Am J Physiol Endocrinol Metab 316: E1136–E1145, 2019.
First published April 19, 2019; doi:10.1152/ajpendo.00548.2018.

RESEARCH ARTICLE

Liver X receptor-α activation enhances cholesterol secretion in lactating mammary epithelium

Diego Y. Grinman,[1] Valeria P. Careaga,[2,3] Elizabeth A. Wellberg,[5] María V. Dansey,[2,4] Edith C. Kordon,[1,4] Steven M. Anderson,[5] Marta S. Maier,[2,3] Gerardo Burton,[2,3] Paul S. MacLean,[6] Michael C. Rudolph,[6]* and Adali Pecci[1,4]*

[1]*Instituto de Fisiología, Biología Molecular y Neurociencias, CONICET, Universidad de Buenos Aires, Buenos Aires, Argentina;* [2]*Unidad De Microanálisis Y Métodos Físicos Aplicados a la Química Orgánica, CONICET, Universidad de Buenos Aires, Buenos Aires, Argentina;* [3]*Universidad de Buenos Aires, Facultad de Ciencias Exactas y Naturales, Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina;* [4]*Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina;* [5]*Department of Pathology, University of Colorado, Anschutz Medical Campus, Denver, Colorado; and* [6]*Division of Endocrinology, Metabolism, and Diabetes, University of Colorado, Anschutz Medical Campus, Denver, Colorado*

Submitted 18 December 2018; accepted in final form 4 April 2019

Grinman DY, Careaga VP, Wellberg EA, Dansey MV, Kordon EC, Anderson SM, Maier MS, Burton G, MacLean PS, Rudolph MC, Pecci A. Liver X receptor-α activation enhances cholesterol secretion in lactating mammary epithelium. *Am J Physiol Endocrinol Metab* 316: E1136–E1145, 2019. First published April 19, 2019; doi:10.1152/ajpendo.00548.2018.—Liver X receptors (LXRs) are ligand-dependent transcription factors activated by cholesterol metabolites. These receptors induce a suite of target genes required for de novo synthesis of triglycerides and cholesterol transport in many tissues. Two different isoforms, LXRα and LXRβ, have been well characterized in liver, adipocytes, macrophages, and intestinal epithelium among others, but their contribution to cholesterol and fatty acid efflux in the lactating mammary epithelium is poorly understood. We hypothesize that LXR regulates lipogenesis during milk fat production in lactation. Global mRNA analysis of mouse mammary epithelial cells (MECs) revealed multiple LXR/RXR targets upregulated sharply early in lactation compared with midpregnancy. LXRα is the primary isoform, and its protein levels increase throughout lactation in MECs. The LXR agonist GW3965 markedly induced several genes involved in cholesterol transport and lipogenesis and enhanced cytoplasmic lipid droplet accumulation in the HC11 MEC cell line. Importantly, in vivo pharmacological activation of LXR increased the milk cholesterol percentage and induced sterol regulatory element-binding protein 1c (Srebp1c) and ATP-binding cassette transporter a7 (Abca7) expression in MECs. Cumulatively, our findings identify LXRα as an important regulator of cholesterol incorporation into the milk through key nodes of de novo lipogenesis, suggesting a potential therapeutic target in women with difficulty initiating lactation.

cholesterol pathway; lactation; LXR; mammary epithelial cells; milk lipid

INTRODUCTION

Newborn nutrition status is a matter of public health concern because breastfed infants are moderately protected against the risks of childhood obesity, type 2 diabetes, and infant mortality (53). For the mother, depending on the duration, lactation is protective against breast cancer risk, type 2 diabetes, and possibly obesity (55). Lactation provides a highly specialized food source that ensures the optimal development of the neonate, and its composition remains dynamic to sustain the energy demands of newborn growth with a near perfect blend of macronutrients (35). Particularly from the neonate's perspective, lipids represent an energy dense nutrient supply used for efficient synthesis of ATP required during postnatal growth and development. Moreover, milk lipids are the source of essential components for building cellular membranes, as well as the substrate for myelin synthesis essential for proper nervous system maturation (23).

Milk fat production at the onset of lactation requires the mammary epithelial cells (MECs) to synchronize tasks including exogenous lipid uptake, processing and intracellular lipid transport, integration with de novo fatty acid synthesis, and packaging into cytoplasmic lipid droplets (CLDs); ultimately, these biosynthetic pathways converge to secrete a complex blend of lipids into the alveolar lumen (33). Lactating mammary epithelium is thought to have consolidated many conserved lipogenic and CLD pathways observed in other tissues, such as the liver and adipose tissues, and has evolved specialized regulation of gene expression common to lipogenesis that is induced in unison at the onset of lactation (43). The appropriate control of these metabolic processes, dependent on maternal diet and physiology, determines the milk fatty acid composition that can directly influence neonatal development (47).

Cholesterol is a critical lipid component of milk, not only because it supplies the offspring with an important structural lipid, but also because of its importance in formation of intracellular CLDs (5). The core of the intracellular CLD harbors densely packed neutral lipids, including cholesterol

* M. C. Rudolph and A. Pecci are co-senior authors and contributed equally to this work.

Address for reprint requests and other correspondence: A. Pecci, Pabellón IFIBYNE, Ciudad Universitaria. Avda. Intendente Güiraldes 2160, Ciudad Autónoma de Buenos Aires, República Argentina, C1428EGA (e-mail: apecci@qb.fcen.uba.ar).

esters (CEs) and triglycerides (TGs) (12). CEs and TGs within the mammary gland are synthesized in the endoplasmic reticulum and packaged into CLDs that migrate to and accumulate at the apical border of mammary acini and are then secreted as a unique triple membrane structure: the milk fat globule (MFG) (29, 31, 32, 51). Hence, milk fat production initiates with the synthesis and incorporation of CEs and TGs into a neutral lipid core.

Liver X receptors (LXRα and LXRβ) belong to the nuclear hormone receptor family of transcription factors, which are known to regulate lipid metabolism and transport in several tissues (4). Similar to other class II nuclear receptors, LXRs regulate transcription as a heterodimer together with the retinoid X receptor (RXR) (18, 53, 60). In the absence of ligands, the heterodimer bound to LXR response elements of target genes recruits corepressors to attenuate transcriptional activation. Upon the binding of agonists, the receptor undergoes conformational changes that result in the release of corepressors and the recruitment of coactivators, leading to transcriptional activation. The endogenous LXRs ligands are oxidative derivatives of cholesterol called oxysterols (17, 18). Increased intracellular levels of cholesterol lead to a proportionate rise in the abundance of oxysterols and consequently increase ligand bound activation of LXRs (49). LXR target genes include the sterol regulatory element-binding protein 1 (Srebp1), fatty acid synthase (Fasn), acetyl CoA carboxylase (Aacac), stearoyl CoA desaturase 1 (Scd1), and some ATP-binding cassette transporters (Abcs), all of which have been shown to be important for the de novo synthesis of fatty acids and cholesterol transport, including during milk production (8, 19, 39, 40, 48, 62).

Several lines of evidence indicate that LXRs play a role in milk fat synthesis and secretion. LXRs mRNAs were shown to be expressed in cow and mouse whole gland lysates in pregnancy and lactation concomitant with the expression of numerous LXR-responsive cholesterol transporters (9, 11, 27, 28, 45). It has also been shown that stimulation of bovine MECs with a synthetic LXR agonist (TO901317) leads to an increase in the de novo synthesis of fatty acids accompanied by increased expression of Srebp1c (11, 25, 30, 37, 57). Previously, we hypothesized that LXRs directly contribute to milk fat production during lactation in the mouse (45). Here, we determined that LXRα is the predominant isoform expressed in isolated MECs and its level increases at midlactation when de novo fatty acid synthesis is maximal (44). We profiled differentially expressed mRNAs between MECs isolated at pregnancy *day 14* and lactation *day 4* and found an LXR/RXR gene signature that includes multiple upregulated LXR target genes. Importantly, short-term pharmacological LXRα activation in the lactating dam increases milk's cholesterol levels along with the expression of Abca7 and Srebp1c. These findings reveal a key role of LXRα in cholesterol synthesis during lactation.

MATERIALS AND METHODS

Reagents. Recombinant Human EGF was purchased from Invitrogen, insulin was obtained from Novo Nordisk, ovine prolactin and dexamethasone were from Sigma, GW3965 was from Medchem Express, and the Compound 1 (CMP1) was prepared as previously described (1). For culture assays, 1,000× solutions were prepared. Steroid stock solutions were prepared in dimethyl sulfoxide.

