ENHANCED LIVER X RECEPTOR AND DECREASED STEROL REGULATORY ELEMENT BINDING TRANSCRIPTION FACTOR 2 ACTIVITIES MAY CONTROL LUTEOLYSIS OF THE HUMAN CORPUS LUTEUM

by

Yafei Xu

A Thesis Submitted to the Faculty of the

SCHOOL OF ANIMAL AND COMPARATIVE BIOMEDICAL SCIENCES

In Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

WITH A MAJOR IN ANIMAL SCIENCES

In the Graduate College

THE UNIVERSITY OF ARIZONA

2017

STATEMENT BY AUTHOR

The thesis titled *Enhanced Liver X Receptor and Decreased Sterol Regulatory Element Binding Transcription Factor 2 Activities May Control Luteolysis of the Human Corpus Luteum* prepared by *Yafei Xu* has been submitted in partial fulfillment of requirements for a master's degree at the University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

SIGNED: *Yafei Xu*

APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

08/18/2017

Randy L. Bogan
Assistant Professor

Date

ACKNOWLEDGEMENTS

I would like to express my deepest appreciation to Dr. Randy Bogan for supporting and encouraging me to have the most wonderful and scientific experiences in Dr. Bogan's lab, the University of Arizona. Your abundant guidance, endless patience and a wealth of academic knowledge in reproductive physiology lead me to be a successful master of science. Dr. Bogan, you are the super hero in my life for the past two years and your greatest characters and spirits in science inspire me in future.

I would like to thank my committee members, Dr. Sean Limesand and Dr. Jose Hernandez-Ledezma. Thank you for giving me the chance and courage to study and research. Your strong support and precious suggestions helped a lot to make this thesis.

Thank you to the staff at the Reproductive Health Center in Tucson and Nickie Seto who performed data from sheep CL. Your hard work made this thesis possible and stronger.

In addition, thank you to the Animal Sciences Graduate Program represented by Dr. Sean Limesand (Program Director), Christina Garcia M.S. (Program Coordinator), and Dr. Benjamin Renquist for their support and guidance.

At last, I would like to thank my parents for your encouragement and endless love. Thank you for giving the power and wings. To my fiancé, Shengxiang, thank you for your brave heart and endless accompany in the past and future. To my cats, thank you for warm stay with me every day and night.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLE

Abstract

The mechanisms causing luteolysis of the primate corpus luteum are unknown. There is an increase in expression of liver x receptor (LXR) target genes and reduced low density lipoprotein receptor (LDLR) during spontaneous luteolysis in primates. The LXRs belong to the nuclear receptor superfamily and increase cholesterol efflux by inducing transcription of their target genes. Uptake of cholesterol into primate luteal cells occurs primarily via LDL, and *LDLR* transcription is regulated by sterol regulatory element binding transcription factor 2 (SREBF2). Luteinizing hormone (LH) and human chorionic gonadotropin (hCG) maintain luteal function by binding to the LH/CG receptor (LHCGR), which stimulates progesterone (P4) synthesis via protein kinase A (PKA). It has also been previously reported that there is an increase in 27-hydroxycholesterol (27OH) concentrations during spontaneous luteolysis in primates. Pregnenolone and P4 inhibit the enzyme activity of CYP27A1 (cytochrome p450, family 27, subfamily A, polypeptide 1), which converts cholesterol into 27OH, an oxysterol that is a natural LXR agonist and SREBF2 inhibitor. Therefore, the overall hypothesis is that LXR-induced cholesterol efflux and reduced LDL uptake via inhibition of SREBF2 activity mediate luteolysis of the human CL.

The objective of study 1 is to determine the effects of LXR activation and SREBF2 inhibition on P4 production, cholesterol metabolism and gene expression; and how hCG signaling via PKA regulates these effects in human luteinized granulosa cells. Basal and hCG-stimulated P4 secretion were significantly decreased by the combined actions of the LXR agonist T0901317 (T09) and the SREBF2

inhibitor fatostatin, which was associated with alterations in cholesterol metabolism leading to reduced intracellular cholesterol storage. Expression of LXR target genes in the presence of T09 was significantly reduced by hCG, while hCG significantly increased *LDLR* expression. These effects of hCG were reversed by a specific PKA inhibitor. Chronic hCG exposure had similar effects on LXR target gene and *LDLR* expression without an exogenous LXR agonist.

The objective of study 2 is to determine the effects of 27OH on P4 production and cholesterol metabolism; and to determine if inhibiting the conversion of cholesterol into pregnenolone increases LXR and decreases SREBF2 target gene expression via CYP27A1 in human luteinized granulosa cells. During luteolysis in primates and sheep, *CYP27A1* expression significantly increased. 27OH significantly decreased hCG-stimulated P4 secretion and enhanced cholesterol efflux. Aminoglutethimide, which inhibits the conversion of cholesterol to pregnenolone, significantly increased *ABCA1* and decreased *LDLR*. Knock-down of CYP27A1 resulted in a significant increase in P4 secretion, but did not prevent aminoglutethimide-induced effects on *ABCA1* and *LDLR*. Knock-down of steroidogenic acute regulatory protein (STAR), which controls cholesterol transport into the mitochondria where CYP27A1 resides, significantly decreased *LDLR* transcription.

Collectively, the data from study 1 support the hypothesis that LXR-induced cholesterol efflux and reduced LDL uptake via inhibition of SREBF2 activity mediates luteolysis in primates, which is reversed by hCG. Data from study 2

indicates that 27OH produced via CYP27A1 may contribute to reductions in P4 synthesis during luteolysis, partially by serving as a dual LXR agonist and SREBF2 inhibitor, although other oxysterols are also likely involved.

1 Chapter 1 – Introduction

1.1 Overview of luteolysis

The corpus luteum (CL) is a transient endocrine structure that forms from the remains of the ovarian follicle after ovulation in female ovaries. Progesterone (P4) produced by the CL is necessary to maintain early pregnancy in humans (1, 2). The structural and functional demise of the CL is called luteolysis. Luteolysis occurs due to a decrease in P4 synthesis (termed functional regression) and degradation and structural remodeling of the CL (structural regression) (3, 4). Functional regression of the CL is a rate-limiting event because P4 itself mediates many processes that maintain the structure and function of the CL (22). In the absence of pregnancy, luteolysis allows a new follicular cycle to begin. However, in women with normal fertility, there exists a high rate (>33%) of early pregnancy loss due to a failure to prevent luteolysis (5).

1.2 Differences in luteolysis between primate and non-primate species

In most mammalian species, the presence of the uterus is necessary for normal luteolysis (6). Hysterectomy of heifers (7), ewes (8), pigs (9), guinea pigs (10), and many other species prolongs luteal function. In most non-primate species, prostaglandin F2α (PGF2α) of uterine origin initiates luteolysis (4, 6). Estradiol produced from the developing preovulatory follicle triggers hypophysial oxytocin release, which stimulates a small pulse of uterine PGF2α (11, 12). A

positive-feedback loop is established with PGF2α causing luteal oxytocin secretion, which results in additional PGF2α release from the uterus (13, 14). However, hysterectomy in primates does not extend the normal lifespan of the CL indicating that the luteolytic signal is not of uterine origin and may be an intraovarian mechanism (15-17). The biological source and role of PGF2α in luteolysis of the primate CL, if any, remains controversial (6). Thus, the specific mechanism causing luteolysis in the primate CL is still undefined.

1.3 Sensitivity of the primate CL to luteinizing hormone during luteolysis

Luteinizing hormone (LH) is necessary to maintain the structure and function of the primate CL. In monkeys, a rapid drop in P4 and premature menses occur with treatment of a GnRH antagonist during the midluteal phase, while replacement with an increasing dose of LH restores P4 secretion and normalizes luteal phase length (18). Pulses of P4 from the CL are directly related to LH pulses, and the sensitivity to LH pulses is reduced as the CL ages (19-22). Increasing LH levels alone is not sufficient to prevent functional regression during the luteal phase (18, 23). A more potent luteinizing hormone/choriogonadotropin receptor (LHCGR) ligand (i.e., human chorionic gonadotropin (hCG)) produced from the placenta during early pregnancy is necessary to prevent luteolysis (18). As LHCGR concentrations do not decrease until after functional regression happens, the loss in luteal sensitivity to LH is not by LHCGR down-regulation (20, 24). Hence, the luteolytic signal in primates must reduce luteal sensitivity to LH.

1.4 Cholesterol efflux and uptake during luteolysis of the primate CL

It has been previously reported that there is an increase in expression of liver x receptor (LXR) target genes and reduced expression of genes regulated by the sterol regulatory element binding transcription factor 2 (SREBF2) during luteolysis in rhesus macaques and sheep (25, 26). The LXRs α (NR1H3) and/or β (NR1H2), belong to the nuclear receptor superfamily and induce transcription of genes involved in cholesterol efflux by binding to numerous precursors or derivatives of cholesterol when cholesterol is in excess (27). The LXRs also inhibit extracellular cholesterol uptake by causing ubiquitin-mediated degradation of the low density lipoprotein receptor (LDLR) (28-30). Collectively, the critical role of LXRs is to decrease intracellular cholesterol concentrations under conditions of cholesterol excess. Conversely, sterol regulatory element binding transcription factor 2 (SREBF2) target genes include the low-density lipoprotein receptor (LDLR) and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), the rate-limiting enzyme in de novo cholesterol synthesis (31, 32). Intracellular cholesterol concentrations are collectively increased by the stimulation of SREBF2 target genes. Therefore, the competing actions of the LXRs and SREBF2 are necessary to maintain cholesterol homeostasis.

1.5 Endogenous LXR agonists and SREBF2 inhibitors during luteolysis of the primate CL

Not only do the LXRs and SREBF2 have reciprocal actions on cholesterol

homeostasis, but their transcriptional activity is also differentially regulated by oxysterols (hydroxylated cholesterol), which are endogenous LXR agonists (27) and SREBF2 inhibitors (32, 33). It has been previously reported that luteal 27-hydroxycholesterol (27OH) concentrations are increased during spontaneous luteolysis in the rhesus macaque CL (30). Products of steroidogenesis including pregnenolone and P4 inhibit the enzyme activity of CYP27A1 (cytochrome p450, family 27, subfamily A, polypeptide 1), which catalyzes the conversion of cholesterol into 27OH (34). Decreasing steroidogenesis may increase CYP27A1 expression and activity, which would promote 27OH synthesis and lead to increased LXR and decreased SREBF2 activity during luteolysis.

1.6 Overall hypothesis

It is known that the CL is a highly steroidogenic gland which produces 40 mg of P4 per day in humans (4, 35). During luteinization, a buildup of intracellular cholesterol stores may be necessary to support the considerable steroid production from the CL (4), while luteolysis may by associated with cholesterol depletion. This indicates that the normal reciprocal relationship between the LXRs and SREBF2 may be disrupted to allow cholesterol accumulation during luteinization, and cholesterol depletion during luteolysis. Therefore, our overall hypothesis is that LXR-induced cholesterol efflux and reduced LDL uptake via inhibition of SREBF2 causes luteolysis of the human CL (Fig. 1.1).

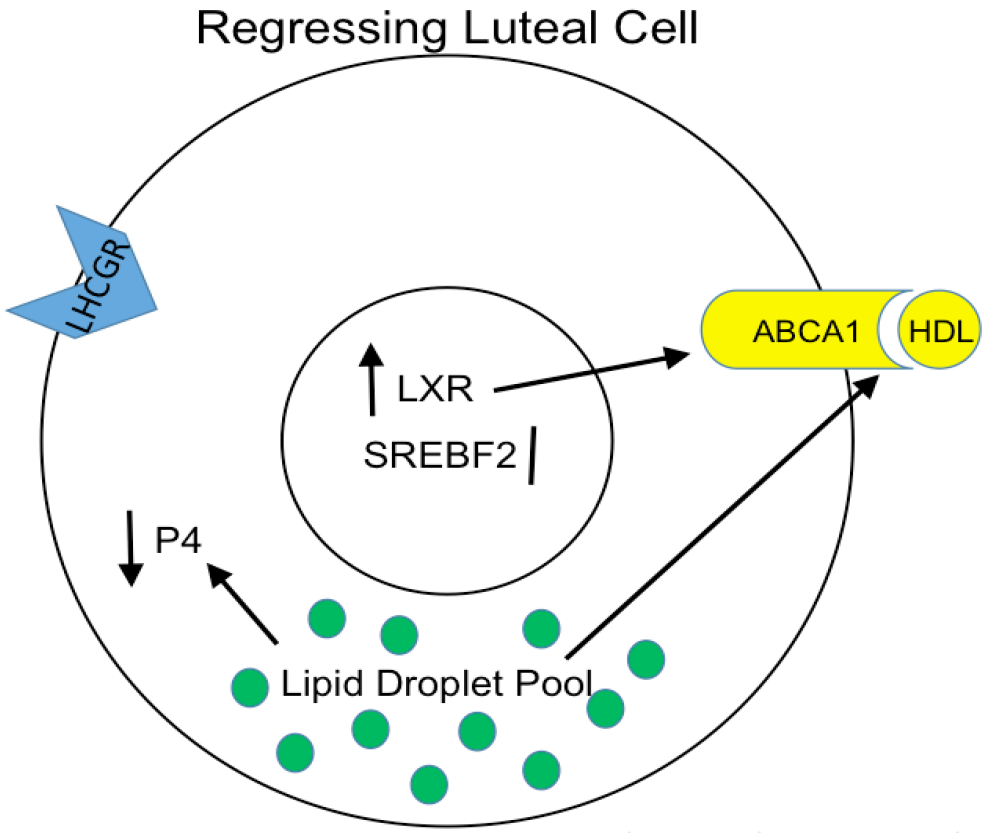

FIG. 1.1: Overall hypothesis. Enhanced LXR and decreased SREBF2 activities may mediate luteolysis by restricting cholesterol availability in human CL.

