Activation of liver X receptor promotes fatty acid synthesis in goat mammary epithelial cells via modulation of *SREBP1* expression

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ABSTRACT

In bovine mammary tissue and cells, liver X receptor (LXR) regulates lipid synthesis mainly via transactivation of the transcription factor sterol regulatory element binding protein 1 (SREBP1). In the present work, we investigated the role of LXR in controlling lipid synthesis via transactivation of SREBP1 in goat primary mammary cells (GMEC). The GMEC were treated with a synthetic agonist of LXR, T0901317, and transactivation and transcription of SREBP1, expression of lipogenic genes, and fatty acid profiling and triacylglycerol (TAG) content of the cells were measured. A mild increase in the mRNA expression level of LXR α (*NR1H3*) was observed following treatment with different concentrations of T0901317, and a dose-dependent increase in mRNA and transactivation of SREBP1 was detected. Activation of LXR resulted in a significant increase in the mRNA expression of most of the measured genes related to de novo synthesis, desaturation, and transport of fatty acids; TAG synthesis; and transcription regulators. Compared with the control, total content of cellular TAG increased by more than 20% with T0901317 treatment. Furthermore, addition of T0901317 increased the proportion of unsaturated fatty acids (e.g., C16:1, C18:1, C20:1, and C22:1), and decreased the proportion of saturated fatty acids (e.g., C16:0, C18:0, C20:0, and C22:0). These results provide evidence that LXR regulates the expression and activity of SREBP1. Our results indicated that LXR participate in regulating the transcription of genes involved in milk fat synthesis in GMEC in an SREBP1-dependent fashion.

Key words: liver X receptor, SREBP1, lactation, lipid synthesis, milk fat

INTRODUCTION

Liver X receptors (LXR) are ligand-dependent nuclear receptors with important roles in regulating lipid homeostasis in rodents and mammals (Chen et al., 2004; Darimont et al., 2006; Oppi-Williams et al., 2013). Liver X receptors form obligate heterodimers with retinoid X receptors (RXR) and are bound and activated by oxysterols (Mangelsdorf et al., 1995; Lehmann et al., 1997). Numerous studies revealed that LXR–RXR heterodimer serves as a key regulator of cholesterol homeostasis through initiating the expression of cholesterol 7 α -hydroxylase, cholesterol ester transfer protein, and ATP-binding cassette transporter 1 (Peet et al., 1998; Russell, 1999; Luo and Tall, 2000; Repa et al., 2000b). Although 2 isoforms of LXR have been characterized in rodents, modulation of LXR α but not LXR β affected the expression of key lipogenic enzymes (Peet et al., 1998; Darimont et al., 2006; Zhang et al., 2014). For ruminants, it has been reported that LXR β is the predominant subtype in regulating lipid synthesis gene expression (Shi et al., 2017).

Sterol regulatory element binding protein 1 (SREBP1) is a transcription factor with key roles in regulating lipid metabolism. To date, 3 isoforms of the SREBP family have been identified in mammals: SREBP1a, SREBP1c, and SREBP2. The SREBP1a and SREBP1c isoforms are alternative transcripts of *SREBF1*, whereas SREBP2 is transcribed from a separate gene (*SREBF2*). Both isoforms of SREBP1 participate in the regulation of fatty acid synthesis (Tontonoz et al., 1993; Foretz et al., 1999; Eberlé et al., 2004), whereas SREBP2 mainly regulates the synthesis of cholesterol (Tontonoz et al., 1993; Shimano, 2001; Horton et al., 2002).

Sterol regulatory element binding proteins are synthesized as precursor proteins residing in the membranes

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of the endoplasmic reticulum and nuclear envelope complexed with the SREBP cleavage activate protein (**SCAP**). When the concentration of cholesterol is high, the SCAP is bound to insulin induced gene (**INSIG**) protein, which anchors the SREBP–SCAP complex to the endoplasmic reticulum or nuclear envelope. When the intracellular cholesterol concentration is low, the INSIG releases the binding of SCAP, allowing the SCAP to chaperone the SREBP to the Golgi apparatus. In the Golgi, the complex goes through 2 steps of proteolytic cleavage by the action of site 1 protease and site 2 protease to release the mature form of SREBP1 protein (N terminus bHLH-Zip active fragment), which contains a nuclear localization signal. The mature SREBP1 enters the cell nucleus to modulate transcription of itself and various lipogenic genes (Horton et al., 2003; Raghov et al., 2008).

