THE SEARCH FOR THE ENDOGENOUS RORg LIGAND

AND

INVESTIGATING THE ROLE OF CYTOCHROME B5 IN

STEROIDOGENESIS AND REGULATION

OF THE CYP17A1 LYASE REACTION

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RORg is an orphan nuclear receptor important in the regulation of immune development and function. RORg regulated TH17 cells have been implicated in the pathology of various autoimmune diseases including multiple sclerosis and rheumatoid arthritis. Targeting RORg through antagonists has emerged as a novel therapeutic tool in the treatment of autoimmune diseases. Identification of the RORg endogenous ligand would offer insight into RORg regulation. It is currently believed that sterols are the endogenous RORg ligands. In these studies I will show that while sterols can bind RORg, they fail to modulate its activity in-vivo or in-vitro. Endogenously extracted

lysophospholipids on the other hand, such as 22:4 LPE, can activate RORg in co-transfection assays and may be RORg ligands. Additionally, 22:4 LPE can increase IL17 production in TH17 cells.

My second project involved investigating the role of cytochrome B5 in steroidogenesis. Cyb5 is involved in regulating electron transfer to numerous P450-mono-oxygenases Cyb5's role in human physiology has been confirmed by the discovery of patients with mutations in Cyb5 that present with isolated 17, 20-lyase deficiency, characterized by low plasma androgens and ambiguous external genitalia. To study the consequences of Cyb5 deficiency in an intact animal and in steroidogenic tissue, I generated mice lacking Cyb5 in the Leydig cell by crossing $Cyb5^{fl/fl}$ and Cre^{SF1} animals. I show that the $Cyb5^{-/-}$ animals were born in a normal Mendelian ratio and had normal fertility with no overt phenotype. Testicular histology revealed no differences between $Cyb5^{-/-}$ and WT animals. Homogenates from $Cyb5^{-/-}$ testes had normal progesterone (P)-to-17α-hydroxyprogesterone (17-OHP) conversion but low 17-OHP-to-androstenedione (A) and testosterone (T) metabolism. The ratio of the hydroxylase to lyase activity was observed to be 1.7 in the WT and 4.5 (3-fold higher) in the $Cyb5^{-/-}$ testes due to deficient lyase activity in the knockout animals. However, steroid production was found to be normal in these animals. Exogenous hCG administration gave a large increase in serum steroids for both the $Cyb5^{-/-}$ and WT animals. In the $Cyb5^{-/-}$ animals, this rise was accompanied by the accumulation of 17-OHP in serum, which led to a 17-OHP/(A+T) ratio that was 44-fold in the KOs. Thus these data demonstrate the physiological significance of Cyb5 in the Cyp17a1 lyase reaction.

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LIST OF ABBREVIATIONS

11-DOC	11-Deoxy Corticosterone
17-OHP	17-Hydroxy Progesterone
22R-HC	22-Hydroxycholesterol
25-HC	25-Hydroxycholesterol
AA	Arachidonic Acid
ABC	ATP Binding Cassette
ACAT	Acyl-CoA:Cholesterol Acyltransferase
ACTH	Adrenocorticotropic Hormone
AF1	Activation Function 1
AF2	Activation Function 2
ALD	Adrenoleukodystrophy
ALPHA Screen	Amplified Luminescent Proximity Homogeneous Assay
APCI	Atmospheric Pressure Chemical Ionization
AR	Androgen Receptor
AS	Aldosterone Synthase
ATP	Adenosine Triphosphate
β-gal	Beta-Galactosidase
BSA	Bovine Serum Albumin
C18-SB	Carbon-18 Stable Bond
CAH	Congenital Adrenal Hyperplasia
CAN	Acetonitrile
CD	Cluster of Differentiation
CIA	Collagen Induced Arthritis
CMC	Critical Micelle Concentration
CMO	Corticosterone Methyl Oxidase Deficiency

CNS	Central Nervous System
Cyb5	Cytochrome B5
DBD	DNA Binding Domain
DC	Dendritic Cell
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
DMAP	4-Dimethylaminopyridine
DMEM	Dulbecco’s Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DP	Double Positive
EAE	Experimental Autoimmune Encephalomyelitis
EDTA	Ethylenediaminetetraacetic Acid
EL4	Mouse Thymocyte Cell Line
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Estrogen Receptor
ER	Endoplasmic Reticulum
ESI	Electrospray Ionization
FA	Fatty Acid
FAD	Flavin Adenine Dinucleotide
FBS	Fetal Bovine Serum
FMN	Flavin Mononucleotide
FSH	Follicle-Stimulating Hormone
FXR	Farnesoid X Receptor
GR	Glucocorticoid Receptor
GST	Glutathione S-Transferases
HCG	Human Chorionic Gonadotropin
HEK293	Human Embryonic Kidney 293
HepG2	Liver Hepatocellular Carcinoma

His	Histidine
HMG-Coa	3-hydroxy-3-methylglutaryl-coenzyme A
HPLC	High Pressure Liquid Chromatography
HS	Horse Serum
HSD	Hydroxysteroid Dehydrogenase
HSL	Hormone Sensitive Lipase
I-BAT	Intestinal Bile Acid Transporter
ID	Internal Diameter
IL	Interleukin
IMM	Inner Mitochondrial Membrane
IPA	n-propanol
IPTG	Isopropyl β-D-1-Thiogalactopyranoside
LBD	Ligand Binding Domain
LDL	Low-Density Lipoprotein
LH	Luteinizing Hormone
LPA	Lysophosphatidic Acid
LPC	Lysophosphatidylcholine
LPCAT	Lysophosphocholine Acyl Transferase
LPE	Lysophosphatidylethanolamine
LPI	Lysophosphatidylinositol
LPS	Lysophosphatidylserine
Luc	Luciferase
LXR	Liver X Receptor
M/Z	Mass/Charge Ratio
MEM	Minimum Essential Media
MeOH	Methanol
MHC	Major Histocompatibility Complex
MOG	Myelin Oligodendrocyte Glycoprotein

MR	Mineralocorticoid Receptor
MS	Mass Spectrometer
MS/MS	Tandem Mass Spec
NH4AC	Ammonium Acetate
NH4OH	Ammonium Hydroxide
OMM	Outer Mitochondrial Membrane
OST	Organ Sulfur Transporter
PA	Phosphatidic Acid
PAF	Platelet Activating Factor
PC	Phosphatidylcholine
PCOS	Polycystic Ovary Syndrome
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PLA1	Phospholipase A1
PLA2	Phospholipase A2
PLD	Phospholipase D
PMSF	Phenylmethanesulfonylfluoride
POR	P450-oxidoreductase
PPAR	Peroxisome Proliferator Activated Receptor
PR	Progesterone Receptor
PS	Phosphatidylserine
PXR	Pregnane X Receptor
RAR	Retinoic Acid Receptor
ROR	RAR-related Orphan Receptor
RORE	RAR-related Orphan Receptor Response Element
RPMI	Roswell Park Memorial Institute Medium
RT	Retention Time
RT-qPCR	RT-Quantitative Polymerase Chain Reaction

RXR	Retinoid X Receptor
S2	Schneider 2 Cells
SAR	Structure Activity Relationship
SCA	Statistical Coupling Analysis
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
SERM	Selective Estrogen Receptor Modulator
SF-1	Steroidogenic Factor 1
SFB	Segmented Filamentous Bacteria
SRC	Steroid Receptor Co-activator
StAR	Steroidogenic Acute Regulatory Protein
Tcomp	T0901317
TCR	T-Cell Receptor
TGF-β	Transforming Growth Factor β
TH1	T-Helper Type 1
TH17	T-Helper Type 17
TH2	T-Helper Type 2
TLR	Toll Like Receptor
TNF-α	Tumor Necrosis Factor Alpha
TR	Thyroid Hormone Receptor
VDR	Vitamin D Receptor

CHAPTER 1

Introduction to RORg

1.1 Introduction to Nuclear Receptors

Nuclear receptors are ligand dependent transcription factors involved in a multitude of biological processes. These transcription factors act by binding directly to DNA leading to gene transcription. Nuclear receptor activity can be controlled by numerous methods, the tissue specific and cell specific regulation of receptor transcription, availability of ligand, and expression of various co-activators and co-repressors that can enhance or repress receptor function. The human nuclear receptor family has 48 members most of which have the same general structure. These consist of an NH2-terminal ligand independent activation function (AF1), a DNA binding domain consisting of two highly conserved zinc finger motifs, a central hinge region, the nuclear receptor LBD that varies greatly between receptors, and finally the COOH domain containing the ligand dependent activation function (AF2) (Mangelsdorf et al. 1995).

Nuclear receptors can be divided into three categories, the traditional endocrine receptors, the adopted orphan receptors whose ligands have recently been identified, and the orphan receptors whose ligands are yet unknown. The traditional endocrine receptors, glucocorticoid (GR), mineralocorticoid (MR), estrogen (ER), androgen (AR), progesterone (PR), vitamin D (VDR), retinoic acid receptor (RAR) and thyroid hormone (TR) receptors were already well known before the discovery of nuclear receptors

because of the myriad of human diseases associated with their malfunction. These receptors bind to their response elements as monomers. They are activated by hormones that are endogenously synthesized ligands that bind with a K_d in the low nanomolar to picomolar range (0.001 – 10 nM). Synthesis of their ligands is under strict endocrine control and generally involves the hypothalamic-pituitary axis. These receptors regulate diverse processes from sexual development to carbohydrate metabolism, and changes in receptor function or ligand availability can cause a number of human diseases. Administration of receptor agonists and antagonists has long been a treatment for these conditions (Chawla et al. 2001).

The second family of nuclear receptors is the adopted orphan receptors. These are receptors for which ligands have recently been identified. These include retinoid X receptor (RXR), liver X receptors (LXRα/LXRβ), peroxisome proliferator-activated receptors (PPARα/PPARγ/PPARδ), pregnane X receptor (PXR) and the farnesoid X receptor (FXR). These receptors all bind DNA as RXR heterodimers. Unlike the steroid receptors that exist in the nucleus or cytoplasm bound to chaperones, the RXR heterodimers exist already bound to their response elements and co-repressors. Ligand binding leads to displacement of the co-repressors and recruitment of co-activators leading to gene transcription. Thus activity of these receptors is not controlled by binding to DNA, rather interaction with associating co-repressors or co-activators. The adopted orphan receptors are lipid modulators that control lipid homeostasis by regulating the absorption, metabolism and synthesis of various lipids. For example LXR regulates cholesterol levels by a number of mechanisms. LXR up-regulates the ATP binding cassette (ABC) transporters that regulate cholesterol efflux and from various tissues

including the small intestine and liver. Additionally, LXR induces expression of CYP7A1, the rate limiting step in bile acid synthesis, increasing conversion of cholesterol to bile acids. The activity of LXR is modulated by oxysterols, a group of cholesterol metabolites with oxygen moieties on the side chain. The RXR heterodimers, like LXR, tend to bind their ligands with a K_d in the micromolar range, in agreement with their action as regulators of dietary lipids, whose endogenous concentrations are often in the micromolar range. The exception to the paradigm of RXR heterodimers being lipid sensors are the three endocrine receptors, TR, RAR and VDR. These three are activated by ligands whose synthesis and availability are tightly controlled, yet require external sources for their synthesis. Unlike the lipid sensors, these receptors function in an endocrine manner, regulating growth and development (Chawla et al. 2001).

The existence of RXR heterodimers suggests that the heterodimers could be activated by either the ligand for RXR or for its heterodimeric partner. Indeed RXR and its heterodimers can exist in three activation states. The first, the permissive state, like the RXR/LXR heterodimers, allows activation by the RXR ligand, the LXR ligand or both (Willy and Mangelsdorf 1997). In contrast to the permissive state, the conditional state does not allow activation by the RXR ligand in the absence of the ligand for its binding partner. An example of this is the RXR/RAR heterodimers, where RAR cannot be activated by the RXR ligand in the absence of the RAR ligand. The third and final example is the RXR/VDR heterodimers that exists in a non-permissive conformation. The RXR ligand is unable to activate the RXR/VDR heterodimer even in the presence of the VDR ligand (Germain et al. 2002). An allosteric network of residues links the LBD of one member of the RXR heterodimers to the other through the dimerization interface,

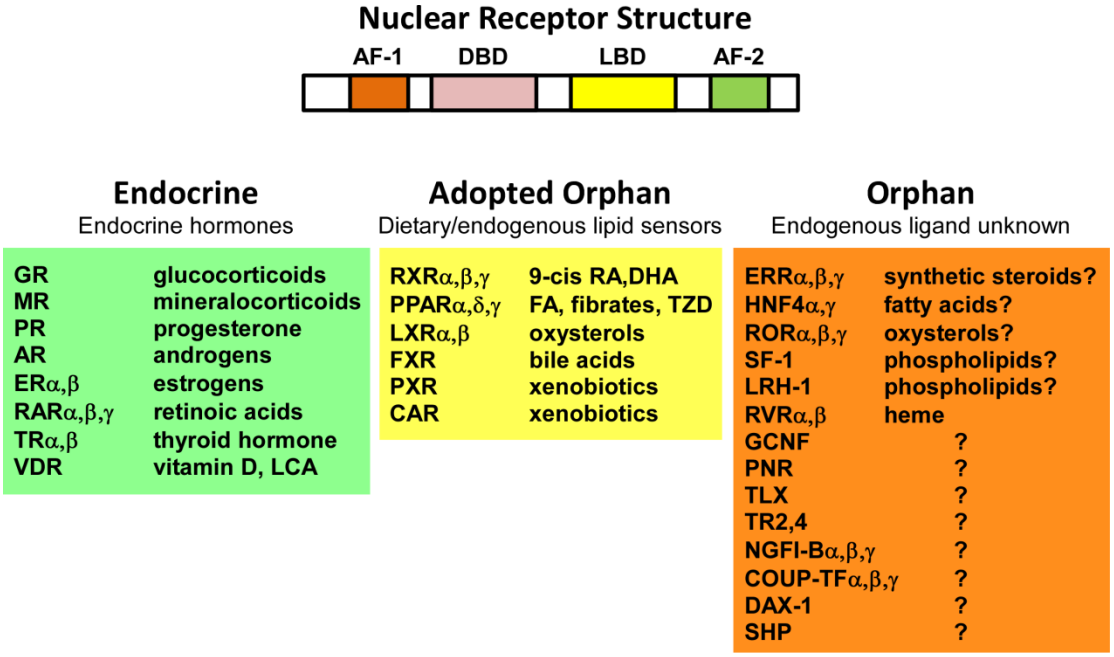


Figure 1.1 The nuclear receptor superfamily. The 48 human nuclear receptors and their endogenous ligands are depicted above. General nuclear receptor structure, consisting of an AF-1 domain, a DNA binding domain (DBD), a ligand bind domain (LBD) and an AF-2 domain is also shown.

thus imparting a permissive, conditional or non-permissive conformation. Using statistical coupling analysis (SCA) to map amino acid interactions, Shulman et al identified residues essential for this allosteric coupling to occur (Shulman et al. 2004). One mutation in the LXR-LBD (E296A) converted the RXR/LXR heterodimer from a permissive to conditional heterodimer. RXR heterodimers are unique in their ability to respond to ligands for both RXR and its binding partner depending on the nature of the interaction between them, permissive, conditional or non-permissive.

The third nuclear receptor family is the orphan nuclear receptors. These include a number of receptors for whom the endogenous ligand is currently unknown. A number of

these receptors, including RORa, RORb and RORg, bind to their response elements as monomers.

1.2 Nuclear Receptor Ligands and their Discovery

Nuclear receptor ligands are hydrophobic molecules that can easily cross the cell membrane and bind to the hydrophobic pocket in the ligand binding domain. Naturally occurring nuclear receptor ligands vary from the cholesterol derived steroids for the steroidogenic receptors, to fatty acids for the PPARs. For the steroid receptors, the ligands have been known since the 1940s, yet the receptors were only recently cloned and characterized. Different ligands can have different affinities for the receptor, with varying potencies and efficacies. Additionally, different ligands may have different actions on various subtypes, isoforms and splice variants of the same receptor.

A ligand can additionally be an agonist in some tissues and an antagonist in others. This is thought to be due to differences in co-activator and co-repressor expression in different tissues. For example, ER agonists/antagonists are known as selective estrogen receptor modulators (SERMs), because they can behave as agonists in some tissues and antagonists in others (Weatherman et al. 1999). Tamoxifen, a drug commonly used for ER positive breast cancer, is an antagonist for ER in breast but an agonist in bone and uterine tissue. A look at the structure of the antagonist vs the agonist reveals that the antagonist generally has the same structure as the agonist, allowing it to fit in the LBD; the most common difference being a large group present in the center of the molecule (Ribeiro et al. 1998). The discovery of endogenous nuclear receptor ligands

can not only help provide a basis for rational design of synthetic agonists that can be used therapeutically in cases of low levels of the endogenous ligand, but also provide a structure upon which novel antagonists can be based. In the case of RORg, the discovery of an agonist would provide a structural framework for antagonist development. Additionally, disrupting agonist synthesis and metabolism would provide an additional route to target RORg activity. Both these methods would be valuable tools in the treatment of TH17 modulated autoimmune diseases.

Nuclear receptor agonists/antagonists and endogenous ligands have been discovered by a number of different approaches. Therapeutic drugs to treat diseases caused by malfunctions in the endocrine receptors were discovered by trial and error, prior to cloning of the receptor. When an agonist is already know, rational design based on LBD structure can be utilized to create more specific agonists. Due to the pro-estrogenic effects of tamoxifen on uterine tissue and its potential to cause uterine cancer, raloxifene was developed as an ER antagonist in breast tissue but with little effect on uterine ER (Scott et al. 1999).

Recent discoveries of ligands for the orphan receptors utilized a co-transfection assay, where either a full length receptor and a plasmid containing the receptor response element upstream of luciferase, or a LBD-Gal4 fusion with a UAS-luc, are co-transfected into a cell line. This allows screening for both agonists and antagonists based on increases or decreases in luciferase production. This method was employed to discover oxysterols as the LXR agonists (Janowski et al. 1996) and bile acids as the FXR agonist (Makishima et al. 1999). A general approach involves testing a compound library against the receptor to develop a structure activity relationship (SAR) that provides insight into

the requirements for compound binding to the LBD. In some cases, such as the *c.elegans* DAF12 receptor, important in larval diapause and adult longevity, the ligand was discovered by an approach that involved utilizing a SAR and discovery of the cytochrome P450 DAF9, required for the final step in DAF12 ligand synthesis (Motola et al. 2006). Often times the function of a nuclear receptor can provide insight into its potential ligands. LXR is a sterol sensor that regulates intra and extracellular sterol levels. The LXR ligand was likely to be a sterol derivative. FXR regulates bile acid homeostasis, and consequently its ligand is a bile acid. Similarly, the PPARs are involved in fatty acid catabolism and storage, and their ligands are free fatty acids. For RORg, its role in immune development and function suggests lipids involved in immune function, such as the eicosanoids and phospholipids, may be potential ligands.

The possibility also exists that some orphan nuclear receptors have no endogenous ligands and their mode of regulation involves either transcriptional control, post transcriptional modulation, or changes in the levels of co-activators and co-repressors. A physiologically abundant lipid such as cholesterol could occupy and stabilize the hydrophobic LBD, allowing receptor function without modulation through changes in ligand availability (Chawla et al. 2001).

1.3 Introduction to RORg

RORg is an orphan nuclear receptor whose endogenous ligand is uncertain. Two isoforms of RORg exists that share a common LBD, with a thymus specific isoform, RORgt, that differs in the $–NH_2$ terminus. The high levels of expression of RORg in the

thymus would suggest an immune phenotype in the knockout mouse (Bookout et al. 2006). RORg or RORgt knock-out mice were found to have similar phenotypes and most of the essential developmental and immune functions of RORg are carried out by the RORgt isoform (Eberl et al. 2004). The RORgt knockout mice were born in a normal mendelian ratio with no visibly discernible phenotype (Sun et al. 2000) . Examination of the thymus showed that it was normal in size, but had a 30-50% reduction in total cell number. This was traced back to a loss of double positive (DP) $CD4^{+}CD8^{+}$ thymocytes. The immature DP cells interact with the major histocompatibility complex (MHC) through their T cell receptors. Too strong an interaction, indicating self-reactivity, and the cell undergoes apoptosis, a process known as negative selection. On the other hand, a cell with a TCR that fails to appropriately interact with an MHC also undergoes apoptosis, a process known as positive selection (Hogquist et al. 2005). This ensures that as the immune cells mature they become self-tolerant. RORgt up regulates the expression of an anti-apoptotic factor BCL-xl that promotes cell survival and provides the cell with adequate time to interact with the MHC (Sun et al. 2000). Placing a constitutively active BCL-xl in $RORgt^{-/-}$ DP thymocytes reverses the observed spontaneous cell death, further supporting a direct modulation by RORg of apoptosis through BCL-xl. The RORgt knockout mice were also found to lack all Peyer's patches and peripheral lymph nodes. The lack of these was traced back to $CD3^{-}CD4^{+}CD45^{+}$ immune progenitor cells that migrate to the periphery in the embryonic stage and aid in lymph node development.

1.4 The Role of RORg in TH17 Cells

Perhaps the most therapeutically relevant role of RORgt is its ability to regulate the differentiation and continued survival of a certain subset of T cells, the TH17 cells (Yang et al. 2008). As immune cells migrate from the thymus to the periphery, they differentiate into different cell fates. Two helper T cell subtypes develop from $CD4^+$ cells, the TH1 and TH2 cells. TH1 cells produce interferon gamma and differentiate in response to IL12. They are involved in the immune response to intracellular pathogens. TH2 cells differentiate in response to IL4 and produce IL4, IL5 and IL13. They are largely responsible for controlling helminthes and are implicated in allergic reactions and asthma (Korn et al. 2009). A third subset of T helper cells has recently been identified. These are the TH17 cells that produce IL17. TGF-β and IL6 production is necessary for differentiation into TH17 cells. The differentiation and sustained response of IL17 cells is controlled by various factors. TGF-β stimulated both Tregs and TH17 cells. However, in the presence of IL6, Treg development is suppressed in favor of TH17 cells (Bettelli et al. 2006). $IL6^{-/-}$ mice lack both TH17 cells in the gut and RORgt expression (Korn et al. 2009). Thus, RORgt expression is dependent on IL6 production. Once TH17 cells have differentiated, a sustained response is dependent on continued production of IL23 (Aggarwal et al. 2003). IL23 production is in turn dependent on RORg, and RORg knockout mice have significantly lower IL23 levels (Nurieva et al. 2007).

