Regulation of lipid synthesis by liver X receptor α and sterol regulatory element-binding protein 1 in mammary epithelial cells

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ABSTRACT

The objectives of this experiment were to characterize the roles of the transcription factors liver X receptor α (LXR α) and sterol regulatory element-binding protein 1 (SREBP1) in the transcriptional regulation of lipid synthesis in a bovine mammary epithelial cell line. Whereas many lipid synthesis genes contain a response element in their promoters for SREBP1, a few also contain a response element for LXR, suggesting that both transcription factors could directly regulate transcription of these genes. However, the promoter of SREBP1 contains a response element for LXR, indicating the additional potential for indirect transcriptional regulation by LXR, through SREBP1, on lipogenic genes. To characterize these effects, small interfering RNA (siRNA) directed against LXR α and SREBP1 were used to knockdown gene expression, and then, in the presence of SREBP1 siRNA, T0901317 (T09) was used to specifically activate LXR α . Reducing LXR α mRNA abundance in mammary alveolar T cells did not alter mRNA abundance of genes involved in de novo lipogenesis or the rate of de novo lipogenesis, suggesting that LXR α is not required for basal transcription of genes required for fatty acid synthesis. Knockdown of SREBP1 reduced the mRNA abundance of acetyl-coenzyme A (CoA) carboxylase, fatty acid synthase, diacylglycerol acyltransferase, and stearoyl-CoA desaturase-1, indicating that these genes are regulated in part by SREBP1. When SREBP1 was reduced, T09 increased the mRNA abundance of acetyl-CoA carboxylase, fatty acid synthase, and diacylglycerol acyltransferase, potentially indicating that these genes are directly regulated by LXR. The results of the present study provide insight into the transcriptional regulatory mechanisms involved in lipid synthesis by mammary epithelial cells, and suggest that several transcription factors may be required for full lipogenic activation.

Key words: liver X receptor, small interfering RNA, sterol regulatory element-binding protein-1, transcriptional regulation

INTRODUCTION

Lipid synthesis involves the de novo synthesis of FA in addition to incorporation of de novo and preformed FA into triglycerides, processes that primarily occur in the liver, adipose, and mammary gland of mammals. Activation of these metabolic pathways requires the coordinated regulation of a network of genes encoding lipogenic enzymes, such as the de novo FA synthesis genes FA synthase (*FASN*) and acetyl-CoA carboxylase (*ACC*); the FA modification gene stearoyl-CoA desaturase-1 (*SCD1*); and triglyceride synthesis genes, including glycerol phosphate acyltransferase (*GPAT*), acylglycerol-3-phosphate O-acyltransferase (*AGPAT*), and diacylglycerol acyltransferase (*DGAT*). Two transcription factors considered critical for the activation of these genes are liver X receptors (LXR) and sterol regulatory element-binding protein 1 (SREBP1).

Liver X receptor α and its isoform LXR β are nuclear receptors responsive to oxysterols or synthetic agonists, such as T0901317 (T09). Upon activation, LXR form heterodimers with the retinoid X receptor before binding to LXR response elements (LXRE) in the promoter of LXR-responsive genes. In mice, the specific knockout of LXR α reduces hepatic mRNA abundance of *FASN* and *SCD1*, but not *ACC* (Peet et al., 1998), whereas in human preadipocytes, LXR α activation with T09 increases *FASN*, *ACC*, and *SCD1* mRNA (Darimont et al., 2006). These findings suggest that LXR could have roles in lipogenic gene regulation, maintaining the expression of some genes and activating others.

In its precursor form, SREBP1 is a membrane-bound transcription factor located in the endoplasmic reticulum that is activated by proteolytic cleavage to its mature form (Wang et al., 1994). Mature SREBP1 translocates to the nucleus where it activates lipogenic genes by binding to the SREBP1 response element (SRE) of target genes, such as *ACC* (Lopez et al., 1996), *FASN* (Magaña and Osborne, 1996), and *GPAT* (Ericsson et al., 1997). In chicken hepatocarcinoma cells, small

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interfering RNA (**siRNA**)-mediated knockdown of SREBP1 reduced *ACC*, but not *FASN* mRNA (Wang et al., 2009). Similar to LXR regulation of lipogenic genes, SREBP1 could be involved in both maintenance and activation of different genes. Additionally, the SREBP1 promoter contains an LXRE (Lengi and Corl, 2010), and SREBP1 mRNA is increased following T09 activation of LXR (Darimont et al., 2006; McFadden and Corl, 2010). Thus, many lipogenic genes can be directly activated by LXR α and SREBP1, and indirectly activated in the absence of an LXRE in the gene promoter by LXR α through SREBP1 activation.