As one of the major lipid-synthesizing organs, the lactating mammary gland synthesizes a great amount of triacylglycerol (**TAG**; Wakil and Abu-Elheiga, 2009; Rudolph et al., 2010). Investigations into the regulation of milk fat synthesis have established SREBP1 as a central hub in the regulation of fatty acid synthesis in the bovine mammary gland (Bauman et al., 2006; Harvatine et al., 2009). In dairy cows, the decrease in activity and expression of SREBP1 by intermediates of the rumen biohydrogenation of PUFA is considered one of the main mechanisms involved in the diet-induced milk fat depression (Bauman et al., 2006). Recent in vitro data provided additional support for the critical role of SREBP1 in controlling expression of genes coding for proteins involved in fatty acid synthesis and uptake in bovine mammary epithelial cells (Ma and Corl, 2012). However, SREBP1 is not the only transcriptional regulator of milk fat synthesis. Evidence has mounted to support a role of a network of transcription factors encompassing peroxisome proliferator activated receptor γ (**PPAR γ**), SREBP1, and LXR α regulating the expression of milk fat synthesis-related genes (Bionaz et al., 2015; Osorio et al., 2016).

The regulatory function of PPAR γ on lipid accumulation has been demonstrated to be mediated by perilipin in mammary epithelial cells of dairy goats (Shi et al., 2014; Kang et al., 2015). The role of LXR in controlling milk fat synthesis was initially demonstrated by work conducted in bovine mammary cells in vitro, where activation of LXR α by the specific agonist T0901317 increased the expression of SREBP1 and augmented de novo fatty acid synthesis (McFadden and Corl, 2010). Since then, much work has been done to clarify the transcriptional regulation mechanism of LXR on SREBP1 and the consequence on milk fat synthesis in ruminants (Wang et al., 2012; Oppi-Williams et al.,

2013; Li et al., 2015b; Yao et al., 2016; Shi et al., 2017). However, much work remains to be done to determine whether the activation of LXR affects the network of genes involved in milk fat synthesis (Bionaz and Loor, 2008). Our hypothesis is that LXR plays a central role in controlling the expression of milk fat synthesis-related genes and increasing intracellular triglycerides synthesis, likely through increasing the expression of *SREBF1*. The objective of the present work was to address the role of LXR in goat milk fat synthesis by using an in vitro approach.

MATERIALS AND METHODS

The Animal Care and Use Committee of Northwest A&F University of Agriculture and Technology (Yangling, Shaanxi, China) approved all experimental procedures involving dairy goats.

Cell Culture and Treatments

Three independent biological replicates were performed for every experiment that is presented. Three healthy 3-yr-old Xinong Saanen dairy goats in the peak lactating period (60 d after parturition) from the experimental farm of Northwest A&F University were selected and used for the present study. The goat mammary epithelial cells (**GMEC**) were isolated and cultured as previously described (Lin et al., 2013). Basal culture medium consisted of Dulbecco's modified Eagle's medium/F12 medium (HyClone Laboratories, Beijing, China) supplemented with 10% fetal bovine serum (HyClone), 5 μ g/mL insulin (Sigma, St. Louis, MO), 5 mg/L hydrocortisone (Sigma), 10 kU/L penicillin–streptomycin (Harbin Pharmaceutical Group Co. Ltd., Harbin, China), and 10 ng/mL epidermal growth factor (Gibco Products International Inc., Langley, OK). The GMEC were cultured at 37°C with 5% CO₂.

Before the experiments, cells (at passage 5) were seeded in 6-well plates (Corning Inc., Corning, NY) at a density of 2×10^4 cells/cm². Twenty-four hours before treatments (approximately 90% confluence), cells were switched to lactogenic medium supplemented with 2.0 μ g/mL prolactin (Sigma) to induce differentiation. Cells were cultured in the lactogenic medium for 24 h before initiation of the treatments. Differentiated GMEC were incubated with T0901317 at 0, 0.01, 0.1, and 1.0 μ M for 48 h (final concentration, dissolved in dimethyl sulfoxide, **DMSO**; Sigma). The control group (0 μ M) was treated with the same amount (1 μ L/mL medium) of DMSO as the T0901317-treated group. All treatments were repeated 3 times.

Total RNA Extraction, Purification, and Quantitative Reverse-Transcription PCR

Total RNA of cells treated with 1 μ M T0901317 or control was isolated using an RNA Prep Pure Cell/Bacteria Kit (Tiangen Biotech Co. Ltd., Beijing, China) according to the manufacturer's procedures. The RNA concentration and purity were measured and determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE). The RNA integrity was assessed by electrophoretic analysis of 28S and 18S rRNA subunits; results showed that the ratio of 28S:18S was approximately 2:1 (Supplemental Figure S1, <https://doi.org/10.3168/jds.2018-15538>). The first-strand cDNA was synthesized (500 ng of RNA/10 μ L reaction) using a PrimeScript RT Reagent Kit (Takara Bio Inc., Otsu, Japan) according to the manufacturer's instructions. Quantitative reverse-transcription PCR (RT-qPCR) was performed using SYBR Premix Ex Taq (Takara Bio Inc.) and 0.5 μ M gene-specific primers in a 10- μ L reaction containing 100 ng of cDNA on a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. The amplification conditions were as follows: 1 cycle at 95°C for 30 s, followed by 39 cycles at 95°C for 5 s and 60°C for 30 s; a dissociation curve of each sample was performed at 95°C for 10 s and then from 65°C to 95°C with an increment of 0.5°C. Gene-specific primers for RT-qPCR were designed using Primer Premier 5 (Premier Biosoft, Palo Alto, CA; Supplemental Table S1, <https://doi.org/10.3168/jds.2018-15538>).