Cell cultures and treatments. HC11 cells, derived from BALB/c mouse normal mammary glands (3) (kindly provided by Dr. Nancy Hynes, Friedrich Meischer Institute, Basel, Switzerland), were routinely grown in RPMI 1640 growth medium (Gibco) supplemented with 10% FBS (Internegocios), containing 5 μg/ml insulin, 10 ng/ml EGF, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Gibco). Under these growth conditions, proliferating/undifferentiated cells were obtained. Upon reaching confluence, cells were grown in EGF-depleted medium (RPMI 1640, 2% FBS, 5 μg/ml insulin, 100 IU/ml penicillin, and 100 μg/ml streptomycin) for 72 h to reach competence (responsiveness to lactogenic stimuli). Functional differentiation of competent cells was then induced by treatment with 100 nM dexamethasone and 1 μg/ml prolactin in EGF-depleted medium for 72 h. Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2.

Lipid droplet analysis. HC11 cells were differentiated in a six-well plate. Upon reaching differentiation, cells were treated with GW3965 (1 μM) and/or the LXR antagonist CMP1 (10 μM) for 72 h, washed three times with 1× PBS, fixed with 4% paraformaldehyde for 20 min at room temperature, and washed three additional times with PBS. Afterwards, cells were incubated in a PBS solution containing BODIPY 493/503 that has affinity for neutral lipids (1μg/ml) (Molecular Probes) for 1 h at 37°C and washed three times with PBS. Images were acquired with a confocal microscope FV1000 (Olympus), using an immersion oil objective UPlanSApo ×60 (NA = 1.35). BODIPY 493/502 was excited using a multiline Ar laser at 488 nm, and the fluorescence was detected with a photomultiplier. The image size was 2,048 × 2,048 pixels^2. Ten 210 × 210 μm^2 fields were randomly captured for each biological sample. The images were binarized and analyzed with the ImageJ software (https://imagej.nih.gov/ij/). CLDs total area per field was quantified automatically, and the number of total cells per field was counted using the *Plug In* "cell counter." The results are expressed as the average value of the total area of fluorescent CLDs (μm^2) in each field and divided by the number of cells in the respective field, as described by Ref. 34.

Animals. C57BL/6j mice were housed in a pathogen-free temperature-controlled environment on a 12:12-h light-dark cycle (lights on 6:30 AM–6:30 PM, 21°C) and fed a standard laboratory chow diet and water ad libitum. Experimental procedures were approved by the Ethics Committees for Animal Experiments of the University of Buenos Aires (Protocol No. 100) and performed in accordance with the standard international animal care protocols (Canadian Council of Animal Care's *1980 Guide to the Care and Use of Experimental Animals*) (CCAC, 1980). Females were mated with males of the same genetic background at 10 wk old. Litters were standardized to six to eight pups for all studies. Pregnancy *day 1* (P1) was designated as the day vaginal plug was observed, and lactation *day 1* (Lac1) was the day that pups were born. In all experiments, animals were euthanized by cervical dislocation.

Milk collection and milk lipids analysis. Milk was collected as described by Schwertfeger et al. (50). Briefly, on Lac10 females were separated from their pups 3 h prior milk collection to allow milk accumulation. Before milking, mice were anesthetized with xylazine (8 mg/kg; Sigma) and ketamine (90 mg/kg; Sigma) and the intraperitoneally injected with 2 units of oxytocin (Sigma) to induce milk let down. The milk was immediately collected from the mammary glands using gentle vacuum suction with a device adapted from Ref. 10 and frozen at −80°C till lipid analysis. For analysis of fat content, whole milk was drawn into capillary tubes and centrifuged at 3,000 rpm for 20 min. The amount of fat collected into the top layer was calculated as a percentage of the total material present in the tube. Milk lipids were extracted from 10 mg freeze-dried samples by chloroform: methanol extraction following the method of Bligh and Dyer (6). To determine the fatty acid composition, fatty acid methyl esters (FAME) were prepared by reaction with 5% HCl in methanol at 70°C for 2 h. After cooling, water was added and FAME were extracted with chloroform. Approximately 1–2 mg of FAME were weighed into

individual vials followed by the addition of internal standard methyl nonadecanoate (C19:0) and analyzed by gas chromatography-mass spectrometry on a Shimadzu GCMS-QP5050 A instrument equipped with a ZB-5 (Phenomenex) column (30 m × 0.25 mm inner diameter, 0.50-μm thickness). Helium was used as carrier gas (0.9 ml/min flow rate). The injector was set a 250°C, and the detector was set at 280°C. Column temperature was programmed to increase from 100 to 200°C at a rate of 6°C/min followed by an isothermal period of 1 min and then heated to 280°C at a rate of 2°C/min followed by an isothermal period of 30 min. Compound identifications were carried out by comparing retention times of FAME standards (Sigma) and mass spectrometric fragmentation patterns. Quantitation was performed by comparing the percentage area of each FAME peak on the chromatogram with that of the internal standard (methyl nonadecanoate; Sigma) of known weight and expressed as percentage of total fatty acids.

Quantification of milk cholesterol. To quantify total cholesterol, an aliquot of 2–7 mg of the lipid samples was saponified with 4% potassium hydroxide in an ethanolic aqueous solution (2:1, vol/vol) at 60°C for 2 h. After cooling at room temperature, the neutral fraction was extracted with *n*-hexane. After addition 5–10 μg of stigmasterol 95% (Sigma) as internal standard, trimethylsilyl derivatives of the neutral fraction were prepared by addition of 20 μl of *N*,*O*-bis(trimethylsilyl)triflouroacetamide with 1% trimethylchlorosilane (Supelco) and heating at 60°C for 20 min. After cooling, the trimethylsilyl derivatives derivatives were dried under a soft stream of nitrogen, dichloromethane was added, and the solution was stored at 4°C. Samples were analyzed by gas chromatography-mass spectrometry within 24 h of derivatization. Helium was used as carrier gas (1 ml/min flow rate). The injector was set a 250°C, and the detector was set at 280°C. Column temperature was programmed to increase from 240°C to 200°C at a rate of 10°C/min followed by an isothermal period of 25 min and then heated to 290°C at a rate of 8°C/min followed by an isothermal period of 30 min.

Enrichment of MECs from whole mammary glands. MECs were prepared using a modified version of the previously described protocol (44). The mammary digestion buffer was prepared and included Kreb-Ringer-HEPES buffer (containing the following in mM: 120 NaCl, 4.7 KCl, 2.2 $CaCl_2$, 10 HEPES, 1.2 KH_2PO_4, and 1.2 $MgSO_4$), 2 mg/ml collagenase A (Roche) and 0.5 mg/ml hyaluronidase (Sigma). Upon animal euthanasia, mammary glands no. 4 and 5 were excised and lymph nodes were removed. Scalpels were used to dice the glands into a fine paste, which were then digested in 10 ml of digestion buffer for 1 h at 37°C in a 200 rpm a shaker. The digested cell suspension was spun at <300 *g* for 3 min and washed three times with ice cold Kreb-Ringer-HEPES buffer. Half of the samples were used for RNA analysis and half for protein analysis. Samples were homogenized using an Ultraturrax T25 (Ika Labortechnik, Staufen, Germany), and lysate was centrifuged at 13,000 *g* for 10 min at 4°C. The isolation procedure reduced the adipocyte marker Perilipin (PLIN) by almost 98%, indicating that the MECs were highly enriched for the epithelial cell population (Supplemental Fig. S1: https://doi.org/10.6084/m9.figshare.7469933.v2).

Protein analysis. Protein extract from the HC11 cells and the MEC-enriched lysate was prepared in RIPA lysis buffer [containing 20 mM Tris·HCl pH 7.4, 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 0.5% deoxycholate, and 0.1% SDS] containing 50 mM NaF, 1 mM Na_3VO_4, and protease inhibitor cocktail (Promega). Lysates were centrifuged at 13,000 g for 10 min at 4°C. Protein concentrations were determined using the Bradford Protein Assay. Proteins were then resolved using SDS-polyacrylamide gels (8–12%). Resolved proteins were transferred to an Amersham Hybond P 0.45 PVDF blotting membrane (GE Healthcare Life Sciences) and immunoblotting was carried out using the Amersham Imager 600RGB (GE Healthcare Life Sciences). The following primary antibodies were used: LXRα (ab106464) and LXRβ (ab24361; Abcam, Cambridge, UK) and HSP90 (no. 4874; Cell Signaling Technology, Beverly, MA).