In response to activation of their TLRs (Toll like receptors) by microbial pathogens, DC cells secrete IL12, IL23 or IL27. The relative amount of these cytokines skews the inflammatory response in favor of TH1 or TH17 cells (Korn et al. 2009). Evidence points to TH17 cells being preferred in response to certain pathogens that require a massive inflammatory response that cannot adequately be generated by TH1

and TH2 cells. When TH17 cells develop in response to self-antigens, they trigger a massive autoimmune response. Skewing of $CD4^{+}$ cells in favor of TH17 cells is implicated in multiple autoimmune diseases. In mouse models of multiple sclerosis such as experimental autoimmune encephalomyelitis (EAE), large numbers of TH17 infiltrates are found in the brain and spinal cord (Komiyama et al. 2006). In patients with psoriasis skin samples reveal skewing towards TH17 cells (Pene et al. 2008). Aspirates from the joints of rheumatoid arthritis patients contain TH17 cells, the quantity of which is prognostic of disease progression (Kirkham et al. 2006). In line with this, conditions that decrease the number of TH17 cells, like $IL6^{-/-}$ or $IL23^{-/-}$ mice, also cure or lessen the symptoms of EAE (Cua et al. 2003) and collagen induced arthritis (CIA) (Nakae et al. 2003). $RORgt^{-/-}$ mice are also resistant to EAE. RORgt is not only required for TH17 differentiation but also for continued production of IL17 (Ivanov et al. 2006). This raises the possibility of RORgt being an important potential therapeutic target for autoimmune diseases. Antagonists of RORgt, which shall be discussed later, similarly attenuate the TH17 response and can cure or lesson the symptoms of EAE (Huh et al. 2011; Solt et al. 2011). These antagonists may enter the clinic soon and be a valuable tool in the fight against autoimmunity.

1.5 RORg Antagonists

The potential to regulate TH17 cell differentiation and activation by modulating RORg activity makes RORg antagonists a potential clinical drug to treat diseases with TH17 pathology. These range from multiple sclerosis, psoriasis, rheumatoid arthritis,

inflammatory bowel disease to asthma (Molet et al. 2001; Fujino et al. 2003; Komiyama et al. 2006; Pene et al. 2008). As such the search for a RORg antagonist was the focus of multiple groups. The products of this search were SR1001 and digoxin (Huh et al. 2011; Ozerlat 2011; Solt et al. 2011).

A screen of known nuclear receptor agonists and antagonists revealed that T0901317 or Tcompound (Tcomp), a potent agonist of the nuclear receptor LXR, was also a potent RORg and RORa inverse agonist (Kumar et al. 2010). It inhibited the two receptors in a dose dependent manner with a K_i in the nanomolar range. Tcomp was also shown to bind directly to RORg in an in-vitro binding assay and cause co-activator displacement and co-repressor recruitment. Based on Tcomp, SR1001 was developed, a compound that retained its ability to inhibit RORg but had no effect on LXRα or LXRβ (Solt et al. 2011). SR1001 could displace radiolabeled 25-hydroxycholesterol from the ligand binding pocket from both RORa and RORg (K_i=172 and 111 nM, respectively). The AF2 domain of the nuclear receptor LBD is found on helix 12 (H12). Ligand binding allows the H12 domain to interact with co-activator LXXL domains (Weatherman et al. 1999). Crystal structures of RORg with SR1001 revealed that SR1001 could block the H12 helix domain from interacting with the co-activator SRC2. When EL4 cells, a murine thymocyte cell line with RORg dependent IL17 production, are given RORg, there is strong suppression of IL17 production. The suppression is RORg dependent, as shown by a knockdown of RORg in these cells. Splenocytes can be induced to differentiate into TH17 cells when cultured with TGF-β and IL6. The addition of SR1001 prior to adding the cytokines prevented TH17 differentiation and IL17 mRNA

production. Thus SR1001 is a potent RORg inverse agonist that can block RORg mediated affects including TH17 differentiation and IL17 production.

Several mouse models of autoimmune diseases exist. One of these, experimental autoimmune encephalomyelitis (EAE), is a well characterized mouse model of multiple sclerosis. Immunization of mice with myelin oligodendrocyte glycoprotein (MOG35–55) leads to TH17 infiltrates in the spinal cords of these animals (Komiyama et al. 2006). Intraperitoneal administration or SR1001 during the incubation phase decreased the number of TH17 cells detected in spinal aspirates (Solt et al. 2011). SR1001 and its derivatives may be clinically relevant in TH17 mediated autoimmune diseases.

A screen by another group identified digoxin as another potential RORg inhibitor (Huh et al. 2011). Unlike SR1001, digoxin inhibition was RORg specific, and digoxin has no effect on RORa activity. Digoxin is a cardiac glycoside already used in the clinic to treat a variety of heart conditions. It has a narrow therapeutic index because of toxicity at high concentrations. Non –toxic digoxin derivatives 20, 22-dihydrodigoxin-21, 23-diol and digoxin-21-salicylidene also inhibited RORg activation and are potential alternatives to digoxin for clinical treatment. Digoxin inhibited differentiation of $CD4^+$ cells into TH17 cells and blocked IL17 production from differentiated TH17 cells. When EAE was induced with MOG, simultaneous digoxin administration decreased spinal TH17 infiltrates and improved clinical outcomes compared to the DMSO control. Digoxin has also been found to prolong allograft survival in mouse heart transplants. TH17 cells have been associated with decreased allograft survival (Antonysamy et al. 1999) and antagonism of IL17A has been shown to improve graft survival (Tang et al. 2001). When mice with cardiac transplants are treated with Digoxin, allograft survival doubles (Wu et

al. 2013). This effect of Digoxin is likely due to its antagonism of RORgt, since mice treated with Digoxin had significantly lower concentrations of TH17 cells in the allograft. Administration of other cardiac glycosides, like LanC, that do not inhibit RORg but have similar effects as Digoxin on cardiac failure, had only a modest increase in graft survival. It is thus likely that Digoxin's effects on graft survival are mediated through its actions on RORg. This makes Digoxin and its derivatives a valuable tool in treating not only autoimmune diseases but also graft vs host disease and graft rejection.

TH17 cells are abundant in the lamina propria of the small intestine and are important in maintaining the integrity of the mucosal barrier. In addition to being mediators of autoimmunity, TH17 cells protect the body from gut bacteria and fungus. The presence of TH17 cells in the lamina propria is dependent on the presence of gut microbiota and TH17 cells are absent in germ free mice (Ivanov et al. 2009). Additionally, certain segmented filamentous bacteria (SFB) when introduced into germ free mice can induce TH17 differentiation, yet other bacteria fail to do so. Removal of intestinal pathogens and prevention of colitis is dependent on TH17 cytokines such as IL22. Commensal gut bacteria such as SFB may protect the intestine from pathogenic colonization by increasing the number of TH17 cells. Survival of mice infected with *C. rodentium* was increased when their intestines had previously been colonized by SFB. The discovery of Digoxin as a RORg antagonist raises the interesting possibility that commensal gut bacteria such as *Eubacteriumlentum*, known to metabolize digoxin, may modulate TH17 cell differentiation and function by metabolizing endogenous RORg antagonists and agonists. This also raises the possibility that the RORg agonist is an intestinal bacteria derived compound. Commensal bacteria, by synthesizing a RORg

agonist, or providing a precursor, may increase TH17 differentiation in the lamina propria of the small intestine.

The search for a RORg antagonist has led to the discovery of molecules such as SR1001 and digoxin that can successfully inhibit RORg dependent TH17 cell differentiation and IL17 production. TH17 cells and immune dysfunction have been implicated in the pathophysiology of multiple autoimmune diseases. Treatment with drugs that antagonize RORg may potentially improve clinical outcomes for patients with autoimmune disease or tissue grafts.

1.6 Oxysterols as RORg Agonists

RORg is considered an orphan nuclear receptor for whom the ligand has not yet identified. However, evidence exists that sterols, particularly oxysterols, may be RORg agonists (Jin et al. 2010; Wang et al. 2010; Wang et al. 2010). When RORg is transfected into mammalian cell based systems it is highly constitutively active, suggesting that mammalian cells contain abundant RORg ligands (Jin et al. 2010). When transfected into a Drosophila S2 insect cell based system lacking sterols or serum, RORg activity is abolished. Addition of sterols or serum to cholesterol deprived S2 cells reconstitutes RORg activity (Huh et al. 2011). This indicates that sterols may be the endogenous RORg ligands.

The RORg LBD has 48% and 46% sequence identity with RORa and RORb respectively. The crystal structure of RORa was the first to be solved (Kallen et al. 2004) and it revealed that cholesterol or cholesterol sulfate occupied the RORa binding

pocket. When cholesterol sulfate, predicted by modeling to be a stronger binder than cholesterol, was added to the purified RORg LBD, it replaced most of the cholesterol from the binding pocket, indicating that cholesterol was interchangeable for a stronger ligand. The shared sequence identity of RORa and RORg LBDs raises the possibility that sterols are also the RORg ligand. Cholesterol, 20α-hydroxycholesterol, 22R-hydroxycholesterol (22R-HC), and 25-hydroxylcholesterol (25-HC) increase the interaction of RORg with the LXXLL motif of co-activator SRC1-2. The potency (EC50) of most oxysterols was in the 20-40 nm range (Jin et al. 2010), compared to the 200nm range for LXRα and LXRβ (Janowski et al. 1999). Thus, while exogenous sterol addition can increase LXRα dependent transcription in mammalian cell systems, the low EC50 of oxysterols for RORg may allow endogenous sterols to saturate RORg activity. The crystal structure of RORg in complex with cholesterol, 22R-HC and 25-HC has been solved. RORg has a well-defined hydrophobic LBD. When the structure is solved in complex with sterols, the AF2 domain on H12 is in the active conformation, in agreement with the hypothesis that sterols are RORg agonists. Additionally, when residue (I397N) is mutated, it creates a hydrogen bond with the C22 hydroxyl of 22R-HC (Jin et al. 2010). This mutation abolishes the ability of sterols other than 22R-HC to bind RORg. The mutated LBD has lower RORg dependent transcription that can be rescued by addition of exogenous 22R-HC. Similarly, mutating L324N that enhances binding of 25-HC significantly induced RORg activity in the presence of exogenous 25-HC. 24-HC, another endogenous oxysterol, has on the other hand been shown to be a RORg inverse agonist (Wang et al. 2010).

These results do not preclude the existence of a yet unknown sterol, or other endogenous molecule, that is a more potent and efficacious RORg ligand. Sterols may indeed constitutively occupy the RORg binding pocket but then be replaced by another ligand to further increase RORg activity. The discovery of digoxin, an endogenously available cardiac glycoside, as a RORg antagonist raises the possibility that RORg may be constitutively active, and endogenous RORg regulation is based on synthesis of antagonists to decrease its high basal activity (Huh et al. 2011).

1.7 Summary

RORg is an orphan nuclear receptor that has been shown to be important in the regulation of immune function. The endogenous ligand for RORg is uncertain, however, certain sterols, though unable to increase RORg activity in cells, can bind RORg in-vitro. Here I will show that sterols, as already published, can bind RORg in in-vitro binding assays and cause co-activator recruitment, yet fail to activate RORg in a co-transfection assay. On the other hand, I will demonstrate that certain endogenously isolated lysophospholipids, such as 22:4 LPE, can activate the Gal4-RORg fusion in HEK293 and HepG2 cells. Additionally, 22:4 LPE treatment leads to IL17 production by the EL4 cell line. I propose that the RORg LBD is promiscuous for a number of sterols, and this promiscuity leads to the high basal activity observed for RORg. In the absence of sterols, RORg activity decreases, because sterols are required for basal RORg activity. Immune stimulation leads to activation of the cytoplasmic and secreted phospholipase A2s (Rosenson 2009), which then form lysophospholipids from membrane phospholipids. In

thymocytes, 22:4 PE is uniquely abundant and may serve as a precursor for 22:4 LPE (Goppelt et al. 1986). These short lived lysophospholipids are rapidly converted back into phospholipids by lysophospholipid acyl-transferase (LPAT). Thus maintaining the levels of lysophospholipids is dependent on continued PLA2 activity and conversion of phospholipids into lysophospholipids. In the absence of a sustained immune signal and elevated PLA2 activity, RORg has basal activity due to the presence of sterols in the binding pocket. Sustained PLA2 activity increases and maintains lysophospholipid levels, which replace sterols in the pocket and further increase RORg activity, TH17 proliferation and IL17 secretion. Thus 22:4 LPE can be thought of as a short lived RORg ligand with a higher potency for RORg than sterols; a 'booster' ligand that can further increase the basal RORg activity that exists in the presence of sterols.

CHAPTER 2

Results

2.1 Introduction

RORg is a member of the nuclear receptor superfamily of ligand regulated transcription factors. A RORg isoform, RORgt, is highly expressed in the thymus and T-cells. RORg knockout mice lack $CD4^+CD8^+$ dual positive thymocytes, lymph nodes, and Peyer's patches in the gut (Sun et al. 2000). RORg is additionally expressed in a subset of $CD4^+$ cells known as TH17 cells, where it increases the differentiation and proliferation of these cells and is important in maintaining IL17 cytokine secretion (Ivanov et al. 2006). TH17 cells are important immune modulators that are implicated in a number of autoimmune diseases including multiple sclerosis, rheumatoid arthritis and Crohn's disease (Kirkham et al. 2006; Komiyama et al. 2006; Pene et al. 2008). In mouse models of autoimmune diseases such as EAE, deletion of RORg or treatment with a RORg antagonist greatly improves clinical outcomes (Huh et al. 2011). Targeting RORg activity, through the use of an antagonist or by decreasing agonist concentrations, is a potential method of ameliorating the symptoms and disease progression of autoimmune diseases.

The endogenous ligand for RORg is uncertain, though sterols are hypothesized to be the endogenous ligands. Sterols can be co-crystalized with the RORg LBD and can bind RORg leading to co-activator recruitment in in-vitro binding assays (Jin et al. 2010).

However, no sterol tested so far has been able to modulate RORg activity in mammalian cells. There are two possibilities, either RORg is constitutively bound to a sterol and not ligand regulated or that the RORg ligand is perhaps an as yet unidentified sterol or another class of lipid.

Here I attempt to discover the identity of the endogenous RORg ligand by two approaches, direct testing of commercially available and synthesized sterols against RORg in a co-transfection assay, and extraction and identification of the ligand from endogenous sources.

2.2.1 Statins Decrease RORg Activity in Co-transfection Assay

It has previously been suggested that sterols could be the endogenous RORg ligands (Wang et al. 2010). RORg crystal structures show either cholesterol or cholesterol sulfate in the binding pocket (Kallen et al. 2004). 25-hydroxycholesterol, an oxygenated sterol, has been shown to bind RORg and cause co-activator recruitment in an in-vitro binding assay known as the ALPHA screen (Jin et al. 2010). To test the effects of sterol depletion on RORg activity, I treated HEK293 and HepG2 cells with 40, 8, 1.6 or 0.32 μM lovastatin or simvastatin. The statins are HMG-COA reductase inhibitors and should reduce sterol synthesis in treated cells (Figure 2.1). Treated and untreated cells were transfected the full length RORgt construct, co-transfected with a vector containing four copies of the ROR response element (RORE) upstream of luciferase. βgal was used as an internal control.

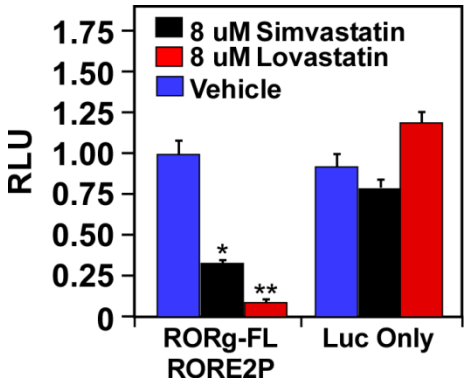


Figure 2.1 Decreased RORg activity in HEK293 cells after statin treatment. HEK293 cells were transfected with RORg-Full Length + RORE2P or luciferase alone. 2 hours after transfection, 8 μM lovastatin, 8 μM simvastatin or vehicle alone were added to the media. Luciferase activity was assayed 24 hours later. Values are means ± SEM. Statistics by two-tailed *t* test. **P*<0.05, ***P*<0.005.

Lovastatin and simvastatin both decreased luciferase levels in the full length RORg. A basal decrease in luciferase levels was observed in the vector only control when statin levels exceeded 20 μM (data not shown), indicating that high concentrations of statin cause a decrease in luciferase production independent of RORg and should be used with caution.

2.2.2 Structure Activity Relationship of Sterols and RORg

A structure activity relationship, or SAR, helps us understand the tolerance level of the RORg binding pocket for changes in sterol structure. To this end, in collaboration with Dr. Fabio Santori at NYU, we tested various sterols for their ability to activate RORg in a co-transfection assay. Dr. Santori has created an S2 cells based system that is largely devoid of endogenously produced cholesterol. When this system is transfected

with the Gal4-RORg fusion and the UAS-Luciferase vector, RORg basal activity, as measured by luciferase production, is very low. The screen for sterols was repeated in HEK293 cells pretreated with lovastatin to decrease basal RORg activity. The majority of the SAR was tested by Dr. Santori and I retested a subset of the active compounds from the screen, such as oxysterols, 4,4-dimethyl-5α-cholesta-8,14,24-trien-3ß-ol (FF-Mas), 5α-cholesta-8,24-dien-3ß-ol (zymosterol) and 4-methylcholesta-8,24-dien-3ß-ol (4α-methylzymosterol).

The SAR is presented in Appendix A. The compounds are organized based on the location of the double bond on the sterol A or B rings. Firstly, it appears that 4-cholesten-3-one, which differs from cholesterol by a ketone on the 3 position instead of a hydroxyl and a double bond at the four position instead of five, activates RORg in S2 cells. Addition of an oxy group to the side chain of 4-cholesten-3-one in some cases led to a robust increase in activity. Various lanosterol derivatives, such as Δ7-lanosterol-aldehyde-mono-f and Δ7–lanosterol-carboxy-dif, also activated RORg in S2 cells. However, in mammalian cells only a small subset of compounds had any effect on RORg. These compounds, such as zymosterol and FF-mas, all have a double bond at the eight position on the B ring. FF-mas additionally has two methyl groups at the 4 position. Note all the compounds that activated RORg in mammalian cells did so only after pretreatment with a statin, indicating that perhaps RORg is constitutively active in the presence of endogenous sterols, or that the endogenous ligand is a sterol not yet identified or tested in the SAR.

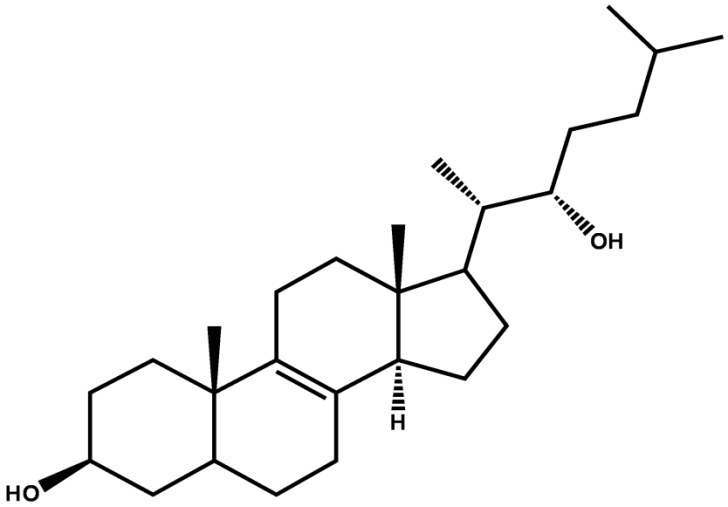


Figure 2.2 5α-Cholest-8-en-3,22-diol. This compound was synthesized based on the information provided by the SAR. A methyl at the four position, a double bond at the eight position, and a hydroxy on the side chain were all indicated by the SAR to be required for RORg activity.

2.2.3 5α-Cholest-8-en-3,22-diol does not Activate RORg

Based on the structure activity relationship, we proceeded to synthesize a sterol variant that could potentially activate RORg. This compound was 5α-Cholest-8-en-3,22-diol. The aim of synthesizing this molecule was to combine all the features indicated to be important for RORg activity by the SAR, including a double bond at the eight position, a methyl at the four position and a hydroxyl group on the side chain. The compound was synthesized by Dr. Kamalesh Sharma in the Richard Auchus laboratory. I tested the compound in HEK293 and HepG2 cells for its ability to activate either the full length or Gal4 fusion RORg protein (data not shown). The compound failed to activate RORg, but was found by our collaborator Dr. Santori to bind RORg in an in-vitro binding

assay (data not shown). It is possible that further modification to these sterols may lead to better binding to RORg, however, since most of these Δ8 sterols are either endogenously absent or of low abundance, I decided to pursue other avenues to identify the ligand.

2.2.4 Extraction of Ligand from Endogenous Tissue

RORgt is predominately expressed in the thymus (Sun et al. 2000). In the thymus it is found in a subset of T cells designated as $CD4^+CD8^+$ or double position (DP) immature T-lymphocytes. These DP cells are most abundant in the neonatal and young ages. By puberty the thymus begins to shrink and is largely replaced by fat. With this in mind, I chose bovine calf thymus as the source material to extract the endogenous RORg ligand. Bovine calf thymus is replete with $CD4^+CD8^+$ cells. Since it has been shown that RORg is essential for $CD4^+CD8^+$ cell survival, bovine calf thymus would likely serve as a rich source of ligand.