1.7 Study 1

In chapter 2 our hypothesis that increased LXR and reduced SREBF2 activity may cause luteolysis in humans, and that hCG prevents luteolysis by reversing these effects (Fig. 1.2), was further tested. We used pharmacologic agents to directly activate the LXRs and inhibit SREBF2 in order to determine the functional effect on cholesterol metabolism and P4 synthesis. We also determined how hCG signaling via PKA regulated these effects of the LXRs and SREBF2. This study helps us to better understand the functional effect of increased LXR and decreased SREBF2 activities during luteolysis, and how hCG prevents luteolysis during early pregnancy.

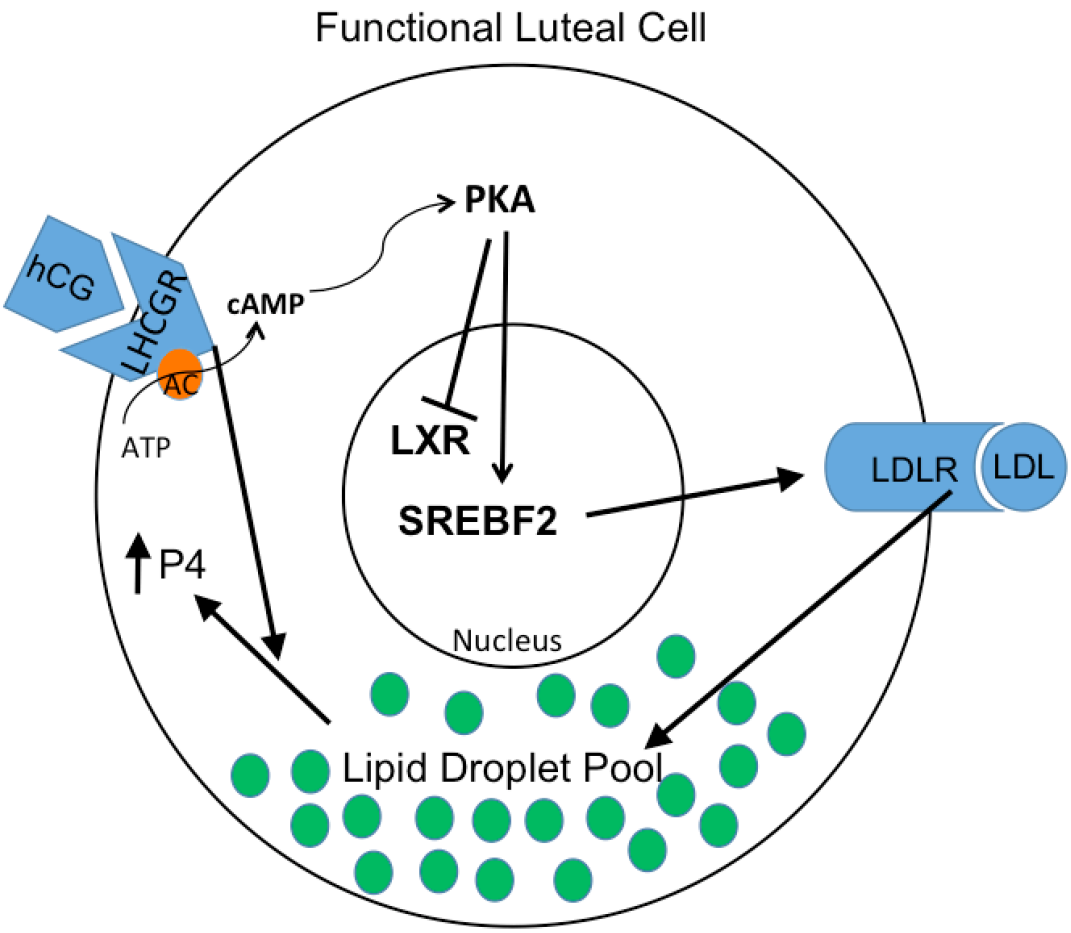

FIG. 1.2: Hypothesis for hCG effects. Inhibited LXR and enhanced SREBF2 by hCG signaling via PKA prevents primate luteolysis.

1.8 Study 2

In chapter 3, our hypothesis that endogenous 27OH produced via CYP27A1 may mediate luteolysis by increasing LXR and reducing SREBF2 activities in the human CL (Fig. 1.3) was tested. Treatment with 27OH was performed to determine its effect on P4 secretion. Furthermore, we used pharmacologic agents to inhibit steroidogenesis, which results in increased enzymatic activity of CYP27A1. We also used small interfering RNA (siRNA) to inhibit CYP27A1, as well as the steroidogenic acute regulatory protein (STAR) that is necessary for cholesterol delivery to CYP27A1. This study helps us to determine the effect of endogenous production of 27OH, which is both an LXR agonist and SREBF2 inhibitor, on luteal function in humans.

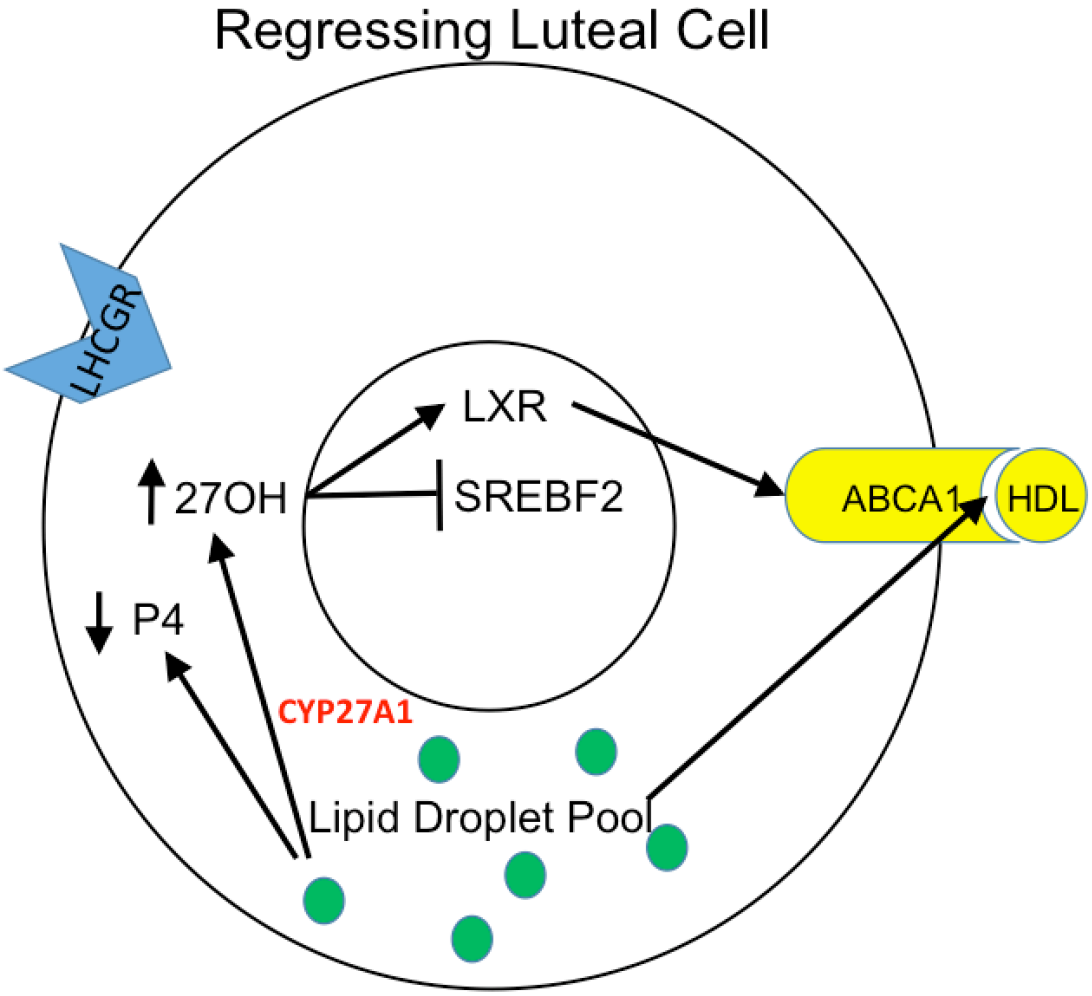

FIG. 1.3: Hypothesis for study 2. Endogenous 27OH produced via CYP27A1 may mediate luteolysis by increasing LXR and inhibiting SREBF2 activities.

2 Chapter 2 - Enhanced Liver X Receptor and Decreased Sterol Regulatory Element Binding Transcription Factor 2 Activities May Control Luteolysis of the Human Corpus Luteum, and are regulated by Human Chorionic Gonadotropin

2.1 Introduction

Luteal cells synthesize and secrete the steroid hormone progesterone (P4) which is necessary to maintain early pregnancy in primates (36). Decreased production of P4 is the key event during luteolysis, which is a functional and structural degradation of the corpus luteum (CL). In most nonprimate species, prostaglandin F2α (PGF2α) of uterine origin has been well understood to initiate luteal regression and luteolysis (6). But in primates, hysterectomy does not extend the normal lifespan of CL (16, 17), indicating intraovarian factors may regulate luteolysis. Thus, the mechanisms causing luteolysis in the primate CL remain undefined.

There is increased expression of liver x receptor (LXR) target genes and decreased low density lipoprotein receptor (LDLR) during spontaneous luteolysis in the primate CL (25). There are two LXR isoforms, α (NR1H3) and β (NR1H2), which are cholesterol sensors belonging to the steroid hormone receptor superfamily. When intracellular cholesterol concentrations rise, they induce transcription of their target genes including ATP binding cassette subfamily A1 (*ABCA1*) and G1 (*ABCG1*), as well as *NR1H3* itself, which results in an enhancement of cholesterol efflux (29, 37,

38). Also, the LXRs inhibit uptake of LDL cholesterol into primate luteal cells by inducing transcription of myosin regulatory light chain interacting protein (*MYLIP*), which causes proteolytic degradation of LDLR (29, 30). In primates, luteal cells primarily depend on LDL-derived cholesterol for steroidogenesis (39). When intracellular cholesterol concentrations decrease, sterol regulatory element binding transcription factor 2 (SREBF2) activates transcription of the rate-limiting enzyme in cholesterol biosynthesis 3-hydroxy-3-methylglutaryl-CoA reductase (*HMGCR*), as well as *LDLR* to increase cholesterol uptake (31, 32, 40). Thus, we hypothesize that limiting the intracellular cholesterol supply via increased LXR and reduced SREBF2 activity could reduce P4 synthesis and cause luteolysis.

It has been well established that during the luteal phase luteinizing hormone (LH) binds to the luteinizing hormone/choriogonadotropin receptor (LHCGR), which maintains P4 secretion by inducing the production of intracellular cyclic AMP (cAMP) and activation of cAMP-dependent protein kinase (PKA) (41-43). During early pregnancy another LHCGR ligand, human chorionic gonadotropin (hCG), is released. Because hCG has a longer half-life relative to LH, it prevents luteolysis by causing sustained activation of the LHCGR (18). Therefore, we hypothesize that hCG signaling via PKA prevents luteolysis by inhibiting the LXRs and stimulating SREBF2.

The objective of this study is to determine the effects of LXR activation and SREBF2 inhibition on P4 production, cholesterol metabolism, and gene expression; and how hCG signaling via PKA regulates these effects. A human luteinized

granulosa cell model was used in this study. Human granulosa cells can be cultured with low doses of hCG for several days to mimic luteinization and produce cells with characteristics highly similar to luteal cells (44).

2.2 Materials and Methods

2.2.1 Isolation of human granulosa cells

The follicular aspirates used in this study were from 41 female patients undergoing oocyte donation or in vitro fertilization (IVF) for male factor or idiopathic infertility at the University of Arizona Assisted Reproduction Center, Tucson, AZ. The patients were 24 to 44 years old at the time of retrieval. The University of Arizona Institutional Review Board (IRB) determined that because follicular aspirates were de-identified before being provided to the investigators, and they are normally a waste byproduct of the IVF procedure; this study was exempt from IRB review because it does not meet the definition of human subjects research as defined in 45 CFR 46.102(f).

