 30 min at room temperature prior to lysate collection. The collected cell lysate was centrifuged at 5000 × g for 10 min to get rid of cell debris. An equal volume of cell lysate and efflux medium was transferred into scintillation vials and mixed with 4 ml of scintillation liquid for β-counting. The percentage of cholesterol efflux and PC efflux, respectively, was calculated by relating the β radioactivity of the corresponding efflux medium to the sum of β radioactivity in efflux medium and in cell lysate. ApoA-1 mediated or HDL mediated efflux was calculated as the difference between percentage of efflux in the presence and absence of acceptor (apoA-1 or HDL). Based on current knowledge concerning the substrate selectivity of the apoA-1/ABCA1 or HDL/ABCG1 pathway, it is assumed that the measured radioactivity during efflux corresponds to the transport of the intact radioactive molecules (i.e. ^{3}H-cholesterol and ^{14}C-phosphatidylcholine, respectively) rather than their metabolites.

2.3.1. Drugs and biochemical targets

Probucol (Sigma) and Benzamil hydrochloride (Sigma) were used to inhibit ABCA1 (Favari et al., 2004; Wu et al., 2004) and ABCG1 activity (Cserepes et al., 2004), respectively. TO901317 (Sigma), a synthetic agonist of LXRα, was used as a promoter of