The RDML files were exported and uploaded into LinRegPCR software for RT-qPCR quantification analysis (Ramakers et al., 2003). LinRegPCR allows users to obtain final values corresponding to a relative abundance of the transcript that can be used for statistical analysis; however, those values were corrected by dividing for a normalization factor. The normalization factor was calculated as the geometrical mean of the RT-qPCR data obtained from LinRegPCR analysis of mitochondrial ribosomal protein L39 (*MRPL39*), ribosomal protein S9 (*RPS9*), and ubiquitously expressed transcript (*UXT*). The stability of the internal control genes and the reliability of the calculated normalization factor were evaluated using geNorm (Vandesompele et al., 2002). The *M*-value was <0.28 for all 3 internal control genes, and the *V*-value was ≤ 0.07 ; thus, all 3 internal controls were very stable and the normalization factor was reliable according to the threshold suggested by Vandesompele et al. (2002; i.e., ≤ 1.5 for *M*-value and ≤ 0.15 for *V*-value).

Oil Red O Staining

After 48 h of incubation with 0 or 1 μ M T0901317, normal GMEC were rinsed twice with cold PBS and fixed with 10% pre-cold paraformaldehyde for 1 h at 4°C. Fixed cells were rinsed twice in PBS and stained with a freshly working solution of Oil Red O (Sigma, St. Louis, MO; 0.5% Oil Red O dissolved in isopropanol: deionized water = 2:1) for 40 min at room temperature under gentle shaking. Finally, cells were rinsed 3 times with cold PBS to remove the Oil Red O residues and observed under a Leica DMI4000B inverted fluorescence microscope (Leica Geosystems Co. Ltd., Norcross, GA; Lin et al., 2013; Li et al., 2015a). Two pictures per well were taken, and quantification of Oil Red O images was carried out using ImageJ software (<http://imagej.nih.gov/ij/>) by transforming the figures in 8-bit (threshold at 200 to 250) and measuring the percentage of area covered by the Oil Red O.

Quantification of Total Cellular TAG

After 48 h of incubation with 0 or 1 μ M T0901317, cells were rinsed 3 times with PBS and then harvested in lysis buffer (50 mM Tris-HCL, pH 7.4, 150 mM NaCl, 1% Triton X-100), and cells were scraped off the culture dish and sonicated to homogenize the cell suspension. Intracellular total TAG was measured according to the GPO-Trinder Triglyceride Assay Kit (Applygen Technologies Inc., Beijing, China). The concentration of TAG was determined on a microtiter plate reader (Bio-Rad) and normalized to total cellular protein assessed using a BCA protein assay (Thermo Fisher Scientific) according to the manufacturer's instructions (Kang et al., 2015).

Fatty Acid Analysis

Normal GMEC seeded in 60-mm culture dish (Corning Inc.) were incubated with DMSO or 1 μ M T0901317 in DMSO for 48 h after 24-h induction of lactation. Total lipid was extracted according to Zhu et al. (2014). Briefly, cells were washed twice with PBS and scraped into a 1.5-mL tube, followed by 5 min of shaking and 5 min of centrifugation at $1,200 \times g$. Total fatty acids were extracted from approximately 100-mg cells using 2 mL of sulfuric acid:methanol (2.5:1 vol/vol) and used for fatty acid analysis by GC-MS (Trace GC ultra-DSQII-AI3000; Thermo Fisher Scientific) using a BP-5MS (30 m $\times$ 0.25 mm $\times$ 0.25 μ m) column. A commercial C19:0 was used as an internal control. The relative proportions of each fatty acid were determined

as percentages of the total peak areas that could be identified (Wang et al., 2012; Takahashi et al., 2013). This experiment was performed with 3 biological replicates.