Follicular aspirates were centrifuged at 500 × g for 5 min at 4ºC. The supernatant was aspirated and the cell pellets were resuspended in nutrient mixture F10 Ham (Ham's F10) with 0.1% (*w:v*) bovine serum albumin (BSA), covered onto a 40% (*v:v*) Percoll gradient (GE Healthcare, Uppsala, Sweden) in Hanks' balanced salt solution (SIGMA-ALDRICH, MO, USA), and centrifuged at 500 × g for 15 min at 4ºC. Supernatant including the granulosa cells was recovered and diluted with Ham's F10 and centrifuged at 500 × g for 5 min at 4ºC. The cell pellet was resuspended and

washed once more. Finally, the cell pellet was resuspended in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F12) with insulin-transferrin-selenium (ITS) supplement (Millipore, CA, USA) and 0.02 IU/ml hCG. Cells were counted by trypan blue (0.2% *v:v*) dye-exclusion method. Plates were coated with 5 μg/ml fibronectin from bovine plasma at 37ºC for 1 hour and washed with sterile PBS. The isolated human granulosa cells were plated in 24-well plates or 96-well plates at the density of 5×10^4 cells/cm^2. Granulosa cells were cultured for 5 days in luteinization media (DMEM/F12 with ITS supplement, 100 units/ml penicillin, 0.1 mg/ml streptomycin (Pen/Strep), and 0.02 IU/ml hCG), incubated at 37ºC and 5% CO_2 in a humidified environment.

2.2.2 Treatment of human luteinized granulosa cells

Cells were treated with vehicle (0.1% *v:v* DMSO), the LXR agonist T0901317 (T09, 0.1 μM), and/or the SREBF2 inhibitor Fatostatin (5 μM) in treatment media (serum free DMEM/F12 containing ITS, Pen/Strep, 20 μg/ml LDL and 10 μg/ml HDL cholesterol). After 16 hours, fresh treatments with or without 2 IU/ml hCG were added for another 4 hours.

To determine whether PKA mediates the hCG effect, cells were treated with vehicle (0.1% v:v DMSO) or T09 (0.1 μM) in treatment media for 16 hours. Fresh treatments were added in the presence and absence of 2 IU/ml hCG and protein kinase inhibitor (PKI, 50 μM) for another 4 hours. To determine the chronic effects of hCG, after 4 days of luteinization 20 μg/ml LDL and 10 μg/ml HDL cholesterol was added

into fresh medium with 0.02 IU/ml hCG for one day. The next day cells were cultured in the presence or absence of 0.2 IU/ml hCG with 20 µg/ml LDL and 10 µg/ml HDL cholesterol, and media was changed daily.

2.2.3 Ovine Luteal Cell Model

Procedures involving sheep were approved by the University of Arizona Institutional Animal Care and Use Committee. The estrous cycle of ewes was synchronized by inserting controlled internal drug release (CIDR) devices for 7 days with a lutalyse (Zoetis Inc., Kalamazoo MI) injection (15 mg/60 kg body weight IM) on day 6. To induce superovulation, at the time of CIDR removal ewes were received 1000 IU pregnant mare serum gonadotropin IM. At 38 hours post-CIDR removal ewes received 750 IU hCG IV to induce final follicular maturation and ovulation. Ovulation was confirmed by analysis of serum P4 concentrations. At 11 days post-CIDR removal (mid-luteal phase), CL were collected from ewes, dispersed with collagenase (Type 2 at 0.2% *w:v*, Worthington Biochemical, Lakewood, NJ) and DNAse I (0.02% *w:v*, Worthington Biochemical), and cultured in DMEM/F12 + ITS. The next day they were treated for 24 hours with T09 (0.1 µM) and/or the adenylate cyclase activator forskolin (10 µM).

2.2.4 Progesterone extractions and assay

Progesterone in culture media was extracted and concentrations were determined by enzymatic immunoassay as previously described (26). Cells were lysed in RIPA buffer containing a protease and phosphatase inhibitor cocktail (Thermo Scientific

Pierce, Rockford, IL), and protein concentrations were determined by a bicinchoninic acid (BCA) protein assay (Thermo Scientific Pierce) following the manufacturer's recommendations. Progesterone concentration was normalized to total protein content in the cells.

2.2.5 Cholesterol efflux assay

Cells were labeled with Bodipy Cholesterol (Avanti Polar Lipids Inc., Alabaster AL, 2 μg/ml) during the 16-hour initial treatments. Cells that were not labeled were used to control for background fluorescence. After labeling, medium was aspirated and cells were washed once with PBS. Cells were then incubated an additional 4 hours in treatment media as described earlier. Media were collected and cells were lysed using 100 μl/well of M-PER reagent (Thermo Scientific). The fluorescence of cells and media was measured, and cholesterol efflux was calculated by the equation 100 × (media fluorescence)/(cell lysate fluorescence + media fluorescence).

2.2.6 LDL uptake assay

Cells were incubated with Dil-LDL (10 μg/ml) instead of unlabeled LDL during the final 4-hour treatment, and cells that did not receive Dil-LDL were used to control for background fluorescence. Next, cells were washed once with PBS and lysed in RIPA buffer containing protease & phosphatase inhibitors. Fluorescence was determined in the cell lysate, and a BCA protein assay was subsequently performed on the lysates. Fluorescence values were normalized to total protein concentration.

2.2.7 Cholesterol extraction and assay

Cells were washed with PBS and scraped into cold PBS containing a protease and phosphatase inhibitor cocktail. The cells were sonicated in an ice water bath using a Branson 250 digital sonifier programmed to cycle at a 10% amplitude with 5 sec on followed by 20 sec off, and a 15 sec total sonication time. Total cholesterol was extracted from cell lysates using the Bligh/Dyer procedure (45), and resuspended in cholesterol assay buffer (0.1 M potassium phosphate, pH 7.0, 50 mM sodium chloride, 5 mM cholic acid and 0.1% Triton X-100). Cholesterol concentrations were determined via a fluorometric enzyme assay using cholesterol esterase (60 units/ml), cholesterol oxidase (200 units/ml), horse radish peroxidase (200 units/ml), and Amplex Red (200 units/ml). A BCA protein assay was performed on the lysates, and cholesterol concentrations were normalized to total protein content.

2.2.8 Quantitative Real-Time PCR (QPCR)

Total RNA was extracted with Trizol reagent and purified over RNeasy columns (Life Technologies) according to manufacturer recommendations. The quantity and purity of RNA was determined by spectrophotometry. The High Capacity cDNA Reverse Transcription Kit (Life Technologies) was used to reverse transcribe RNA into cDNA. The corresponding forward and reverse primers and Taqman probe sequences are listed in Table 1. Reactions for QPCR were performed in a StepOne Plus Real-Time PCR system (Life Technologies) using Taqman MGB probe or SYBR green detection. Relative mRNA abundance was determined by extrapolation of Ct

values from a standard curve of serial cDNA dilutions, and normalized to glucose-6-phosphate dehydrogenase (*G6PD*).

Table 1 - Primer and Taqman MGB probe sequences used in QPCR analyses

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	MGB Probe (5'-3') or SYBR
ABCA1	TCCAGGCCAGTACGGAATTC	TCCTCGCCAAACCAGTAGGA	CTGGTATTTTCCTTGCACCAA
ABCG1	GACCAGCTTTACGTCCTGAGTCA	GTTCAGACCCAAATCCCTCAAAT	AAAGTCTGCAATCTTGTGCC
MYLIP	GCCACCCAGTCAGGAAAGAAT	GCCCTGGTGCTGATCATTTT	CAGCATCGTGCTCTT
LDLR	CTGGTCAGATGAACCCATCAAAG	GCCGATCTTAAGGTCATTGCA	CCAACGAATGCTTGGAC
NR1H2	ATCGTGGACTTCGCTAAGCAA	GATCTCGATAGTGGATGCCTTCA	TGCCTGGTTTCCTGC
NR1H3	TCCCCATGACCGACTGATG	CAGACGCAGTGCAAACACTTG	TCCCACGGATGCTAAT
HMGCR	AACACGATGCATAGCCATCCT	AAGGCCAGCAATACCCAAAA	SYBR Green
STAR	GTGGGTGCCTTCCAGAAATATAGT	TGACTGGTGCCTATGAAAGCAA	SYBR Green
SREBF2	ATCGCTCCTCCATCAATGACA	TCCTCAGAACGCCAGACTTGT	SYBR Green
G6PD	GAAGCCGGGCATGTTCTTC	TAGGCGTCAGGGAGCTTCAC	SYBR Green

2.2.9 Statistical Analysis

Each individual experiment was repeated on at least three cases with cells from different patients. Statistical analysis was performed with mixed effects regression analysis (Stata Version 14) using treatment as the fixed effect and cell replicate as the random effect followed by a Bonferroni multiple testing procedure for pairwise comparisons, and data were log-transformed if necessary to stabilize variance. Data are expressed as mean plus the standard error of the mean. Statistically significant comparisons ($p < 0.05$) are indicated when columns do not share a common letter.

2.3 Results

2.3.1 Effect of LXR agonist and/or SREBF2 inhibitor on P4 secretion and cholesterol metabolism

Treatment with T09 and fatostatin in the absence of hCG caused an additive significant decrease ($P < 0.05$) in P4 secretion from human luteinized granulosa cells compared with the DMSO vehicle control (Fig. 2.1A). In the presence of hCG, P4 secretion was significantly ($p< 0.05$) reduced approximately 39% by combined T09 and fatostatin treatment. In the presence or absence of hCG, cholesterol efflux was significantly ($p< 0.05$) enhanced by T09 or T09 combined with fatostatin treatment, while fatostatin itself significantly ($p< 0.05$) reduced cholesterol efflux (Fig. 2.1B). The uptake of LDL was significantly lower for T09 compared to fatostatin treatment, with combined treatment not being different from DMSO (Fig. 2.1C). Total intracellular cholesterol concentrations were significantly ($p< 0.05$) decreased with T09 and fatostatin combined treatment relative to DMSO in the presence of hCG. In general hCG decreased total cholesterol concentrations, with a significant decrease caused by hCG occurring in the T09 and T09 with fatostatin treatment groups (Fig. 2.1D).

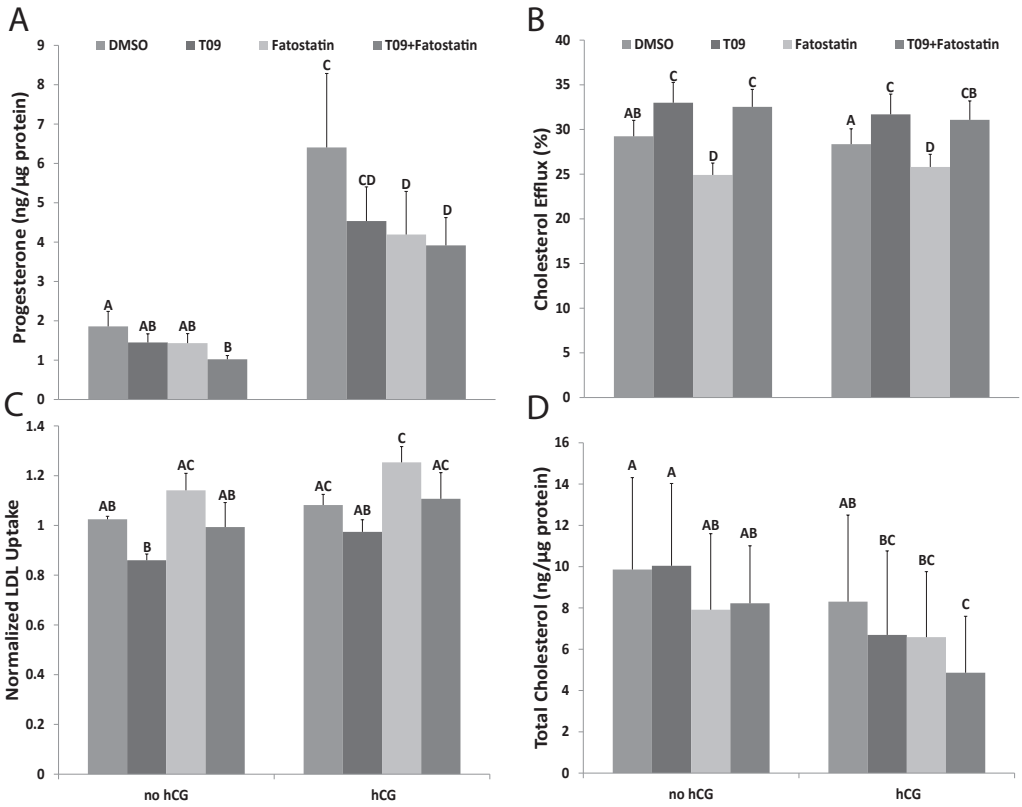

FIG. 2.1: Effects of T09 and/or Fatostatin with or without hCG on P4 secretion and cholesterol metabolism in human luteinized granulosa cells. Panel A is P4 concentrations in spent media, panel B shows the percentage of cholesterol efflux, panel C is LDL uptake, and panel D is the total intracellular cholesterol concentration. Error bars indicate one standard error of the mean (SEM). Columns without a common letter are significantly different ($p < 0.05$).