Lipids from bovine calf thymus were extracted using a folch procedure as described in the materials and methods (Figure 2.3). I then separated the lipids by class on a silica column. Lipids were adsorbed on the column and eluted in order of increasing polarity. The first fraction collected was the chloroform fraction that contains fatty acids, triglycerides and some sterols. The second fraction, the acetone fraction, contains the remaining sterols and importantly the oxysterols that are potential RORg ligands. The third and final fraction, methanol, contained all the phospholipids. Ten grams of lipid

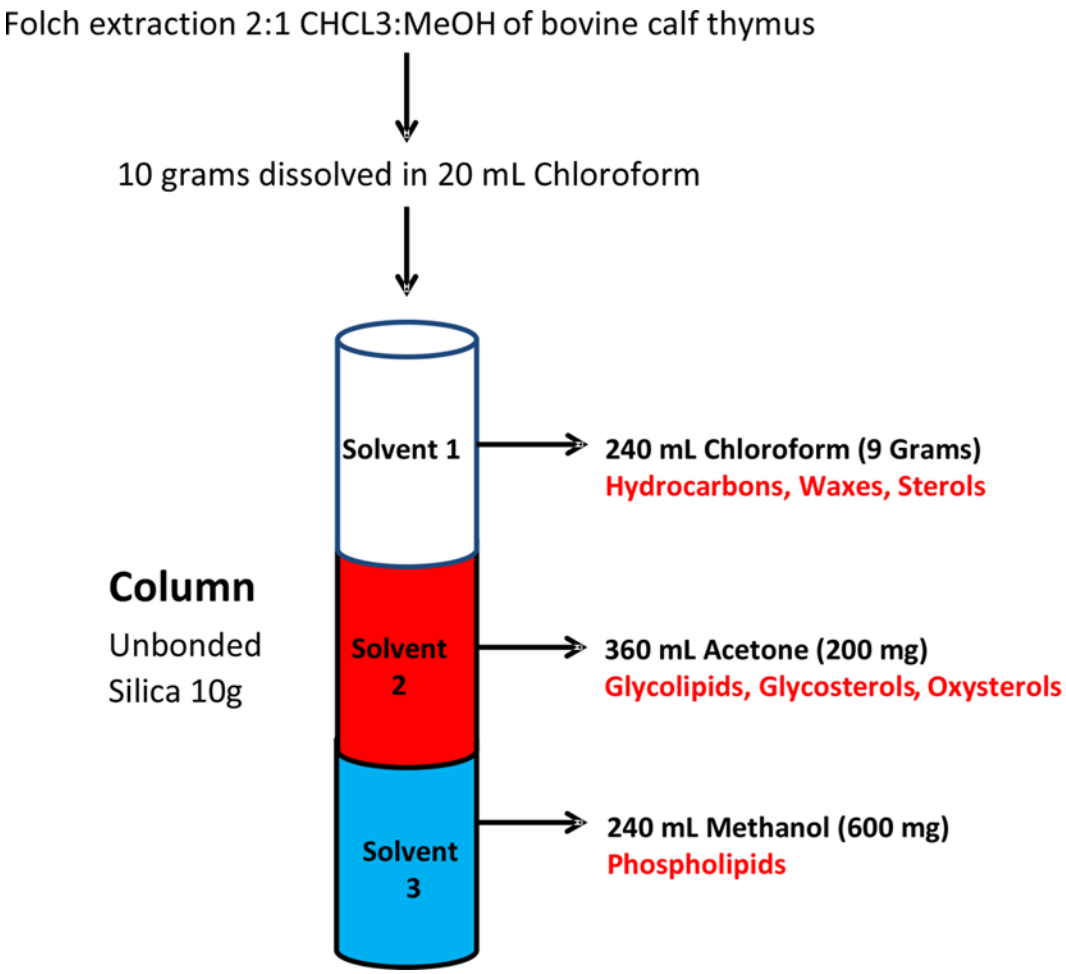


Figure 2.3 Extraction of lipids from bovine calf thymus. Twenty three kilograms of bovine calf thymus was homogenized in 10 L/Kg of 2:1 chloroform: methanol. Addition of 0.2 volumes of 0.9% NaCl resulted in phase separation. Lower organic phase was isolated and solvent evaporated with a rotary evaporator. Resulting lipids were loaded 10 grams at a time onto silica columns followed by elution of lipids with 240 mL chloroform, 360 mL acetone and 240 mL methanol.

were loaded on the column for each elution, of which the majority, nine grams, eluted in the chloroform fraction. 200 mg eluted in the acetone fraction and 600 mg in the methanol fraction. Initial tests of the fractions in a co-transfection assay failed to yield any positive results due to high toxicity. I proceeded to further purify and analyze the fraction by HPLC/MS, as described in the following sections.

2.2.5 Acetone/Chloroform Fractions Fail to Activate RORg in a Co-transfection Assay

The chloroform and acetone fractions contained the majority of the sterols from calf thymus. Since sterols are the putative RORg ligands, I began by analyzing these fractions. The fractions were further separated by reverse phase HPLC using a C-18 semi-prep column (9.8 mm ID, 250 mm length). Each run consisted of a water: methanol gradient from 70% MeOH to 100% MeOH over 30 minutes, followed by 30 minutes of 100% MeOH (Figure 2.4). Fractions were collected every two minutes, dried down under a stream of nitrogen and reconstituted in 1:1 chloroform: methanol. Aliquots of these fractions were then tested for their ability to activate the Gal4-RORg fusion in a co-transfection assay. The acetone fractions failed to activate RORg in HepG2 or HEK293 cells (Figure 2.5). To confirm the presence of sterols in the acetone fractions, I tested them for LXRβ-Gal4 agonist activity. The acetone fractions activated LXR as expected, indicating the presence of sterols in the fraction. TH17 cells are abundant in the small intestine and are potentially another source of RORg ligands (Sun et al. 2000). Lipids

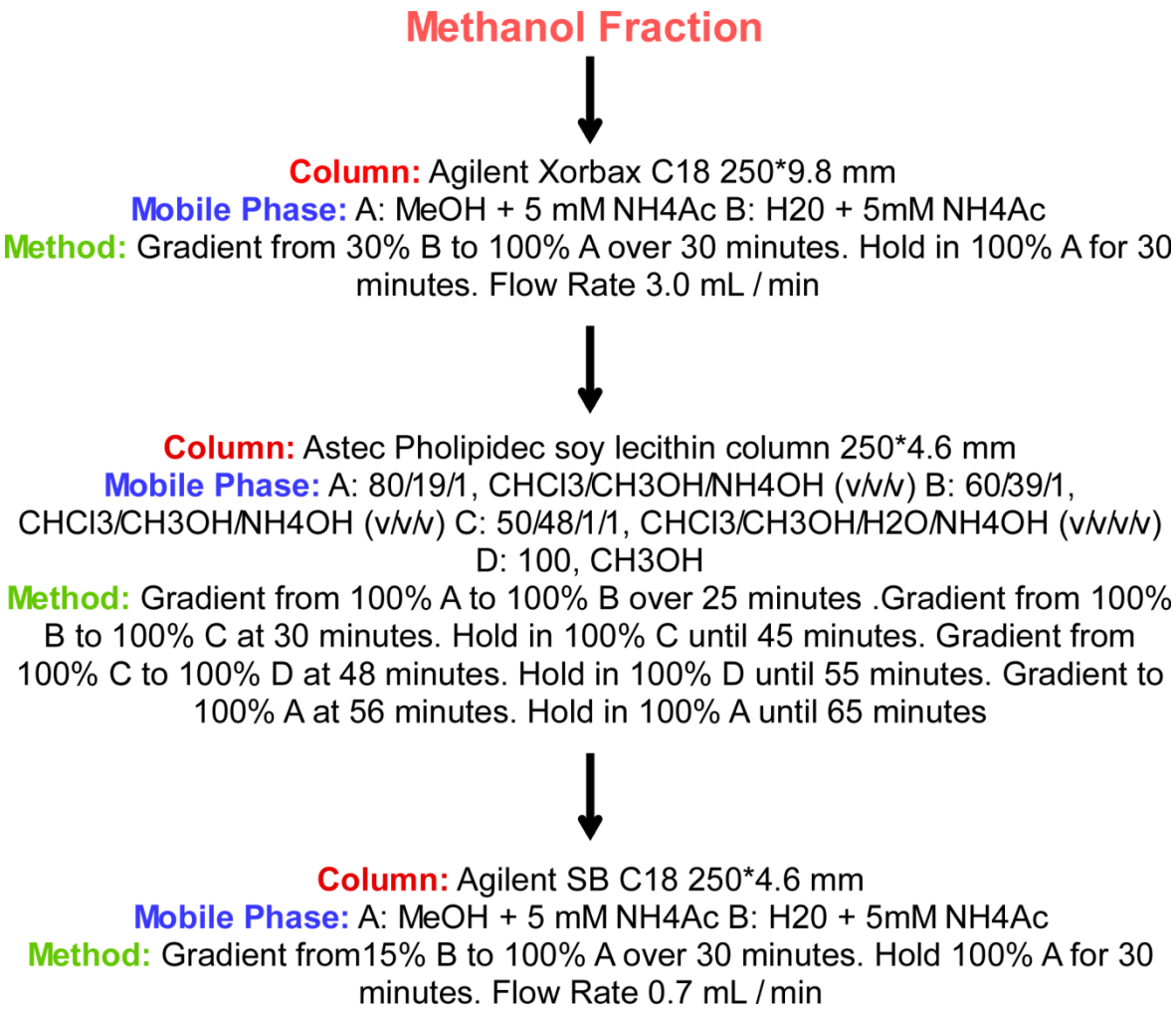


Figure 2.4 HPLC separations of lipid fractions. The acetone, chloroform and methanol fractions were further purified using reverse phase HPLC. All fractions were run through a C18 semi-prep with thirty fractions taken every two minutes. HPLC fractions from the acetone and chloroform fractions failed to activate RORg in a co-transfection assay. The methanol fraction activated RORg and was further purified according to the schematic above utilizing the columns and elution conditions described.

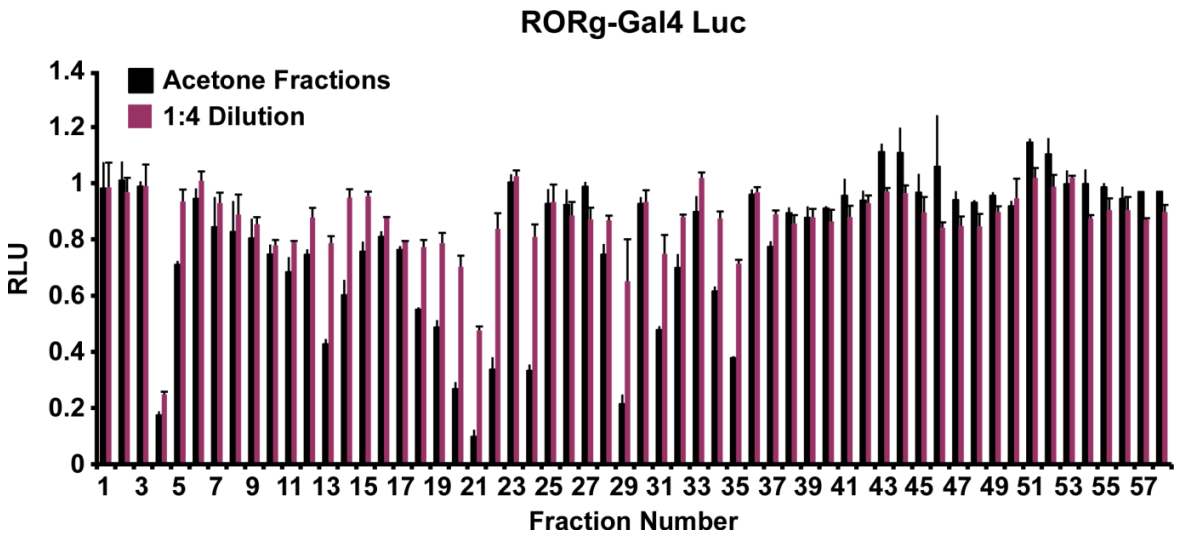


Figure 2.5 Test of acetone fractions for RORg activity. Lipids in the acetone fraction were further separated using a C18 reverse phase HPLC column. HepG2 cells were transfected with Gal4-RORg and UAS-Luc vectors. Twenty four hours post transfection aliquots of the fractions dissolved in media were added to cells. Luciferase activity was measure 24 hours later. Activity was normalized to βgal. Values are means ± SEM.

from the small intestine were extracted and separated by class as described before (Figure 2.3).

The acetone eluent was fractioned by HPLC and the fractions tested against Gal4-RORg in a co-transfection assay. Intestinal sterol extracts failed to activate RORg while robustly activating LXR (data not shown). Thus both thymic and intestinal sterol extracts failed to elicit any RORg activity. These data, along with the lack of activity seen while constructing the sterol-RORg SAR, suggests that perhaps sterols do not endogenously activate RORg. It is also possible that endogenous sterol levels are sufficient to saturate RORg activity, leaving open the possibility that RORg is not modulated by a ligand but rather transcriptionally or by post transcriptional modifications such as phosphorylation. Further studies would be required to test these possibilities.

2.2.6 Sterols Bind RORg in an In-Vitro Binding Assay

Cell culture based co-transfection assays suffer from several pitfalls. The cells may rapidly metabolize any potential ligand added to the cell. Additionally, the lack of a transporter required for the ligand to cross the cell membrane would fail to allow ligand receptor binding. The latter is seen with conjugated bile acids that fail to activate FXR in the absence of the bile acid transporter I-BAT (Makishima et al. 1999). Sulfated sterols and certain bile acids require the organosulfur transporters (OST) to cross into the cytoplasm (Rao et al. 2008). To overcome these pitfalls, I utilized an in-vitro binding assay to test for direct binding of compounds to RORg. The system, known as an ALPHA screen, consists of His-tagged RORgt protein bound to nickel chelated donor beads. Biotinylated SRC3-1b peptide is bound to an acceptor bead. Ligand binding leads to co-activator recruitment and energy transfer between the acceptor and donor bead that produces a luminescent signal in the 520-620nm range (Figure 2.6). Previously it has been shown that sterols can cause co-activator recruitment to RORg in the ALPHA screen (Jin et al. 2010). To confirm a functional screen, I tested 25-hydroxycholesterol for its ability to activate RORg. As expected, 25-hydroxycholesterol bound RORg and caused co-activator recruitment (Figure 2.6), but failed to produce a signal in the absence of RORg protein (Figure 2.6) or SRC3-1b peptide (data not shown). The screen was subsequently used to test the lipids extracted from bovine calf thymus. Both the chloroform and acetone fractions produced a signal in the ALPHA screen (data not shown). However, signal was detected even in the absence of protein, indicating that lipids in the extracts were causing nonspecific bead interaction. The presence of lipids

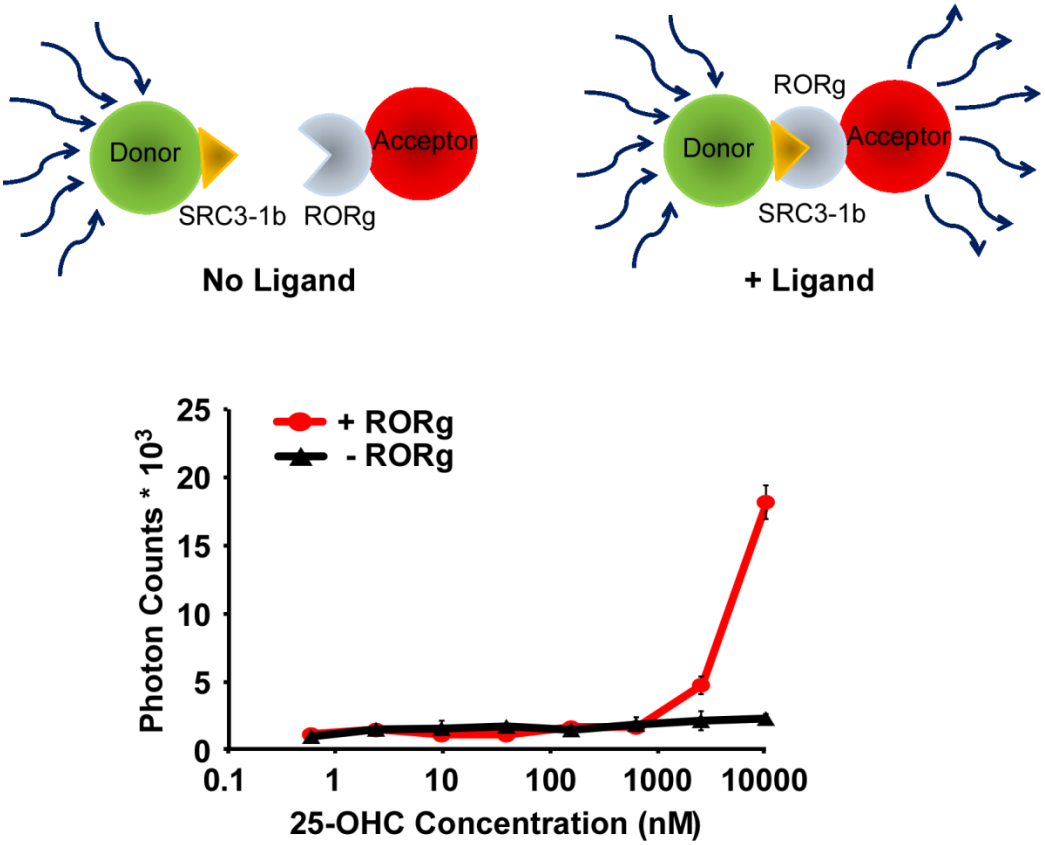


Figure 2.6 25-hydroxycholesterol binds RORg in an ALPHA screen. His-RORgt purified from bacteria was bound to nickel chelated donor beads. Biotinylated SRC3-1b (Biotin-GHKKLLQLLTS-COOH) was bound to the acceptor bead. 25-OHC at given concentrations was added to a mix of beads in the presence or absence of RORg protein. Values are means ± SEM.

that cause nonspecific bead interaction and a false positive signal precluded the use of the ALPHA screen as a tool to directly screen for the ligand from endogenous extracts.

2.2.7 Methanol Fraction Activates Gal4-RORg in a Co-transfection Assay

As previously described, I had separated the bovine calf thymus into three fractions based on lipid class. The first two of these, the chloroform and acetone fraction, failed to activate RORg in a co-transfection assay. The remaining methanol fraction was similarly separated using semi-prep reverse phase HPLC with a C18 column (Figure 2.4). These fractions were tested in a co-transfection assay using the Gal4-RORg fusion. Three fractions subsequently referred to as fractions twelve, thirteen and fourteen, induced RORg dependent expression of the UAS-Luc reporter (Figure 2.7A). T0901317, commonly referred to as Tcompound, is an LXRα/β agonist. Recently it has been shown that Tcomp is also a strong RORg antagonist (Kumar et al. 2010). To test the ability of the methanol fractions to rescue Tcomp inhibition of RORg, I transfected HepG2 cells with Gal4-RORg and UAS-Luc. Cells were subsequently treated with 10 μM Tcomp and the methanol fractions. I utilized two luciferase reporter systems, the stable TKLUC system and Luc2P, a luciferase with a short degron attached to the end that induces rapid degradation and turnover of the luciferase protein. The Luc2P system gives a lower signal but is more sensitive for compounds that are rapidly degraded by cells due to a lower background. Upon Tcomp treatment fractions thirteen and fourteen rescued inhibition in the Luc2P system but failed to do so for TKLUC. Two additional fractions that were not active in the absence of Tcomp, fractions eighteen and nineteen, could rescue Tcomp inhibition of RORg in both the TKLUC and Luc2P systems (Figure 2.7B).

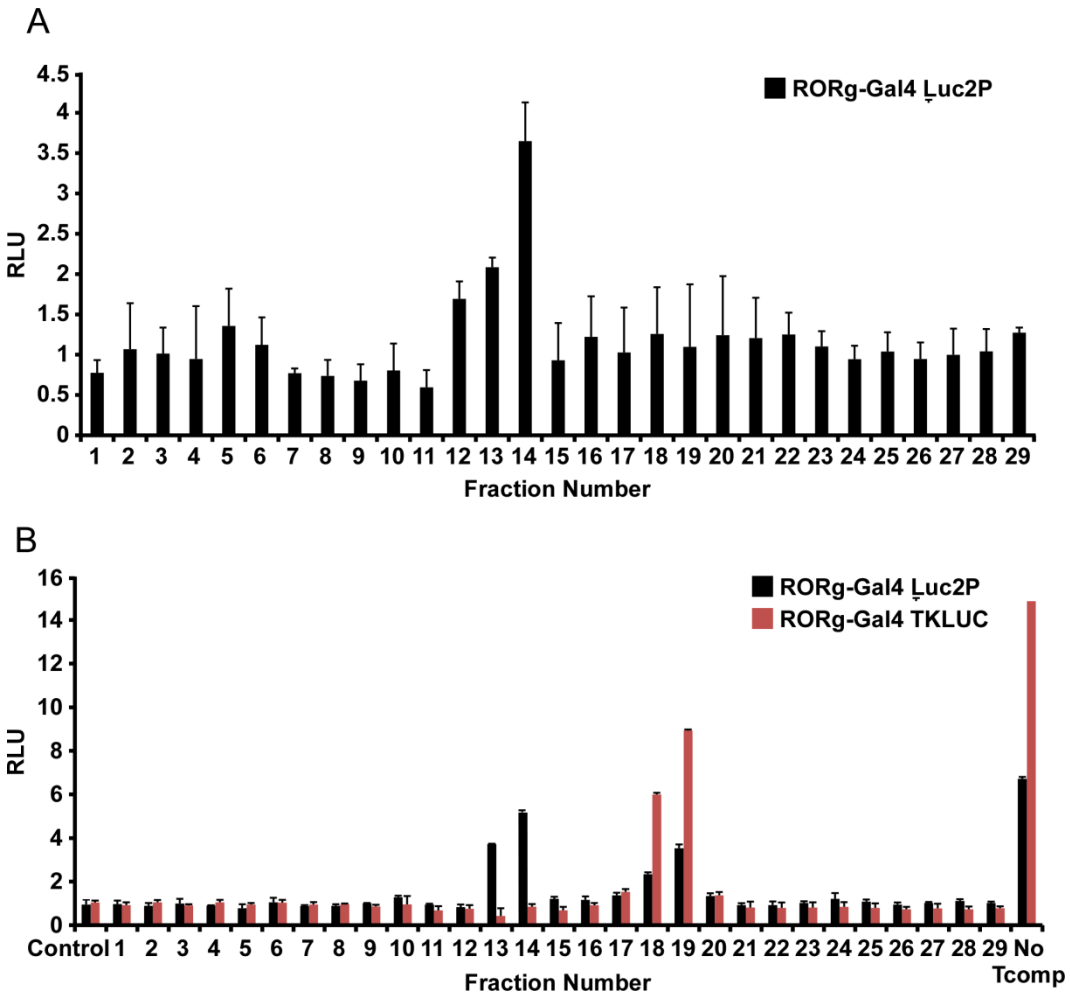


Figure 2.7 Methanol fractions activate RORg in a co-transfection assay. (A) Gal4-RORg was transfected along with UAS-Luc2P into HepG2 cells. Cells were treated with methanol fractions and luciferase activity measured 24 hours later. (**B)** HepG2 cells were transfected with Gal4-RORg and either UAS-TKLUC or UAS-Luc2P. Post transfection, cells were treated with 10 μM *T*0901317 and methanol fractions. Luciferase activity was measured 24 hours later. βgal was used to control for transfection efficiency and detect compound toxicity. Values are means ± SEM.

2.2.8 Mass Spectrometric Analysis of Active Fractions 13 and 14

Fractions 13 and 14 were able to activate RORg in a standard co-transfection assay and rescue Tcomp inhibition of RORg (Figure 2.7). No compound or fraction tested in our SAR table or from endogenous extracts had been able to induce RORg activity without prior treatment with a RORg inhibitor or a statin. To elucidate their composition, fractions 13 and 14 were analyzed using a single quadruple mass spectrometer and tandem MS/MS. All three fractions were found to contain various amounts of different lysophospholipids (Figure 2.8). Lysophospholipids are phospholipids lacking the A1 or A2 fatty acid chain. Various head groups can occupy the final position on the glycerol backbone. The most common of these head groups are choline, ethanolamine, inositol, serine or glycerol attached to a phosphate (Figure 2.9). The fractions contained large quantities of lysophosphocholine (data not shown) and smaller amounts of lysophosphoethanolamine, lysophosphoinositol, lysophosphoserine and lysophosphoglycerol (Figure 2.8). Additionally, the fractions contained minor amounts of lysophosphatidic acid, which lacks a head group. It is important to note that the MS analysis focused on known lysophospholipids, additional compounds whose MS/MS spectra could not be matched to known compounds were also found in the fractions. These shall be discussed in a later section. Compounds that did not ionize under the conditions employed would also not be detected. This leaves open the possibility that a lysophospholipid with an obscure or unknown modification may exist in these fractions. Preliminary analysis was carried out on our single quadruple MS followed by

phospholipid head group and fatty acid chain confirmation by Dr. Pavlina Ivanova in the Alex Brown laboratory at Vanderbilt.