In dairy cows, SREBP1 is considered a primary regulator of lipid synthesis in the mammary gland, as diets that induce milk fat depression also reduce SREBP1 expression (Harvatine and Bauman, 2006). Additionally, LXR α is considered a potentially important regulator of milk fat synthesis, as expression of LXR α is increased during the transition from pregnancy to lactation (Mani et al., 2009), and stimulation of bovine mammary epithelial cells with T09 increases de novo FA synthesis (McFadden and Corl, 2010). Therefore, the main objective of these experiments was to investigate the regulation of genes involved in milk fat synthesis by SREBP1 and LXR α in bovine mammary alveolar T (**MAC-T**) cells, to characterize the role of LXR α in mammary epithelial cell lipid synthesis.

MATERIALS AND METHODS

Cell Culture and Transfection

The MAC-T cells used in this study were routinely grown at 37°C and 5% CO₂ in growth media [Dulbecco's modified Eagle medium-high glucose (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (Atlanta Biologicals Inc., Lawrenceville, GA), 10 kU of penicillin/mL, 10 mg of streptomycin/mL, and 25 μ g/mL of amphotericin (Sigma-Aldrich)]. For transfections, cells were seeded into 6 well plates at a density of 2×10^4 cells/cm² and incubated in growth media overnight. Cells were transfected with 100 nM siRNA (Dharmacon, Thermo Fisher Scientific Inc., Waltham, MA) and 4 μ L of transfection reagent (DharmaFECT transfection reagent no. 2; Thermo Scientific Inc.) in serum and antibiotic-free transfection media [Dulbecco's modified Eagle medium-high glucose supplemented with 0.1 μ g of insulin/mL and 1.5 μ g/mL of prolactin (Sigma-Aldrich)]. For the first experiment, treatments included specific anti-LXR α siRNA (**LX**; an equal mix of 3 sequences) and siRNA with a random sequence (**RN**). For the second experiment, treatments consisted of anti-SREBP1 siRNA in transfection media (**SR**; an equal mix of 2 sequences) or transfection media supple-

Table 1. Sequences of small interfering RNA oligonucleotides

Target ¹	Sequence
LXR α 1	GCUAAAUGAUGCUGAGUUUUU
LXR α 2	UGCAGGAGAUUCGUGGAUUUUU
LXR α 3	GCUGAAGACGUCUGCGAUUUU
SREBP1 1	CCACAACGCCATCGAGAAA
SREBP1 2	GCACCGAGGCCAAGTTGAA

¹LXR = liver X receptor; SREBP = sterol regulatory element-binding protein.

mented with T09 [**SRT**; 2.5 μ M T09 (Sigma-Aldrich)], the RN treatment, and the RN treatment in T09-supplemented media (**RNT**). Sequences of the siRNA oligonucleotides are shown in Table 1. All experiments were repeated 3 to 4 times and cells were harvested at 48 h for gene and protein expression analysis, or for use in acetate incorporation assays.

RNA Extraction and Real-Time Quantitative PCR

Total RNA was extracted using RNAzol (Molecular Research Center Inc., Cincinnati, OH) according to the manufacturer's instructions and quantified using a spectrophotometer (NanoDrop; Thermo Fisher Scientific Inc.). Adequate RNA purity was determined by samples having a 260/280 ratio greater than 1.80, and following this, RNA was reverse transcribed to cDNA (1 μ g per 20- μ L reaction; Omniscript RT kit; Qiagen Inc., Valencia, CA). Quantification of transcripts was performed using SYBR Green master mix (GoTaq qPCR kit; Promega Corp., Madison, WI) and 0.25 μ M gene-specific primers (Table 2) in a 25- μ L reaction containing 125 ng of cDNA. Reactions were incubated in a thermocycler (7300 real-time PCR machine; Applied Biosystems Inc., Foster City, CA) at 95°C for 15 min, followed by 35 cycles of 95°C (30 s), 60.5°C (15 s), and 72°C (1 min). Each reaction was performed in duplicate wells. β -Actin was used as the endogenous control gene and cycle threshold (**C_t**) values were not influenced by treatments. Melt curves were analyzed for all reactions. Fold change was calculated using $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001) with the RN treatment as the calibrator.