2.3.2 Effect of hCG, LXR agonist and/or SREBF2 inhibitor on expression of LXR and SREBF2 target genes

In the absence of hCG, the LXR target genes *ABCA1* and *ABCG1* were significantly ($p< 0.05$) increased by T09 approximately 4.9 and 7.4-fold, respectively, while *MYLIP* was also significantly ($p< 0.05$) increased by T09. There was a significant increase in *NR1H3* induced by T09 and fatostatin combined treatment. Also, T09 and fatostatin combined treatment caused a significantly ($p<0.05$) higher increase in *ABCA1* and *ABCG1* expression compared to T09 alone. Human chorionic gonadotropin significantly ($p< 0.05$) inhibited T09-induced *ABCA1*, *ABCG1*, and *MYLIP* expression in the presence and absence of fatostatin co-treatment (Fig. 2.2A). Treatment with hCG significantly ($p< 0.05$) increased expression of *LDLR* 7.6-fold ($p< 0.05$), while fatostatin itself had no effect. Human chorionic gonadotropin also significantly ($p< 0.05$) increased expression of *HMGCR* in the presence of fatostatin (Fig. 2.2B).

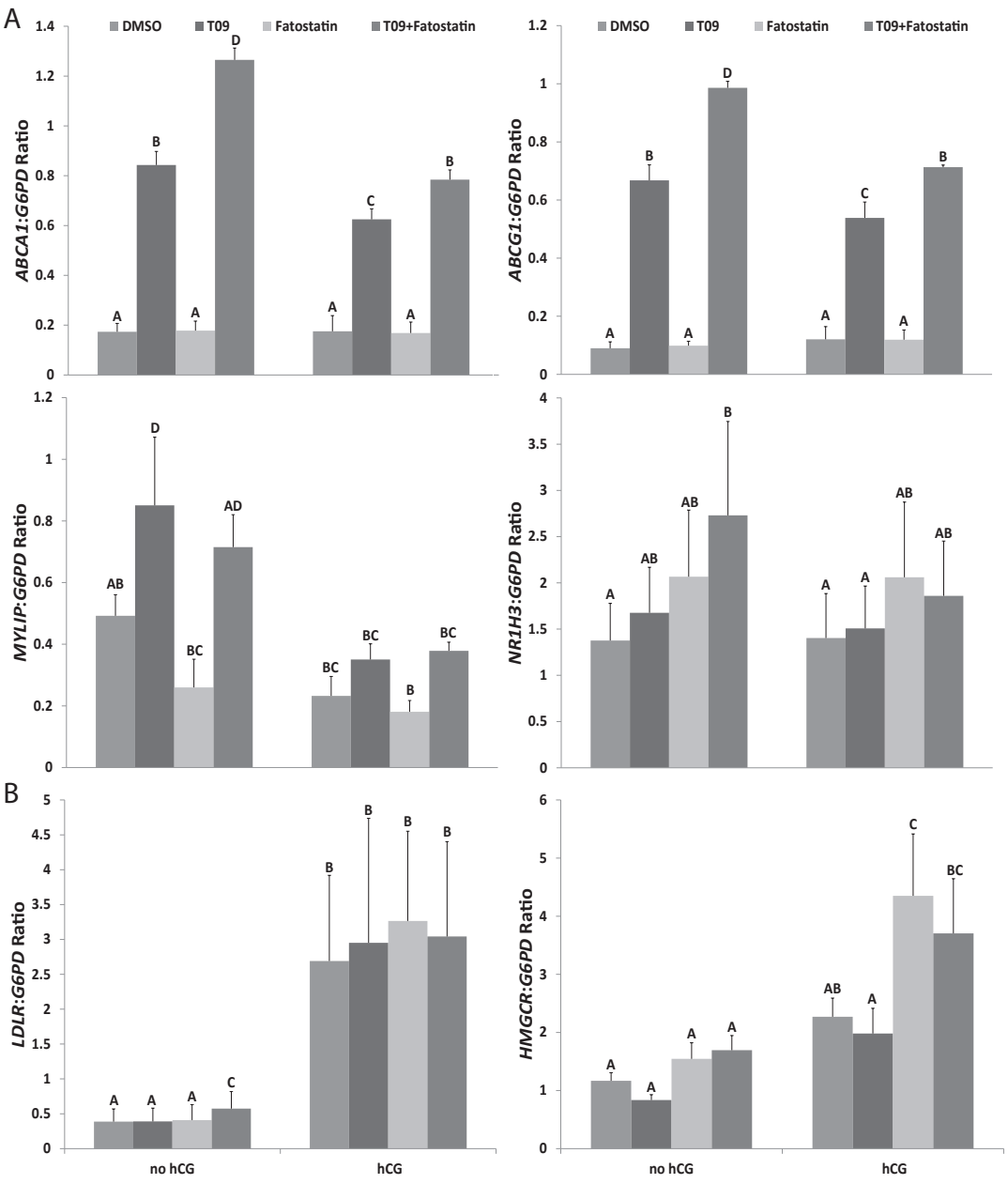

FIG. 2.2: Effects of T09 and/or fatostatin with and without hCG on the expression of LXR and SREBF2 target genes in human luteinized granulosa cells. Panel A shows QPCR data for the four LXR target genes: *ABCA1*, *ABCG1*, *NR1H3*, and *MYLIP*. Panel B is QPCR data for the two SREBF2 target genes *HMGCR* and *LDLR*. *G6PD* is the housekeeping control. Error bars indicate one SEM, and columns without common letter are significantly different ($p < 0.05$).

2.3.3 Effects of PKA inhibitor and/or hCG on LXR target genes and LDLR expression

While *ABCA1* expression in the presence of T09 was significantly ($p < 0.05$) decreased by hCG, there was no effect of hCG on T09-induced *ABCA1* expression during PKI co-treatment. Human chorionic gonadotropin significantly ($p < 0.05$) increased *LDLR* expression, which was prevented by PKI (Fig. 2.3).

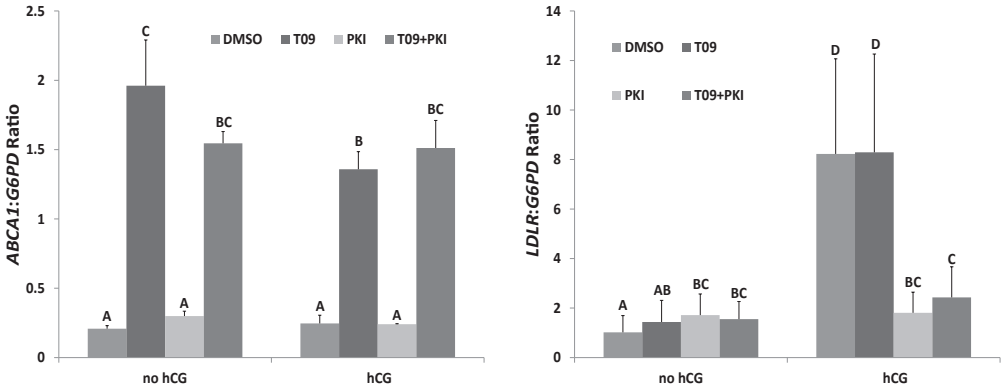

FIG. 2.3: Effects of hCG signaling via PKA on expression of *ABCA1* and *LDLR* in human luteinized granulosa cells. Error bars indicate one SEM, and columns without common letters are significantly different ($p<0.05$).

2.3.4 Effects of Chronic hCG exposure on expression of LXR target genes and *LDLR*

In the absence of an exogenous LXR agonist, 0.2 IU/ml hCG exposure for 3 days significantly ($p < 0.05$) inhibited *ABCA1* expression compared to cells not receiving hCG for 3 days. Furthermore, there was a time-dependent effect of hCG with *ABCA1* expression being significantly ($P < 0.05$) decreased at day 3 compared to day 1 of hCG treatment, and a significant ($p < 0.05$) increase in *ABCA1* from day 2 to day 3 in cells not receiving hCG (Fig. 2.4). The expression of *LDLR* was maintained at similar levels throughout the 3 days of hCG treatment with days 1 and 2 having significantly ($p < 0.05$) higher *LDLR* concentrations than the same days in cells not receiving hCG (Fig. 2.4).

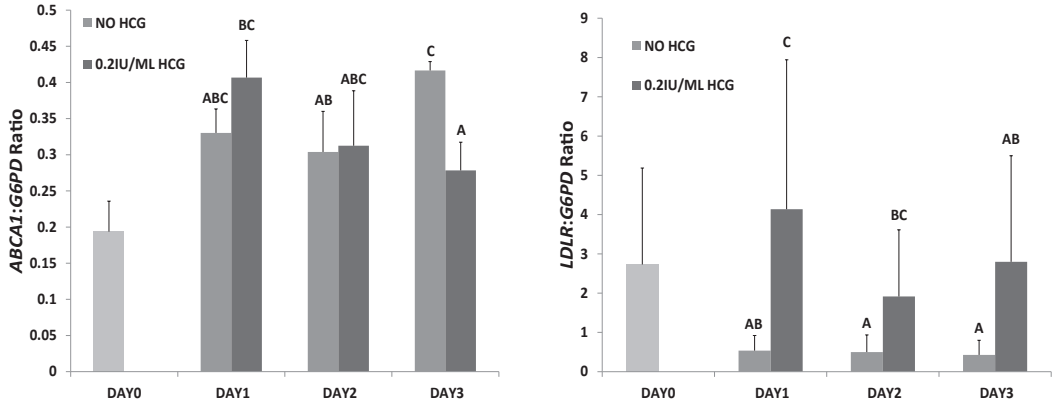

FIG. 2.4: Effects of chronic hCG exposure on *ABCA1* and *LDLR* gene transcription in human luteinized granulosa cells. The day 0 timepoint was not included in statistical analysis and is shown as a reference for the baseline level of gene expression at the conclusion of the luteinization period. Error bars indicate one SEM, and columns without common letters are significantly different ($p < 0.05$).

2.3.5 LXR regulation of *STAR* transcription in humans and sheep

In human luteinized granulosa cells, T09 did not increase *STAR* transcription while hCG significantly ($p<0.05$) increased *STAR* expression (Fig. 2.5A). In contrast, T09 significantly ($p< 0.05$) increased *STAR* expression in the presence and absence of pharmacologic activation of PKA via forskolin in ovine mixed luteal cells (Fig. 2.5B). These changes in *STAR* expression were associated with a significant ($p< 0.05$) increase in basal P4 secretion, and a tendency for an enhancement of forskolin-induced P4 synthesis in ovine luteal cells.

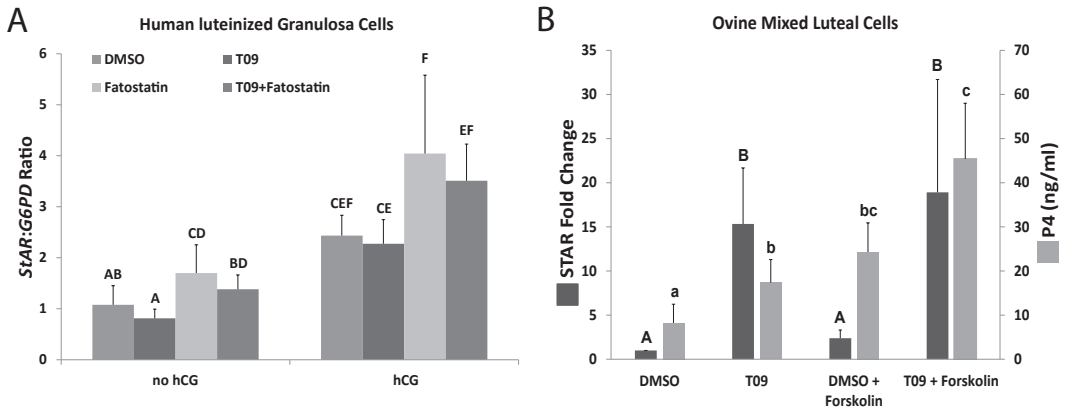

FIG. 2.5: Effects of LXR agonist on *STAR* transcription in human luteinized granulosa cells and ovine mixed luteal cells. Panel A is QPCR data for *STAR* expression in human luteinized granulosa cells. Panel B shows expression of *STAR* and P4 production in response to the LXR agonist T09 and the adenylyl cyclase activator forskolin in ovine mixed luteal cells. Error bars indicate one SEM. and columns without common letters of the same case are significantly different ($p< 0.05$).

2.4 Discussion

Previous data has shown an association of increased LXR target gene expression and reduced LDLR with luteolysis in primates (25). However, functional evidence of whether these changes in gene expression could actually inhibit P4 production was limited. In this study, we used the LXR agonist T09 and the SREBF2 antagonist fatostatin to mimic the changes in gene expression observed during luteolysis. Indeed, T09 and fatostatin collectively were able to significantly decrease basal and hCG-stimulated P4 secretion, indicating that increased LXR and decreased SREBF2 activity may decrease P4 synthesis in the primate CL. As expected, alterations in cholesterol efflux, LDL uptake, and/or total cholesterol concentrations were observed that are consistent with the anti-steroidogenic effect being mediated principally by a reduction in cholesterol availability for steroidogenesis. Additionally, the expression of known LXR target genes increased with T09 as expected, and interestingly fatostatin co-treatment further increased *ABCA1*, *ABCG1*, and *NR1H3* expression compared to T09 alone. The reason for this interaction between T09 and fatostatin on LXR target gene expression is not clear.