Plasmid Construction, Transfection, and Luciferase Assay

Goat SREBP1c promoter [−2,012 bp ~+ 1 bp; 2 sterol response elements were located in −97 bp to −116 bp and −809 bp to −817 bp, respectively; 2 LXR response elements were located in −220 bp to −235 bp and −271 bp to −286 bp, respectively; +1 indicates the transcriptional start site (TSS), − means the upstream sequence of TSS, and + means the downstream sequence of TSS] was cloned from goat genomic DNA using LA Taq Polymerase and 2× GC buffer I (Takara Bio Inc.) following a previously published protocol (Lengi and Corl, 2010). Primers used were as follows: forward, 5'-GGGAGATTTGAAGGTAGAGC-3'; reverse, 5'-GGCACCGAGGATGGAAC-3'. The resulting amplicon was cloned into pMD19-T vector for sequencing and then subcloned into the pGL3-Basic vector (Promega, Madison, WI) by using *KpnI* (Takara Bio Inc.) and *HindIII* (Takara Bio Inc.) enzyme sites.

The DNA transfection of GMEC was performed with X-Treme Gene HP DNA Transfection Reagent (Roche China Ltd., Shanghai, China). The GMEC at 80% confluence in a 48-well plate were transiently transfected with 200 ng of pGL3-SREBP1c reporter plasmid along with 4 ng of *Renilla* vector as a control to normalize transfection efficiency (Kang et al., 2015) and then incubated with T0901317 at final concentration of 0, 0.01, 0.1, 1, 2, and 10 μ M T0901317. After 48 h of incubation, cells were harvested and lysed for luciferase activity measurement using a Dual-Luciferase Reporter Assay System according to the manufacturer's instructions (Promega). Each treatment was repeated in 3 replicates.

Statistical Analysis

All treatments were conducted in triplicate. Data from each sample were divided by the mean of the control group to obtain a mean of 1 for the control group and a fold change relative to the control for the treatments. This transformation allowed us to better visualize the results, maintaining the variation in the control as untransformed data for statistical comparisons. Transformed data were checked for normal distribution using the Univariate procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC) prior to the statistical analysis. For unequally spaced dose-effect analysis, we used the IML procedure with ORPOL in association

with GLM with orthogonal contrasts to obtain linear and quadratic effects. For the RT-qPCR analysis, the data were further transformed in natural logarithm before statistical analysis. This was performed using the GLM procedure of SAS with treatment as main effect and replicate as random effect. Differences were considered statistically significant when $P \leq 0.05$, and data were reported as least squares means $\pm$ standard error.

RESULTS

Activation of LXR by T0901317 Increases Transcription of SREBP1

After treatment with T0901317, the transcription activity of SREBP1 increased in a dose-dependent manner as observed in Figure 1. The increase reached a peak and a plateau with 1 μ M T0901317.

When normal (i.e., nontransfected) GMEC were treated with 1 μ M T0901317, we detected a moderate but significant increase in mRNA expression level of LXR α (Figure 2A) and a large increase in mRNA abundance of *SREBF1*. The increase in SREBF1 was almost exclusively due to the increase in expression of *SREBF1A* (Figure 2B and C).

Activation of LXR Enhances the Expression of Fatty Acid- and TAG Synthesis-Related Genes in GMEC

The mRNA abundance of solute carrier family 27 member 6 (*SLC27A6*), fatty acid binding protein 3 (*FABP3*), and acyl-CoA synthase long-chain family member 1 (*ACSL1*), coding for proteins responsible for long-chain fatty acid transport and activation, were all upregulated by the agonist of LXR (Figure 3A), with the largest increase observed in stearoyl-coenzyme A desaturase 1 (*SCD1*). Similarly, the mRNA abundance of all the genes measured coding for enzymes involved in de novo fatty acid synthesis was upregulated by the activation of LXR (Figure 3B). These included fatty acid synthase (*FASN*), acetyl-CoA carboxylase α (*ACACA*), ATP citrate lyase (*ACLY*), acyl-CoA synthetase short-chain family member 2 (*ACSS2*), and *SCD1*. The elongation of very long chain fatty acids family member 6 (*ELOVL6*) and isocitrate dehydrogenase 1 (*IDH1*), important for the provision of NADPH for fatty acid synthesis, was not affected by the treatment.

As summarized in Figure 3C, treatment with 1 μ M T0901317 increased the mRNA expression of genes responsible for TAG synthesis and lipid droplet formation and secretion: lipin 1 (*LPIN1*), diacylglycerol O-acyltransferase 2 (*DGAT2*), perilipin 2 (*PLIN2*,

Figure 1. T0901317, a synthetic agonist of liver X receptor (LXR), increases the transactivation of *SREBF1* in a dose-dependent manner. Goat mammary epithelial cells were transfected with an SREBP1c promoter plasmid driving luciferase expression and incubated with 0, 0.01, 0.1, 1, 2, and 10 μ M T0901317 for 48 h. Significant linear ($P < 0.0001$) and quadratic ($P = 0.04$) effects were detected. RLU = relative light unit. Values are presented as LSM $\pm$ SEM. Different letters (a–c) denote significant difference at $P < 0.05$.

also known as *ADRP*) and 3 (*PLIN3*, also known as TIP47), and adipose triglyceride lipase (*ATGL*), responsible for the lipid droplet TG depletion. Among the same category of genes, glycerol-3-phosphate acyltransferase 1 (*GPAM*), diacylglycerol O-acyltransferase 1 (*DGAT1*), and xanthine dehydrogenase (*XDH*) were not significantly affected by the activation of LXR (Figure 3C).