Gene-specific primers for the transcripts used in the study are shown in Table 2. Primer pair specificity was validated by sequencing products generated from PCR reactions. Primer pair efficiencies were calculated by generating a standard curve with 5-fold serial dilutions of cDNA for each primer pair. Each reaction was performed in triplicate. The slope of the curve was calculated for each primer pair, and efficiency was calculated as efficiency = $10^{(-1/\text{slope})}$, with an efficiency of 2 (indicating exact doubling for each cycle) equaling

Table 2. Sequences of primers used for real-time PCR amplification

Identification ¹	Forward primer	Reverse primer	Efficiency of primer pair, %
LXR α	GACCGACTGATGTTCCCAAG	ACAGAAGACACGGAGGAGGA	95.1
LXR β	TGTTGTCTTACACCCGCATC	CAGTCCCTTCTACCCCAGT	104.5
SREBP1	ATGCCATCGAGAAACGCTAC	GTCCGCAGACTCAGGTTCTC	100.0
<i>ABCG1</i>	GACTCGGTCTCTACGCAC	CGGAGAAACACGTCCATCTC	99.4
<i>FASN</i>	AACACCATGTGGCAGGAAGT	TGTAGCCGTTTCACCAAGTAA	99.6
<i>ACC1</i>	GGGTGAAAGACTGGGTTGAA	GACAGAGCACGGATGTGATG	100.5
<i>AGPAT</i>	AAGCAAGTTGCCATCCTCA	AAACTGTGGCTCCAATTTCTGA	96.6
<i>DGAT</i>	GACACAGACAAGGACGGAGA	CAGCATCACACACACCAA	103.8
<i>GPAT</i>	ATTGACCCTTGGCAGGATAG	AACAGCACCTTCCCACAAAG	100.0
<i>LPL</i>	GAGCCAAAAGAAGCAGCAAG	AGGCAGGGTAAAAGGGATGT	99.3
<i>SCD1</i>	CCCTTTCCTTGAGCTGTCTG	ATGCTGACTCTCTCCCCTGA	98.2
Actin	CTCTTCCAGCCTTCCTTCCT	GGGCAGTGATCTCTTTCTGC	99.2

¹LXR = liver X receptor; SREBP = sterol regulatory element-binding protein; *ABCG* = ATP-binding cassette protein G; *FASN* = FA synthase; *ACC* = acetyl-CoA carboxylase; *AGPAT* = acylglycerol-3-phosphate O-acyltransferase; *DGAT* = diacylglycerol acyltransferase; *GPAT* = glycerol phosphate acyltransferase; *LPL* = lipoprotein lipase; *SCD* = stearoyl CoA desaturase.

100% efficiency. Primer pair efficiencies were between 95 and 105% for each primer pair (Table 2).

Immunoblotting

Western blot analysis of total ACC1 (rabbit anti-human ACC polyclonal antibody, 3662; Cell Signaling Technology Inc., Danvers, MA), SCD1 (custom synthesized rabbit anti-bovine antibody; Pacific Immunology Corp., Ramona, CA), FAS (rabbit anti-human FAS monoclonal antibody, 3180; Cell Signaling Technology Inc.), and SREBP1 (mouse anti-human SREBP1 monoclonal antibody SC-13551; Santa Cruz Biotechnology Inc., Santa Cruz, CA) was performed. The authors were unable to validate an antibody against bovine LXR α . Media were removed and cells washed once with Dulbecco's modified PBS (DPBS; 5.36 mM KCl, 2.94 mM KH₂PO₄, 273.78 mM NaCl, and 16.2 mM Na₂HPO₄; pH 7.4) before harvesting protein in 50 μ L of lysis buffer [50 mM Tris-HCl, pH 7.4; 0.5% (vol/vol) Triton X-100; 300 mM NaCl; 2 mM EDTA, pH 8.0; and 2% (vol/vol) protease inhibitor cocktail (Sigma-Aldrich)]. Cells were incubated on ice for 10 min, centrifuged at 16,000 $\times g$ for 15 min (4°C), and the resulting supernatant retained. Protein concentrations were quantified by the Bradford assay (Quick Start Bradford protein assay; Bio-Rad Laboratories Inc., Hercules, CA), adjusted to 2 μ g/ μ L by addition of cold lysis buffer, combined with an equivalent volume of 2 $\times$ Laemmli sample buffer (Sigma-Aldrich), and boiled at 95°C for 10 min.