Surprisingly, the expression of SREBF2 target genes *HMGCR* and *LDLR* was not significantly decreased by the SREBF2 inhibitor fatostatin. Analysis of total cholesterol concentrations support that fatostatin inhibited P4 by decreasing cholesterol availability. Sterol regulatory element-binding proteins (SREBPs) play a central role in cell metabolism by controlling synthesis of fatty acids, triglycerides,

and cholesterol (32). Mammalian SREBP isoforms SREBP-1a and SREBP-1c are encoded by *SREBF1*, and SREBP-2 is encoded by *SREBF2* (31). SREBP-1a activates fatty acid and cholesterol synthesis while SREBP-1c preferentially targets genes involved in fatty acid synthesis, and SREBP-2 primarily transcribes genes necessary for cholesterol synthesis and uptake (32). Therefore, we elected to focus on SREBF2 in the current study. Fatostatin was originally identified as an inhibitor of all SREBPs by blocking their translocation from the endoplasmic reticulum to the Golgi, which is necessary for their activation (46). This results in decreased expression of SREBP target genes, and reduces the levels of intracellular fatty acids and cholesterol (46, 47). Considering that fatostatin did not decrease *HMGCR* and *LDLR*, the decreased intracellular cholesterol and P4 secretion may be regulated by other target genes of SREBF1 and/or SREBF2. Also, compensatory mechanisms may have obscured an effect of fatostatin on gene expression. For example, the reduced intracellular cholesterol concentrations caused by fatostatin may have activated homeostatic mechanisms that restored *HMGCR* and *LDLR* expression via a non-SREBP mechanism. A homeostatic feedback mechanism is consistent with our findings of a tendency for fatostatin to increase LDL uptake, as well as to significantly inhibit cholesterol efflux. Therefore, it appears that the anti-steroidogenic effect of fatostatin is mediated via reduced cholesterol availability, but the extent to which this is due to inhibition of SREBPs or possibly other off-target effects is unclear.

If increased LXR and decreased SREBF2 activity mediates luteolysis, because hCG prevents luteolysis it would be predicted to decrease LXR and increase SREBF2

activities via PKA. Proteolytically cleaved SREBF2 has been reported to increase with hCG treatment in rat ovaries, and *LDLR* transcription was enhanced by LH in porcine granulosa cells (48, 49). In this study, hCG significantly enhanced the SREBF2 target genes *HMGCR* and *LDLR* while inhibiting the T09-induced expression of the LXR target genes *ABCA1*, *ABCG1*, and *MYLIP*. In the presence of hCG, P4 secretion was significantly stimulated as expected. Although hCG had no significant effect on cholesterol efflux and LDL uptake, total intracellular cholesterol was decreased by hCG. This indicates that hCG-induced steroidogenesis places a large demand for cholesterol on the CL. The PKA inhibitor PKI is a potent, competitive, synthetic peptide inhibitor of PKA derived from the active domain of the naturally-occurring heat-stable inhibitor protein PKI (50). It is more specific for PKA than another commonly used PKA inhibitor H-89 that inhibits additional kinases besides PKA (51, 52). In the presence of PKI, the effects of hCG to decrease T09-induced *ABCA1* expression and increase *LDLR* were reversed, indicating that hCG mediates its effects on the LXRs and SREBF2 through PKA. However, it should also be noted that hCG signaling via PKA has actions that will increase LXR and decrease SREBF2 activities as well. For example, PKA activates hormone sensitive lipase, which frees cholesterol from cholesterol esters stored in lipid droplets and thus causes an indirect increase in endogenous LXR agonists and SREBF2 inhibitors (49). Also, the large increase in *LDLR* expression caused by hCG will likely lead to enhanced cholesterol uptake into the cell, which due to the normal homeostatic control of intracellular cholesterol concentrations (33), will consequently increase

LXR and reduce SREBF2 activity. Thus, while hCG may indirectly increase LXR and reduce SREBF2 activity due to its effects that increase intracellular cholesterol availability, data in the current study demonstrate that it simultaneously limits LXR and maintains SREBF2 activity, which may be a mechanism to keep the cholesterol supply high by escaping the normal control of cholesterol homeostasis. The effects of chronic hCG exposure further support this model of hCG/PKA regulation of the LXRs and SREBF2 as short term exposure to hCG in the absence of an exogenous LXR agonist resulted in higher *ABCA1* expression than long-term hCG exposure, and hCG withdrawal resulted in a dramatic loss in *LDLR*.

The PKA-induced transport of cholesterol across the mitochondrial membrane is the rate-limiting step in steroidogenesis and is controlled by STAR (53). In mice, *STAR* is an LXR target gene as its expression is increased by an LXR agonist, which results in enhanced P4 production (48, 49). In this study, we found a similar effect in ovine mixed luteal cells. There was a significant increase in basal and forskolin-induced *STAR* expression caused by T09. Basal P4 secretion was also significantly increased by T09 in ovine luteal cells. However, we also found that *STAR* was not induced by T09, and T09 contributed to significant reductions in P4 synthesis in human luteinized granulosa cells. In a previous study, a 7-day treatment with T09 tended to slightly reduce *STAR* expression and induced a significant dose-dependent reduction in P4 secretion by human luteinized granulosa cells (54). This indicates that there may be a key difference between species in LXR regulation of luteal function with the LXRs stimulating P4 secretion via induction of *STAR*

expression in nonprimate cells, but not in primates.

Collectively, these data support the hypothesis that decreased P4 secretion during luteolysis is caused by enhanced LXR-mediated cholesterol efflux and reduced LDL uptake via inhibition of SREBF2. During early pregnancy hCG signaling via PKA can prevent these effects, as well as LH during the luteal phase. The molecular mechanisms mediating the effect of hCG still need to be elucidated. Some possible mechanisms include direct phosphorylation of the LXRs, SREBF2, or their transcriptional co-activators or co-repressors; alterations in the availability of endogenous LXR agonists and SREBF2 inhibitors; or changes in mRNA stability. The effect of LXR activation and SREBF2 inhibition on luteal function, and the mechanisms that normally lead to the apparent increase in LXR and decrease in SREBF2 activity during luteolysis, also need to be determined in *vivo*.

3 Chapter 3 - Endogenous 27-hydroxycholesterol produced by CYP27A1 may mediate luteolysis in the human corpus luteum

3.1 Introduction

The loss of P4 synthesis is the key event during luteolysis, a functional and structural degradation of the corpus luteum (CL). There is increased expression of liver x receptor (LXR) target genes and decreased low density lipoprotein receptor (*LDLR*) during spontaneous luteolysis in the primate CL (25). The LXRs belong to the steroid hormone receptor superfamily, which induce transcription of their target genes to stimulate cholesterol efflux (29, 37, 38). Sterol regulatory element binding transcription factor 2 (SREBF2) increases intracellular cholesterol concentrations by activating transcription of *LDLR* to increase cholesterol uptake, as well as the rate-limiting enzyme in cholesterol biosynthesis 3-hydroxy-3-methylglutaryl-CoA reductase (*HMGCR*) (31, 32, 40). Increased efflux and reduced uptake of cholesterol within the primate CL may restrict P4 production during luteolysis (25, 30).

Hydroxylated cholesterol (‘‘oxysterols’’) or biosynthetic precursors of cholesterol are the known endogenous LXR agonists that have been identified (27) including: 22R-hydoxycholesterol (byproduct of steroidogenesis); 24S-hydroxycholesterol (24SOH), 25-hydroxycholesterol (25OH), and 27-hydroxycholesterol (27OH) (enzymatically produced); 24,25-epoxy cholesterol (produced via a shunt in the cholesterol biosynthetic pathway); and desmosterol (the

immediate precursor to cholesterol) (27). The enzymatically-generated oxysterols including 24SOH, 25OH, and 27OH also prevent SREBF2 processing and translocation to the nucleus (32, 33). It has been previously reported that luteal 27OH concentrations are increased during spontaneous luteolysis in the rhesus macaque CL (30). Therefore, 27OH is an attractive candidate as the endogenous LXR agonist and SREBF2 inhibitor that stimulates the LXRs and reduces SREBF2 activity during luteolysis.

It is known that products of steroidogenesis including pregnenolone and P4 inhibit the enzyme activity of CYP27A1 (cytochrome p450, family 27, subfamily A, polypeptide 1), which converts cholesterol to 27OH (34). Decreasing steroidogenesis may increase *CYP27A1* expression and activity, which would promote 27OH synthesis and lead to increased LXR and decreased SREBF2 activity during luteolysis. Therefore, we hypothesize that 27OH produced via CYP27A1 may mediate luteolysis by inducing LXR and reducing SREBF2 activities. The objective of this study is to determine the effects of 27OH on P4 production and cholesterol metabolism; and to determine if inhibiting the conversion of cholesterol to pregnenolone increases LXR and decreases SREBF2 target gene expression via CYP27A1 in human luteinized granulosa cells.

3.2 Materials and Methods

3.2.1 Isolation of human granulosa cells

Follicular aspirates from a total of 30 women were used in this study. Human granulosa cells were isolated and cultured with luteinizing media as described in Chapter 2, except that the ITS supplement in luteinization media was substituted with 10% FBS for all experiments excluding those involving 27OH supplementation.

3.2.2 Treatment of human luteinized granulosa cells

To determine the effect of 27OH, cells were treated with vehicle (0.1% *v:v* DMSO) or 27OH (2 μM) in treatment media (serum free DMEM/F12 containing ITS, Pen/Strep, 20 μg/ml LDL and 10 μg/ml HDL cholesterol). After 16 hours, fresh treatments with or without 2 IU/ml hCG were added for another 4 hours.

To determine the effect of steroidogenesis on LXR and SREBF2 activity, cells were treated with vehicle (0.1% *v:v* DMSO), aminoglutethimide (500 μM), trilostane (10 μM) and aminoglutethimide (500 μM) with 0.1 or 1 μM P4 replacement in treatment media for 24 hours. Media was replaced with fresh treatments, and cells were incubated for an additional 4 hours until cell harvest.

3.2.3 Small interfering RNA (siRNA) transfection

After 4 days of luteinization, siRNA transfection was performed. Pre-designed Silencer® Select siRNAs (ThermoFisher Scientific) against human *STAR* (siRNA ID s13527), *CYP27A1* (siRNA ID s3887), or a non-targeting negative control siRNA

(catalog 4390843) were used. A 100 nM concentration of siRNA was diluted with Opti-MEM media (ThermoFisher Scientific), mixed with 2 µl per well (24-well plate) of TransIT-siQUEST transfection reagent (Mirus Bio LLC), and incubated for 30 minutes at room temperature before being added to cells in luteinization media for 24 hours. Cells were then treated as described previously. Knockdown efficiency for each target was confirmed by reverse transcription quantitative real-time PCR.

3.2.4 Induced and spontaneous luteolysis sheep model

All procedures involving animals were approved by the University of Arizona Institutional Animal Care and Use Committee. Procedures for collection of CL from sheep during either prostaglandin F2α (PGF2α)-induced or spontaneous luteolysis have been described previously (26).

3.2.5 Microarray Database Mining

The raw data files (.CEL) from two publically-available microarray databases in rhesus macaque CL (24, 55) were loaded into the Affymetrix Expression Console version 1.0. Expression files (.CHP) were generated after using the robust multichip average (RMA) algorithm for normalization. Normalized expression data was then searched for probeset ID MmuSTS.4195.1.S1_at, which corresponds to *CYP27A1*.

3.2.6 P4, cholesterol efflux, LDL uptake, and QPCR assays; and Statistical Analysis

The methods for these assays, as well as the statistical analysis of all experiments in this study, are the same as described in Chapter 2.

3.3 Results

3.3.1 Expression of *CYP27A1* during luteolysis in rhesus macaques and sheep

The concentration of *CYP27A1* mRNA was evaluated within macaque CL at specific stages during the luteal phase (Fig. 3.1A). There was a significant ($p < 0.05$) increase in *CYP27A1* from the mid-late to the functionally regressed late stage, and a further significant ($p < 0.05$) increase by the very late stage (menses) (Fig. 3.1A). During PGF2α-induced luteolysis in sheep, the expression of *CYP27A1* was significantly ($P < 0.05$) increased 24 and 48 h after PGF2α-treatment (Fig. 3.1B). During spontaneous lutelysis in sheep, *CYP27A1* was also significantly ($P < 0.05$) increased compared to mid-luteal phase CL (Fig. 3.1B)

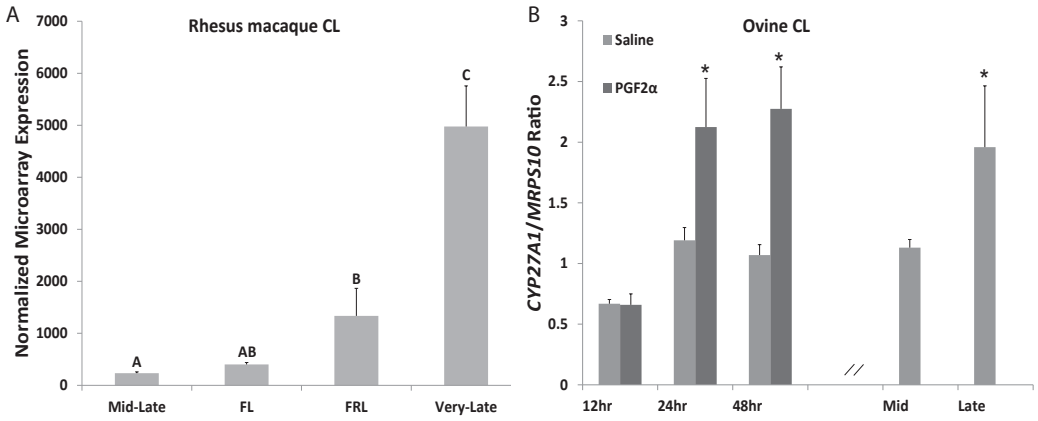

FIG. 3.1: Expression of *CYP27A1* during luteolysis in rhesus macaques and sheep. Panel A shows transcription of *CYP27A1* in rhesus macaque CL collected during the luteal phase (mid-late = days 10-12; FL, functional late = days 14-16 and P4 > 1.5 ng/ml; FRL, functional regressed late = days 14-16 and P4 < 0.5 ng/ml; very late = menses, days 18-19). Columns without a common letter are significantly ($p < 0.05$) different. Panel B is effect of luteolysis on mRNA of *CYP27A1* in ovine CL. The x axis is separated between the induced and spontaneous luteolysis models. Asterisks denote significant ($p < 0.05$) difference between saline and PGF2α treatments at the corresponding time point (induced luteolysis) or between mid- and late luteal phase CL (spontaneous luteolysis). *MRPS10* is the housekeeping control. Error bars indicate one standard error of the mean (SEM).