Among genes involved in transcriptional regulation, only *INSIG1* was significantly upregulated by 1 μ M T0901317 treatment, whereas *SCAP* and *PPARA* were not affected and *PPARG* tended ($P = 0.07$) to be decreased by the activation of LXR (Figure 3D).

T0901317 Increases the Content of Cellular TAG and MUFA

The activation of LXR by T0901317 increased the amount of lipid droplet containing TAG in cells, as observed with Oil Red O staining (Figure 4A–C). The quantification of TAG revealed a >1.2-fold increase in cellular TAG by 1 μ M T0901317 treatment (Figure 4D).

Compared with the control group, T0901317 had a significant effect on the proportion of each long-chain

fatty acid in the cells. The proportion of the saturated C18:0 decreased and that of long-chain MUFA, C16:1, *cis*-9-C18:1, C20:1, and C22:1 increased (Table 1). However, the proportion of C18:2 decreased numerically and proportion of PUFA decreased significantly with T0901317 treatment (Table 1).

DISCUSSION

Studies conducted in rodents have demonstrated that LXR isoforms and SREBP1c work synergistically to increase the synthesis of liver TAG (Repa et al., 2000b; Okazaki et al., 2010). On one hand, LXR enhances fatty acid synthesis largely by activating the transcription of SREBP1c, which in turn transactivates lipogenic genes (Liang et al., 2002; Chen et al., 2004); on the other hand, LXR also directly stimulates the transcription of certain lipogenic genes, including *ACACA* in chick embryos (Talukdar and Hillgartner, 2006) and *FASN* in human cells (Joseph et al., 2002). However, compared with the control group, the increase in mRNA abundance of *ACACA*, *SCD1*, and *FASN* after treatment with the LXR agonist T0901317 was significantly dampened in SREBP1c knockout mice (Liang et al., 2002). In the same study, the treatment with T0901317

Figure 2. Activation of liver X receptor (LXR) by T0901317, a synthetic agonist of liver X receptor, increases the transcription of *SREBF1*. Goat mammary epithelial cells were incubated for 48 h with dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO; control) or 1 μ M T0901317. (A) The mRNA expression level of *NR1H3* (LXR α gene). (B) The mRNA expression level of *SREBF1A*. (C) The mRNA expression level of *SREBF1*. No significant linear effect was observed, but a significant quadratic effect ($P < 0.05$) was detected for all 3 transcripts. Values are presented as LSM $\pm$ SEM. Different letters (a–c) denote significant difference at $P < 0.05$.

failed to affect the transcription of *Gpam* in SREBP1c knockout mice. Mice deficient in LXR α also exhibit reduced expression of SREBP1c and lipogenic genes such as *Scd1* and *Fasn* in the liver (Peet et al., 1998). Taken together, these data indicate that LXR activates fatty acid and TAG synthesis directly or indirectly through SREBP1. Similar to the above findings, the activation of LXR directly increased the expression of lipogenic genes in bovine mammary epithelial cells in an SREBP1-independent fashion (Oppi-Williams et al., 2013). Several studies demonstrated a direct effect of LXR on the expression of *SREBF1* and the downstream genes *FASN* and *SCD1* in goat mammary cells (Li et al., 2015b; Yao et al., 2016). More recent is the demonstration that the LXR β also control the expression of SREBP1c and FASN in goat mammary cells (Shi et al., 2017). In rodents, studies underscored a more important role of LXR α and not LXR β in regulating lipid metabolism (Repa et al., 2000a; Zhang et al., 2014). We conclude that the leading cause of this difference may be aroused by differences in species and tissues; this needs to be further investigated.

LXR Controls the Expression of SREBP1 in GMEC

Results from the present study clearly demonstrated that activation of LXR increased the expression of *SREBF1* in a dose-dependent manner, with a relatively modest effect on the mRNA expression level of *NR1H3*, the LXR α -coding gene. The highest transactivation of SREBP1c and expression of *SREBF1* was observed when the concentration of T0901317 was 1 μ M. Studies on other species have used 2 μ M (Lengi and Corl, 2010; McFadden and Corl, 2010) and 10 μ M (Han et al., 2009; Vedin et al., 2009) T0901317 to activate lipogenic synthesis. The reason for the differences might be due to species differences. However, our data clearly underscore a major role of LXR in controlling the expression of *SREBF1* in GMEC, confirming the previous data obtained in bovines and goats. This conclusion is also supported by the presence of 2 LXR binding sites in the promoter region of SREBP1c in bovines and goats (Lengi and Corl, 2010; Xu et al., 2016a).