For ACC1, 45 μ g of protein per sample was resolved on a 7.5% SDS-polyacrylamide gel (Lonza PAGER Gold Plus Precast Gels; Lonza Rockland Inc., Rockland, ME); for SCD1, 30 μ g of protein was resolved on a 12% gel; for FAS, 30 μ g of protein was resolved on a 7.5% gel; and for SREBP1, 40 μ g of protein was resolved on a 12% SDS gel before transfer to a polyvinylidene

difluoride (PVDF) membrane (Amersham Hybond-P; GE Healthcare UK Ltd., Buckinghamshire, UK). Membranes were blocked with Tris-buffered saline (TBS) that included 0.1% Tween-20 and 5% dried nonfat milk (ACC1, SCD1, and SREBP1) or TBS with 2% BSA (FAS) for 1 h at room temperature. Membranes were then incubated overnight with primary antibody (ACC1, 1:1000; SCD1, 1:1000; SREBP1, 1:1000; and FAS, 1:2000), followed by incubation with an appropriate secondary antibody (Santa Cruz Biotechnology Inc.) for 1 h, and protein bands visualized with a chemiluminescent detection system (ECL Plus Western Blotting Detection System; GE Healthcare UK Ltd.). Following SCD1 visualization, membranes were stripped and probed with an antibody directed against β -actin (Sigma-Aldrich; monoclonal rabbit anti-mouse β -actin; 1:4000). For all antibodies, bands were visualized and quantified using a ChemiDoc XRS digital imaging system (Bio-Rad Laboratories Inc.).

Acetate Incorporation Assay

Following transfection, half of the media from each well was removed and replaced with media containing sodium acetate (300 mM) and [¹⁴C] acetic acid (37,000 Bq/well; American Radiolabeled Chemicals Inc., St. Louis, MO). Following incubation (4 h, 37°C, 5% CO₂), media were removed and cells rinsed with a balanced salt solution before lysis with 0.1% SDS in DPBS. Lipids were extracted from the lysate with 3:2 hexane isopropanol, and total radioactivity in the organic phase quantified by liquid scintillation counting.

Statistical Analysis

The mixed models procedure of SAS (SAS Enterprise Guide v.4.2; SAS Institute Inc., Cary, NC) was used

for all analyses. For all transcript abundance data, $2^{-\Delta\Delta Ct}$ values were analyzed. The effect of LXR α siRNA on transcript abundance and acetate incorporation in MAC-T cells was assessed using a mixed model ANOVA for the main effect of treatment (RN or LX).

The effect of SREBP1 siRNA on transcript abundance, protein expression, and acetate incorporation in MAC-T cells was assessed using a mixed model ANOVA for the main effects of treatment (RN or SR) and T09 and the treatment by T09 interaction. Where a significant treatment by T09 interaction was detected, means were separated using the Tukey procedure. Densitometry data for *FASN* were log transformed to improve normality of residuals.

RESULTS

Treatment of MAC-T cells with LXR α siRNA reduced *LXR α* ($P < 0.001$) and *LXR β* ($P < 0.001$) mRNA abundance by 80 and 50% (Figure 1), respectively, while not altering acetate incorporation ($P > 0.3$; Figure 1). Knockdown of LXR α did not alter *ABCG1* mRNA abundance ($P > 0.4$), however it did increase *LPL* ($P = 0.002$), *SCD1* ($P = 0.001$), and *SREBP1* ($P = 0.017$) mRNA abundance (Table 3). Neither the de novo FA synthesis genes *ACC1* and *FASN*, nor the triglyceride synthesis genes *AGPAT*, *GPAT*, and *DGAT* were altered by reduced LXR α ($P > 0.2$; Table 3).

A treatment by T09 interaction existed for mRNA abundance of *SREBP1* ($P < 0.001$; Figure 2), whereby SREBP1 siRNA decreased *SREBP1* mRNA in both SR- and SRT-treated cells ($P < 0.001$) and T09 increased *SREBP1* mRNA in RN-treated cells only (P

Table 3. Mean abundance of transcripts in mammary alveolar T cells (MAC-T) transfected with nontargeting small interfering RNA (siRNA; RN) or liver X receptor α (LXR α) siRNA (LX; $n = 5$)

Transcript ¹	Treatment		SEM ²	P-value
	RN	LX		
ABCG1	1.00	1.12	0.10	0.43
SREBP1	1.00	1.41	0.09	0.017
LPL	1.02	2.24	0.19	0.002
ACC1	1.01	0.86	0.07	0.20
FASN	1.00	0.88	0.07	0.29
GPAT	1.00	0.89	0.10	0.44
AGPAT	1.02	0.86	0.09	0.26
DGAT	1.00	1.06	0.05	0.40
SCD1	1.00	1.30	0.04	0.001

¹ABCG = ATP-binding cassette protein G; SREBP = sterol regulatory element-binding protein G; LPL = lipoprotein lipase; ACC = acetyl-CoA carboxylase; FASN = FA synthase; AGPAT = acylglycerol-3-phosphate O-acyltransferase; GPAT = glycerol phosphate acyltransferase; DGAT = diacylglycerol acyltransferase; SCD = stearoyl-CoA desaturase.