RNA isolation and RT-real time PCR. HC11 samples were lysated in 500 μl TRIzol (Invitrogen) while mammary gland derived MECs were homogenized in 1 ml TRIzol using an Ultraturrax T25 (Ika Labortechnik). Lysates were cleared at 13,000 *g* for 10 min at 4°C. The RNA was isolated according to the manufacturer's instructions. Total RNA was quantitated using the Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific). For all samples, the ratio of absorbance at 260 nm to absorbance at 280 nm was >1.8. For quantitative real-time PCR analysis, cDNA was synthesized using 1 μg of total RNA and 200 units of the Moloney Murine Leukemia Virus Reverse Transcriptase Enzyme (Promega) in 20 μl of total volume according to manufacturer's instructions. For the quantitative PCR the cDNA was diluted to 20 ng/ul, and 5 μl of the diluted cDNA was used per reaction. All reactions were conducted in a volume of 25 μl containing 2–4 mM $MgCl_2$ (Invitrogen), 0.2 mM dNTPs (Invitrogen), 0.8 mM of the primer pair, 0.625 U Taq polymerase (Invitrogen), and 0.025 μl of a 1/30,000 Sybr Green (Roche, Indianapolis, IN) dilution. Quantitavie PCR data were collected on the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). Primer sequences are available on request. PCR products representing the unique product (the amplicon) for each gene were amplified from cDNA as above, and amplicons were purified using QIAquick PCR Purification Kit (Qiagen). Concentrations of purified double stranded amplicons for each gene were measured using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). mRNA copy numbers were calculated using each amplicon standard regression curve from the known quantity of the amplicon as previously described (46).

Global gene expression profiling. Microarray data were generated and analyzed as previously described (42, 58). Briefly, 250 ng of total RNA were used as input to the Whole-Transcript Expression RNA kit (Life Technologies, Carlsbad, CA), samples were hybridized to mouse 1.1 ST Gene Arrays (Affymetrix, Santa Clara, CA), and arrays were washed, stained, and imaged using the Affymetrix GeneAtlas Personal Microarray System. Raw data were gas chromatography-robust multiarray averaging normalized and log2 transformed, and statistics were performed using the Partek Genomic Suite (Partek, St. Louis, MO). Significant and differentially expressed genes were uploaded to Ingenuity Pathway Analysis Software (Qiagen) for biological interpretation. Data can be accessed through the NCBI GEO using GSE121819.

Statistical analysis. Results were expressed as means ± SE of at least 3 independent experiments. Statistical analyses were performed with STATISTICA 6.0 (StatSoft) and consisted of one-way ANOVA followed by Tukey's or Dunnett's multiple comparisons tests as indicated in the figure legends. Before statistical analysis, Q-Q plot and Shapiro Wilks test were performed for normality. Homoscedasticity was assessed with Levene's test. In figures, different letters or asterisks mean significant differences ($P < 0.05$) between means or versus control, respectively. For microarray analysis, one-way ANOVA followed by Bonferroni multiple comparisons test with a false discovery rate of 0.01 was performed using Partek Genomics Suite version 6.6 software (Partek, St. Louis, MO).

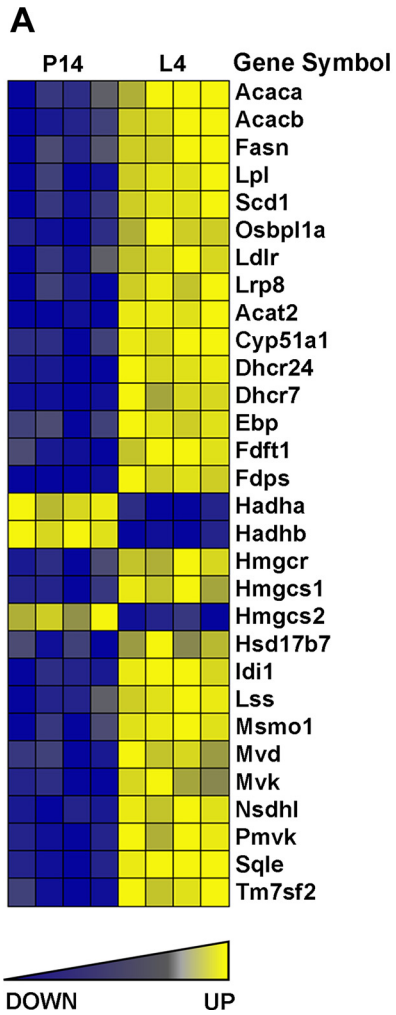
RESULTS

Lipogenic genes and LXRα expression are upregulated in mammary epithelial cells during lactation. Two principle stages of the mammary gland were analyzed by global gene expression profiling using isolated MECs, functional differentiation at P14 and copious milk production at Lac4. With the use of a 1.3-fold cutoff and a false discovery rate multiple testing significance of 0.025, >2,400 genes were differentially expressed. This list of significantly changed transcripts was used to seed Ingenuity Pathway Analysis to identify key

pathways involved in the MECs transition from P14 to Lac4. Notably, the enzymatic superpathway for cholesterol biosynthesis (Fig. 1*A*) and the mRNA signature for LXR/RXR activation had strong positive z-scores (activated) using an unsupervised method (Supplemental Table S1: https://doi.org/10.6084/m9.figshare.7471178.v1). Several lipogenic genes were upregulated in Lac4 MECs relative to P14, many of which are involved in the cholesterol biosynthetic pathway, including acetyl-Co A acetyltransferase 2 (Acat2), mevalonate (diphospho) decarboxylase (Mvd), mevalonate kinase (Mvk), phosphomevalonate kinase (Pmvk), 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (Hmgcs1), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (Hmgcr), squalene epoxidase (Sqle), lanosterol synthase (Lss), isopentenyl-diphosphate delta isomerase (Idi1), farnesyl diphosphate farnesyl transferase 1 (Fdft1), farnesyl diphosphate synthetase (Fdps), 24-dehydrocholesterol reductase (Dhcr24) and 7-dehydrocholesterol reductase (Dhcr7). Moreover, the expression of Ldl receptor and Lrp8, which mediate cholesterol uptake also increased, while the ATP-binding cassette transporters involved in cholesterol secretion (Abca1 and Abcg1) decreased, suggesting that cholesterol loading of MECs happens early in lactation (Fig. 1*A*). While genes involved in fatty acid breakdown (i.e., Hadha/b and Hmgcs2) were also decreased, expression of known LXR target genes that participate in the de novo synthesis and transport of fatty acids (i.e., Lpl, Acaca, Fasn, Cd36 and Scd1) were upregulated, supporting the involvement of the LXR pathway with potential regulation of the lipogenic program of MECs during lactation.

The protein level of the LXRs was evaluated by Western blot analysis specifically in adipocyte-depleted MECs from late pregnant mice and at multiple time points throughout lactation. LXRα protein was expressed in P17 MECs and its levels rose consistently to midlactation, reaching significance by Lac10 relative to P17 (Fig. 1*B*, Up). Additionally, the nuclear localization of LXRα was confirmed in MECs from Lac4 dams (Supplemental Fig. S1: https://doi.org/10.6084/m9.figshare.7469933.v2). LXRβ protein was undetectable in all samples analyzed (Grinman, DY, unpublished observation), supporting a role of for LXRα as the primary isoform in the mammary epithelium. This observation was further reinforced by the significantly lower (-4.81 ± 0.24-fold) abundance of Lxrβ mRNA copy number compared with Lxrα in Lac10 MECs (Fig. 1*B*, Down).

LXR activation increases cytoplasmic lipid droplet accumulation. A well-established mammary epithelial cell line HC11 was used to address whether LXR activation stimulates lipogenesis in vitro. The predominant LXR isoform expressed in differentiated HC11 cells is Lxrα (Supplemental Fig. S2: https://doi.org/10.6084/m9.figshare.7471505.v1), with the mean tran-



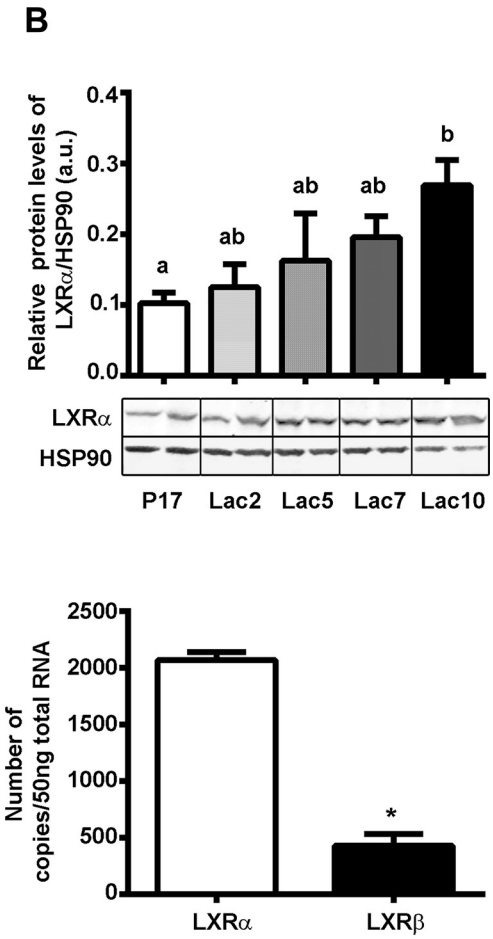


Fig. 1. Liver X receptor-α (LXRα) is the prominent isoform upregulated during lactation in isolated mammary epithelial cells. *A*: global mRNA profiling of isolated mammary epithelial cells (MECs) indicates multiple components of the cholesterol biosynthesis superpathway are robustly upregulated between functional differentiation in pregnancy [pregnant *day 14* (P14)] and following secretory activation in established lactation [lactation *day 4* (L4)] ($n = 4$ per time point; 1-way ANOVA, multiple testing correction false discovery rate $P = 0.025$). *B*, *top*: LXRα protein levels measured along a time series in isolated MEC fractions from late pregnancy (P17) to mid-lactation (Lac10) dams ($n = 5$ per time point; representative immunoblot in duplicate MEC preparations is shown below with HSP90 as the loading control). *B*, *bottom*: absolute mRNA copy number for LXRα and LXRβ quantified in 50 ng of cDNA from Lac10 MECs. In all cases, bars are means ± SE. Bars with different letters or $^*P < 0.05$ are significantly different.