2.2.9 MS Analysis of Fractions 18 and 19

Fractions 18 and 19 were found to rescue Tcomp inhibition of RORg in a co-transfection assay. I examined the composition of these fractions using HPLC/MS as previously described. These fractions, in contrast to the lysophospholipids found in fractions 12, 13 and 14, were composed of various phospholipids, with phosphocholine being the most abundant (Figure 2.10). I proceeded to test various commercially available phospholipids for their ability to rescue Tcomp inhibition of RORg. Multiple phospholipids rescued Tcomp inhibition of RORg in a dose dependent manner (Figure 2.11B). For a phospholipid to rescue Tcomp inhibition of RORg, I found that it required an unsaturated fatty acid at the A1 or A2 position of the glycerol molecule. While 16:0 18:1 PC could rescue RORg dependent luciferase activity, 16:0 18:0 LPC failed to do so (Figure 2.11A). Phospholipids with a choline head group were best at rescuing inhibition, though ethanolamine head groups showed a partial rescue. To eliminate the possibility of rescue of inhibition by sequestration of the antagonist by phospholipid vesicles, I tested the ability of phospholipids to inhibit Tcomp activation of LXRβ. Phospholipids failed to inhibit Tcomp activation of LXRβ (Figure 2.11B), indicating a direct action of phospholipids on RORg itself. Phospholipids, while unable to activate RORg, could rescue Tcomp inhibition of RORg.

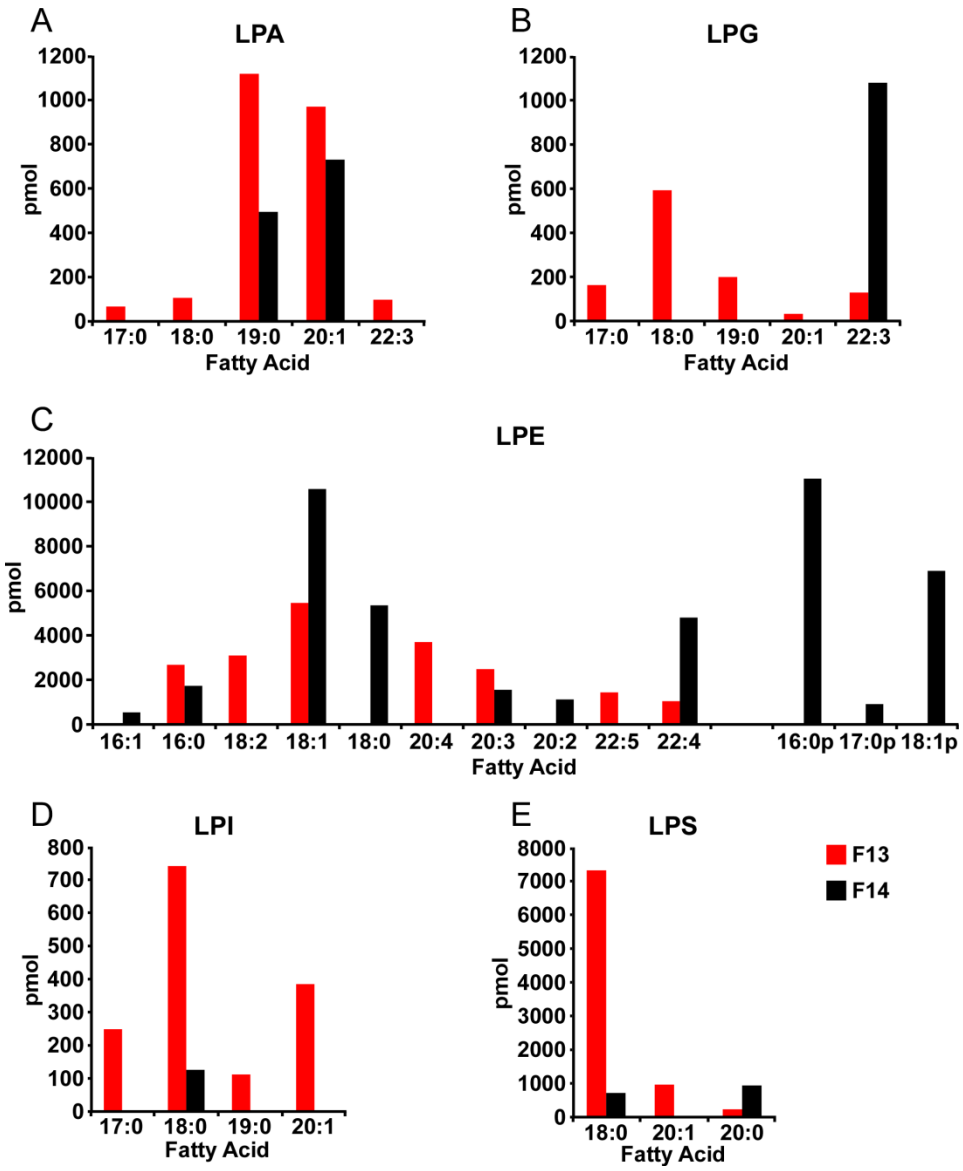


Figure 2.8 Composition of methanol fractions 13 and 14. The methanol fractions 13 and 14 were analyzed using HPLC coupled MS/MS. LPA=lysophosphatidic acid, LPG=lysophosphoglycerol, LPE=lysophosphoethanolamine, LPI=lysophosphoinositol, LPS=lysophosphoserine

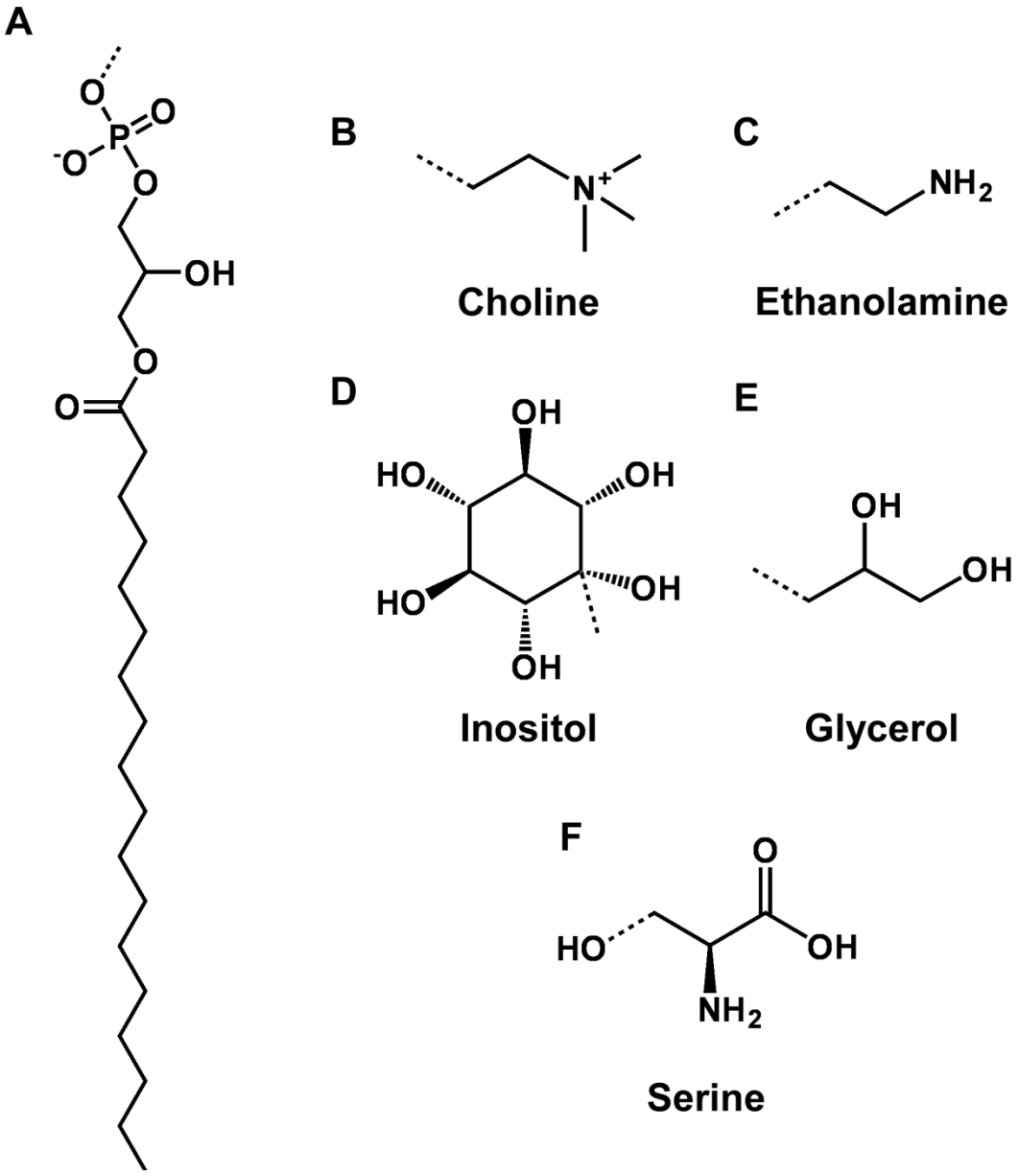


Figure 2.9 Structure of lysophospholipids. (A) A lysophospholipid consists of a glycerol backbone with a fatty acid at one of the free -OHs on the glycerol, one free –OH with no group attached, and a phosphate group on the remaining –OH. The phosphate group can either have no head group (lysophosphatidic acid), or a choline **(B)**, ethanolamine **(C)**, inositol **(D)**, glycerol **(E)** or serine **(F)** moiety attached to it.

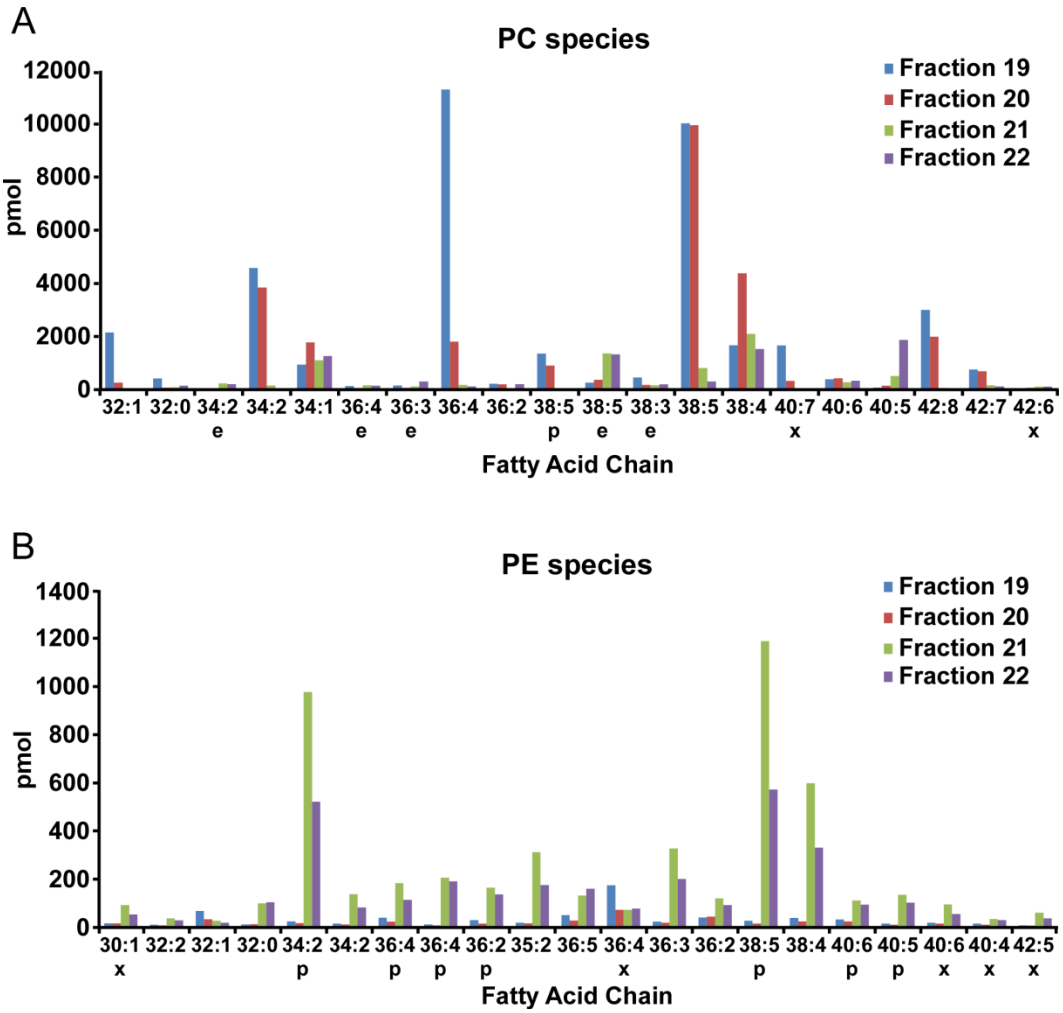


Figure 2.10 Composition of methanol fractions 18 and 19. The methanol 18 and 19 were further purified on a reverse phase SB-C18 column. The active fractions from these, nineteen and twenty for fraction eighteen, and twenty-one and twenty-two for fraction nineteen, were analyzed by HPLC-MS/MS. PC = phosphatidylcholines, PE = phosphatidylethanolamine.

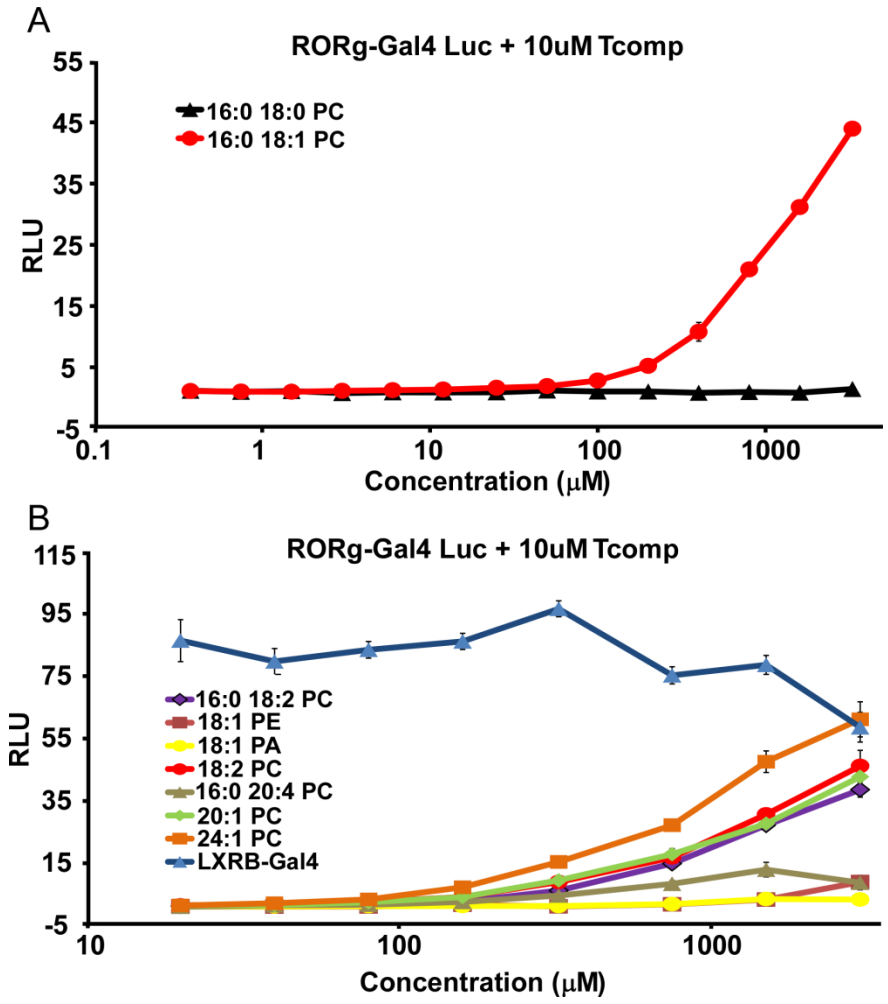


Figure 2.11 Phospholipids can rescue Tcomp inhibition of RORg. HepG2 cells were transfected with Gal4-RORg and UAS-TKLUC. Cells were treated with 10 μM Tcomp and dosed with **(A)** 16:0 18:0 PC or 16:0 18:1 PC **(B)** the indicated phospholipids. Gal4-LXRβ transfected cells were treated with 10 μM Tcomp and various doses of 16:0 18:1 PC. Values are means ± SEM.

2.2.10 Lysophospholipid Structure Activity Relationship

I have shown that an endogenous extract containing mainly lysophospholipids can activate RORg in a co-transfection assay. Numerous commercially available lysophospholipids were tested in co-transfection assays for RORg activation. Of these 18:1 LPC, 18:1 LPE and 16:0 LPE activated RORg at supraphysiologic doses (Appendix B). Activation was dose responsive, but failed to saturate due to compound toxicity. Of the three, 18:1 LPE had the strongest activation, in line with evidence that the active fraction contained compounds with a phosphoethanolamine head group. Fractions thirteen and fourteen of the methanol eluent have been shown to activate RORg in a co-transfection assay. These fractions contained large amounts of lysophospholipids with varying head groups. To narrow down the candidate compound, I further purified these fractions by HPLC. A gradient of chloroform: methanol was employed to successfully separate various lysophospholipids based on head group (Figure 2.4). By sequential runs on reverse and normal phase columns, followed by testing for activity in a RORg co-transfection assay, activity was narrowed down to the lysophosphatidylethanolamines (see materials and methods for details). The LPEs in the active fraction had fatty acids of varying chain lengths, including 22:4, 20:2 and 20:5. These compounds were not commercially available and were synthesized as described in the materials and methods. A completed SAR (Appendix B), including commercially available and synthesized phospholipids, revealed that both 20:2 and 22:4 LPE activate RORg at nanomolar doses (Figure 2.12A). Changing the head group to a PC while retaining the same fatty acid had

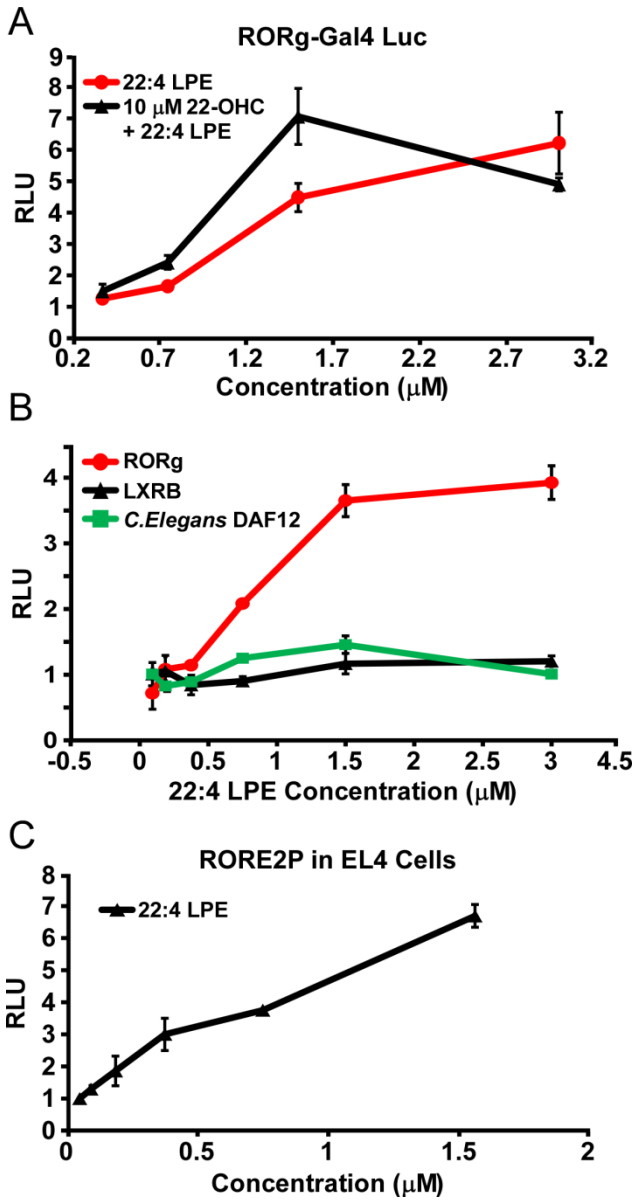


Figure 2.12 22:4 LPE activates RORg (A) HEK293 cells were transfected with Gal4-RORg and UAS-TKLUC. Cells were treated with 22:4 LPE or 22:4 LPE + 10 μM 22-hydroxycholesterol. **(B)** HEK293 cells were transfected with Gal4-RORg, Gal4-LXRβ or Gal4-Daf12 along with UAS-TKLUC. Cells were treated with 22.4 LPE and luciferase activity measure 24 hours later. Tcomp and dafachronic acid were used as LXR and Daf12 positive controls (data not shown). **(C)** EL4 cells were transfected with the RORE2P vector driven by endogenous RORγ and treated with 22:4 LPE for 24 hours. Values are means ± SEM.

varying effects on the EC50 (Appendix B). LPA variants with these fatty acids failed to activate RORg, indicating a requirement for either a choline or ethanolamine head group.

2.2.11 22:4 LPE can Rescue T-comp Repression of RORg

Tcompound is a known LXR agonist. It has been shown that Tcomp can also serve as a potent RORg antagonist. To confirm these findings, I added various concentrations of Tcomp to HEK293 and HepG2 cells co-transfected with either the Gal4-RORg fusion or full length RORg vectors and either the UAS-Luc or RORE-Luc. As expected, Tcomp repressed RORg dependent luciferase production in a dose dependent manner (Figure 2.13A). When 25-hydroxycholesterol was added in addition to Tcomp, it failed to rescue repression of luciferase production (Figure 2.13B). The inability of an oxysterol to displace Tcomp from the binding pocket further suggests that sterols may not be the endogenous ligands. On the other hand, lysophospholipids such as 22:4 LPE could rescue Tcomp inhibition of RORg in a dose dependent manner (Figure 2.13B).

2.2.12 22:4 LPE Causes IL17 Production in EL4 Cells.