²Pooled SEM.

Figure 1. Transcript fold change of liver X receptor α (LXR α) and LXR β and [14 C]-labeled acetate incorporation into mammary alveolar T (MAC-T) cells transfected with nontargeting small interfering RNA (siRNA; RN) or LXR α siRNA (LX; $n = 4$). Data are presented as means $\pm$ pooled SEM.

< 0.001). For SREBP1 protein levels, a main effect of treatment was observed, whereby SREBP1 siRNA reduced mature SREBP1 protein abundance ($P < 0.001$; Figure 2) with no effect of T09 ($P > 0.1$) in either RN- or SR-treated cells ($P > 0.1$). Acetate incorporation was reduced by SREBP1 knockdown ($P < 0.001$; Figure 2), but was increased by T09 in both SR- and RN-treated ($P < 0.001$) cells.

A treatment by T09 interaction existed for abundance of *ABCG1* mRNA ($P = 0.012$), whereby the SR treat-

ment did not influence *ABCG1* mRNA in the absence of T09 ($P > 0.5$) but in the presence of T09, was lower in SR-treated than RN-treated cells ($P = 0.055$; Table 4). Knockdown of SREBP1 reduced mRNA abundance of the de novo lipogenesis genes, *ACC1* ($P < 0.001$; Figure 3) and *FASN* ($P < 0.001$; Figure 4), while having no effect on *LPL* ($P > 0.1$; Table 4). Protein abundance of *ACC1* ($P = 0.049$; Figure 3) and *FASN* ($P = 0.007$; Figure 4) were decreased by SREBP1 knockdown. Inclusion of T09 in culture media increased *ACC1* (Figure

Figure 2. Transcript fold change (top left panel) and protein (top right panel) abundance of sterol regulatory element-binding protein 1 (SREBP1), and [14 C]-labeled acetate incorporation (lower left panel) in mammary alveolar T (MAC-T) cells transfected with nontargeting small interfering RNA (siRNA; RN) or SREBP1 siRNA (SR; $n = 4$). Cell culture media included either 0 or 2.5 μ M synthetic agonist T0901317 (T09). Data are presented as means $\pm$ pooled SEM. Different letters (a–c) indicate differences ($P < 0.001$). TRT = treatment.

Table 4. Mean abundance ($\pm$ SEM) of transcripts in mammary alveolar T (MAC-T) cells transfected with nontargeting small interfering RNA (siRNA; RN) or sterol regulatory element-binding protein 1 (SREBP1) siRNA (SR), in media that included either 0 or 2.5 μ M synthetic agonist T0901317 (T09; RNT or SRT; n = 4)

Transcript ¹	Treatment ²				P-value		
	RN	SR	RNT	SRT ³	TRT ⁴	T09	TRT $\times$ T09
ABCG1	1.00 $\pm$ 0.26 ^a	1.45 $\pm$ 0.26 ^a	6.21 $\pm$ 0.26 ^b	5.09 $\pm$ 0.30 ^c	0.20	<0.001	0.012
LXR α	1.00 $\pm$ 0.13	0.92 $\pm$ 0.13	1.15 $\pm$ 0.13	0.84 $\pm$ 0.13	0.22	0.82	0.43
LXR β	1.00 $\pm$ 0.08	1.50 $\pm$ 0.08	0.91 $\pm$ 0.08	1.34 $\pm$ 0.10	<0.001	0.16	0.70
LPL	1.00 $\pm$ 0.08	1.27 $\pm$ 0.08	1.30 $\pm$ 0.08	1.38 $\pm$ 0.08	0.11	0.078	0.32
GPAT	1.00 $\pm$ 0.15	0.99 $\pm$ 0.15	1.32 $\pm$ 0.15	1.13 $\pm$ 0.18	0.53	0.18	0.56
AGPAT	1.00 $\pm$ 0.08	1.25 $\pm$ 0.08	0.95 $\pm$ 0.08	1.08 $\pm$ 0.10	0.079	0.32	0.62
DGAT	1.00 $\pm$ 0.10	1.45 $\pm$ 0.10	0.89 $\pm$ 0.10	1.09 $\pm$ 0.11	0.005	0.023	0.18

^{a-c}Unlike superscripts within a row indicate that means are different, $P < 0.05$.