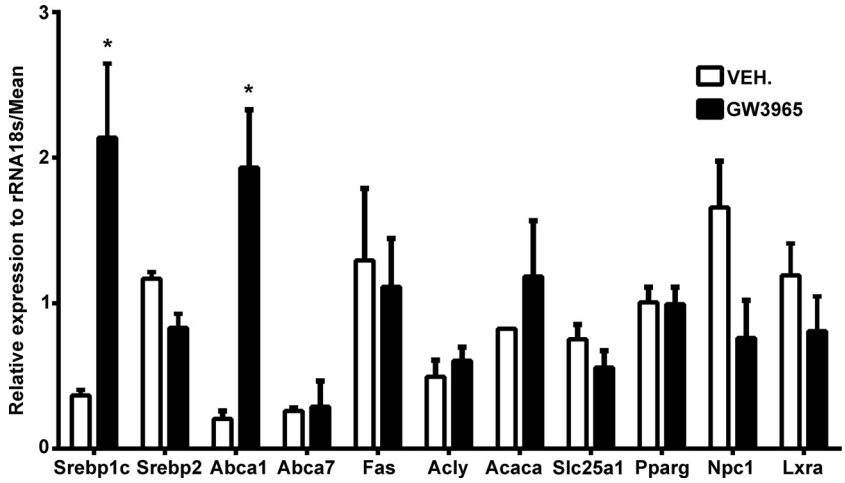


Fig. 2. In vitro liver X receptor-α (LXRα) activation induces the expression of sterol regulatory element-binding protein 1c (SREBP1c) and ATP-binding cassette A1 (ABCA1) genes. Abundance of different ABC transporters and lipogenic genes were measured by quantitative RT-PCR from differentiated HC11 cells treated with GW3965 (1 μM) or vehicle (DMSO) for 6 h. Copy number of each gene relative to rRNA18s is plotted as the ratio to the mean of all samples for each gene to display expression levels on a comparable scale. Bars are means ± SE (*$P < 0.05$, $n = 3$).

script copy number ~3,000-fold higher than that of Lxrβ in each of the three differentiation stages. This observation in HC11 cell culture is consistent with the difference observed in vivo from isolated MECs and supports a role for the LXRα isoform in mammary epithelial cells. To evaluate the effects of LXRα activation in differentiated HC11, cells were treated with the LXR agonist GW3965. Levels of mRNAs for the Srebp1c transcription factor and Abca1 transporter significantly increased upon ligand stimulation under fully differentiated conditions (Fig. 2), whereas the mRNA levels of Abca7, Fasn, Acly, Acaca, Slc25a1, Pparγ, and Npc1 remain unchanged. Moreover, LXRα activation enhanced the mean CLD area by 33% (Fig. 3), indicating the in vitro induction of lipogenesis in MECs over 72 h of treatment. GW3965 dependent CLD accumulation was completely blocked in the presence of LXR antagonist CMP1 with GW3965 treatment. Treatment with CMP1 alone did not affect the CLDs accumulation, suggesting that this compound does not alter CLD stability per se.

LXR activation promotes milk cholesterol secretion during lactation. In vivo, lactation day 9 dams treated with the LXRα agonist GW3965 acutely for 24 h had significantly upregulated LXRα (Fig. 4), indicating possible autoregulation of the LXR promoter in MECs. Moreover, the Abca7 cholesterol transporter was significantly induced following 24 h in vivo GW3965 treatment, potentially identifying a novel LXR target gene in MECs. Consistent with differentiated HC11 cells, the transcription factor Srebp1c was

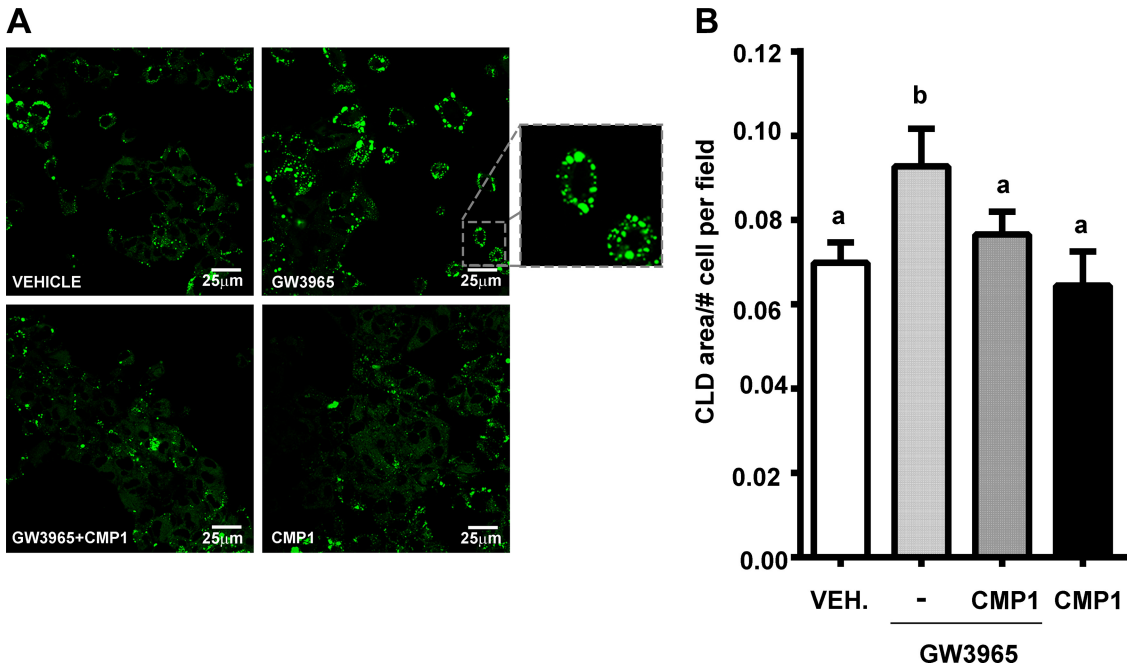


Fig. 3. Liver X receptor-α (LXRα) activation enhances cytoplasmic lipid droplets accumulation in differentiated HC11 cells. Differentiated HC11 cells were incubated with vehicle (VEH.), LXR agonist GW3965 (1 μM), LXR antagonist Compound 1 (CMP1) (10 μM), or both (GW3965 + CMP1) for 72 h and then stained with BODIPY 493/503. (A) Representative confocal images. *B*: quantification of the cytoplasmic lipid droplet (CLD) area. Values are expressed as means ± SE of the total area of fluorescent CLDs (μm^2) in each field divided by the number of cells in the respective field counted manually using the ImageJ *Plug In* "cell counter." Treatments with different letters are significantly different ($P < 0.05$, $n = 3$).