22:4 fatty acid, or adrenic acid, is a low abundance fatty acid. However, the bovine calf thymus is uniquely enriched in 22:n phosphatidylethanolamine species

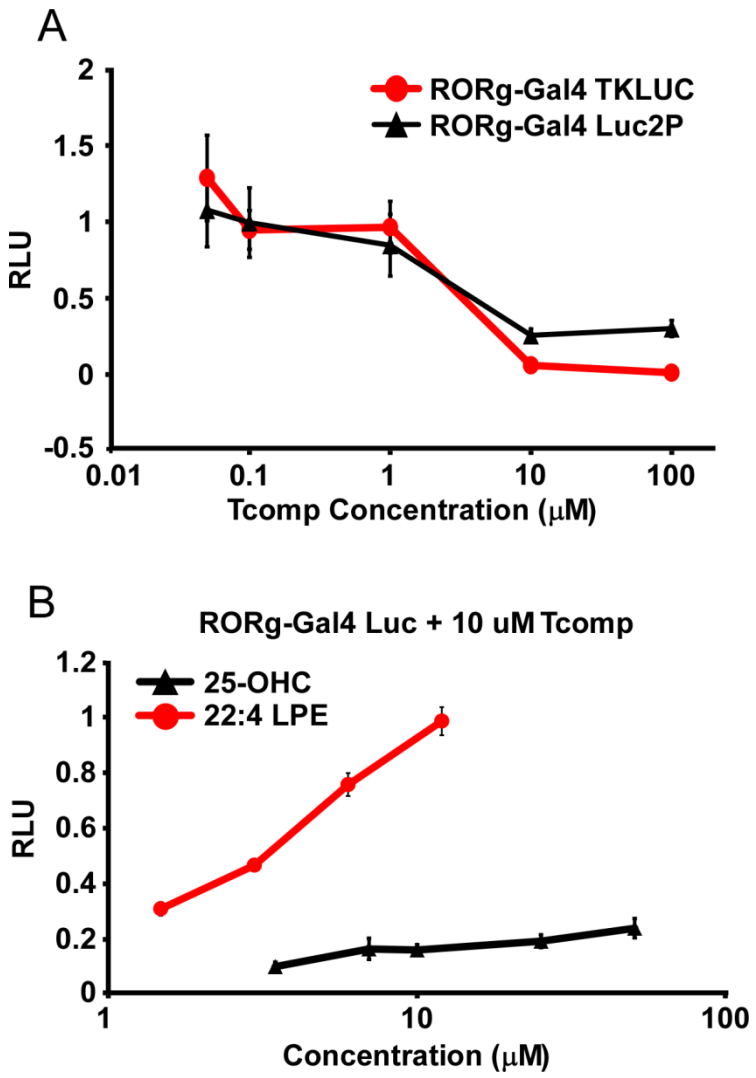


Figure 2.13 22:4 LPE but not oxysterols rescue Tcomp inhibition of RORg. (A) HepG2 cells were transfected with Gal4-RORg and UAS-TKLUC or UAS-Luc2P. Cells were treated with Tcomp and luciferase activity measured 24 hours later. **(B)** HepG2 cells transfected with Gal4-RORg were treated with 10 μM Tcomp and either 25-OH cholesterol or 22:4 LPE. Values are means ± SEM.

(Van Blitterswijk et al. 1982). I have shown that extracts from bovine calf thymus that activate RORg in a co-transfection assay are also abundant in 22:4 LPE (Figure 2.8C). It is likely that the enrichment of 22:n fatty acids in only the PE fraction of phospholipids serves as a pool for 22:4 LPE production. The ratio of 22:4 fatty acid to 18:1 fatty acid is high only in the LPE fraction of lysophospholipids in the thymus (Figure 2.8). 22:4 LPE activates RORg in the nanomolar range (Figure 2.12A). This activity is specific for RORg and 22:4 LPE fails to activate LXRβ or the *C.elegans* Daf12 receptor (Figure 2.12B). However, 22:4 LPE also fails to increase luciferase production in the presence of the full length RORg vector and RORE-luc in HepG2 and HEK293 cells (data not shown). It is feasible that this lack of activity is due to the absence of a necessary co-factor, or due to the rapid metabolism of the ligand. EL4 cells are derived from murine T-lymphocytes and have been shown to have RORg dependent IL17 production. I dosed EL4 cells with 22:4 LPE and assayed IL17a production 24 hours later using RT-qPCR. 22:4 LPE had a robust, dose responsive induction of IL17 mRNA production (Figure 2.14A). 20:3 LPE, which fails to activate the RORg-Gal4 fusion, failed to increase IL17 mRNA levels. Increase in IL17 protein level in response to 22:4 LPE was confirmed by ELISA (Figure 2.14C). To further test the ability of 22:4 LPE to activate the full length receptor in EL4 cells, the RORE-Luc vector was transfected into EL4 cells by electroporation. Upon addition of 22:4 LPE an increase in luciferase production was observed, indicating that the endogenously expressed RORg protein could bind to and activate the transfected RORE-Luc in the presence of 22:4 LPE (Figure 2.12C). Interestingly, 22-hydroxycholesterol caused both IL17 mRNA and protein production in EL4 cells (Figure 2.14 B and C).

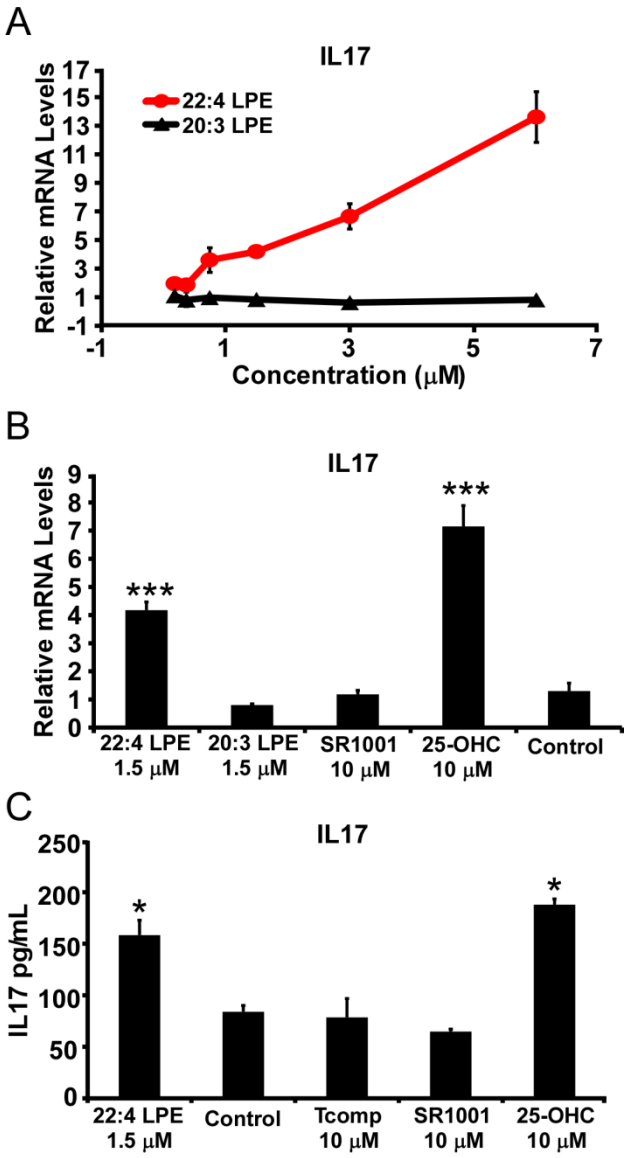


Figure 2.14 22:4 LPE causes IL17 production in EL4 cells. (A) EL4 cells were treated with 22:4 LPE or 20:3 LPE and IL17 mRNA levels measured 24 hours later **(B)** EL4 cells were treated with 1.5 μM 22:4 LPE, 1.5 μM 20:3 LPE, 10 μM SR1001, 10 μM 25-hydroxycholesterol or vehicle alone and IL17 mRNA levels assayed 24 hours later. **(C)** EL4 cells were treated with 1.5 μM 22:4 LPE, 10 μM Tcomp, 10 μM SR1001 or vehicle alone. IL17 protein levels were measured by elisa 24 hours post treatment. Values are means ± SEM. Statistics by two-tailed *t* test. **P*<0.05, ****P*<0.0005.

2.2.13 22:4 LPE Metabolites in HEK293 and HepG2 Cells

22:4 LPE is an abundant lysophospholipid in the thymus. I have shown that it activates RORg in a standard co-transfection assay. Lysophospholipids are rapidly degraded upon addition to cells. The major pathway of degradation is acylation of the lysophospholipid A2 chain by the enzyme LPCAT (lysophosphocholine acyl transferase) (Illingworth and Portman 1972; Jackson et al. 2008). Additional minor pathways involve addition of an acetyl group to the A2 position to form platelet activating factor (PAF), or degradation to lysophosphatidic acid (LPA) by lysophospholipase D, a special form phospholipase D (PLD) that can act on lysophospholipids (Baker and Chang 1999). On the other hand, lysophospholipids are formed through the action of the phospholipases A1 and A2 on phospholipids.

Attempts to modulate synthesis or degradation of the lysophospholipids in HepG2 and HEK293 cells produced no positive results (data not shown). S32826 is a potent inhibitor of the extracellular lysophospholipase D autotaxin (Giganti et al. 2008). Addition of S32826 to HepG2 cells had no effect on RORγ activity. When I added exogenous autotaxin to HepG2 cells, which should have decreased lysophospholipid levels, I saw no decrease in RORg activity. sPLA2 is the major secreted PLA2 and is responsible for increasing lysophospholipid levels. sPLA2 protein is elevated in the joints of patients with rheumatoid arthritis (Boilard et al. 2010). This raised the possibility that sPLA2 was increasing the levels of lysophospholipids, thus increasing RORg activity and consequently TH17 cell proliferation and IL17 secretion in joints of patients with rheumatoid arthritis. The sPLA2 inhibitors Thioetheremide-PC and CAY10590 (Yu et al.

1990) both failed to modulate RORg activity. Stimulation of endogenous sPLA2 secretion by a combination of IL6 and TNF-α (Crowl et al. 1991) had only a modest effect on RORg activity. A 40 fold increase in sPLA2 mRNA levels upon cytokine administration was confirmed by qPCR. Thus increasing or decreasing sPLA2 activity did not have a discernible effect on RORg.

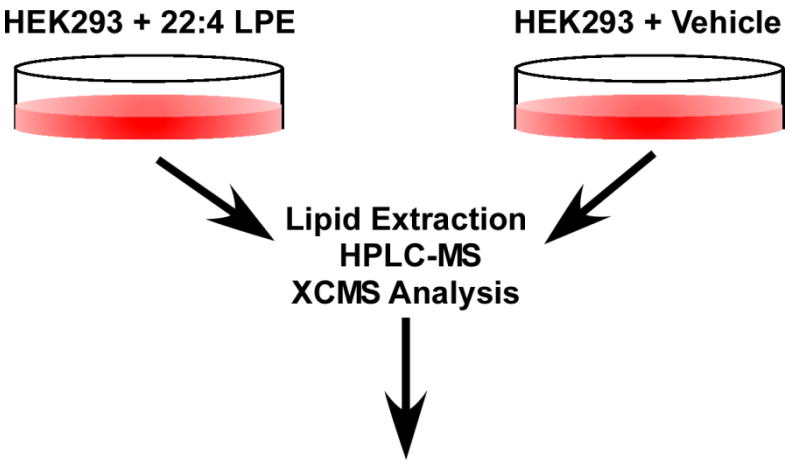
To determine the fate of lysophospholipids when added to cells, I added 22:4 LPE to both HepG2 and HEK293 cells. Cells and growth media were harvested and lipids extracted every two hours. I injected the lipids into a HPLC/MS and monitored the degradation of 22:4 LPE over time. Within two hours a 20% reduction in LPE levels was observed, with levels dropping to 20% of original dose at 24 hours. Simultaneously, HepG2 and HEK293 cells were transfected with Gal4-RORg and UAS-Luc and treated with 22:4 LPE. Luciferase activity was measured every two hours. While 22:4 LPE was rapidly degraded upon addition to cells, maximal RORg activity was observed at 24 hours post treatment (data not shown). This indicates that perhaps 22:4 LPE is metabolized into a RORg ligand.

To determine the identity of the unknown metabolite, I treated HEK293 cells with 22:4 LPE or vehicle control. The cells and media were (n=6 of each group) harvested and lipids extracted followed by determination of lipid contents by HPLC-MS. Lipids were scanned from an m/z of 100-1000. The lipid runs were analyzed by the Scripps XCMS software and multiple runs compared for differences in lipid levels (Figure 2.15). The largest fold change observed was for an m/z of 522. This compound eluted further than known lysophospholipids on our pholipidec column. The m/z of 22:4 LPE is 530, indicating that the metabolite has a loss of a functional group and addition of

a different group, with a net mass change of eight. This compound will be sent to our collaborators in the Brown lab (Vanderbilt) for further analysis.

To further test the theory that a 22:4 LPE metabolite was the active compound and a RORg ligand, I treated HEK293 cells with 22:4 LPE for twenty four hours followed by lipid extraction from the media and cells. The extracted lipids were fractionated on the pholipidec column (Figure 2.4) and tested for RORg activity in a co-transfection assay (Figure 2.16). As expected, activity no longer migrated with 22:4 LPE, which elutes at 12-15 minutes, but now moved to fraction twelve, corresponding to an elution time of 33-36 minutes. Accounting for slightly varying elution times run to run and the delay in the fraction collector, this corresponds to the peak m/z 522 that elutes at 30 minutes (Figure 2.15).

Another possibility is that lysophospholipids simply serve as precursors to eicosanoids through fatty acid cleavage from the side chain. With this is mind, I treated Gal4-RORg transfected HepG2 cells with 20:2 and 22:4 fatty acids (Figure 2.17). Neither fatty acid (for other FAs tried see Appendix B) activated RORg, ruling out the possibility that eicosanoid production was the source of 22:4 LPE activity.



Fold Change	MZ	RT
2.7	522	29.1
2.2	130	12.4
2.2	708	20.5
2.2	711	0.6
1.9	715	0.5
1.9	494	29.4
1.9	656	3.3
1.8	468	30.2
1.7	127	15.5
1.7	713	0.6
1.7	730	3.3

Figure 2.15 22:4 LPE metabolites formed in HEK293 cells. HEK293 cells were treated with 750 nM 22:4 LPE or vehicle (n=6 per group). Lipids were extracted and injected on an HPLC-MS system. MS data was analyzed using the XCMS software suit to look for differences in peak intensity between the two groups. Peaks are given in order of decreasing fold change between 22:4 LPE and vehicle treated cells. RT = retention time, MZ = mass/charge ratio.

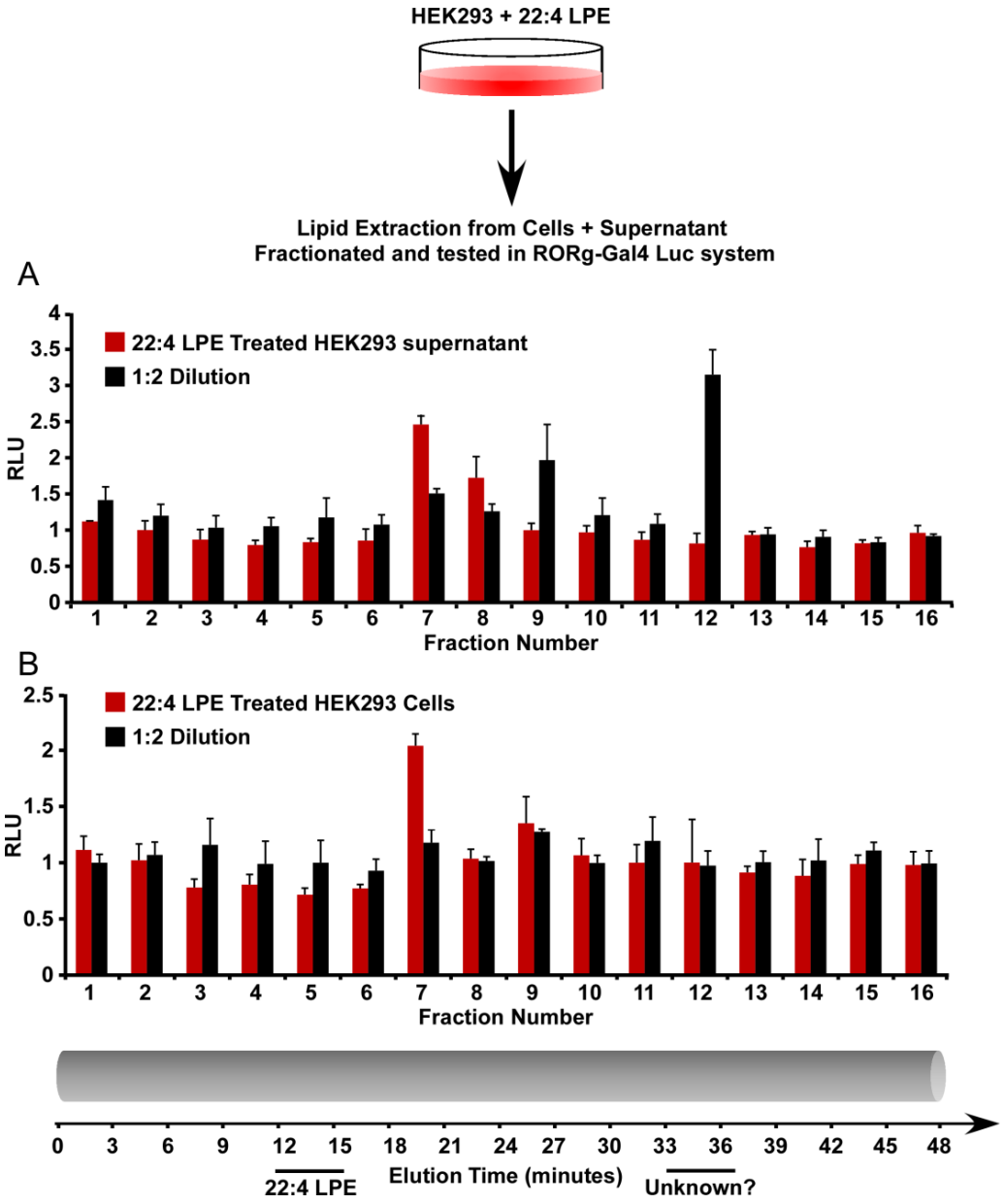


Figure 2.16 22:4 LPE is converted into metabolites that activate RORg. HEK293 cells were treated with 750 nM 22:4 LPE for 24 hours. Cells and supernatant (cell media) were extracted and fractioned by HPLC. HPLC fractions were dried down, resuspended in media and applied to HEK293 cells transfected with Gal4-RORg and UAS-Luc. 22:4 LPE elution time relative to elution time of unknown metabolite is shown below. Values are means ± SEM.

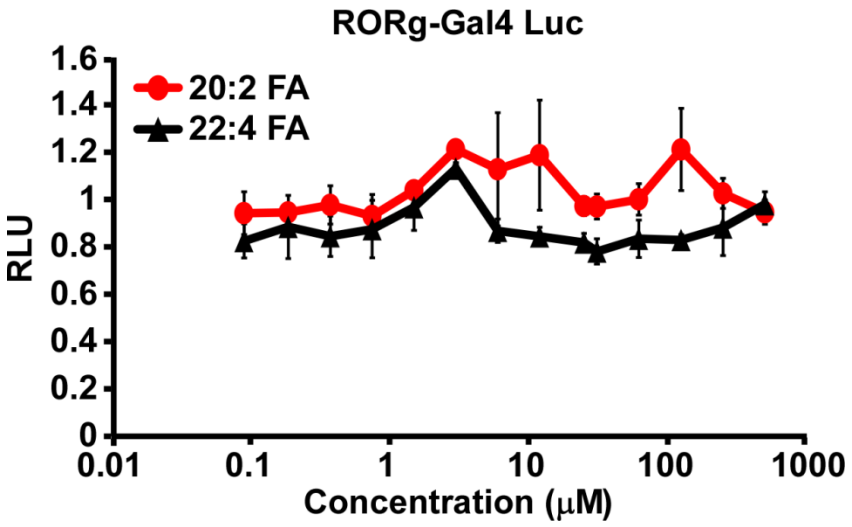


Figure 2.17 Fatty acids fail to activate RORg. HepG2 cells were transfected with Gal4-RORg and UAS-Luc. 20:2 and 22:4 FFA were applied to cells at given doses and luciferase activity measured 24 hours later. Values are means ± SEM

2.2.14 Lysophospholipids Do Not Bind RORg in the ALPHA Screen

I had previously described an ALPHA screen assay that allows detection of co-activator recruitment due to ligand binding (Figure 2.6). Various lysophospholipids were tested in the ALPHA screen for their ability to bind the RORg LBD (Figure 2.18). 18:1 LPC, 18:1 LPA and 20:4 LPE did not bind RORg in the ALPHA screen. 18:1 LPE appears to bind, however it also caused a non-specific interaction between the beads. 18:1 LPI and LPS produced no signal until their concentration crossed the CMC. At that point

they produced a robust signal that was not dose responsive. This is likely due to a non-specific interaction of the micelles with the RORg protein and ALPHA screen beads. Attempts to add detergents and alter buffer conditions to prevent micelle formation failed. The failure of LPEs to bind RORg may be due to a number of reasons, a lower phospholipid CMC in the ALPHA screen buffer as compared to a cell; requirement for a yet unidentified carrier protein to aid in the interaction of RORg with LPEs; the cellular conversion of 22:4 LPE into the true ligand or 22:4 LPE action on RORg modulated through an indirect mechanism that does not involve ligand binding.

2.2.15 Unknown Lysophospholipids Observed in the MS Spectra of Active Fractions

Endogenous extracts from bovine calf thymus contain lipids that can activate RORg in a co-transfection assay. I have shown that one of the active fractions is enriched in LPEs, specifically 22:4 LPE, which activates RORg in the nanomolar range. However, other active fractions that lacked 22:4 LPE also exist. One of these contains a compound that produces a peak with a predominant ion of m/z 538 observed in negative ion mode. MS/MS fragmentation pattern reveals the presence of an ethanolamine head group and a 18:0 fatty acid at the A1 position. The remaining mass could only be accounted for by a small modification at the A2 position. Oxidative cleavage of the A2 fatty acid can generate a large number of products with modified sn-2 residues (Figure 2.19A) (Prescott et al. 2000). Since the identity of the A1 fatty acid, the glycerol moiety and the head group is known, only a mass of 60 remains to be assigned. There are limited ways to

assign a mass of 60 to a group at the A2 position. One A2 modification that has been identified is a COO-C-OH group (Figure 2.18B). It is possible that the 22:4 LPE metabolite detected (Section 2.2.11) also contains a minor modification at the A2 position. Isolation of the metabolite, followed by MS and NMR analysis and synthesis of the potential compound, would be required to confirm this hypothesis.