¹ABCG = ATP-binding cassette protein G; LXR = liver X receptor; LPL = lipoprotein lipase; AGPAT = acylglycerol-3-phosphate O-acyltransferase; GPAT = glycerol phosphate acyltransferase; DGAT = diacylglycerol acyltransferase.

²RN = nontargeting; SR = SREBP1 siRNA; RNT = RN treatment in T09-supplemented media; SRT = SR media supplemented with T09.

³For SRT treatment, n = 3 for ABCG1, LXR β , GPAT, and DGAT.

⁴TRT = treatment.

3) and *FASN* (Figure 4) mRNA in both SREBP1 siRNA-treated and RN-treated cells ($P < 0.001$), whereas neither *ACC1* ($P > 0.2$; Figure 3) nor *FASN* ($P > 0.08$; Figure 4) protein abundance was influenced by T09.

Knockdown of SREBP1 increased mRNA abundance of the triglyceride synthesis gene *DGAT* ($P = 0.005$), while having no effect on *GPAT* ($P > 0.5$) or *AGPAT* ($P > 0.07$; Table 4). Inclusion of T09 in culture medium had no effect on *GPAT* ($P > 0.1$) or *AGPAT* ($P > 0.3$) but increased *DGAT* mRNA in cells treated with SREBP1 and nontargeting siRNA ($P = 0.023$; Table 4). The mRNA ($P < 0.001$; Figure 5) and protein ($P < 0.001$; Figure 5) abundance of *SCD1* were decreased by SREBP1 knockdown, and neither mRNA nor protein was influenced in either treatment by addition of T09 to culture medium ($P > 0.1$). Abundance of *LXR α* was not influenced by either SREBP1 knockdown ($P > 0.2$) or T09 ($P > 0.8$), whereas *LXR β* mRNA was increased by SREBP1 knockdown ($P < 0.001$) but was not altered by T09 ($P > 0.1$; Table 4).

DISCUSSION

Mammary gland fat synthesis occurs in mammary epithelial cells, with lipids being secreted as membrane-bound fat globules. The transcription factor SREBP1 is considered a global regulator of lipid metabolism, and inhibiting SREBP1 activation reduces lipogenesis and lipogenic gene expression in the liver of mice (Kuriyama et al., 2005). Transcription of SREBP1 is stimulated by insulin (Foufelle and Ferre, 2002) and this stimulation is potentially mediated, in part, by LXR α (Fiévet and Staels, 2009), as disruption of the LXRE site in the SREBP1 promoter does not reduce basal transcription, but does prevent insulin-stimulated SREBP1 expres-

sion (Chen et al., 2004). Both SREBP1 and LXR α are considered important regulators of lipogenic gene expression in dairy cows and the mRNA abundance of both are increased with the onset of lactation (Mani et al., 2009). Further, it is now known that the bovine SREBP1 promoter contains LXRE sites that are critical for T09-stimulated activation (Lengi and Corl, 2010).

The LXR α nuclear receptor was initially identified as being responsible for increasing lipogenic gene expression in response to the presence of oxidized cholesterol derivatives. These derivatives bind LXR α and activate it, through conformational changes, to dimerize with the retinoid-X receptor and then bind to LXRE in the promoter region of target genes. Although it is likely that the primary method by which LXR α increases lipogenic genes is through SREBP1 (Repa et al., 2000; Schultz et al., 2000), the promoter of some lipogenic genes, including *FASN*, contain an LXRE (Joseph et al., 2002), potentially indicating that LXR α is as influential as SREBP1 in stimulating lipogenic genes. Thus, the primary objective of these experiments was to determine the relative importance of LXR α and SREBP1 on lipogenic gene expression and de novo FA synthesis in MAC-T cells.

Reducing *LXR α* mRNA abundance in MAC-T cells resulted in the primary finding that both the abundance of transcripts for proteins involved in de novo lipogenesis and the rate of de novo lipogenesis, as indicated by acetate incorporation, were unaltered. The de novo lipogenesis genes *ACC1* and *FASN* are under the control of both LXR α and SREBP1 (Lopez et al., 1996; Magaña and Osborne, 1996; McFadden and Corl, 2010), with previous work in bovine mammary epithelial cells demonstrating that T09 activation strongly increases their gene expression (McFadden and Corl,

Figure 3. Transcript fold change and protein abundance of acetyl-CoA carboxylase 1 (ACC1) in mammary alveolar T (MAC-T) cells transfected with nontargeting small interfering RNA (siRNA; RN) or sterol regulatory element-binding protein 1 (SREBP1) siRNA (SR; n = 4). Cell culture media included either 0 or 2.5 μM synthetic agonist T0901317 (T09). Data are presented as means ± pooled SEM. TRT = treatment.