3.3.2 Effect of 27OH on P4 secretion, cholesterol efflux, LDL uptake, and expression of genes involved in cholesterol efflux and uptake

In the presence of hCG, P4 secretion was significantly ($P < 0.05$) decreased by 27OH. In the absence of hCG, 27OH had no effect on P4 synthesis (Fig. 3.2). Cholesterol efflux was significantly ($P < 0.05$) increased by 27OH in the presence of hCG. 27OH tended to decrease LDL uptake. 27OH significantly ($P < 0.05$) increased *ABCG1* mRNA concentration. However, 27OH had no effect on *ABCA1* and *LDLR*.

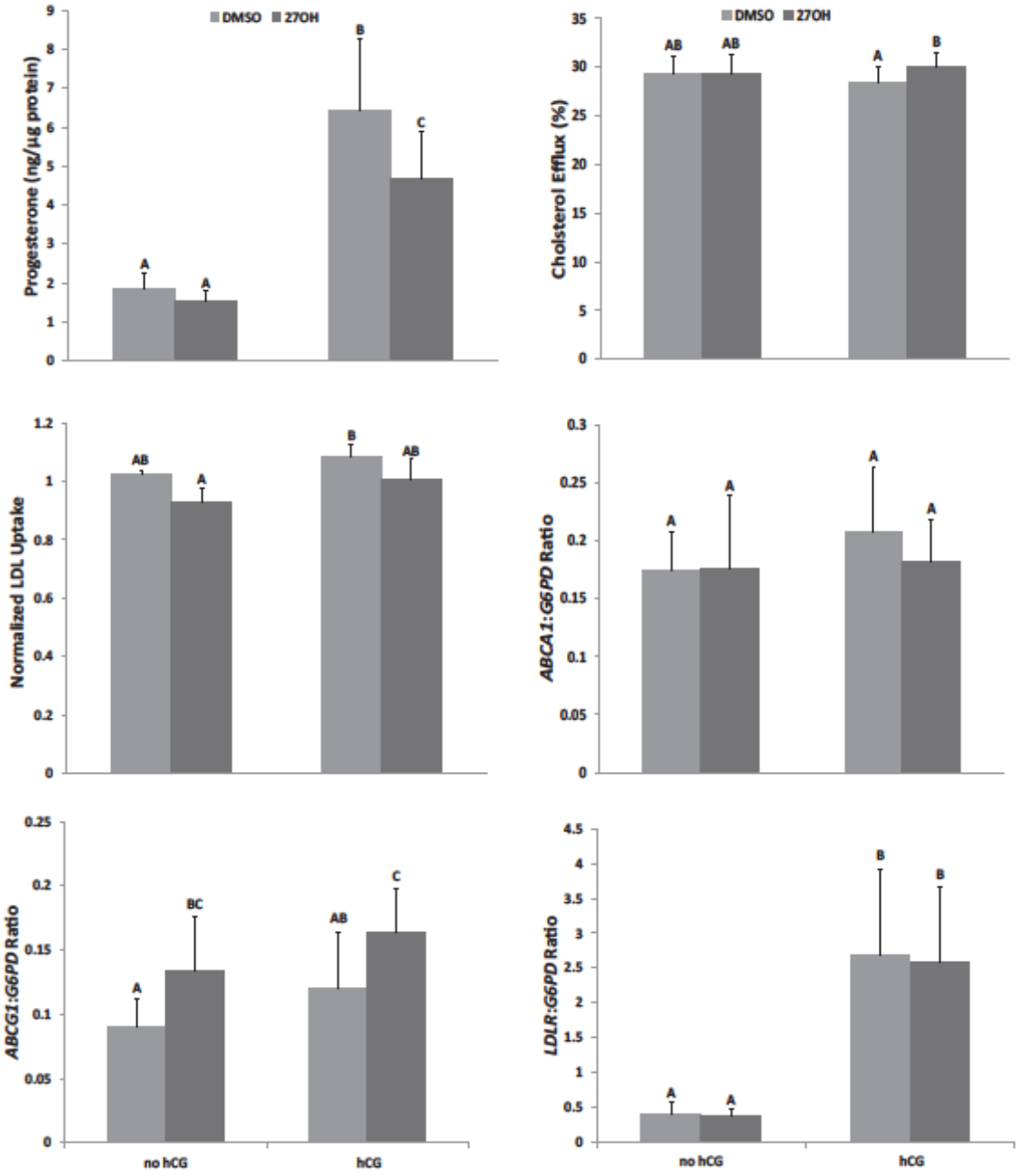
DMSO
27OH
Progesterone (ng/µg protein)
Cholsterol Efflux (%)
Normalized LDL Uptake
ABCA1:G6PD Ratio
ABCG1:G6PD Ratio
LDLR:G6PD Ratio
no hCG
hCG

FIG. 3.2: The effects of 27OH on P4 secretion, cholesterol efflux, LDL uptake and expression of genes involved in cholesterol metabolism in human luteinized granulosa cells. P4 concentrations in spent media, the percentage of cholesterol efflux, LDL uptake and *ABCA1*, *ABCG1*, and *LDLR* expression are shown. Error bars indicate one SEM. Columns without a common letter are significantly different ($p < 0.05$).

3.3.3 Effect of steroidogenesis inhibitors on LXR and SREBF2 target gene expression

Aminoglutethimide blocks the conversion of cholesterol to pregnenolone (34). Trilostane blocks the conversion of pregnenolone into P4 (56). The LXR target gene *ABCA1* was significantly ($P < 0.05$) increased, while the SREBF2 target genes *LDLR* and *HMGCR* were significantly decreased by aminoglutethimide. Replacement with 0.1 μM or 1 μM of P4 did not alter the aminoglutethimide effect. Trilostane had no effect on *ABCA1*, *LDLR*, or *HMGCR*. Transcription of *SREBF2* was significantly decreased by aminoglutethimide, while trilostane did not affect *SREBF2* expression. The transcription of *CYP27A1* was significantly ($P < 0.05$) decreased by aminoglutethimide and P4 replacement did not alter the aminoglutethimide effect. Trilostane significantly ($P < 0.05$) increased *CYP27A1* mRNA concentration (Fig. 3.3).

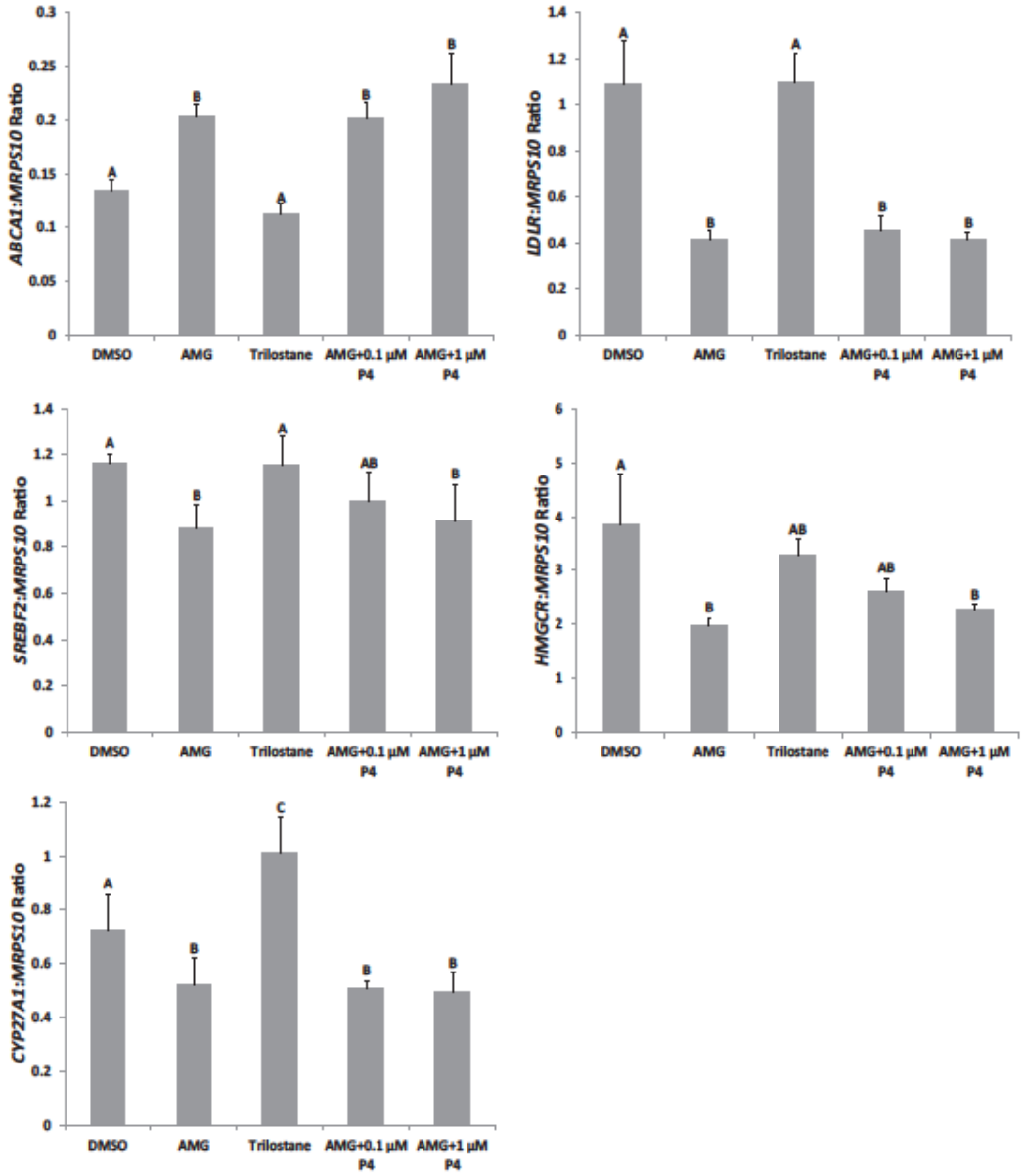

FIG. 3.3: The effects of steroidogenesis inhibitors on expression of genes involved in cholesterol metabolism in human luteinized granulosa cells. QPCR data for *ABCA1*, *LDLR*, *SREBF2*, *HMGCR*, and *CYP27A1* are shown. *MRPS10* is the housekeeping control. Error bars indicate one SEM, and columns without a common letter are significantly different ($p < 0.05$).

3.3.4 Cholesterol efflux and LDL uptake following aminoglutethimide treatment with or without hCG

In the absence of hCG, aminoglutethimide significantly ($P < 0.05$) decreased cholesterol efflux and LDL uptake in human luteinized granulosa cells. In the presence of hCG, aminoglutethimide significantly ($P < 0.05$) decreased cholesterol efflux while LDL uptake was no longer significantly decreased by aminoglutethimide (Fig. 3.4).

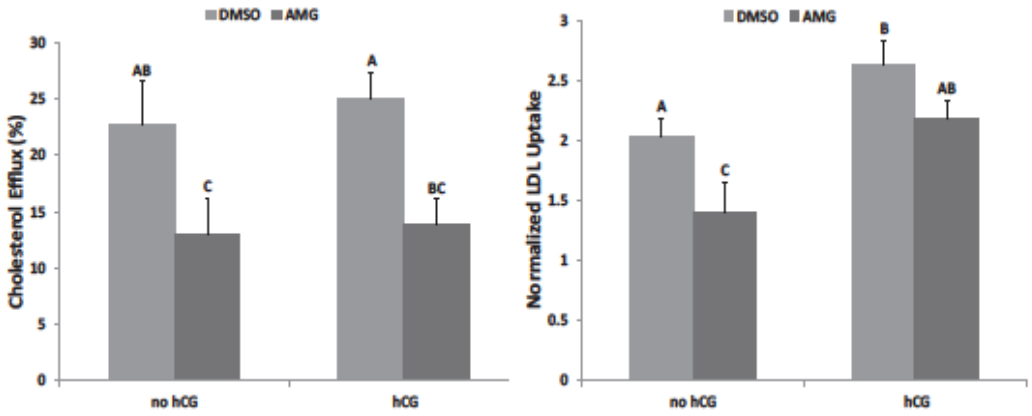

FIG. 3.4: The effects of aminoglutethimide on cholesterol efflux and LDL uptake in human luteinized granulosa cells. Percentage of cholesterol efflux and normalized LDL uptake are shown. Error bars indicate one SEM, and columns without a common letter are significantly different ($p< 0.05$).