The SREBP activity is regulated at 2 major levels: transcriptional and posttranslational. Especially important for the latter is the proteolytic cleavage of SREBP precursors to generate the mature (and active) SREBP (Eberlé et al., 2004). Studies on several species demonstrated that insulin regulates the activity of SREBP1 mainly at the transcriptional level (Dif et al., 2006; Owen et al., 2012) and likely through LXR (Chen et al., 2004), whereas other synthetic and natural molecules, such as PUFA, inhibit SREBP1 activity (and, as consequence, expression) by blocking its

proteolytic cleavage (Harvatine et al., 2009; Kamisuki et al., 2009; Takeuchi et al., 2010). In our study, results of luciferase assay and RT-qPCR suggested an increase of *SREBF1* transcription. To verify whether the change in activation and transcription results in higher protein abundance and activation of SREBP1 (i.e., abundance of the cleaved protein in the nucleus), we ran a Western blot analysis to measure the mature form of SREBP1 using one biological replicate (see description of the method and figure in Supplemental Figure S2, <https://doi.org/10.3168/jds.2018-15538>). The data indicated a dose effect on the mature SREBP1 protein abundance

(Supplemental Figure S2); thus, the data indicated both transcriptional and posttranscriptional regulation of SREBP1 by LXR. This is consistent with similar work conducted on bovine cells (McFadden and Corl, 2010). In addition, the expression of the inhibitor of SREBP1 activity, *INSIG1*, which is also a target gene of SREBP1 (Horton et al., 2003; Xu et al., 2016b), increased only 2.5-fold, indicating that there may be a less than proportional increase in retention of immature SREBP1 in the endoplasmic reticulum. The above observations may support a larger nuclear presence of SREBP1 due to an increase in transcription, but we

Figure 3. Activation of liver X receptor (LXR) by T0901317, a synthetic agonist of liver X receptor, upregulates the expression of genes coding for key proteins involved in (A) long-chain fatty acid (LCFA) activation and transport, (B) de novo fatty acid (FA) synthesis, (C) triglycerides (TAG) synthesis, and (D) transcription regulation. Values are presented as LSM $\pm$ SEM. *P < 0.05; **P < 0.01.

Figure 4. Activation of liver X receptor (LXR) by T0901317, a synthetic agonist of liver X receptor, promotes milk fat droplet and triacylglycerol accumulation in goat mammary epithelial cells. Lipid droplets were stained by Oil Red O: (A) 0 μM T0901317; (B) 1 μM T0901317. Scale bar = 100 μm. (C) Percentage area covered with Oil Red O staining as measured using ImageJ software (<http://imagej.nih.gov/ij/>). (D) Total cellular triacylglycerol (TAG) content. Values are presented as LSM ± SEM. Different letters (a,b) denote significant difference at $P < 0.05$.

cannot exclude the possibility that T0901317 indirectly accelerates the proteolytic cleavage process of SREBP1 or enhances the stability of nuclear SREBP1.

LXR Directly and Indirectly Regulates the Expression of Milk Fat Synthesis-Related Genes and Intracellular TAG Synthesis in GMEC

Milk lipid synthesis and secretion is a complex process that requires the orchestration of a wide variety of proteins (Bionaz and Looor, 2008). Studies to date support SREBP1 playing a primary role in regulating the expression of genes coding for those proteins in human and bovine mammary tissue (Harvatine et al., 2009; Mohammad and Haymond, 2013; Osorio et al., 2016). A role of other transcription factors, especially the ligand-dependent nuclear receptors PPAR γ (Bionaz et al., 2013) and LXR (Oppi-Williams et al., 2013; Li et al., 2015b), in controlling the expression of genes involved in milk fat synthesis in bovines and caprines is emerging (Osorio et al., 2016). The receptor PPAR γ is a putative or demonstrated upstream transcriptional regulator of most milk fat synthesis-related genes (Bionaz et al., 2013), whereas LXR appears to control milk

fat synthesis chiefly through the regulation of *SREBF1* expression. Indeed, this was demonstrated by using *SREBF1*-specific small interfering RNA (siRNA) in bovine mammary immortalized epithelial cells (Ma and Corl, 2012). Transfection of *NR1H3*-specific siRNA in Mac-T cells did not affect the mRNA abundance of genes coding for proteins involved in the de novo fatty acid synthesis, indicating that LXR α is not required for basal transcription of genes related to fatty acid synthesis (Oppi-Williams et al., 2013). In contrast, treatment with T0901317 enhanced de novo fatty acid synthesis in bovine mammary epithelial cells (McFadden and Corl, 2010). Furthermore, the activation of LXR α in the presence of an *SREBF1*-specific siRNA increased the mRNA abundance of *ACACA*, *FASN*, and *DGAT1* but not *SCD1* in Mac-T cells (Oppi-Williams et al., 2013), indicating that LXR can regulate milk fat synthesis in an SREBP1-independent fashion. The data also indicate that *SCD1* expression is exclusively regulated by SREBP1. Expression of *SCD1* was abolished after T0901317 treatment in SREBP1c $^{-/-}$ mice (Liang et al., 2002). The >7-fold increase in mRNA abundance of *SCD1* in our study after T0901317 treatment supports *SCD1* being an SREBP1 target gene also in GMEC.