2010). We found that *ACC1* and *FASN* were unaltered by LXRα reduction, which likely indicates that basal gene expression is maintained by other transcription

Figure 4. Transcript fold change and protein abundance of FA synthase (FASN) in mammary alveolar T (MAC-T) cells transfected with nontargeting small interfering RNA (siRNA; RN) or sterol regulatory element-binding protein 1 (SREBP1) siRNA (SR; n = 4). Cell culture media included either 0 or 2.5 μM synthetic agonist T0901317 (T09). Data are presented as means ± SEM (mRNA) or geometric mean ± pooled SEM (protein). TRT = treatment.

factors, such as SREBP1, which was increased in response to LXRα knockdown. Alternatively, it is possible that LXRβ, which was only reduced by 50% in the LX-treated cells, maintained lipogenic gene expression

Figure 5. Transcript fold change of stearoyl-CoA desaturase 1 (SCD1) in mammary alveolar T (MAC-T) cells transfected with nontargeting small interfering RNA (siRNA; RN) or sterol regulatory element-binding protein 1 (SREBP1) siRNA (SR; $n = 4$). Cell culture media included either 0 or 2.5 μ M synthetic agonist T0901317 (T09). Data are presented as means $\pm$ pooled SEM. TRT = treatment.

with reduced abundance of LXR α . The reduction of LXR β is potentially due to sequence similarity between the 2 isoforms. Both α and β isoforms dimerize with retinoid X receptor (RXR) and bind LXRE (Baranowski, 2008), indicating that complete knockdown of both may be required to determine the full effect of LXR on fat metabolism in MAC-T cells.

In opposition to the lack of change in transcripts for proteins involved in de novo lipogenesis, we observed increased SREBP1, *LPL*, and *SCD1* mRNA in cells with reduced abundance of LXR α . As the bovine SREBP1 promoter contains an SRE in addition to 2 LXRE (Lengi and Corl, 2010), it is possible that positive feedback signaling by SREBP1 itself contributed to increased SREBP1 mRNA abundance. The increase in SREBP1 might have then stimulated *LPL* and *SCD1* gene expression, increasing the abundance of these genes independent of LXR α activation. As shown in rodent species, gene expression of *LPL* is primarily regulated by SREBP1 (Schoonjans et al., 2000), and previous work in bovine mammary epithelial cells shows regulation of *SCD1* by SREBP1 (Ma and Corl, 2012).

Activation of LXR by T09 increased SREBP1 mRNA and mature protein by 135 and 42%, respectively, a finding that supports previous work demonstrating the presence of an LXRE in the SREBP1 promoter (Lengi and Corl, 2010). This increase was paralleled by increased mRNA abundance of *ABCG1*, *ACC1*, and *FASN* by 521, 64, and 26%, respectively. As *ABCG1* contains an LXRE but not an SRE, this increase is likely due to direct transcriptional activation by LXR (Sabol et al., 2005). In opposition to this, *ACC1* is highly responsive to SREBP1 stimulation, selectively responsive to LXR α at the PII promoter only, and unresponsive to LXR β at all promoters (Zhao et al., 2010). As it is likely that all 3 promoters are relevant to *ACC1* transcription in mammary epithelial cells of dairy cows (Molenaar et al., 2003), it is possible that LXR α activated *ACC1* transcription directly through the PII promoter or indirectly through SREBP1. Similarly, the *FASN* promoter contains both LXRE and SRE sites, and gene expression is acutely stimulated by both T09 (McFadden and Corl, 2010) and insulin (Barthel et al., 2002). Similar to our *ACC1* mRNA findings, the observed increase in *FASN* mRNA due to T09 stimulation could have been due to either direct LXR activation by T09 or the increase in mature SREBP1 protein levels. Intriguingly, the increases in *FASN* and *ACC1* did not translate to increased protein levels. Lastly, T09 increased de novo lipogenesis, a finding in agreement with previous studies (McFadden and Corl, 2010).