2.3 Summary

RORg activity can be decreased by the addition of cholesterol synthesis inhibitors. However, despite binding in the ALPHA screen, all sterols tested failed to activate RORg in a co-transfection assay in mammalian cell lines. Extractions of lipids from bovine calf thymus also failed to reveal any sterols that could potentially modulate RORg activity. Endogenous phospholipid extracts did have RORg activity, however only against the Gal4-RORg fusion protein. The identity of the activating phospholipid was confirmed as 22:4 LPE through mass spectrometric analysis. 22:4 LPE activates RORg in HEK293 and HepG2 cells at nanomolar doses. It can also increase IL17 secretion in the TH17 like EL4 murine thymocyte cell line. Upon addition to cells, 22:4 LPE is rapidly metabolized to an unknown compound that may be the actual ligand. This may explain 22:4 LPEs failure to bind RORg in the ALPHA screen or activate the full length RORg vector. Additional work to elucidate the structure of this metabolite and additional active compounds found in endogenous extracts is still required.

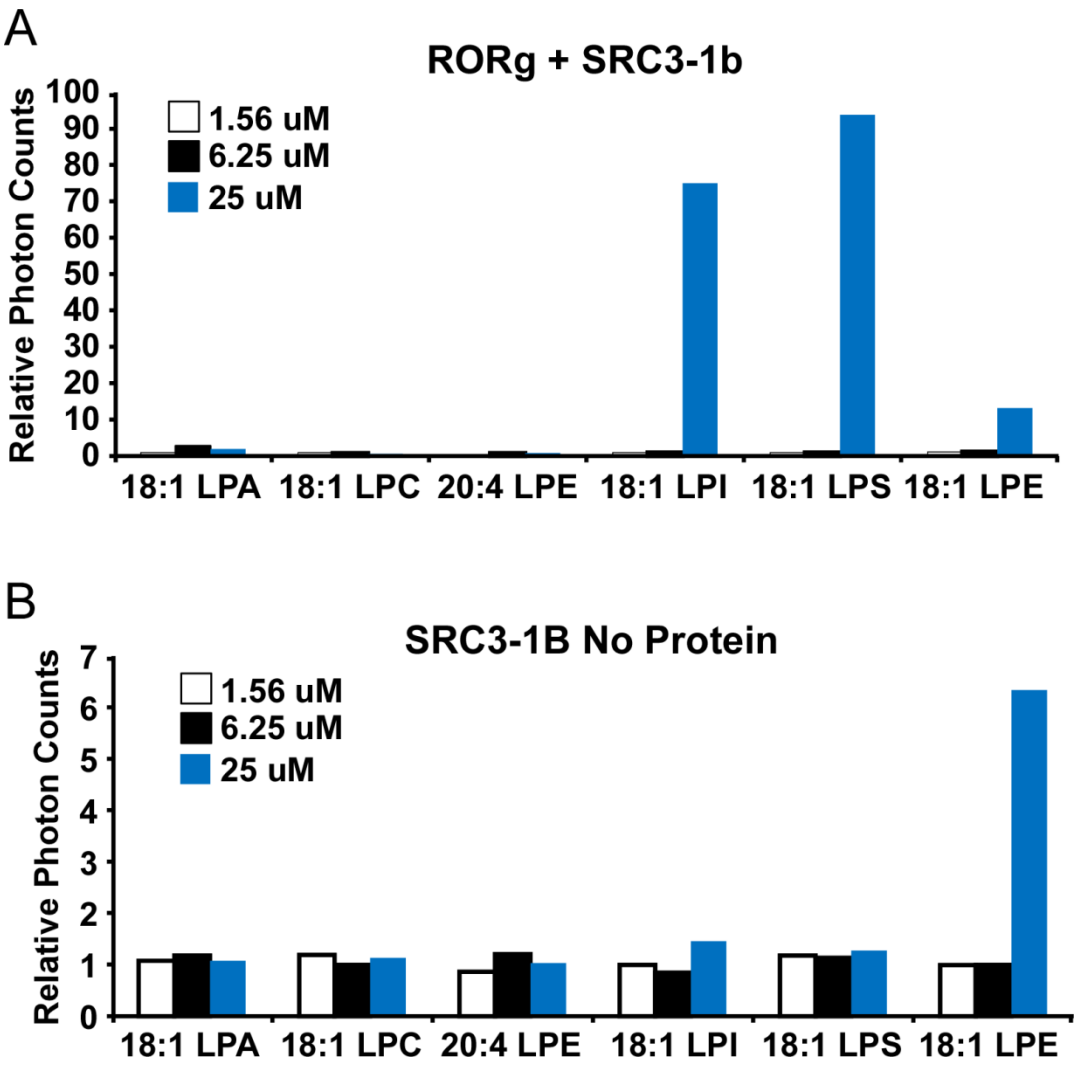


Figure 2.18 Lysophospholipids do not cause RORg co-activator recruitment in the ALPHA screen assay. RORg-HisGST protein and b-SRC3-1b were added to ALPHA screen donor and acceptor beads. 18:1 LPA, 18:1 LPC, 20:4 LPE, 18:1 LPI, 18:1 LPS and 18:1 LPE were added at given doses and incubated for 1 hour at room temperature followed by measurement of total photon counts.

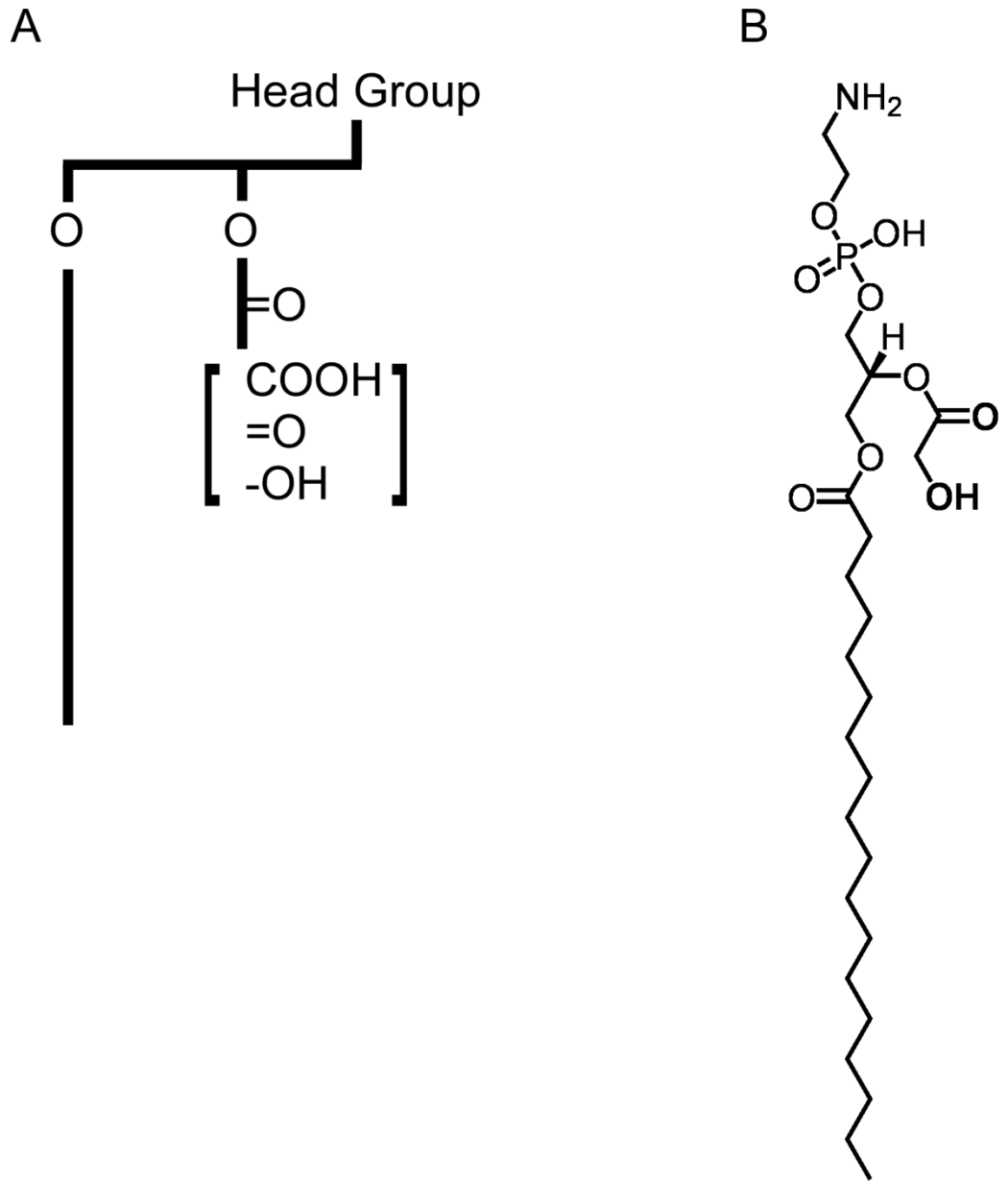


Figure 2.19 Potential structure of unknown phospholipids in active fractions. (A) Modifications on the A2 chain of phospholipids that are known to exist (B) Possible structure of the unknown phospholipid in a fraction that can activate RORg. MS/MS fragmentation analysis revealed an 18:0 fatty acid at the A1 position, a phosphoethanolamine head group and a mass of 539.

CHAPTER 3

Discussion

3.1 RORg May be Constitutively Active

It has long been thought that certain nuclear receptors may be constitutively active (Chawla et al. 2001). Nuclear receptor LBDs usually have a very hydrophobic core. The core is stabilized by the presence of a hydrophobic small molecule. In the case of some nuclear receptors, an abundant endogenous lipid such as cholesterol may permanently occupy the pocket. Receptor activity in such cases would not be ligand dependent but modulated transcriptionally or by post translational modifications. Additionally, cellular co-activator and co-repressor levels could be altered to modulate activity.

There are several lines of evidence that support the hypothesis that RORg is constitutively active and that the LBD is always occupied by a sterol. I and other groups have shown that basal RORg activity is high in mammalian cells. The RORg LBD can bind multiple sterols in in-vitro binding assays such as the ALPHA screen (Figure 2.6) (Jin et al. 2010). Statins, which are HMG-COA inhibitors and decrease cellular sterol levels, also decrease RORg activity (Figure 2.1). Upon treatment with statins, several sterols rescue inhibition of RORg (Appendix A). However, despite an extensive search, no sterol I have tested, or in the published literature, is capable of significantly increasing RORg activity in mammalian cell lines.

RORg binds oxysterols with a K_d in the 20 nM range whereas oxysterols bind LXRα/β with a K_d in the 200 nM range (Janowski et al. 1999; Jin et al. 2010). The affinity of RORg for cholesterol is also an order of magnitude higher than for the LXRs. Thus I propose that RORg has a much higher affinity for sterols than the sterol receptor LXR and that cellular sterol concentrations are sufficient to permanently saturate the receptor. RORg also appears to be more promiscuous in its choice of sterols, allowing anything from oxysterols to cholesterol biosynthesis intermediates to bind the LBD.

3.2 22:4 LPE as the RORg ligand

Taken together these studies suggest that RORg activity may be modulated by lysophospholipids and not sterols. I propose that sterols do indeed always occupy the RORg pocket and provide a high basal activity, but these can subsequently be replaced by lysophospholipids that can further increase RORg activity. Several lines of evidence support this. Firstly, there is very little evidence that oxysterols modulate endogenous RORg activity. Secondly, RORg promiscuously binds several sterols from multiple sterol classes (Appendix A) making it unlikely that change in the endogenous concentrations of any of these would have a significant impact on activity. Finally, no sterol tested so far has been able to significantly increase RORg activity in a co-transfection assay.

Lysophospholipids are a class of compounds created by the cleavage of the A1 or A2 chain of phospholipids by phospholipase A1 or A2 respectively. Cytosolic PLA2 (cPLA2) is an intracellular PLA2 whose activity is modulated by intracellular calcium concentrations. cPLA2 is an important immune modulator; several immunogenic signals

can increase cellular calcium levels or cPLA2 phosphorylation (Leslie 1997). cPLA2 cleaves the A2 chain of phosphocholine yielding arachidonic acid (AA) and lysophosphatidylcholine. Arachidonic acid is further converted into a number of pro-inflammatory eicosanoids. In addition to cPLA2, there are a number of secreted PLA2s (sPLA2) that are implicated in multiple disease processes including atherosclerosis (Jonsson-Rylander et al. 2008). In multiple sclerosis and mouse models of multiple sclerosis such as EAE, sPLA2 levels are significantly elevated in cerebrospinal fluid (Cunningham et al. 2006). sPLA2 activity was also 4-5 fold higher in the urine of patients with multiple sclerosis. Interestingly, EAE and multiple sclerosis are TH17 mediated diseases whose clinical outcomes are significantly improved by a RORg antagonist (Solt et al. 2011). Patients with rheumatoid arthritis, another TH17 and RORγ mediated disease, express large quantities of sPLA2s in the synovial fluid. Treatment with sPLA2 inhibitors improves clinical outcomes in EAE (Cunningham et al. 2006), atherosclerosis (Rosenson 2009) and rheumatoid arthritis (Bradley et al. 2005).

Undoubtedly a large component of sPLA2 action is through the generation and AA and its subsequent conversion to various inflammatory mediators such as eicosanoids. However, it is interesting that both sPLA2 inhibitors and RORg inhibitors have similar effects and drastically improving clinical outcomes in TH17 modulated immune diseases. This raises the possibility that sPLA2 action and RORg activity are related. The byproduct of all PLA2 action is lysophospholipids (Figure 3.1). For every molecule of AA generated, a molecule of a lysophospholipid is made. I propose that the action of sPLA2 generates AA that is converted to eicosanoids that module the

inflammatory process. In addition, the lysophospholipids generated as a consequence of increased sPLA2 activity activate RORg, increasing TH17 cell proliferation.

These studies have shown that lysophospholipids, especially 22:4 LPE, can activate the RORg-Gal4 fusion in both HEK293 and HepG2 cells (Figure 2.11). The action of 22:4 LPE is specific for RORg-Gal4 and it fails to activate LXRβ or the *C.elegans* Daf-12 receptor. 22:4 LPE can also completely rescue Tcomp inhibition of RORg, unlike oxysterols (Figure 2.12). A complete dose response curve cannot be generated due to the toxicity of 22:4 LPE at low doses (~10 μM). This may explain the inability of 22:4 LPE to activate the RORg-full length construct. While 22:4 LPE may have similar efficacies for the full length and Gal4 fusions, its potency on the full length construct may be lower than on the Gal4 fusion, thus not having any detectable activity over background at low doses. The reasons for the toxicity of 22:4 LPE are unclear, as 22:6 LPE and 18:1 LPE that have lesser and greater CMCs respectively (critical micelle concentrations) are both less toxic. A cell surface G-protein coupled LPE receptor has been proposed to exist, however it does not show specificity for LPE fatty acid chain length or unsaturation (Makide et al. 2009), while changing the LPE fatty acid chain length has a dramatic effect on RORg activity (Appendix B). I have also shown that 22:4 LPE, in contrast to 20:3 LPE, can increase IL17 production in EL4 cells, (Figure 2.14), though it has yet to be shown to be RORg dependent.

22:4 fatty acid is a low abundance fatty acid that constitutes less than 1% of the total phospholipid fatty acid pool. In thymocytes however, 22:4 PE is enriched and forms 10% of the PE fraction (Van Blitterswijk et al. 1982; Goppelt et al. 1986). This increased 22:4 PE pool in thymocytes could serve as a pool for 22:4 LPE generation and RORg

activation. The reasons for the enrichment of large amounts of the low abundance 22:4 fatty acid in the PE fraction of thymocytes are currently unknown, as are the source of the 22:4 fatty acid, whether it is endogenously synthesized or obtained from an external source. This abundance of 22:4 LPE in thymocytes may also explain the failure of exogenous sPLA2 addition or PLA2 inhibition in modulating RORg activity in HepG2 and HEK293 cells, since both cell lines lack significant amounts of 22:4 PE to serve as a substrate for the enzyme. Those experiments would need to be repeated with cells cultured in media containing abundant 22:4 fatty acid or 22:4 PE, to increase 22:4 PE concentrations in the cell membrane.

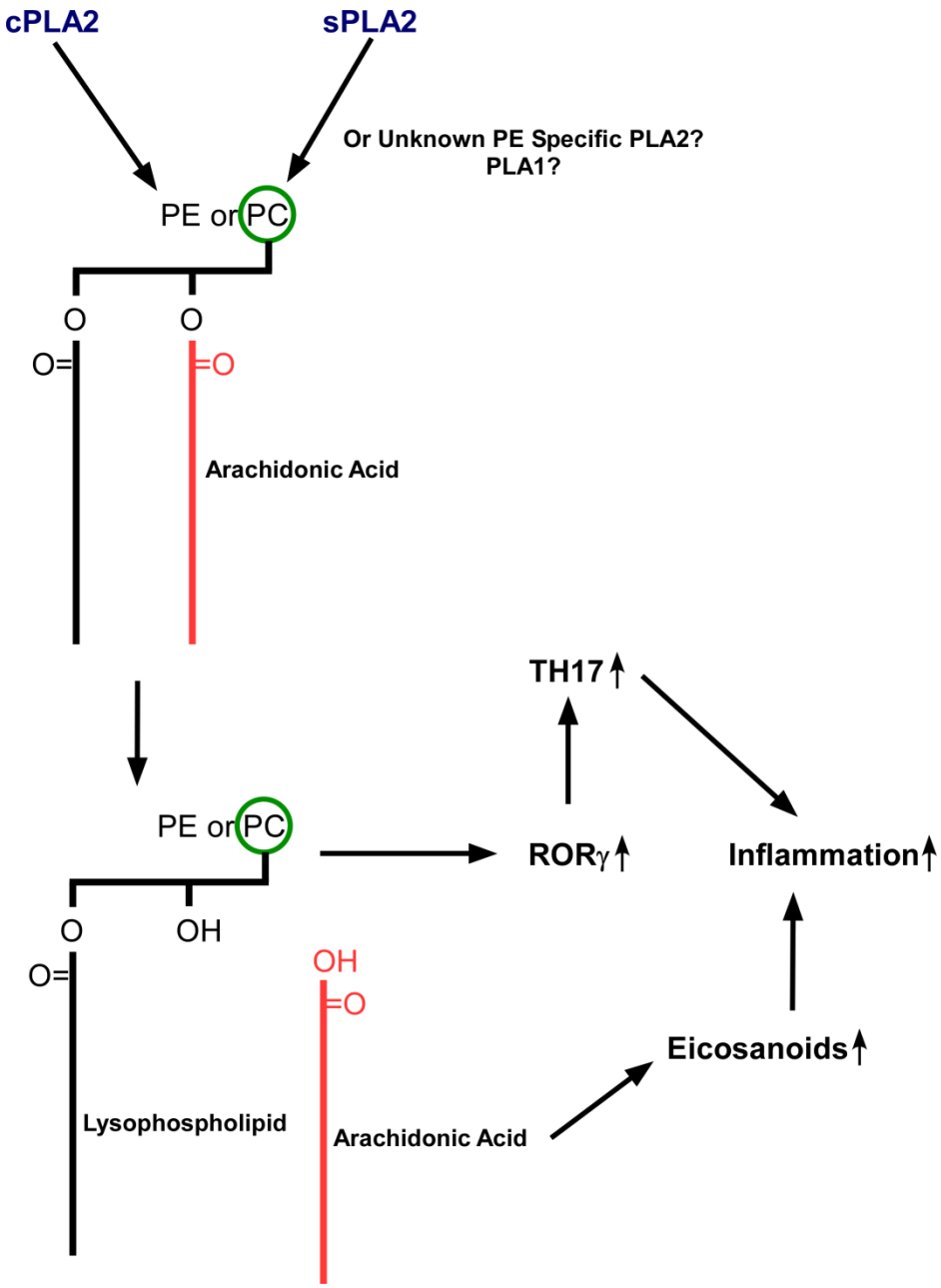


Figure 3.1 Proposed mechanism of RORg ligand synthesis. Immunogenic activation and secretion of either cPLA2 or sPLA2 increases cleavage of phospholipid side chains to release arachidonic acid (AA) and lysophospholipids. AA is further metabolized into the pro-inflammatory eicosanoids whereas the lysophospholipids can activate RORg, increasing TH17 cell proliferation and IL17 secretion

CHAPTER 4

Introduction to Cytochrome B5

4.1 Introduction to Steroidogenesis

Steroid hormones are cholesterol derived compounds that regulate a multitude of physiological processes from sexual development and function, to salt and glucose homeostasis (Miller and Auchus 2011). The process of converting cholesterol to steroids is known as steroidogenesis. The initial step of steroidogenesis is the cleavage of the C27 cholesterol side chain to yield the C21 pregnanes. These can then be further hydroxylated and converted to the C21 mineralocorticoids and glucocorticoids. Further excision of the side chain of the C21 pregnanes yields the C19 male and C18 female sex hormones (Figure 4.1). The process of steroidogenesis is tightly regulated in fetal and adult life. It involves the delivery of cholesterol to the mitochondria followed by its conversion into the various steroids by a series of enzymes. These enzymes include the P450s and hydroxysteroid dehydrogenases (HSDs). This introduction will briefly discuss the various enzymes involved in steroidogenesis and the diseases caused by mutations in these enzymes with a special emphasis on CYP17A1 and cytochrome B5.

4.2 Trafficking of Cholesterol

Steroidogenesis begins with the uptake of cholesterol by steroidogenic cells and its transfer to the mitochondria, where the initial steps of steroidogenesis are performed.

While the human adrenal, testis and ovaries can synthesize cholesterol de novo, the majority of it is derived from the uptake of circulating LDL (Gwynne and Strauss 1982). This cholesterol is then converted to cholesterol esters by acyl-coenzyme A: cholesterol acyltransferase (ACAT) and stored in lipid droplets. When required, cholesterol can be released from these cholesterol esters by hormone sensitive lipase (HSL). In the adrenals, adrenocorticotropic hormone (ACTH), a hormone derived from the anterior pituitary, stimulates steroidogenesis by inhibiting the esterification of cholesterol and increasing the activity of HSL. In addition, ACTH increases uptake of circulating LDL, increasing the total cholesterol available to the cell. This cholesterol is then delivered to the outer mitochondrial membrane (OMM). Cholesterol is practically insoluble in water and the transfer of cholesterol from the OMM to the inner mitochondrial membrane (IMM), where the first enzyme of steroidogenesis is located, requires StAR (Steroidogenic acute regulatory protein) (Miller 2007). StAR is a 37-kDa protein expressed in the adrenals and the gonads. It includes a mitochondrial leader sequence that is cleaved off at the mitochondria to yield a 30-kDA protein. StAR rapidly shuttles cholesterol from the OMM to the IMM. In the absence of StAR the first step of steroidogenesis proceeds at roughly 14% of its normal rate (Lin et al. 1995). In the placenta, StAR is absent and its role in trafficking is performed by a StAR related protein, MLN64 (Bose et al. 2000). This explains why humans and rodents lacking StAR can still reach term, since the placenta can continue to make progesterone to maintain the pregnancy.