In agreement with these results, we observed large changes in expression of de novo fatty acid synthesis-related genes (e.g., *FASN*, *ACACA*, and *SCD1*) after T0901317 addition.

It has been previously reported that LXR (Calkin and Tontonoz, 2010; Zhang et al., 2012) and SREBP1 (Ishimoto et al., 2009; Li et al., 2014) activation significantly induces the accumulation of TAG in hepatocytes and cancer cells. Similar to these previous data, our study revealed an overall increase in expression of genes coding for key enzymes responsible for the synthesis of TAG and a larger intracellular accumulation of TAG by T0901317 treatment. Among the TAG synthesis-related genes, *LPIN1* and *DGAT2* were the most affected by LXR activation (Figure 3C).

Lipin 1 modulates TAG accumulation in hepatoblastoma cells (Ishimoto et al., 2009). The same study also identified an SREBP1 response element in the promoter region of *LPIN1*, indicating direct regulation of *LPIN1* by SREBP1. Together with this finding, the increase in expression of *LPIN1* after activation of LXR in our study supports this gene being an SREBP1 target in GMEC.

Table 1. Effects of liver X receptor (LXR) activation by T0901317, a synthetic agonist of LXR, on intracellular fatty acid composition in goat mammary epithelial cells

Fatty acid ¹ (%)	Treatment ²		P-value
	Control	T0901317	
C16:0	15.44 ^A ± 1.12	13.57 ^b ± 0.48	0.17
C16:1	1.15 ^B ± 0.07	2.44 ^A ± 0.18	<0.01
C18:0	9.44 ^A ± 0.61	5.49 ^B ± 0.57	<0.01
<i>cis</i> -9 C18:1	27.13 ± 0.55	30.15 ± 0.85	0.05
C18:1 (total)	30.54 ^B ± 0.41	36.81 ^A ± 0.29	<0.01
C18:2	1.12 ± 0.12	0.73 ± 0.07	0.07
C20:1	0.95 ^B ± 0.06	1.63 ^A ± 0.09	<0.01
C22:1	0.24 ^b ± 0.01	0.44 ^a ± 0.03	0.01
LCPUFA ³ (total)	13.98 ^a ± 0.92	8.71 ^b ± 0.18	0.01
C20:3	1.14 ^a ± 0.06	0.73 ^b ± 0.03	0.01
C20:4	5.45 ^a ± 0.32	3.33 ^b ± 0.28	0.02
C20:5	0.29 ^A ± 0.02	0.13 ^B ± 0.01	<0.01
C22:4	1.10 ^a ± 0.07	0.76 ^b ± 0.04	0.01
C22:5	2.96 ^A ± 0.14	2.07 ^B ± 0.19	<0.01
C22:6	3.05 ^a ± 0.32	1.70 ^b ± 0.10	0.02

^{a,b,A,B}Difference determined using Tukey's test; letters in lowercase (a,b) and uppercase (A,B) denote $P < 0.05$ and $P < 0.01$, respectively.

¹Fatty acid composition was calculated as percentage of total fatty acids.

²Differentiated goat mammary epithelial cells were incubated with T0901317 at 0, 0.01, 0.1, and 1.0 μM for 48 h (final concentration, dissolved in dimethyl sulfoxide; Sigma, St. Louis, MO). The control group (0 μM) was treated with the same amount (1 $\mu\text{L}/\text{mL}$ medium) of dimethyl sulfoxide as the T0901317-treated group.

³Long-chain PUFA contained C20:3, C20:4, C20:5, C22:4, C22:5, and C22:6.

As a key metabolic enzyme, DGAT catalyzes the conversion of diacylglycerol and fatty acyl-CoA into TAG. Two isoforms of DGAT have been characterized in the mammary gland: *DGAT1* and *DGAT2*. The relative mRNA abundance of *DGAT2* was very low in bovine mammary tissue and its expression was not affected by lactation, whereas *DGAT1* had a relatively high abundance of mRNA and its expression increased as lactation progressed (Bionaz and Looor, 2008), and DGAT1 genetic variants are reported to be strongly associated with fat yield and composition in cows (Bovenhuis et al., 2015; Tăbăran et al., 2015). In the present study, we found that both *DGAT1* and *DGAT2* had a similar mRNA abundance in GMEC (Supplemental Table S1, <https://doi.org/10.3168/jds.2018-15538>), and the expression of *DGAT2* increased in T0901317-treated cells compared with the control. It is now possible to conclude with our data whether activation of LXR increased transcription of the *DGAT2* isoform directly or indirectly (i.e., through SREBP1).