With SREBP1 knockdown, *SREBP1* mRNA was reduced by 85% and addition of T09 to the treatment media reduced this knockdown to 67%. The effect of

T09 on mRNA did not translate to an effect on mature protein levels, however, and mature SREBP1 remained undetectable in both SR- and SRT-treated cells. This indicates that any effects of T09 on other genes in cells treated with SREBP1 siRNA are likely due to LXR α directly binding the promoter and not to SREBP1. Further, although LXR α mRNA was not altered in response to SREBP1 knockdown, LXR β mRNA was increased, regardless of T09, by an average of 42%. It is, therefore, possible that LXR β played a role in any mRNA increases due to SR or SRT treatment. The increase in the LXR β isoform mRNA could be due to feedback signaling or a compensatory response to maintain lipogenic gene expression.

De novo lipogenesis was decreased in SREBP1 siRNA-treated cells, but, interestingly, was recovered with LXR activation. Similarly, both *ACC1* and *FASN* mRNA were reduced by SREBP1 knockdown and increased by T09 addition, suggesting that both transcription factors are significant regulators of lipogenesis, with SREBP1 necessary for maintaining basal levels and LXR capable of stimulation. However, T09 was unable to increase protein levels of FAS and ACC1 in cells with reduced SREBP1; thus, the mechanism of increased acetate incorporation is unknown.

Following elongation, long-chain FA are desaturated by SCD1 before triglyceride synthesis. Our finding that *SCD1* mRNA was reduced by SREBP1 knockdown is in agreement with previous studies in MAC-T cells (Ma and Corl, 2012) and suggests that SREBP1 is responsible for activating *SCD1* transcription in bovine cells. Data from other species suggests that *SCD1* is regulated by both SREBP1 and LXR, with *SCD1* being responsive to LXR activation by T09 in mouse liver (Weiss et al., 2011) and also increased by treatment that elevates *SREBP1* mRNA levels (Yin et al., 2007). Our results from both the LXR α and SREBP1 knockdown experiments indicate that SREBP1 has primary regulatory responsibility for *SCD1* transcriptional regulation.

The final step of lipogenesis, triglyceride production, is facilitated by the enzymes GPAT, AGPAT, and DGAT, which attach long-chain FA in a successive manner to glycerol. In the present experiment, *GPAT* mRNA levels were not altered by either SREBP1 siRNA or T09. This disagrees with previous work demonstrating that SREBP1 siRNA reduced *GPAT* mRNA by approximately 20% (Ma and Corl, 2012), which suggested that SREBP1 is important in *GPAT* regulation. As T09 treatment had no effect on *GPAT* mRNA, it is unlikely that LXR is involved in transcriptional regulation of GPAT. Similarly, T09 did not influence *AGPAT* mRNA in RN-treated cells, suggesting that LXR is not involved in the transcriptional regulation of this gene. However, T09 did decrease *DGAT* mRNA abundance.

Knockdown of SREBP1 increased mRNA levels of both *AGPAT* and *DGAT*, a finding that is in agreement with previous research (Ma and Corl, 2012). As the LXR β isoform mRNA was also increased by SREBP1 knockdown, it is possible that LXR β could play a role in mediating these changes, depending on its activation.

Although much is known about how dietary factors affect milk fat production, little is known about the transcriptional programs that mediate these effects. For instance, supplementation of diets with fish or plant oils results in milk fat depression, and this correlates with lower mRNA abundance of *ACC1*, *FASN*, *GPAT*, and *AGPAT* (Peterson et al., 2003). It is known that biohydrogenation of PUFA in the rumen leads to the production of intermediates, including *trans*-10,*cis*-12 conjugated linoleic acid (CLA), and when MAC-T cells are treated with this CLA isoform, it reduces cleavage of SREBP1 to its mature form, and reduces mRNA of *ACC1*, *FASN*, and *SCD1* (Peterson et al., 2004). Diet-induced milk fat depression and *trans*-10,*cis*-12 CLA treatment also reduced SREBP1 mRNA in mammary tissue (Harvatine and Bauman, 2006). Similar to CLA treatment of mammary epithelial cells, use of siRNA in the present experiment reduced mature SREBP1 protein, de novo lipogenesis, and mRNA levels of *ACC1*, *FASN*, and *SCD1*, but increased *ACC1* and *FASN* transcripts in response to LXR activation with reduced abundance of SREBP1, indicating a potential role for LXR in their regulation. Reductions in mammary tissue SREBP1 associated with diet-induced or CLA-induced milk fat depression could result, as proposed by Harvatine and Bauman (2006), from direct regulation of SREBP1 gene transcription by SREBP1 itself, as an SREBP1 response element is present in the promoter. Additionally, an LXR response element is present in the SREBP1 promoter (Lengi and Corl, 2010), and LXR may have a role in the transcriptional regulation of SREBP1 and lipogenic genes with promoters that include an LXR response element.

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