Disorders of cholesterol trafficking to the mitochondria include adrenoleukodystrophy (ALD). ALD is commonly caused by a mutation in an ATP-binding cassette transporter, ABCD1 (ALDP) (Watkins et al. 1995). ABCD1 is

responsible for trafficking very long chain fatty acids to the peroxisome for β-oxidation. Thus mutations in ABCD1 cause the accumulation of very long chain fatty acids, especially in the adrenal cortex and Leydig and/or Sertoli cells of the testis. ALD causes both neuronal leukodystrophy with CNS symptoms and adrenal insufficiency. A spectrum of diseases caused by defects in cholesterol trafficking include Wolman disease, caused by mutations in the lysosomal cholesterol esterase (Anderson et al. 1994) and Smith-Lemli-Opitz syndrome, caused by mutations in the sterol Δ7 reductase gene DHCR7 (Correa-Cerro and Porter 2005).

Mutations in StAR lead to the genetic disorder of lipoid congenital adrenal hyperplasia (lipoid-CAH) (Tee et al. 1995), which is characterized by nearly absent steroid levels, high ACTH and enlarged adrenals laden with cholesterol. The symptoms of lipoid-CAH led to a two hit model of disease progression (Bose et al. 1996). First, StAR is absent leading to a defect in fetal adrenal steroidogenesis. Since feedback inhibition of steroids is required to modulate ACTH and LH secretions from the pituitary, the absence of steroids leads to an increase in both hormones. ACTH then promotes the uptake of cholesterol by the adrenal cells. As the cells accumulate excess cholesterol esters, further damage occurs, leading to apoptosis. In male patients, the Leydig cells that secrete testosterone are destroyed early, leading to insufficient steroids for male external genital development (Ogata et al. 1989). Thus genotypic males are born with a blind vaginal pouch. However the Sertoli cells of the testis survive, secreting Mullerian inhibitory hormone, preventing the development of a cervix, uterus or fallopian tubes.

Genetic females are born with normal internal and external genitalia since the fetal ovary doesn't produce any steroids. However, upon reaching puberty, individual

follicles are recruited to produce steroids. During the early phase the ovary is functional and it is only over time with cholesterol ester toxicity that steroidogenesis becomes impaired (Bose et al. 1997). Thus women are partially feminized but unable to ovulate and conceive.

4.3 The cytochrome P450 Enzymes

The cytochrome P450 enzymes, commonly abbreviated as CYP, catalyze the oxygenation of many substances. The P450s are prominently involved in steroidogenesis and the detoxification of endogenous and exogenous substances. The P450s derive electrons from NADPH through an intermediary protein, ferrodoxin for mitochondrial P450s and P450 oxidoreductase (POR) for P450s located in the endoplasmic reticulum (ER) (Miller 2005). Human beings have 57 P450s, seven of which are found in the mitochondria. Of these seven, CYP11A1, CYP11B1 and CYP11B2 are involved in steroidogenesis. Fifty P450s are found in the ER, of which three, CYP17A1, CYP21A2 and CYP19A1 are involved in steroidogenesis (Miller and Auchus 2011).

The first and rate limiting step of steroidogenesis is catalyzed by CYP11A1 that cleaves the side chain of C27 cholesterol to yield the C21 steroid pregnenolone. CYP11A1 is located on the IMM, and delivery of cholesterol to the IMM by StAR is essential for its proper function. CYP11A1 begins by hydroxylating cholesterol on the 22 position, then the 20 position. The 20(R),22(R)-dihydroxycholesterol is then cleaved by the same enzyme to yield pregnenolone (Simpson 1979). The expression of CYP11A1 generally renders a tissue steroidogenic (Hu et al. 2002), and its expression in the gonads

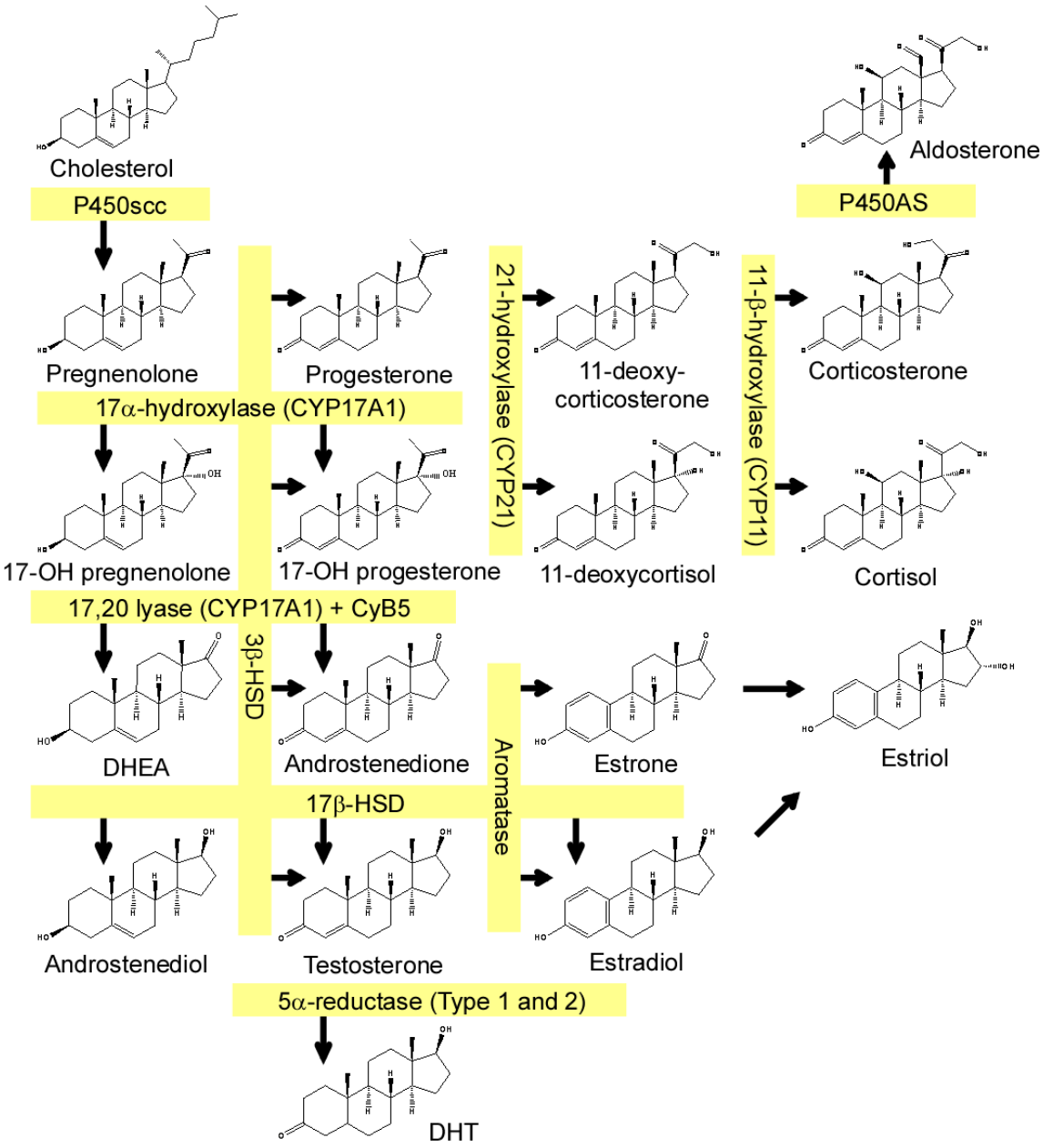


Figure 4.1 The major pathways of steroidogenesis. The major steroidogenic enzymes, their substrates, products and the reactions they catalyze are described above. Cyb5 is required for the CYP17A1 17,20 lyase reaction but not the 17-hydroxylase reaction.

and adrenals is dependent on the expression of the nuclear receptor steroidogenic factor 1 (SF1) (Parker and Schimmer 1997). In addition CYP11A1 expression can be induced by cAMP, produced by ACTH stimulation of the adrenal (John et al. 1986). Since CYP11A1 is the rate limiting step in steroidogenesis, the more copies of the enzyme present in a cell, the greater its steroidogenic potential. P450scc deficiency is a relatively rare form of lipoid-CAH, StAR deficiency being the primary cause (Kim et al. 2008). Similar to StAR deficiency, CYP11A1 loss leads to elimination of almost all steroidogenesis. However, complete absence of CYP11A1 does not occur, and CYP11A1 deficiency is always partial and not complete. Because StAR is not expressed in the placenta, StAR mutant lipoid-CAH fetuses continue to produce progesterone to maintain the pregnancy. However lipoid-CAH caused by loss of CYP11A1 knocks out placental steroid production. Few fetuses come to term, perhaps due to continued maternal progesterone production.

The next step of steroidogenesis is catalyzed by the CYP17A1 that interacts with POR to hydroxylate progesterone and pregnenolone. It also perform the 17,20-lyase reaction, converting the C21 steroids 17-hydroxyprogesterone (17-OHP) and 17-hydroxypregnenolone into the C19 steroids dehydroepiandrosterone and androstenedione, respectively. The 17,20-lyase reaction is greatly enhanced by the presence of cytochrome B5. This P450 shall be discussed in detail in a later section.

The hydroxylated C21 steroids can proceed to be converted to C19 androgens by CYP17A1 or can alternatively be further hydroxylated and eventually be converted to the mineralocorticoids or glucocorticoids (Figure 4.1). The adrenal glomerulosa and fasciculata express the microsomal P450 CYP21A1. There are two tandem CYP21 genes,

CYP21A2 which is functional in humans (Chang and Chung 1995) and CYP21A1, which functions in mice (Parker et al. 1985), but is a non-functional pseudogene in human beings. CYP21A2 catalyses the conversion of progesterone into 11-deoxycorticosterone (11-DOC) and 17-OHP into 11-deoxycortisol. These precursors are then further converted to aldosterone and cortisol, respectively. Because the CYP21 genes are located in the major histocompatibility (MHC) locus, these genes undergo frequent mutations and recombinations (White and Speiser 2000). Thus, 21-hydroxylase deficiency is the most common cause of CAH, due primarily to gene conversion events with CYP21A1P. Patients usually present with severe mineralocorticoid and glucocorticoid deficiency characterized by hyponatremia, hyperkalemia and hypotension (Merke et al. 2000), which can be fatal if not caught and treated early. Lack of cortisol increases ACTH secretion by the pituitary, which in turn increases adrenal steroid production. Since the CYP21A2 enzyme is compromised, 17-OHP and 17-OH pregnenolone accumulate and are converted to DHEA and androstenedione, then to androgens, which leads to virilization of female fetuses (Speiser et al. 1992). A normal adrenal produces much more cortisol than aldosterone, thus CYP21A2 deficiency usually leads to low aldosterone and salt wasting with elevated levels of 17-OHP and the sex steroids.

CYPc11 are mitochondrial P450s, involved in the final steps of mineralocorticoid and glucocorticoid synthesis. In humans, this gene has two isoforms, CYPc11B, expressed in the adrenal fasciculata, and CYPc11AS, expressed in the adrenal zona glomerulosa (Mornet et al. 1989). The mouse genome also has two identical CYP11 genes performing the same functions as in humans (Payne and Hales 2004). CYP11B performs the conversion of 11-deoxycortisol to cortisol and 11-DOC to corticosterone. It

is deficient in the 18-methyloxidase activity found in CYPc11AS, required to convert 18-hydroxycorticosterone to aldosterone. CYP11B deficiency is a minor cause of CAH (White et al. 1991). Mutations in CYP11B cause accumulation of 11-DOC and 11-deoxycortisol in the fasciculata (Zachmann et al. 1983). The normally functioning CYPc11AS remains able to make aldosterone in the glomerulosa, however 11-DOC can act as a mineralocorticoid, and the high levels seen in CYP11B deficient patients lead to salt retention and hypertension, thus suppressing renin and aldosterone. Similar to 21-hydroxylase deficiency, defects in cortisol production leads to elevated ACTH levels and an increase in male sex steroid production, which leads to virilization of female infants. In the glomerulosa, CYPc11AS performs the same functions as CYPc11B, along with 18-hydroxylation and 18-methyl oxidation of corticosterone into aldosterone. Deficiency of this enzyme is referred to as aldosterone synthase deficiency. There are two forms, the first, corticosterone methyl oxidase deficiency I (CMOI), results from a complete loss of both the 18-hydroxylase and 18-methyl oxidase activity (Ulick et al. 1992). CMOI is characterized by salt wasting and elevated levels of corticosterone with low levels of 18-OH corticosterone and aldosterone. CMOII, on the other hand, is a selective defect in the 18-methyl oxidase activity of CYPc11AS, resulting in elevated 18-OH corticosterone (Pascoe et al. 1992).

The final P450 involved in steroidogenesis is CYP19A1, which catalyzes the aromatization of the male sex steroids into the female estrogens. This single enzyme can both demethylate the side chain and aromatize the A-ring, hence its common name, aromatase (Simpson et al. 1994). CYP19A1 is expressed in many tissues including the steroidogenic ovary and placenta and non steroidogenic tissues such as fat and bone. An

aromatase knockout mouse has been made, and aromatase deficiency is found in some rare patients. In both cases, embryonic and fetal development are normal, indicating that aromatase and fetal/placental estrogen production is not essential for proper development (Conte et al. 1994). The placenta usually produces large amounts of estriol and estradiol that proceed to enter maternal circulation. The fetal adrenal usually produces large amounts of DHEA that enters circulation and is subsequently aromatized by the placenta. Fetal lack of CYP19A1 prevents placental aromatization of fetal DHEA, allowing androgens to enter the maternal circulation and virilize the mother.

4.4 The Hydroxysteroid Dehydrogenases

In addition to the cytochrome P450s, steroidogenesis involves a number of hydroxysteroid dehydrogenases. These enzymes either reduce a ketone to an alcohol or oxidize an alcohol to a ketone. This process involves the donation of a hydrogen by NADPH or the transfer of a hydrogen to NAD+ (Agarwal and Auchus 2005). In vitro, these enzymes can be made to function as either dehydrogenases or reductases, but in tissues they are usually unidirectional, depending on the abundance of NADPH or NAD+ (Agarwal and Auchus 2005) and their relative affinities for NADPH and NAD(+).

3βHSD converts pregnenolone to progesterone and 17-hydroxy pregnenolone to 17-OHP. This process involves converting the hydroxyl on the 3 position to a ketone group and the simultaneous isomerization of the Δ5 double bond to the Δ4 position, thus converting Δ5 steroids to Δ4 steroids. Since the 3βHSD reaction has a higher K_m than the 17-hydroxylation carried out by CYP17, Δ5 steroids are more abundant than Δ4 steroids

(Auchus et al. 1998; Lee et al. 1999). In humans there are two 3βHSD isoforms, 3βHSD1 expressed in the placenta, liver, brain and breast (Lachance et al. 1990) and 3βHSD2 expressed in the adrenals and gonads (Lorence et al. 1990). Since progesterone production from pregnenolone is dependent on 3βHSD and placental progesterone production is required to maintain a pregnancy, 3βHSD1 mutations have not been identified. Patients with 3βHSD2 mutations have a block in conversion of Δ5 precursors to active steroid hormones. This defect leads to accumulation of Δ5 steroids such as DHEA, that can then be converted to testosterone primarily by liver 3βHSD1. Thus, 46XX fetuses are virilized slightly in utero (Moisan et al. 1999). However, the peripheral conversion to testosterone is not adequate to properly develop the male external genitalia. The block in conversion to Δ4 steroids also leads to a mineralocorticoid and glucocorticoid deficiency.

17βHSDs are a group of enzymes that catalyze the conversion of DHEA to androstenediol, androstenedione to testosterone and estrone to estradiol and vice versa. There are multiple 17βHSD isoforms, including 14 in humans (Peltoketo et al. 1988). 17βHSD1 has a strong preference of substrates with an aromatic A ring and is expressed primarily in the placenta and ovary, converting estrone to estradiol (Tremblay et al. 1989). Unlike 17βHSD1, 17βHSD2 inactivates both androgens and estrogens, thus catalyzing the reverse reactions of 17βHSD1. 17βHSD2 is expressed in the endothelial cells of placental intravillous vessels where it can protect the fetal circulation from maternal sex steroids (Takeyama et al. 1998). 17βHSD3 is the testicular isoform of 17βHSD that converts DHEA to androstenediol and androstenedione to testosterone

(Andersson et al. 1996). 17βHSD3 deficient females are normal, but lack of testicular androgen production prevents proper development of male external genitalia in deficient males. (Geissler et al. 1994).

4.5 The 17-Hydroxylase and 17,20-Lyase enzyme : CYP17A1

CYP17A1 is a single enzyme that catalyzes both the 17-hydroxylation of progesterone and pregnenolone and the 17, 20-lyase reaction, further converting them to androstenedione and DHEA respectively. It is primary expressed in the adrenals and gonads. The lyase activity has a 50 fold greater preference for Δ5 steroids vs Δ4 steroids, leading to higher concentrations of the Δ5 sterols in both the adrenals and the gonads (Auchus et al. 1998). The hydroxylation reaction is less dependent on electron transfer from NADPH via POR than the lyase reaction. Additionally, cytochrome B5 (cyb5) causes a 10 fold increase in the lyase reaction, with no change in the preference for Δ5 sterols (Auchus et al. 1998). This allosteric stimulation allows the hydroxylase and lyase reactions to be modulated separately, despite being performed by a single enzyme. Levels of NADPH, POR and cyb5 determine the efficiency of the hydroxylase vs lyase reaction. CYP17A1 is absent in the zona glomerulosa of the adrenal, its hydroxylase activity is present in the fasciculata and reticularis, while the lyase activity is present only in the reticularis (Miller and Auchus 2011). Thus presence or absence of CYP17A1 and its hydroxylase and lyase activities determines the fate of steroidogenesis in the zones of the adrenal; progesterone-derived mineralocorticoids in the glomerulosa, 17-OHP-derived glucocorticoids in the fasciculata and DHEA in the reticularis.

Both isolated 17,20-lyase deficiency and 17α-hydroxylase/17,20-lyase deficiency have been found in patients (Zachmann et al. 1972). This observation initially led to the incorrect assumption that they were two separate enzymes. After discovering that a single enzyme (CYP17A1) could carry out both the lyase and hydroxylase reactions (Nakajin et al. 1981), genetic screening identified CYP17A1 mutations that lack the lyase activity while retaining the hydroxylase activity. Two mutations, R347H and R358Q, which mutate the POR binding site on the protein, both cause isolated lyase deficiency (Geller et al. 1997). The lyase reaction is more dependent on electron transfer through POR explaining this phenotype. Consistent with this model, mutations in POR or cyb5 can also present as isolated 17,20-lyase deficiency (Hershkovitz et al. 2008). Mutations that inhibit binding of 17-hydroxypregnenolone also cause selective lyase deficiency. The mutantation E305G still binds 17-OHP; however the Δ5 pathway does not produce sufficient androgens for normal male external genital development (Sherbet et al. 2003).

Males with a combined lyase/hydroxylase deficiency present with ambiguous external genitalia and absent plasma 17-hydroxylated C21 steroids. This leads to a glucocorticoid deficiency and increases in the levels of 11-DOC and corticosterone (Scaroni et al. 1986). The elevated 11-DOC leads to hypertension, since 11-DOC is a mineralocorticoid agonist. Females present with normal genital development but fail to produce DHEA during adrenarche and estrogen during puberty and also have hypertension (Biglieri et al. 1966).

4.6 Electron Transfer to cytochrome P450s

Transfer of electrons from NADPH to P450 mono-oxygenases is essential for their enzymatic action. In the mitochondria, this role is carried out by the iron/sulfur protein ferrodoxin and the flavoprotein ferrodoxin reductase (Miller 2005). Electrons are transferred through a chain, from NADPH to ferrodoxin reductase, from ferrodoxin reductase to ferrodoxin and finally to the P450.

Microsomal P450s, on the other hand receive electrons from a distinct flavoprotein, P450 oxidoreductase (POR). POR is analogous to fusion protein of ferrodoxin reductase and ferrodoxin. POR has two lobes, one containing an FAD binding domain that interacts with NADPH, and another containing an FMN (flavin mononucleotide) binding domain that interacts with the redox partner-binding site of the P450 (Ellis et al. 2009). The FMN and FAD domains are connected by a linker region that allows substantial movement. It is currently believed that POR exists in a compact conformation, with FMN and FAD domains close together, facilitating electron transfer from FAD to FMN. A conformational change creates a more extended state that allows transfer of the electrons from the FAD to P450s. Negatively charged acidic residues on the FAD domain interact with positively charged basic domains on the P450. Mutations in these binding sites, which prevent interaction of POR with CYP17A1, are one of the causes of isolated lyase deficiency (Auchus and Miller 1999).

Patients with POR deficiency have been identified and usually present with combined CYP17A1 and CYP21A2 deficiencies, since POR is required for both enzymes (Malunowicz et al. 1987). The defect in 21-hydroxylase activity leads to moderate

accumulation of 17-OHP but low cortisol levels. Administration of cosyntropin (ACTH analog) increases 17-OHP levels with little change in cortisol levels. Since the 17,20-lyase reaction is more dependent on POR than the 17-hydroxylase reaction, C-19 steroid levels are low. Males have underdeveloped external genitalia whereas females may be mildly virilized (Fluck and Miller 2006). The virilization of females is presumed to be due to failure of placental aromatase, a POR dependent enzyme, to aromatize the large amounts of DHEA normally made by the fetal adrenal (Miller 1998). A second mechanism for the virilization of females could be the conversion of progesterone and 17-OHP to DHT via 5α-pregnane intermediates (discussed in detail in a later section).

4.7 Introduction to Cytochrome B5

Cytochrome B5 or Cyb5 is important in regulating a number of P450 activities. Cyb5 is a 15.2-kDa hemoprotein located in the endoplasmic reticulum. Similar to ferrodoxin, it receives electrons from NADPH through an intermediary that can be either NADH-cytochrome-b5-reductase, or NADPH-POR. In fatty acid synthesis, cyb5 is an electron acceptor for microsomal desaturases, and cyb5 deficient mice have skin defects due to decreased de-novo unsaturated fatty acid synthesis (Finn et al. 2011). In blood, soluble cyb5 is required by methemoglobin reductase to convert methemoglobin back to hemoglobin; consequently, isolated 17,20 lyase deficiency due to cyb5 mutations can be differentiated from isolated lyase deficiency due to CYP17 (Van Den Akker et al. 2002) or POR (Hershkovitz et al. 2008) mutations by increased methemoglobin concentrations in patient blood (Vergeres and Waskell 1995).

The role of cyb5 as an enhancer of P450 mono-oxygenase is still controversial. In P450s such as the CYP4A subfamily, which consist of eicosanoid and fatty acid hydroxylases, cyb5 can increase or decrease the K_m of the reaction depending on the substrate involved (Loughran et al. 2001). This is also seen with CYP17A1, where cyb5 greatly stimulates the 17, 20-lyase reaction when 17-OHP or 17-OH pregnenolone are substrates, but is not required for 5α-reduced substrates (Gupta et al. 2003).