3.3.5 Effect of *CYP27A1* knockdown on P4 production and aminoglutethimide-induced changes in gene expression

The expression of *CYP27A1* mRNA was significantly ($P < 0.05$) decreased by transfection with the *CYP27A1* siRNA. Secretion of P4 was significantly ($P < 0.05$) increased in the presence or absence of hCG in *CYP27A1* knockdown cells. Aminoglutethimide significantly increased *ABCA1* and decreased *LDLR* in control cells, which was not prevented by *CYP27A1* knockdown (Fig. 3.5).

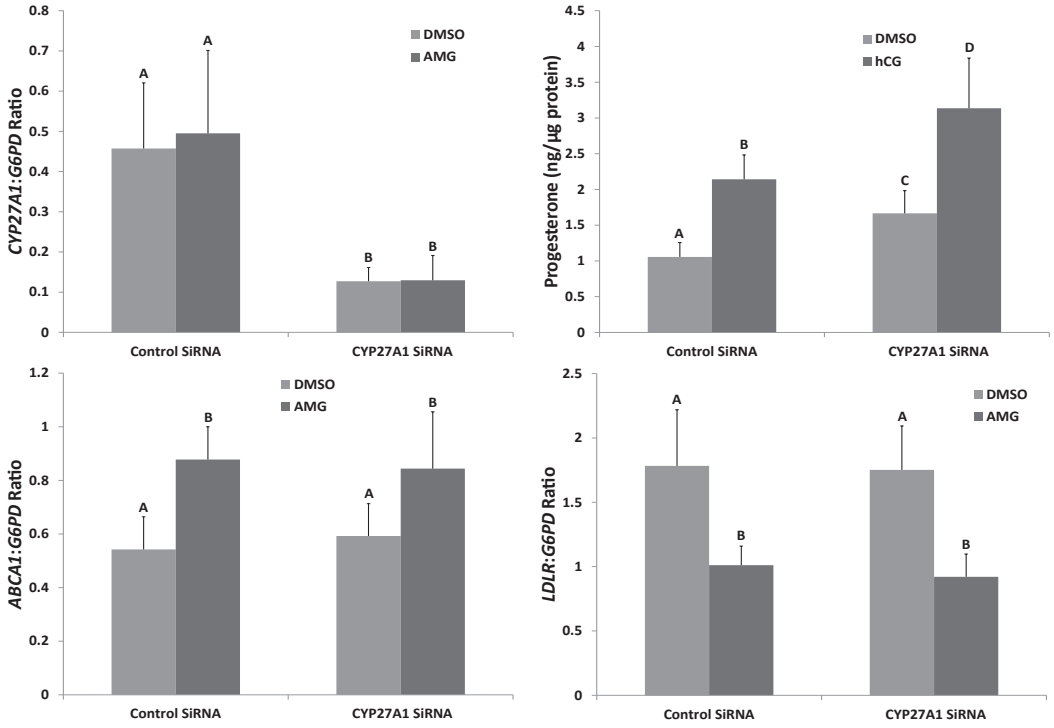

FIG. 3.5: The effect of CYP27A1 knockdown on P4 secretion, *ABCA*1 and *LDLR* expression. QPCR data for *CYP27A1*, production of P4, and *ABCA1* and *LDLR* expression in response to aminoglutethimide and CYP27A1 siRNA are shown. Error bars indicate one SEM. and columns without common letters are significantly different ($p < 0.05$).

3.3.6 Effect of *STAR* knockdown on aminoglutethimide-induced changes in gene expression

The expression of *STAR* was significantly ($P < 0.05$) decreased by transfection with the *STAR* siRNA compared to cells transfected with control siRNA in the absence of aminoglutethimide. In the presence of aminoglutethimide there was no knockdown of *STAR,* which was due to a single replicate that had higher *STAR* expression in the knockdown compared to control cells. Synthesis of P4 was significantly ($P < 0.05$) decreased in *STAR* knockdown cells compared to the control siRNA in the absence of aminoglutethimide. Aminoglutethimide significantly ($P < 0.05$) decreased P4 secretion in cells treated with control siRNA, and was not significantly different from *STAR* knockdown cells. The LXR target gene *ABCA1* was significantly ($P < 0.05$) increased by aminoglutethimide, while the *STAR* siRNA did not prevent this effect on *ABCA1* transcription. The *LDLR* was significantly ($P < 0.05$) decreased by aminoglutethimide in control cells. Knockdown of *STAR* significantly ($P < 0.05$) decreased *LDLR* transcription, and was not different from aminoglutethimide treatment (Fig. 3.6).

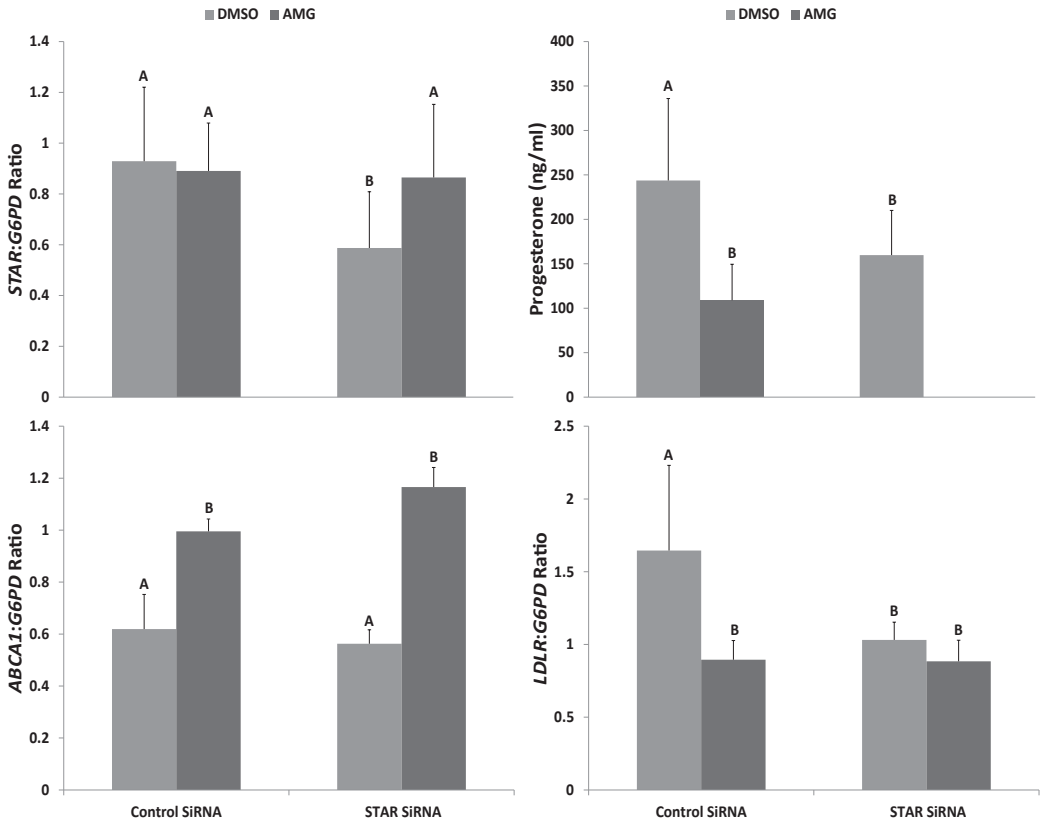

FIG. 3.6: The effect STAR knockdown on *ABCA1* and *LDLR* expression. QPCR data for *STAR* expression, production of P4, and *ABCA1* and *LDLR* expression in response to aminoglutethimide and STAR siRNA are shown. Error bars indicate one SEM. and columns without common letters are significantly different ($p < 0.05$).

3.4 Discussion

The expression of *CYP27A1* is significantly ($P < 0.05$) increased in both rhesus and ovine CL during luteolysis. This is consistent with a previous report that luteal 27OH concentrations are increased during spontaneous luteolysis in the rhesus macaque CL (30). Products of steroidogenesis including pregnenolone and P4 inhibit the enzyme activity of CYP27A1, thus limiting 27OH synthesis in the functional CL (34). In this study, 27OH significantly decreased P4 secretion, which was associated with increased cholesterol efflux and a slight decrease in LDL uptake in human luteinized granulosa cells. The effects of 27OH on cholesterol efflux, uptake, and LXR and SREBF2 target gene expression were modest. In a previous report, a 5 μM dose of 27OH significantly ($P < 0.05$) decreased *LDLR* mRNA and LDLR protein while 1 μM did not significantly alter LDLR in macaque luteal cells (30). In this study, we used a 2 μM dose of 27OH, which is likely the reason why there was no significant effect on *LDLR* mRNA and LDL uptake and only modest effects on efflux.

Aminoglutethimide blocks the conversion of cholesterol to pregnenolone (34) by inhibiting the cholesterol side-chain cleavage enzyme P450scc, a member of cytochrome P450 superfamily of enzymes. Although aminoglutethimide blocks the first step in steroidogenesis (e.g. conversion of cholesterol to pregnenolone), the synthesis of all hormonally active steroids will consequently be decreased. Trilostane inhibits P4 production by blocking the conversion of pregnenolone into P4 (56). Pregnenolone and P4 inhibit the enzyme activity of CYP27A1 (34). Therefore,

aminoglutethimide would be expected to increase the enzyme activity of CYP27A1 by inhibiting production of pregnenolone and P4. However, because trilostane only blocks P4 production and does not affect pregnenolone synthesis, it would not be expected to result in increased CYP27A1 enzyme activity. Inhibiting P4 production by trilostane did not affect the expression of *ABCA1* and *LDLR*. However, *ABCA1* was significantly increased while *LDLR* was significantly decreased by aminoglutethimide. Aminoglutethimide acts within the mitochondria where CYP27A1 is located while trilostane inhibits the enzyme 3β-HSD in smooth endoplasmic reticulum. This indicates that inhibiting cholesterol conversion to pregnenolone in the mitochondria, but not inhibition of downstream steps in steroidogenesis, results in increased LXR and decreased SREBF2 activity. Replacing P4 or pregnenolone (data not shown) did not reverse the effects of aminoglutethimide. This is possibly because the steroids were added to the culture media and were not able to enter the mitochondria where CYP27A1 is located. It was surprising that *CYP27A1* mRNA was significantly decreased by aminoglutethimide, which contrasts with the significant increase in *CYP27A1* expression observed during luteolysis in primates and sheep. However, we did not quantify CYP27A1 enzyme activity, which has previously been shown to be inhibited by pregnenolone and P4 (34). Therefore, aminoglutethimide likely still resulted in increased 27OH formation in spite of the decrease in *CYP27A1* mRNA. Furthermore, trilostane significantly increased *CYP27A1* transcription, which is consistent with *CYP27A1* expression during luteolysis in primates and sheep *in vivo* and indicates that P4 may repress *CYP27A1*

transcription.

We successfully knocked down *CYP27A1* expression by approximately 70% in human luteinized granulosa cells. This resulted in a significant increase in P4 secretion, which is complementary to the significant decrease in hCG-stimulated P4 secretion caused by 27OH. However, knockdown of *CYP27A1* did not prevent the aminoglutethimide-induced increase in *ABCA1* and decrease in *LDLR*. This indicates that another oxysterol could be mediating the aminoglutethimide effect instead of, or in addition to, 27OH. *STAR* was knocked down by a modest 30-40% in human luteinized granulosa cells. However, the knockdown of STAR was secondarily validated by the significant decrease in P4 concentrations. STAR plays a critical role in the PKA-induced transport of cholesterol across the mitochondrial membrane, the rate-limiting step in steroidogenesis (53). While knockdown of *STAR* would prevent cholesterol transport into the mitochondria where CYP27A1 resides, it also may result in accumulation of cholesterol in the cytoplasm. Cholesterol 25-hydroxylase is a multi-transmembrane endoplasmic reticulum (ER) protein that catalyzes the production of 25OH, another oxysterol that inhibits the cleavage of sterol regulatory element binding proteins (SREBPs) in the endoplasmic reticulum (57, 58). When levels of cholesterol are high cholesterol binding to the sterol-sensing domain of SREBP cleavage activating protein (SCAP), or 25OH binding to insulin-induced gene (INSIG) proteins, promotes complex formation between INSIG proteins and SCAP–SREBP. This blocks transport of SCAP–SREBP to the Golgi, which is necessary for proteolytic processing and activation of SREBP transcriptional activity (58).

Therefore, because expression of the SREBF2 target gene *LDLR* was significantly decreased in STAR siRNA transfected cells, this indicates that the interruption of cholesterol transport into the mitochondria caused by inhibition of STAR function results in reduced SREBF2 activity due to the accumulation of cholesterol and/or 25OH.