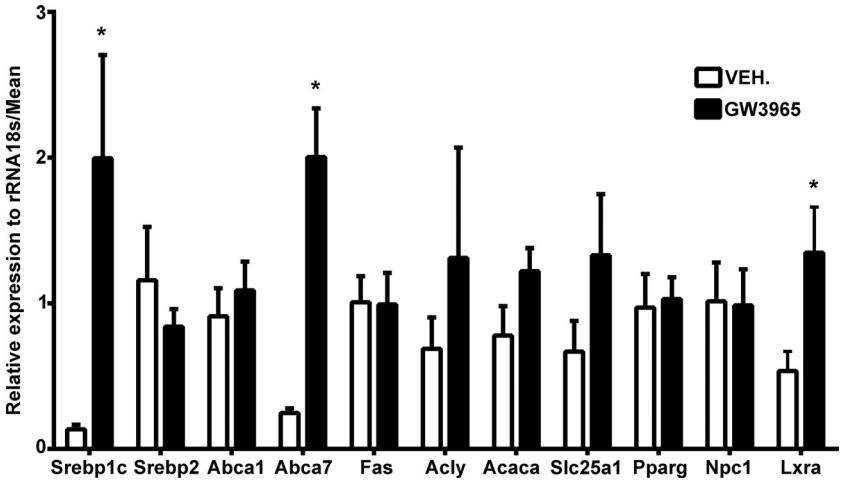


Fig. 4. Liver X receptor-α (LXRα) activation induces the expression of ATP-binding cassette A7 (ABCA7) and sterol regulatory element-binding protein 1c (SREBP1c) in vivo. Abundance of different ABC transporters and lipogenic genes were measured by quantitative RT-PCR from isolated mammary epithelial cells (MECs) following 24 h of either GW3965 (30 mg/kg) or vehicle (10% DMSO in saline) at lactation *day 9*. Copy number of each gene relative to rRNA18s is plotted as the ratio to the mean of all samples for each gene to display expression levels on a comparable scale. Bars are means ± SE (*$P < 0.05$, $n = 5$).

markedly upregulated in vivo, and de novo fatty acid synthesis pathway genes Acly, Acaca, and Slc25a1 tended to increase at 24-h following a single dose of GW3965 treatment in lactating dams.

The acute LXR activation significantly increased the percentage of total cholesterol (2.6 ± 0.4-fold) in the milk fat (Fig. 5*A*), indicating that activated LXRα can enhance cholesterol incorporation into the milk. Based on the sharp induction of Srebp1c and increasing trend for de novo fatty acid synthesis enzyme genes, we hypothesized that GW3965 stimulation of LXR would increase the proportion of de novo-derived medium chain fatty acids in the milk lipids. Unexpectedly, acute GW3965 administration did not influence the milk fatty acid profile (Fig. 5*B*) nor the relative abundance of medium chain fatty acids (Fig. 5*B*, *inset*). Consistent with this observation, levels for FASN mRNA (Fig. 4) and protein (Grinman, DY, unpublished observation) were unchanged in isolated MECs following acute GW3965 treatment. Additionally, preformed fatty acid composition did not vary in the milk fat, suggesting 24 h following a single dose of GW3965 did not influence their uptake and incorporation into TG. The increased milk cholesterol following GW3965 treatment was independent of the total milk fat at ~25% of milk volume (Fig. 5*C*, *left*), which was supported by the dry mass of total lipid per mass of wet milk (Fig. 5*C*, *right*), indicating acute GW3965 treatment did not affect the total milk fat amount.

DISCUSSION

During lactation, the functional mammary gland is charged with the essential task to synthesize and secrete energy dense lipids in the form of TG and CEs. Several studies have shown coordinated induction of genes involved in cholesterol and fatty acid synthesis occurring at the transition between functional differentiation of MECs in pregnancy and secretory activation that triggers copious milk fat production in mice (2, 13, 43, 45). Previous evidence regarding levels of LXR mRNA and protein patterns in the mammary gland, particularly in MECs, has been inconsistent. Here, we provide strong evidence for the MEC-specific contribution of activated LXRα in the regulation of cholesterol incorporation in milk fat during established lactation in mice.

In line with previous findings (2, 13, 41, 44, 45), we observed a robust induction of lipogenic genes between functionally differentiated MECs (P14) and MECs undergoing copious milk fat synthesis (L4). During mouse peak lactation (Lac10), the energy demands for maintaining growth of the litter become maximal, and lipids provide on average more than 80% of the energy available to the young (21). For example, de novo fatty acid synthesis reaches its highest point at Lac10 (44), which coincides with the maximal LXRα levels we observed in MECs progressively from late pregnancy (P17) to peak lactation (Lac10). Acute administration of synthetic LXR agonist GW3965 at Lac9 strongly upregulated Srebp1c and tended to enhance expression of Acly, Acaca, and Slc25a1, suggesting a possible feed-forward loop to balance uptake of cholesterol with de novo fatty acid levels during TG synthesis. Although acute treatment of Lac9 dams with GW3965 increased milk total cholesterol ~2.6-fold, which was coincident with a previously undescribed MECs-specific upregulation of Abca7 (Fig. 3*B*), we observed no effect (< 7% increase, $P = 0.2$) on de novo or preformed fatty acid composition.

Others have shown that ABCA7 protein was expressed abundantly in the MECs at peak lactation in mice (Lac9–12), with a generalized cellular localization that was neither apical or basal (27). Although the exact function(s) of ABCA7 are not well defined, ABCA7 activity has been connected with cellular efflux pathways for cholesterol and phospholipids as well as lipoprotein particle binding (20). ABCA7 stimulates diffusion-mediated cholesterol release mainly to a partially lipidated discoidal form of APOE (7), while ABCA1 is an essential molecule for HDL generation and mediates cholesterol efflux to lipid-free acceptors, such as APOA-1 and APOE (38, 56). These differences, along with the observation that liver-specific LXRα targets Abca1 and Abcg1 are downregulated, underscores a possible mammary specific adaptation for LXR function during lactation such as cholesterol loading. Collectively, we propose that the LXRα/RXR pathway reinforces cholesterol and de novo fatty acid pathways during peak lactation, in part by Abca7 and Srebp1c induction, ensuring milk lipids are balanced and TG synthesis is sufficient to meet the energy and structural lipid demand for neonatal growth. This idea is further supported by our observation that LXRα activation increased accumulation of CLD, which could be accompanied by an enrichment of cholesterol inside these structures, resulting in more cholesterol secreted within the MFGs. If such enrichment takes place, it is likely mainly due to intracellular