Expression of *AGPAT6* was increased upon transactivation of LXR. The *Agpat6* has a high expression in the mammary epithelium of mice, and *Agpat6*^{-/-} mice exhibited a striking defect in lactation accompanied by a dramatic decrease in the size and number of lipid droplets within mammary epithelial cells and depletion of diacylglycerol and TAG in the milk (Beigneux et al., 2006). Different from our observation, the use of siRNA specific for *SREBF1* did not affect *AGPAT6* expression in bovine mammary cells (Ma and Corl, 2012; Oppi-Williams et al., 2013).

In addition, the expression of genes involved in lipid droplet formation, with the exclusion of *XHD*, was increased when LXR was activated. Perilipin 2 and perilipin 3 are 2 milk fat droplet proteins (Ogg et al., 2004; Ma and Corl, 2012); perilipin 2 is reported to be colocalized on the surface of lipid droplets (McIntosh et al., 2012) and is essential for the formation of milk fat lipid droplets and lactation, as demonstrated in mice (Russell et al., 2011). Studies to date have revealed that *PLIN2* is mainly regulated transcriptionally by PPAR and LXR in monogastrics (Fan et al., 2009; Kotokorpi et al., 2010; Kang et al., 2015), indicating that the regulatory role of SREBP1 in milk fat droplet formation or secretion is not as strong as fatty acid synthesis, desaturation, elongation, and TAG synthesis. Using the free web-based tool Lasagna (Lee and Huang, 2013), we analyzed the promoter region of sheep *PLIN2* for SREBP1 and LXR (NR1H2:RXR in Jaspas core matrices) response elements. We observed that the most significant enriched response elements were related to LXR, but the software also revealed several significant enriched response elements for SREBP1.

LXR and the Cross-Talk Among Transcriptional Regulators

Data generated in bovine mammary tissue indicated potential cross-talk between several transcriptional factors in the control of the milk fat synthesis transcriptional network (Bionaz and Loor, 2008; Bionaz et al., 2013). A role of PPAR γ in controlling expression of genes related to milk fat synthesis is emerging (Bionaz et al., 2013), but no role of PPAR α in mammary tissue has been reported for bovines or goats even though the mRNA abundance of *PPARA* is greater than the mRNA abundance of *PPARG* in bovine mammary tissue (Bionaz et al., 2013). Our data (Supplemental Table S1, <https://doi.org/10.3168/jds.2018-15538>) support a similar mRNA abundance proportion between PPAR isotypes in GMEC; however, our data indicated that expression of the 2 PPAR isotypes is not controlled by LXR, and a tendency for a contrasting effect of LXR and PPAR γ is present in GMEC. However, the significance of this observation is unclear.

LXR Activation Promotes Formation of UFA in GMEC

Mammary tissue actively desaturates long-chain fatty acids and produces UFA, especially long-chain MUFA, from long-chain SFA (Bauman et al., 2006). The Δ^9 -desaturase protein coded by *SCD1* plays a central role in such activity. A strong association between SCD activity and concentration of MUFA in milk was detected in bovines (Conte et al., 2010). In our previous study in GMEC, we observed that the upregulation in expression of *SCD1* was strongly associated with an increase in the total proportion of C16:1 and C18:1 (Shi et al., 2012; Takahashi et al., 2013). In the present study, activation of LXR strongly upregulated the transcription of many milk fat synthesis-related genes, but the larger effect was observed for the expression of *SCD1* (Figure 3). Thus, the increase in the proportion of C16:1 and C18:1 and decreased proportion of C16:0 and C18:0 by activation of LXR in the present study can be due to an increase in activity of *SCD1*.

CONCLUSIONS

In summary, our data indicate that activation of LXR by T0901317 enhanced transcription of SREBP1, increased the transcription of most of the milk fat synthesis-related genes, augmented the intracellular amount of TAG, and increased the desaturation of long-chain fatty acids in GMEC. The data suggest that most of the observed effects after activation of LXR are SREBP1 mediated. A model summarizing the findings from the present experiment is reported in

Supplemental Figure S3 (<https://doi.org/10.3168/jds.2018-15538>). Overall, results from the present study provide strong evidence that LXR and SREBP1 are key regulators of de novo fatty acid and TAG synthesis in goat mammary tissue. In addition, the use of reporter construct and measurement of gene expression using a specific LXR activator can provide insight into the role of LXR in milk fat synthesis. However, the use of point mutations or specific deletions can provide more robust proof.

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