Overall, these data support the hypothesis that CYP27A1-derived 27OH may contribute to the P4 decrease during luteolysis, partially by activating the LXRs and inhibiting SREBF2. However, other oxysterols may also be involved. The roles of other endogenous oxysterols in mediating cholesterol metabolism and P4 production during luteolysis await further clarification.

4 References

1. Csapo AI, Pulkkinen MO, Ruttner B, Sauvage J, Wiest W. The significance of the human corpus luteum in pregnancy maintenance: I. Preliminary studies. American journal of obstetrics and gynecology. 1972;112(8):1061-7.

2. Csapo AI, Pulkkinen MO, Wiest W. Effects of luteectomy and progesterone replacement therapy in early pregnant patients. American journal of obstetrics and gynecology. 1973;115(6):759-65.

3. Hoyer PB. Regulation of luteal regression: the ewe as a model. Journal of the Society for Gynecologic Investigation. 1998;5(2):49-57.

4. Stouffer RL. Structure, function, and regulation of the corpus luteum. Knobil and Neill's physiology of reproduction: Elsevier Inc.; 2006.

5. Baird DD, Weinberg CR, McConnaughey DR, Wilcox AJ. Rescue of the corpus luteum in human pregnancy. Biology of reproduction. 2003;68(2):448-56.

6. Niswender GD, Juengel JL, Silva PJ, Rollyson MK, McIntush EW. Mechanisms controlling the function and life span of the corpus luteum. Physiological reviews. 2000;80(1):1-29.

7. Anderson L, Neal F, Melampy R. Hysterectomy and ovarian function in beef heifers. American journal of veterinary research. 1962;23:794.

8. Wiltbank J, Casida L. Alteration of ovarian activity by hysterectomy. Journal of Animal Science. 1956;15(1):134-40.

9. Anderson L, Rathmacher R, Melampy R. The uterus and unilateral regression of

corpora lutea in the pig. American Journal of Physiology--Legacy Content. 1966;210(3):611-4.

10. Heap R, Perry J, Rowlands I. Corpus luteum function in the guinea-pig; arterial and luteal progesterone levels, and the effects of hysterectomy and hypophysectomy. Journal of reproduction and fertility. 1967;13(3):537-53.

11. Fairclough R, Moore L, McGowan L, Peterson A, Smith J, Tervit H, et al. Temporal relationship between plasma concentrations of 13, 14-dihydro-15-keto-prostaglandin F and neurophysin I/II around luteolysis in sheep. Prostaglandins. 1980;20(2):199-208.

12. McCracken JA, Custer EE, Eldering JA, Robinson AG. The central oxytocin pulse generator: a pacemaker for the ovarian cycle. Acta neurobiologiae experimentalis. 1996;56(3):819-32.

13. Tsai S-J, Wiltbank MC. Prostaglandin F2α induces expression of prostaglandin G/H synthase-2 in the ovine corpus luteum: a potential positive feedback loop during luteolysis. Biology of reproduction. 1997;57(5):1016-22.

14. Silvia W, Lewis G, McCracken J, Thatcher W, Wilson Jr L. Hormonal regulation of uterine secretion of prostaglandin F2α during luteolysis in ruminants. Biology of reproduction. 1991;45(5):655-63.

15. Auletta FJ, Speroff L, Caldwell BV. Prostaglandin F2α induced steroidogenesis and luteolysis in the primate corpus luteum. The Journal of Clinical Endocrinology & Metabolism. 1973;36(2):405-7.

16. Daniel Castracane V, Moore GT, Shaikh AA. Ovarian function in

hysterectomized Macaca fascicularis. Biology of reproduction. 1979;20(3):462-72.

17. Ranney B, Abu-Ghazaleh S. The future function and fortune of ovarian tissue which is retained in vivo during hysterectomy. American journal of obstetrics and gynecology. 1977;128(6):626-34.

18. Duffy DM, Stewart DR, Stouffer RL. Titrating luteinizing hormone replacement to sustain the structure and function of the corpus luteum after gonadotropin-releasing hormone antagonist treatment in rhesus monkeys. The Journal of Clinical Endocrinology & Metabolism. 1999;84(1):342-9.

19. Brannian JD, Stouffer RL. Progesterone production by monkey luteal cell subpopulations at different stages of the menstrual cycle: changes in agonist responsiveness. Biology of reproduction. 1991;44(1):141-9.

20. CAMERON JL, STOUFFER RL. Gonadotropin receptors of the primate corpus luteum. II. Changes in available luteinizing hormone-and chorionic gonadotropin-binding sites in macaque luteal membranes during the nonfertile menstrual cycle. Endocrinology. 1982;110(6):2068-73.

21. EYSTER KM, OTTOBRE JS, STOUFFER RL. Adenylate cyclase in the corpus luteum of the rhesus monkey. III. Changes in basal and gonadotropin-sensitive activities during the luteal phase of the menstrual cycle. Endocrinology. 1985;117(4):1571-7.

22. Ellinwood WE, Norman RL, Spies HG. Changing frequency of pulsatile luteinizing hormone and progesterone secretion during the luteal phase of the menstrual cycle of rhesus monkeys. Biology of reproduction. 1984;31(4):714-22.

23. HUTCHISON JS, NELSON PB, ZELEZNIK AJ. Effects of different gonadotropin pulse frequencies on corpus luteum function during the menstrual cycle of rhesus monkeys. Endocrinology. 1986;119(5):1964-71.

24. Bogan RL, Murphy MJ, Hennebold JD. Dynamic changes in gene expression that occur during the period of spontaneous functional regression in the rhesus macaque corpus luteum. Endocrinology. 2008;150(3):1521-9.

25. Bogan RL, Hennebold JD. The reverse cholesterol transport system as a potential mediator of luteolysis in the primate corpus luteum. Reproduction. 2010;139(1):163-76.

26. Seto NL, Bogan RL. Decreased Cholesterol Uptake and Increased Liver X Receptor-Mediated Cholesterol Efflux Pathways During Prostaglandin F2 Alpha-Induced and Spontaneous Luteolysis in Sheep. Biology of reproduction. 2015;92(5):128, 1-9.

27. Wójcicka G, Jamroz-Wiśniewska A, Horoszewicz K, Bełtowski J. Liver X receptors (LXRs). Part I: Structure, function, regulation of activity, and role in lipid metabolism Receptory wątrobowe X (LXR). Część I: Budowa, funkcja, regulacja aktywności i znaczenie w metabolizmie lipidów. Postepy Hig Med Dosw(online). 2007;61:736-59.

28. Nilsson M, Stulnig TM, Lin C-Y, Yeo AL, Nowotny P, Liu ET, et al. Liver X receptors regulate adrenal steroidogenesis and hypothalamic-pituitary-adrenal feedback. Molecular Endocrinology. 2007;21(1):126-37.

29. Zelcer N, Hong C, Boyadjian R, Tontonoz P. LXR regulates cholesterol uptake

through Idol-dependent ubiquitination of the LDL receptor. Science. 2009;325(5936):100-4.

30. Bogan RL, DeBarber AE, Hennebold JD. Liver x receptor modulation of gene expression leading to proluteolytic effects in primate luteal cells. Biology of reproduction. 2012;86(3):89, 1-9.

31. Horton JD, Goldstein JL, Brown MS. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. The Journal of clinical investigation. 2002;109(9):1125.

32. Goldstein JL, DeBose-Boyd RA, Brown MS. Protein sensors for membrane sterols. Cell. 2006;124(1):35-46.

33. Hong C, Tontonoz P. Liver X receptors in lipid metabolism: opportunities for drug discovery. Nature reviews Drug discovery. 2014;13(6):433-44.

34. RENNERT H, FISCHER RT, ALVAREZ JG, TRZASKOS JM, STRAUSS III JF. Generation of regulatory oxysterols: 26-hydroxylation of cholesterol by ovarian mitochondria. Endocrinology. 1990;127(2):738-46.

35. Lipsett M. Steroid hormones. Reproductive endocrinology. 1986:140-5.

36. Csapo AI, Pulkkinen MO, Ruttner B, Sauvage JP, Wiest WG. The significance of the human corpus luteum in pregnancy maintenance. I. Preliminary studies. Am J Obstet Gynecol. 1972;112(8):1061-7.

37. Laffitte BA, Joseph SB, Walczak R, Pei L, Wilpitz DC, Collins JL, et al. Autoregulation of the human liver X receptor α promoter. Molecular and cellular biology. 2001;21(22):7558-68.

38. Lund EG, Menke JG, Sparrow CP. Liver X receptor agonists as potential therapeutic agents for dyslipidemia and atherosclerosis. Arteriosclerosis, thrombosis, and vascular biology. 2003;23(7):1169-77.

39. Grummer R, Carroll D. A review of lipoprotein cholesterol metabolism: importance to ovarian function. Journal of Animal Science. 1988;66(12):3160-73.

40. Shimomura I, Shimano H, Horton JD, Goldstein JL, Brown MS. Differential expression of exons 1a and 1c in mRNAs for sterol regulatory element binding protein-1 in human and mouse organs and cultured cells. Journal of Clinical Investigation. 1997;99(5):838.

41. Ascoli M, Fanelli F, Segaloff DL. The lutropin/choriogonadotropin receptor, a 2002 perspective. Endocrine reviews. 2002;23(2):141-74.

42. Marsh JM, Savard K. The stimulation of progesterone synthesis in bovine corpora lutea by adenosine 3', 5'-monophosphate (1). Steroids. 1966;8(2):133-48.

43. Leung PC, Steele GL. Intracellular signaling in the gonads. Endocrine reviews. 1992;13(3):476-98.

44. Stewart DR, VandeVoort CA. Simulation of human luteal endocrine function with granulosa lutein cell culture. The Journal of Clinical Endocrinology & Metabolism. 1997;82(9):3078-83.

45. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Canadian journal of biochemistry and physiology. 1959;37(8):911-7.

46. Kamisuki S, Mao Q, Abu-Elheiga L, Gu Z, Kugimiya A, Kwon Y, et al. A small molecule that blocks fat synthesis by inhibiting the activation of SREBP. Chemistry

& biology. 2009;16(8):882-92.

47. Li X, Chen Y-T, Hu P, Huang W-C. Fatostatin displays high antitumor activity in prostate cancer by blocking SREBP-regulated metabolic pathways and androgen receptor signaling. Molecular cancer therapeutics. 2014;13(4):855-66.

48. Cummins CL, Volle DH, Zhang Y, McDonald JG, Sion B, Lefrançois-Martinez A-M, et al. Liver X receptors regulate adrenal cholesterol balance. Journal of Clinical Investigation. 2006;116(7):1902.

49. Manna PR, Cohen-Tannoudji J, Counis R, Garner CW, Huhtaniemi I, Kraemer FB, et al. Mechanisms of Action of Hormone-sensitive Lipase in Mouse Leydig Cells ITS ROLE IN THE REGULATION OF THE STEROIDOGENIC ACUTE REGULATORY PROTEIN. Journal of Biological Chemistry. 2013;288(12):8505-18.

50. Cheng H-C, Kemp B, Pearson R, Smith AJ, Misconi L, Van Patten SM, et al. A potent synthetic peptide inhibitor of the cAMP-dependent protein kinase. Journal of Biological Chemistry. 1986;261(3):989-92.

51. Marunaka Y, Niisato N. H89, an inhibitor of protein kinase A (PKA), stimulates Na+ transport by translocating an epithelial Na+ channel (ENaC) in fetal rat alveolar type II epithelium. Biochemical pharmacology. 2003;66(6):1083-9.

52. Lochner A, Moolman J. The many faces of H89: a review. Cardiovascular Therapeutics. 2006;24(3-4):261-74.

53. Manna PR, Stetson CL, Slominski AT, Pruitt K. Role of the steroidogenic acute regulatory protein in health and disease. Endocrine. 2016;51(1):7-21.

54. Drouineaud V, Sagot P, Garrido C, Logette E, Deckert V, Gambert P, et al.

Inhibition of progesterone production in human luteinized granulosa cells treated with LXR agonists. Molecular human reproduction. 2007;13(6):373-9.

55. Bogan RL, Murphy MJ, Stouffer RL, Hennebold JD. Systematic determination of differential gene expression in the primate corpus luteum during the luteal phase of the menstrual cycle. Molecular Endocrinology. 2008;22(5):1260-73.

56. KOMANICKY P, SPARK RF, MELBY JC. Treatment of Cushing's syndrome with trilostane (WIN 24,540), an inhibitor of adrenal steroid biosynthesis. The Journal of Clinical Endocrinology & Metabolism. 1978;47(5):1042-51.

57. Adams CM, Reitz J, De Brabander JK, Feramisco JD, Li L, Brown MS, et al. Cholesterol and 25-hydroxycholesterol inhibit activation of SREBPs by different mechanisms, both involving SCAP and Insigs. Journal of Biological Chemistry. 2004;279(50):52772-80.

58. Cyster JG, Dang EV, Reboldi A, Yi T. 25-Hydroxycholesterols in innate and adaptive immunity. Nature reviews Immunology. 2014;14(11):731.