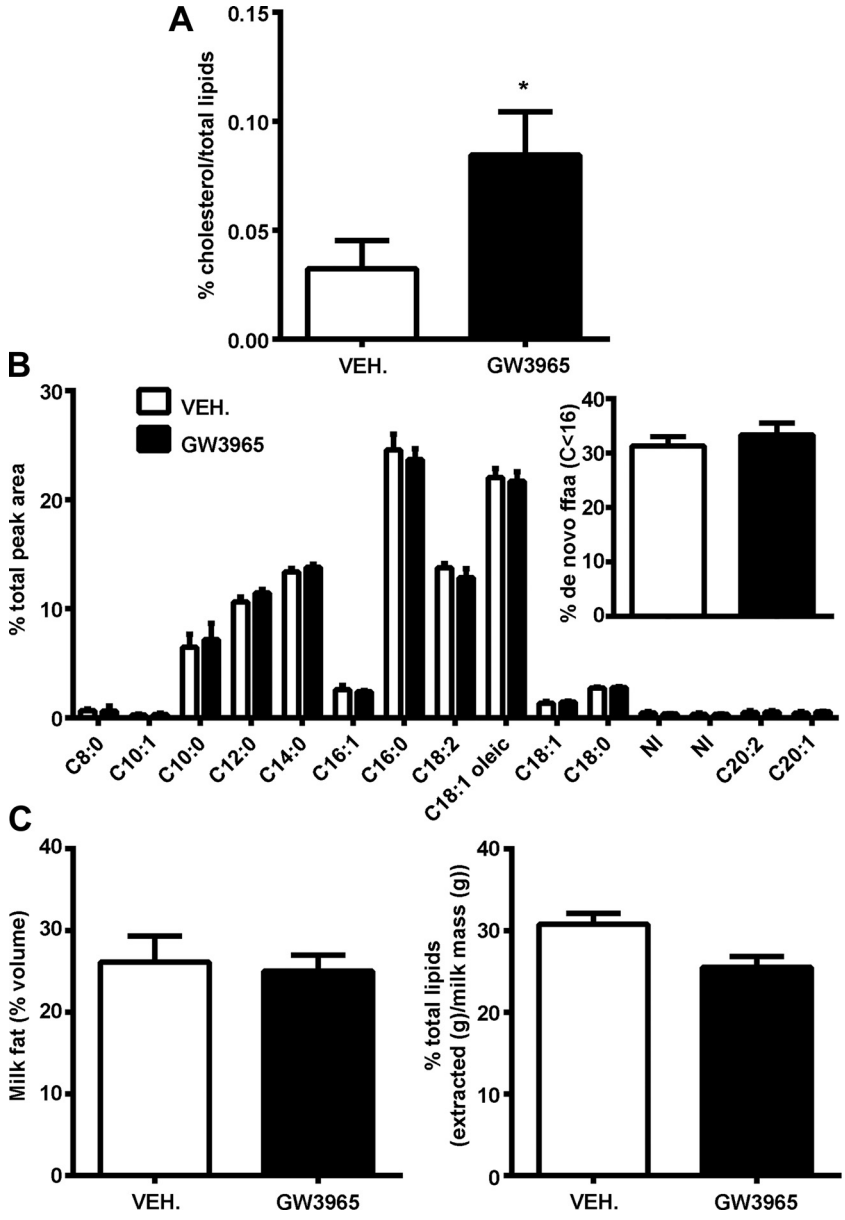


Fig. 5. In vivo liver X receptor-α (LXRα) activation leads to a significant increase in the percentage of cholesterol in milk fat. LAC9 dams were injected intraperitoneally with GW3965 (30 mg/kg) and euthanized 24 h later. *A*: cholesterol milk levels from saponified and trimethylsilyl derivatized lipids from GW3965- or vehicle-treated dams assessed by gas chromatography-mass spectrometry (GC-MS). *B*: fatty acid methyl esters profile quantified by GC-MS from the transmethylated total lipid fraction, indicating that acute treatment with GW3965 did not change the portion of de novo synthesized medium chain fatty acids (inset) or preformed fatty acids. *C*: the milk fat percent per volume (creamocrit; *left*) and the %total lipid extracted (g) per wet milk mass (g) (*right*) were unaffected following GW3965 treatment. NI, nonidentified. Bars are means ± SE (*$P < 0.05$, $n = 5$).

cholesterol redistribution toward the lipid droplets (24), rather than an increase in the de novo cholesterol synthesis, since we did not detect differential expression levels of sterol response element-binding protein 2, a key regulator of sterol synthesis, in response to ligand treatment.

What triggers the LXRα expression increase in the mammary epithelium during lactation remains to be elucidated. That LXRα protein levels steadily increase up to peak lactation, and LXRα mRNA is induced following GW3965 treatment suggests its expression is likely regulated independently of lactogenic hormones. As a consequence of the cellular cholesterol accumulation occurring in Lac4 MECs, generation of downstream cholesterol metabolites could build up, including the endogenous ligands LXRα, oxysterols. Of note, the murine Lxrα gene was shown to be auto regulated in adipose tissue via an LXRE motif present in the mouse Lxrα promoter that is conserved in the human gene (54). Moreover, autoregulation of the human LXRα was also described in primary human macrophages (22, 59). Furthermore, sharp upregulation of many cholesterol biosynthetic genes early in established lactation (Lac4) along with increased expression of Ldlr and Lrp8 (Fig. 1*A*) supports that MECs might use these proteins to load cholesterol into the MECs following secretory activation. Indeed, Osbpl1a was sharply induced (2.64-fold) at the transition from functional differentiation to established lactation in isolated MECs, which is known to bind 25-hydroxycholesterol and cholesterol (52). Therefore, we speculate the consistently increasing LXRα protein in MECs following secretory activation may represent a positive self-feedback loop to maximize the cholesterol and fatty acid levels during peak lactation. LXRα autoregulation would be important then to increase the levels of the receptor over a threshold necessary to induce the batch of target genes involved in lipid transport later in lactation (54). Remarkably, the steady rise of LXRα protein levels throughout lactation mirrors the growth trajectory and subsequent energy demands of the growing pups.

MECs appear to have a specialized subset of LXR/RXR target genes (i.e., Lpl, Acaca, Fasn, Cd36, and Scd1) independent of ones identified in liver or adipose tissues (i.e., Abca1, Abcg1, and Mylip). However, following acute GW3965 treatment, there was a dramatic increase in Srebp1c levels, the isoform most expressed in the mouse mammary gland during pregnancy and lactation. Therefore, we cannot exclude the possibility that Srebp1c mediates LXR dependent upregulation of Abca7. Previous observations in bovine and goat mammary epithelial cells treated in vitro with TO901317, an LXR synthetic agonist (11, 26, 30, 36, 37, 61), demonstrated that LXRα activation increases the expression of specific ABC transporters and lipogenic genes resulting in enhanced cholesterol efflux and de novo lipogenesis. Although Abca7 expression regulation was shown to be unresponsive to LXR ligands in mouse macrophages and fibroblasts (16, 20), it can be controlled by sterol response element-binding proteins 2 through a sterol-responsive/regulatory element (16).

In summary, we provide new evidence that highlights the role of LXRα in controlling the cholesterol transport to the milk and showed for the first time that in vivo activation of this receptor modulates the levels of milk cholesterol without affecting fatty acid profile. Although further investigation using conditional knock out of LXRα during lactation would provide valuable information, LXRα/RXR arises as a key pathway that ensures the cholesterol availability necessary for neutral lipid packaging into CLD and free cholesterol secretion delivered through the milk. Two major implications derive from these findings. On one hand, LXRs are potentially attractive druggable targets for the treatment of several diseases, i.e., atherosclerosis, cardiovascular diseases (14) and, more importantly, mastitis (9, 15). Therefore, critical evaluation regarding the administration of LXR agonists should be made when designing therapies for those lactating mothers, since it could impact the milk cholesterol composition and consequently have implications in neonate's development while breastfeeding. On the other hand, although this study was limited by observations at 24 h, our results could also suggest that selective LXRα agonist treatment, when administered in an acute manner, might help to overcome the difficulties that obese and type 2 diabetic mothers experience with lactation initiation and maintenance. Further characterization is needed to understand if the acute GW treatment has effects at later times and in metabolic tissues other than the lactating mammary epithelium.

ACKNOWLEDGMENTS

We thank Lirane Moutinho for technical assistance with animal handling and milk collection. D. Y. Grinman was a CONICET, and BEC.ar research fellows. V. P. Careaga, M. V. Dansey, E. C. Kordon, M. S. Maier, G. Burton, and A. Pecci are CONICET members.

Present address for D. Y. Grinman: Section of Endocrinology and Metabolism, Dept. of Internal Medicine, School of Medicine, Yale Univ., New Haven, CT.

GRANTS

A. Pecci was supported by ANPCyT (PICT 2014-630); CONICET-Argentina (PIP-11220110100702); and University of Buenos Aires (20020130100363BA). G. Burton was supported by ANPCyT (PICT 2014-0626) and University of Buenos Aires (20020130100367BA). M. C. Rudolph was supported by NIH Grant K01-DK-109079, BIRCWH Scholar Award K12 HD057022, Colorado Nutrition, and Obesity Research Center (NIH P30-DK-048520); and P. S. MacLeon was supported by NIH Grants P01-HD-038129 and R01-HD-075285.

DISCLAIMERS

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

D.Y.G., M.V.D., E.C.K., P.S.M., M.C.R. and A.P. conceived and designed research; D.Y.G, V.P.C. and E.A.W. performed experiments; D.Y.G., M.C.R. and A.P. analyzed data; D.Y.G., P.S.M., M.C.R. and A.P. interpreted results of experiments; D.Y.G and M.C.R. prepared figures; D.Y.G., M.C.R. and A.P. drafted manuscript; D.Y.G., V.P.C., M.V.D., M.S.M., G.B., P.S.M., M.C.R. and A.P. edited and revised manuscript; D.Y.G., V.P.C., E.A.W., M.V.D., E.C.K., S.M.A., M.S.M., G.B., P.S.M., M.C.R. and A.P. approved final version of manuscript.

REFERENCES

1. **Álvarez LD, Dansey MV, Grinman DY, Navalesi D, Samaja GA, Del Fueyo MC, Bastiaensen N, Houtman R, Estrin DA, Veleiro AS, Pecci A, Burton G.** Destabilization of the torsioned conformation of a ligand side chain inverts the LXRβ activity. *Biochim Biophys Acta* 1851: 1577–1586, 2015. doi:10.1016/j.bbalip.2015.09.007.
2. **Anderson SM, Rudolph MC, McManaman JL, Neville MC.** Key stages in mammary gland development. Secretory activation in the mammary gland: it's not just about milk protein synthesis! *Breast Cancer Res* 9: 204, 2007. doi:10.1186/bcr1653.
3. **Ball RK, Friis RR, Schoenenberger CA, Doppler W, Groner B.** Prolactin regulation of beta-casein gene expression and of a cytosolic 120-kd protein in a cloned mouse mammary epithelial cell line. *EMBO J* 7: 2089–2095, 1988. doi:10.1002/j.1460-2075.1988.tb03048.x.
4. **Baranowski M.** Biological role of liver X receptors. *J Physiol Pharmacol* 59, *Suppl* 7: 31–55, 2008..
5. **Barbosa AD, Siniossoglou S.** Function of lipid droplet-organelle interactions in lipid homeostasis. *Biochim Biophys Acta Mol Cell Res* 1864: 1459–1468, 2017. doi:10.1016/j.bbamcr.2017.04.001.
6. **Bligh EG, Dyer WJ.** A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 37: 911–917, 1959. doi:10.1139/y59-099.
7. **Chan SL, Kim WS, Kwok JB, Hill AF, Cappai R, Rye K-A, Garner B.** ATP-binding cassette transporter A7 regulates processing of amyloid precursor protein in vitro. *J Neurochem* 106: 793–804, 2008. doi:10.1111/j.1471-4159.2008.05433.x.
8. **Costet P, Luo Y, Wang N, Tall AR.** Sterol-dependent transactivation of the human ABC1 promoter by LXR/RXR. *J Biol Chem* 275: 28240–28245, 2000. doi:10.1074/jbc.M003337200.
9. **Fu Y, Tian Y, Wei Z, Liu H, Song X, Liu W, Zhang W, Wang W, Cao Y, Zhang N.** Liver X receptor agonist prevents LPS-induced mastitis in mice. *Int Immunopharmacol* 22: 379–383, 2014. doi:10.1016/j.intimp.2014.07.015.
10. **Fukuta K.** Collection of body fluids. In: *The Laboratory Mouse*. Amsterdam: Elsevier, p. 543–554.
11. **Harvatine KJ, Boisclair YR, Bauman DE.** Liver x receptors stimulate lipogenesis in bovine mammary epithelial cell culture but do not appear to be involved in diet-induced milk fat depression in cows. *Physiol Rep* 2: e00266, 2014. doi:10.1002/phy2.266.
12. **Hashemi HF, Goodman JM.** The life cycle of lipid droplets. *Curr Opin Cell Biol* 33: 119–124, 2015. doi:10.1016/j.ceb.2015.02.002.
13. **Heinz RE, Rudolph MC, Ramanathan P, Spoelstra NS, Butterfield KT, Webb PG, Babbs BL, Gao H, Chen S, Gordon MA, Anderson SM, Neville MC, Gu H, Richer JK.** Constitutive expression of microRNA-150 in mammary epithelium suppresses secretory activation and impairs de novo lipogenesis. *Development* 143: 4236–4248, 2016. doi:10.1242/dev.139642.
14. **Hong C, Tontonoz P.** Liver X receptors in lipid metabolism: opportunities for drug discovery. *Nat Rev Drug Discov* 13: 433–444, 2014. doi:10.1038/nrd4280.
15. **Hu X, Zhang N, Fu Y.** Role of liver X receptor in mastitis therapy and regulation of milk fat synthesis. *J Mammary Gland Biol Neoplasia*, 2018. doi:10.1007/s10911-018-9403-5.
16. **Iwamoto N, Abe-Dohmae S, Sato R, Yokoyama S.** ABCA7 expression is regulated by cellular cholesterol through the SREBP2 pathway and

associated with phagocytosis. *J Lipid Res* 47: 1915–1927, 2006. doi:10.1194/jlr.M600127-JLR200.

17. **Janowski BA, Grogan MJ, Jones SA, Wisely GB, Kliewer SA, Corey EJ, Mangelsdorf DJ.** Structural requirements of ligands for the oxysterol liver X receptors LXRalpha and LXRbeta. *Proc Natl Acad Sci USA* 96: 266–271, 1999. doi:10.1073/pnas.96.1.266.
18. **Janowski BA, Willy PJ, Devi TR, Falck JR, Mangelsdorf DJ.** An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. *Nature* 383: 728–731, 1996. doi:10.1038/383728a0.
19. **Kennedy MA, Venkateswaran A, Tarr PT, Xenarios I, Kudoh J, Shimizu N, Edwards PA.** Characterization of the human ABCG1 gene: liver X receptor activates an internal promoter that produces a novel transcript encoding an alternative form of the protein. *J Biol Chem* 276: 39438–39447, 2001. doi:10.1074/jbc.M105863200.
20. **Kim WS, Fitzgerald ML, Kang K, Okuhira K, Bell SA, Manning JJ, Koehn SL, Lu N, Moore KJ, Freeman MW.** Abca7 null mice retain normal macrophage phosphatidylcholine and cholesterol efflux activity despite alterations in adipose mass and serum cholesterol levels. *J Biol Chem* 280: 3989–3995, 2005. doi:10.1074/jbc.M412602200.
21. **Kounig B, Riester J, Markl H.** Maternal care in house mice (Mus musculus): II. The energy cost of lactation as a function of litter size. *J Zool (Lond)* 216: 195–210, 1988. doi:10.1111/j.1469-7998.1988.tb02425.x.
22. **Laffitte BA, Joseph SB, Walczak R, Pei L, Wilpitz DC, Collins JL, Tontonoz P.** Autoregulation of the human liver X receptor alpha promoter. *Mol Cell Biol* 21: 7558–7568, 2001. doi:10.1128/MCB.21.22.7558-7568.2001.
23. **Larque E, Demmelmair H, Koletzko B.** Perinatal supply and metabolism of long-chain polyunsaturated fatty acids: importance for the early development of the nervous system. *Ann N Y Acad Sci* 967: 299–310, 2002. doi:10.1111/j.1749-6632.2002.tb04285.x.
24. **Le Lay S, Robichon C, Le Liepvre X, Dagher G, Ferre P, Dugail I.** Regulation of ABCA1 expression and cholesterol efflux during adipose differentiation of 3T3-L1 cells. *J Lipid Res* 44: 1499–1507, 2003. doi:10.1194/jlr.M200466-JLR200.
25. **Lengi AJ, Corl BA.** Short communication: identification of the bovine sterol regulatory element binding protein-1c promoter and its activation by liver X receptor. *J Dairy Sci* 93: 5831–5836, 2010. doi:10.3168/jds.2010-3236.
26. **Li J, Luo J, Zhu J, Sun Y, Yao D, Shi H, Wang W.** Regulation of the fatty acid synthase promoter by liver X receptor α through direct and indirect mechanisms in goat mammary epithelial cells. *Comp Biochem Physiol B Biochem Mol Biol* 184: 44–51, 2015. doi:10.1016/j.cbpb.2015.02.005.
27. **Mani O, Körner M, Sorensen MT, Sejrsen K, Wotzkow C, Ontsouka CE, Friis RR, Bruckmaier RM, Albrecht C.** Expression, localization, and functional model of cholesterol transporters in lactating and nonlactating mammary tissues of murine, bovine, and human origin. *Am J Physiol Regul Integr Comp Physiol* 299: R642–R654, 2010. doi:10.1152/ajpregu.00723.2009.
28. **Mani O, Sorensen MT, Sejrsen K, Bruckmaier RM, Albrecht C.** Differential expression and localization of lipid transporters in the bovine mammary gland during the pregnancy-lactation cycle. *J Dairy Sci* 92: 3744–3756, 2009. doi:10.3168/jds.2009-2063.
29. **Martin S, Parton RG.** Caveolin, cholesterol, and lipid bodies. *Semin Cell Dev Biol* 16: 163–174, 2005. doi:10.1016/j.semcdb.2005.01.007.
30. **McFadden JW, Corl BA.** Activation of liver X receptor (LXR) enhances de novo fatty acid synthesis in bovine mammary epithelial cells. *J Dairy Sci* 93: 4651–4658, 2010. doi:10.3168/jds.2010-3202.
31. **McManaman JL.** Milk lipid secretion: recent biomolecular aspects. *Biomol Concepts* 3: 581–591, 2012. doi:10.1515/bmc-2012-0025.
32. **McManaman JL.** Lipid transport in the lactating mammary gland. *J Mammary Gland Biol Neoplasia* 19: 35–42, 2014. doi:10.1007/s10911-014-9318-8.
33. **McManaman JL, Neville MC.** Mammary physiology and milk secretion. *Adv Drug Deliv Rev* 55: 629–641, 2003. doi:10.1016/S0169-409X(03)00033-4.
34. **Melo RCN, D'Ávila H, Bozza PT, Weller PF.** Imaging lipid bodies within leukocytes with different light microscopy techniques. *Methods Mol Biol* 689: 149–161, 2011. doi:10.1007/978-1-60761-950-5_9
35. **Neville MC.** Lactation and its hormonal Control. In: *Knobil and Neill's Physiology of Reproduction*. Amsterdam: Elsevier, 2014, p. 2993–3054.
36. **Ontsouka CE, Huang X, Aliyev E, Albrecht C.** In vitro characterization and endocrine regulation of cholesterol and phospholipid transport in the mammary gland. *Mol Cell Endocrinol* 439: 35–45, 2017. doi:10.1016/j.mce.2016.10.016.
37. **Oppi-Williams C, Suagee JKK, Corl BA.** Regulation of lipid synthesis by liver X receptor α and sterol regulatory element-binding protein 1 in mammary epithelial cells. *J Dairy Sci* 96: 112–121, 2013. doi:10.3168/jds.2011-5276.
38. **Oram JF.** HDL apolipoproteins and ABCA1: partners in the removal of excess cellular cholesterol. *Arterioscler Thromb Vasc Biol* 23: 720–727, 2003. doi:10.1161/01.ATV.0000054662.44688.9A.
39. **Repa JJ, Turley SD, Lobaccaro JA, Medina J, Li L, Lustig K, Shan B, Heyman RA, Dietschy JM, Mangelsdorf DJ.** Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers. *Science* 289: 1524–1529, 2000. doi:10.1126/science.289.5484.1524.
40. **Repa JJ, Liang G, Ou J, Bashmakov Y, Lobaccaro JM, Shimomura I, Shan B, Brown MS, Goldstein JL, Mangelsdorf DJ.** Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta. *Genes Dev* 14: 2819–2830, 2000. doi:10.1101/gad.844900.
41. **Rodriguez-Cruz M, Sánchez R, Sánchez AM, Kelleher SL, Sánchez-Muñoz F, Maldonado J, López-Alarcón M.** Participation of mammary gland in long-chain polyunsaturated fatty acid synthesis during pregnancy and lactation in rats. *Biochim Biophys Acta* 1811: 284–293, 2011. doi:10.1016/j.bbalip.2011.01.007.
42. **Rudolph MC, Jackman MR, Presby DM, Houck JA, Webb PG, Johnson GC, Soderborg TK, de la Houssaye BA, Yang IV, Friedman JE, MacLean PS.** Low neonatal plasma n-6/n-3 PUFA ratios regulate offspring adipogenic potential and condition adult obesity resistance. *Diabetes* 67: 651–661, 2018. doi:10.2337/db17-0890.
43. **Rudolph MC, McManaman JL, Phang T, Russell T, Kominsky DJ, Serkova NJ, Stein T, Anderson SM, Neville MC.** Metabolic regulation in the lactating mammary gland: a lipid synthesizing machine. *Physiol Genomics* 28: 323–336, 2007. doi:10.1152/physiolgenomics.00020.2006.
44. **Rudolph MC, Monks J, Burns V, Phistry M, Marians R, Foote MR, Bauman DE, Anderson SM, Neville MC.** Sterol regulatory element binding protein and dietary lipid regulation of fatty acid synthesis in the mammary epithelium. *Am J Physiol Endocrinol Metab* 299: E918–E927, 2010. doi:10.1152/ajpendo.00376.2010.
45. **Rudolph MC, Neville MC, Anderson SM.** Lipid synthesis in lactation: diet and the fatty acid switch. *J Mammary Gland Biol Neoplasia* 12: 269–281, 2007. doi:10.1007/s10911-007-9061-5.
46. **Rudolph MC, Wellberg EA, Lewis AS, Terrell KL, Merz AL, Maluf NK, Serkova NJ, Anderson SM.** Thyroid hormone responsive protein Spot14 enhances catalysis of fatty acid synthase in lactating mammary epithelium. *J Lipid Res* 55: 1052–1065, 2014. doi:10.1194/jlr.M044487.
47. **Rudolph MC, Young BE, Lemas DJ, Palmer CE, Hernandez TL, Barbour LA, Friedman JE, Krebs NF, MacLean PS.** Early infant adipose deposition is positively associated with the n-6 to n-3 fatty acid ratio in human milk independent of maternal BMI. *Int J Obes* 41: 510–517, 2017. doi:10.1038/ijo.2016.211.
48. **Sabol SL, Brewer HB Jr, Santamarina-Fojo S.** The human ABCG1 gene: identification of LXR response elements that modulate expression in macrophages and liver. *J Lipid Res* 46: 2151–2167, 2005. doi:10.1194/jlr.M500080-JLR200.
49. **Schroepfer GJ Jr.** Oxysterols: modulators of cholesterol metabolism and other processes. *Physiol Rev* 80: 361–554, 2000. doi:10.1152/physrev.2000.80.1.361.
50. **Schwertfeger KL, McManaman JL, Palmer CA, Neville MC, Anderson SM.** Expression of constitutively activated Akt in the mammary gland leads to excess lipid synthesis during pregnancy and lactation. *J Lipid Res* 44: 1100–1112, 2003. doi:10.1194/jlr.M300045-JLR200.
51. **Stein O, Stein Y; Electron Microscopic Radioautographic Study of the Mouse Lactating Mammary Gland.** Lipid synthesis, intracellular transport, and secretion. II. Electron microscopic radioautographic study of the mouse lactating mammary gland. *J Cell Biol* 34: 251–263, 1967. doi:10.1083/jcb.34.1.251.
52. **Suchanek M, Hynynen R, Wohlfahrt G, Lehto M, Johansson M, Saarinen H, Radzikowska A, Thiele C, Olkkonen VM.** The mammalian oxysterol-binding protein-related proteins (ORPs) bind 25-hydroxycholesterol in an evolutionarily conserved pocket. *Biochem J* 405: 473–480, 2007. doi:10.1042/BJ20070176.
53. **Teboul M, Enmark E, Li Q, Wikström AC, Pelto-Huikko M, Gustafsson JA.** OR-1, a member of the nuclear receptor superfamily that interacts with the 9-cis-retinoic acid receptor. *Proc Natl Acad Sci USA* 92: 2096–2100, 1995. doi:10.1073/pnas.92.6.2096.

54. **Ulven SM, Dalen KT, Gustafsson J-A, Nebb HI.** Tissue-specific autoregulation of the LXRalpha gene facilitates induction of apoE in mouse adipose tissue. *J Lipid Res* 45: 2052–2062, 2004. doi:10.1194/jlr.M400119-JLR200.
55. **Victora CG, Bahl R, Barros AJD, França GVA, Horton S, Krasevec J, Murch S, Sankar MJ, Walker N, Rollins NC; Lancet Breastfeeding Series Group.** Breastfeeding in the 21st century: epidemiology, mechanisms, and lifelong effect. *Lancet* 387: 475–490, 2016. doi:10.1016/S0140-6736(15)01024-7.
56. **Wang N, Tall AR.** Regulation and mechanisms of ATP-binding cassette transporter A1-mediated cellular cholesterol efflux. *Arterioscler Thromb Vasc Biol* 23: 1178–1184, 2003. doi:10.1161/01.ATV.0000075912.83860.26.
57. **Wang W, Luo J, Zhong Y, Lin XZ, Shi HB, Zhu JJ, Li J, Sun YT, Zhao WS.** Goat liver X receptor α, molecular cloning, functional characterization and regulating fatty acid synthesis in epithelial cells of goat mammary glands. *Gene* 505: 114–120, 2012. doi:10.1016/j.gene.2012.05.028.
58. **Wellberg EA, Rudolph MC, Lewis AS, Padilla-Just N, Jedlicka P, Anderson SM.** Modulation of tumor fatty acids, through overexpression or loss of thyroid hormone responsive protein spot 14 is associated with altered growth and metastasis. *Breast Cancer Res* 16: 481, 2014. doi:10.1186/s13058-014-0481-z.
59. **Whitney KD, Watson MA, Goodwin B, Galardi CM, Maglich JM, Wilson JG, Willson TM, Collins JL, Kliewer SA.** Liver X receptor (LXR) regulation of the LXRalpha gene in human macrophages. *J Biol Chem* 276: 43509–43515, 2001. doi:10.1074/jbc.M106155200.
60. **Willy PJ, Umesono K, Ong ES, Evans RM, Heyman RA, Mangelsdorf DJ.** LXR, a nuclear receptor that defines a distinct retinoid response pathway. *Genes Dev* 9: 1033–1045, 1995. doi:10.1101/gad.9.9.1033.
61. **Yao DW, Luo J, He QY, Xu HF, Li J, Shi HB, Wang H, Chen Z, Loor JJ.** Liver X receptor α promotes the synthesis of monounsaturated fatty acids in goat mammary epithelial cells via the control of stearoyl-coenzyme A desaturase 1 in an SREBP-1-dependent manner. *J Dairy Sci* 99: 6391–6402, 2016. doi:10.3168/jds.2016-10990.
62. **Yoshikawa T, Shimano H, Amemiya-Kudo M, Yahagi N, Hasty AH, Matsuzaka T, Okazaki H, Tamura Y, Iizuka Y, Ohashi K, Osuga J, Harada K, Gotoda T, Kimura S, Ishibashi S, Yamada N.** Identification of liver X receptor-retinoid X receptor as an activator of the sterol regulatory element-binding protein 1c gene promoter. *Mol Cell Biol* 21: 2991–3000, 2001. doi:10.1128/MCB.21.9.2991-3000.2001.