Cyb5 is a small, cylindrical membrane protein consisting of 6 helices and 5 β-strands. The protein contains two domains, an amino terminal hydrophilic domain towards the cytosolic side of the ER, and a smaller hydrophobic membrane-binding domain on the carboxyl end that anchors the protein to the ER. The larger amino terminal domain contains a hydrophobic pocket in which a heme iron is coordinated with two conserved histidine side chains, H68 and H44 (Schenkman and Jansson 2003). In between the two domains is a proline-rich hinge region that can be cleaved by proteases to release a soluble b5.

While cyb5 can accept electrons from POR, whether electron transfer is the primary method by which it stimulates CYP17A1 lyase activity is still a matter of debate. Multiple mechanisms of action of cyb5 have been proposed, including a direct transfer of electrons from POR to P450 through cyb5 or complexing of cyb5 with the P450 mono-oxygenase to simultaneously accept two electrons from POR. The latter mechanism obviates the need for the P450 to accept one electron, dissociate from POR, then re-associate and accept another electron, thus greatly increase the speed of the reaction (Schenkman and Jansson 2003). Some evidence, mainly from the actions of apo-b5 (lacking the heme and unable to undergo electron transfer) on stimulating testosterone

6β-hydroxylation, has suggested that perhaps some of cyb5s roles are as an allosteric effector (Yamazaki et al. 1996). Similarly, cyb5 can also stimulate CYP17A1 activity without direct electron transfer (Auchus et al. 1998). The interaction of cyb5 with CYP17A1 requires residues E48 and E49 and mutating those residues abolishes its activity on CYP17A1, while maintain its electron transfer abilities (Naffin-Olivos and Auchus 2006).

Cyb5 mutations have been detected in patients presenting with isolated 17, 20-lyase deficiency but no mutations in CYP17A1 or POR. One such mutation that has been found is p.H44L (Idkowiak et al. 2012). The patient presented with ambiguous genitalia, low urine androgen metabolites but normal mineralocorticoids and glucocorticoids, supporting a block in the CYP17A1 lyase reaction. When CYP17A1 enzyme assays were conducted in vitro, addition of cyb5 but not cyb5 with the p.H44L mutation, could increase the lyase activity.

4.8 5α-Reductase and the Alternate Pathway of Steroidogenesis

The 5α-reductases reduce the Δ4 double bond of steroids, delivering a hydride to the alpha face. The 5α-reductases have markedly different patterns of expression and functions in humans vs rodents. In humans there are two isoforms, SRD5A1, found in the scalp and peripheral tissues, and SRD5A2, found in the testis and prostate (Jenkins et al. 1992). The most important role of 5α-reductases in humans is the conversion of testosterone into the more potent androgen dihydrotestosterone (DHT). DHT is essential

for male sexual differentiation. SRD5A2 is the only isoform expressed in the fetus and individuals with SRD5A2 deficiency may have normal male genitalia, ambiguous genitalia or female genitalia, depending on the level of enzyme malfunction (Wilson et al. 1993). During puberty, expression of high levels of SRD5A1 in the liver can convert circulating testosterone into DHT leading to virilization. SRD5A2 is also the principal isoform expressed in the prostate, and inhibitors such as finasteride and dutasteride are used to treat conditions such as prostate hyperplasia and adenocarcinoma (McConnell et al. 1998).

The 5α-reductases can also convert progesterone and 17-OHP into their 5α-reduced analogues. In humans this process is utilized to degrade steroids in the liver for eventual elimination. In mice the 5α-reductases are known to also be involved in an alternate pathway of steroid synthesis (Ghayee and Auchus 2007). In immature mouse testis, androstanediol is the predominant steroid (Chase and Payne 1983). Its major pathway of synthesis involves conversion from DHT by 3α-HSD. Alternatively, progesterone can be 5α-reduced to form 5α–dihydroprogesterone. It is further converted by 3α-HSD into 5α-pregnane-3-ol-20-one which is a substrate for CYP17A1 that first hydroxylates it to 5α-pregnane-3,17-diol-20-one. The CYP17A1 lyase reaction then cleaves the side chain to yield androsterone that is subsequently converted to 5α-androstane-3,17–diol (androstanediol) (Mahendroo et al. 2004) (Figure 4.2). Importantly, the lyase reaction when using 5α-reduced pregnanes as a substrate does not require cytochrome b5, unlike when 17-OHP or 17-hydroxypregnenolone are substrates (Gupta et al. 2003). In the presence of b5, the CYP17A1 lyase prefers 17-OHP and 17-hydroxy

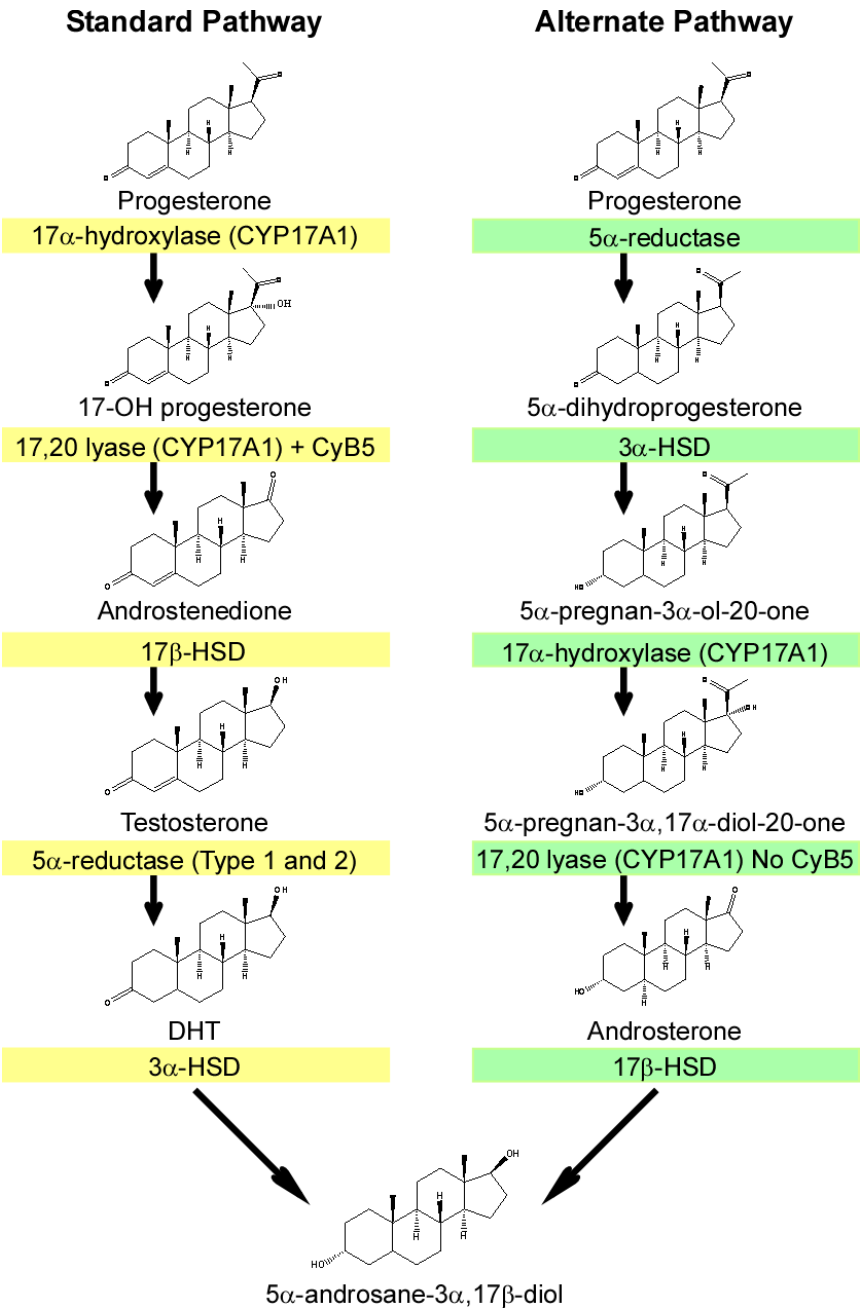


Figure 4.2 The alternate pathway of steroidogenesis. The 5α-reductases can utilize progesterone and 17-OHP as substrates to produce 5α-reduced pregnanes that are further converted to androsterone and androstanediol. The 17,20-lyase activity of CYP17A1 does not require cyb5 when 5α-reduced pregnanes are substrates.

pregnenolone as substrates, explaining why conversion to androstenedione and testosterone is the predominant pathway of androstanediol synthesis in mice.

SRD5A1 can only be detected in the Leydig cells of the mouse testis after day 24-26 (Mahendroo et al. 2004). While SRD5A1 has been shown to be important in the production of 5α- reduced pregnanes, SRD5A2 does not appear to be important in this pathway. In the tammar wallaby, unlike the mouse, CYP17A1 prefers 5α-reduced metabolites of progesterone as a substrate and the alternate pathway of androstanediol production is the predominant pathway (Wilson et al. 2003). Additionally, androstanediol is the steroid responsible for tammar wallaby male phenotypic differentiation, though it is rapidly converted to DHT and may perhaps simply be a DHT precursor (Wilson et al. 2002). Androstanediol and the alternate pathway may thus have no role in mice other than serving as an alternate route of DHT synthesis. Unlike in humans, DHT has no role in mouse phenotypic development and mice lacking SRD5A1 and SRD5A2 have fully formed genitalia and only slight reductions in prostate and seminal vesicle weight (Mahendroo et al. 2001). In cyb5$^{-/-}$ mice however, where the system is stressed and straining to produce adequate levels of testosterone, knocking down the 5α-reductases and the alternate pathway of DHT synthesis may elicit a phenotype, as DHT might be compensating for low testosterone during embryonic development.

4.9 The Alternate Pathway of Steroidogenesis in Humans

In humans, first trimester expression of 5α-reductase enzymes has been detected in the testis and adrenal (Thigpen et al. 1993). However, it is unlikely the 5α-reductase is

involved in the backdoor pathway during fetal male external genital development, and it is more likely that it is instead involved in the classical pathway of converting testosterone to DHT. The backdoor pathway could be important in cases of androgen excess and when the classical pathway is compromised due to enzyme deficiencies (Ghayee and Auchus 2007). Polycystic ovary syndrome (PCOS) is characterized by excessive amounts of androgen synthesis and hirsutism of affected patients. The ovaries of patients with PCOS have elevated levels of 17-OHP, indicating perhaps a defect in the CYP17A1 lyase activity. SRD5A1 is known to be expressed in the ovary (Milewich et al. 1995) and it is possible that the androgen excess in PCOS derives from the conversion of excess 17-OHP to DHT via the backdoor pathway. Consistent with this, large amounts of 5α-pregnane-3,20-dione are present in the serum and follicular fluid of women with PCOS (Agarwal et al. 1996) .

Both CYP21A2 and 3βHSD deficiencies can lead to excess androgen production. Lack of CYP21A2 leads to an increase in the levels of progesterone and 17-OHP that are subsequently converted to testosterone and DHT. In 3βHSD2 deficiency, there is an overproduction of DHEA that is converted by peripheral 3βHSD1 into testosterone. However, despite the production of testosterone in both cases, the 3βHSD deficient females virilize only slightly in comparison to the CYP21A2 deficient females. This could be explained by SRD5A1 conversion of the 17-OHP into DHT via the backdoor pathway, which cannot be utilized in 3βHSD2 deficiency, in which pregnenolone and 17-hydroxypregnenolone accumulate, but neither is a substrate for the SRD5A1 (Ghayee and Auchus 2007) . The most convincing evidence of the backdoor

pathway being utilized in humans is POR deficiency. POR deficiency affects various microsomal P450s including CYP17A1 and CYP21A2. This defect leads to accumulation of 17-OHP. The CYP17A1 lyase reaction is more dependent on electron transfer from P450 and cyb5 than the hydroxylase reaction. Thus the accumulating 17-OHP cannot be converted to androstenedione and testosterone, leading to low testosterone levels. Females with POR deficiency, paradoxically, are virilized in utero. This is plausibly due to the backdoor pathway converting 17-OHP to 5α-pregnane-3α,17α-diol-20-one, which is an excellent substrate for CYP17A1 lyase and not as dependent on POR and cyb5 (Arlt et al. 2004). Elevated urinary 5α-pregnane-3α,17α-diol-20-one and androsterone found in the urine of mothers carrying fetuses with POR deficiency, further support this hypothesis (Shackleton et al. 2004).

CHAPTER 5

Results

5.1 Introduction

Cytochrome b5 (cyb5) is a 15.2-kDa hemoprotein located on the endoplasmic reticulum where it is important in regulating the activities of various P450s (Schenkman and Jansson 2003). Cyb5 can accept electrons from POR or cytochrome-b5-redutase and transfer them to a P450. It may also have an allosteric role in regulating the interaction between POR and P450s, since apo-b5 (lacking the heme group) can also stimulate the activity of certain P450s (Yamazaki et al. 1996). The focus of these studies is on cyb5's role in stimulating the 17, 20-lyase activity of CYP17A1. CYP17A1 is an important enzyme in steroidogenesis that can perform two distinct chemical reactions, the 17-hydroxylation with progesterone and pregnenolone and the 17,20 lyase reaction that cleaves the side chain converting 17-OHP to androstenedione and 17-OH pregnenolone to DHEA (Miller and Auchus 2011). The lyase reaction converts the C21 pregnanes to the C19 sex steroids. The lyase reaction is more dependent on electron transfer from POR than the hydroxylase reaction, and mutations in POR or cyb5 that hinder the transfer can cause isolated 17, 20-lyase deficiency (Hershkovitz et al. 2008; Idkowiak et al. 2012). Human patients present with ambiguous genitalia, elevated plasma 17-OHP and decreased androgen levels.

Mice lacking cyb5 have profound changes in hepatic drug metabolism and skin disorders related to their inability to synthesize unsaturated fatty acids (McLaughlin et al. 2010; Finn et al. 2011). Here I investigate the role of cyb5 in the formation of testicular androgens and male phenotypic development through the conditional knock out of testicular cyb5 in a mouse model.

5.2.1 Testis-Specific Cyb5 Knockout

Cyb5 is expressed in numerous tissues such as the liver, where it stimulates the activity of various P450s involved in drug metabolism (Finn et al. 2008). To elucidate the role of cyb5 in male sexual development and function, I created testis and adrenal specific cyb5 knockout mice by crossing $cyb5^{flox/flox}$ mice with CRE^{SF1}. SF1 is a nuclear receptor expressed mainly in the Leydig cells of the testis, in the ovaries, the adrenal cortex, precursors of pituitary gonadotropic cells and in the ventro-medial hypothalamus (VMH) (Val et al. 2003; Bookout et al. 2006). Out of the tissues targeted by SF-1-Cre in male mice, cyb5 only has a defined role in the testicular Leydig cells, because mouse adrenals contain little CYP17A1. Knockdown of cyb5 was confirmed in the testis by qPCR and western blot using liver as a control (Figure 5.1A and B). The $cyb5^{flox/flox} * CRE^{SF1}$ male mice were observed to be phenotypically normal; with no differences in external sexual development between wild type and knock out. Testicular histology revealed no defects in testicular development (Figure 5.1D). To determine if the cyb5 knockouts had any defects in their ability to breed, we placed knockouts or wild type male mice with wild type females and measured litter size and frequency (Figure 5.1C).

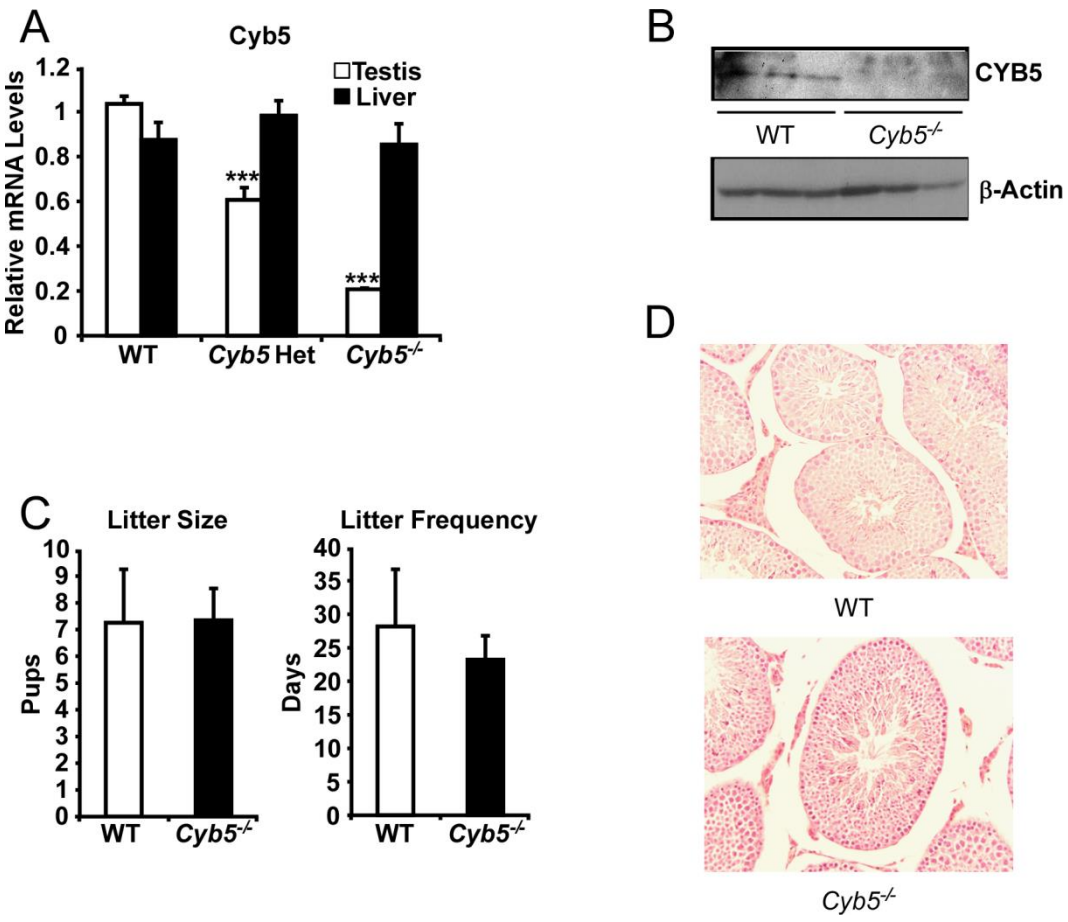


Figure 5.1 Cyb5$^{-/-}$ mice have no changes in fertility or testicular histology. (A) mRNA was extracted from testis and liver of cyb5$^{-/-}$, cyb5$^{-/wt}$ and wild type mice and cyb5 mRNA levels measured. **(B)** Cyb5 protein levels were measured by western blot in testis from wild type and cyb5$^{-/-}$ mice utilizing an abcam anti-cyb5antibody **(C)** Cyb5$^{-/-}$ or wild type males were placed with wild type females and monitored daily. Litter size and frequency were recorded. **(D)** Testis from cyb5$^{-/-}$ and wild type litter mates were fixed in formalin and H&E stained. Values are means ± SEM. Statistics by two-tailed *t* test. ***P<0.0005

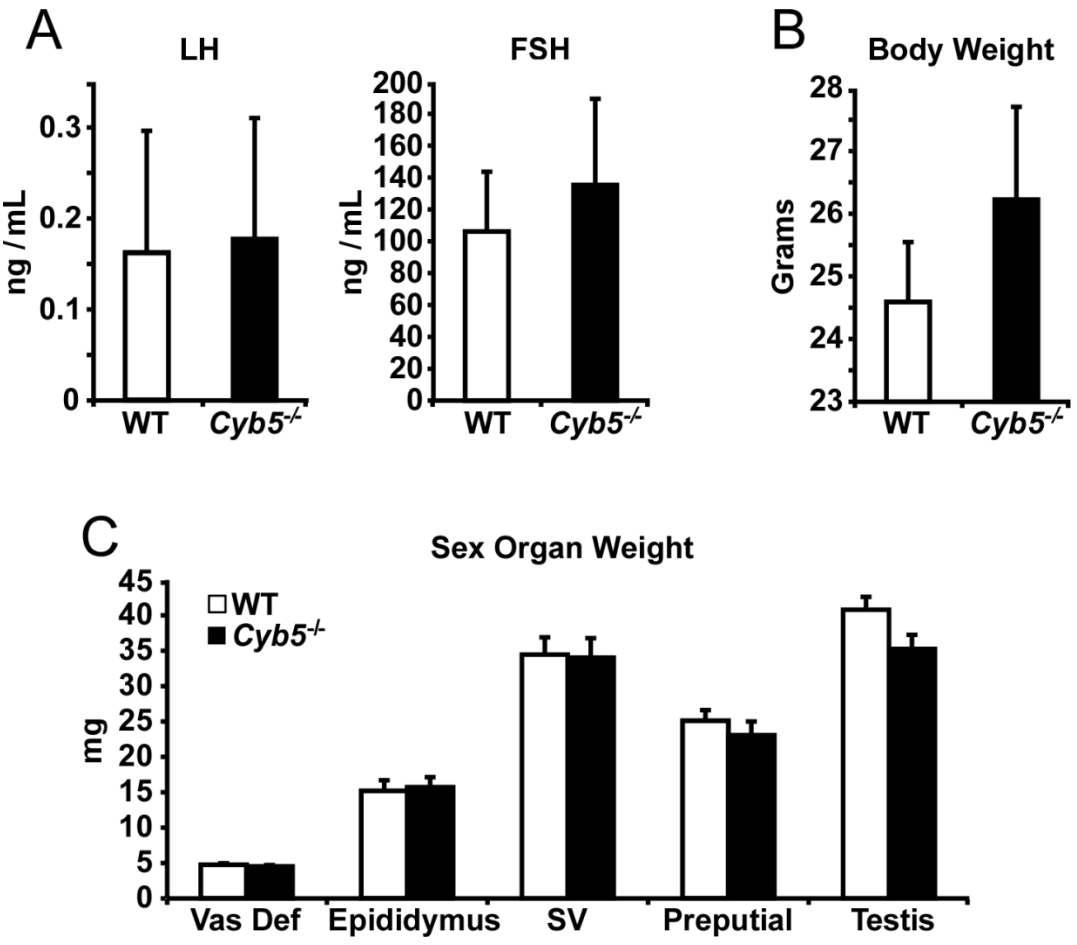


Figure 5.2 Cyb5$^{-/-}$ mice show no changes in serum FSH/LH levels or sex organ weight (A) Two month old cyb5$^{-/-}$ mice and wild type littermates (n=10 per group) were sacrificed and plasma collected. FSH/LH levels were measured by radioimmunoassay. **(B)** Body weight of two months old cyb5$^{-/-}$ and wild type littermates was recorded (n=10 per group) **(C)** Cyb5$^{-/-}$ or wild type males (n=10 per group) were sacrificed and weights of the vas deferens, epididymis, seminal vesicles (SV), preputial glands and testis measured. Values are means ± SEM. Statistics by two-tailed *t* test.

No differences were observed between the two cohorts, indicating that cyb5 knockouts had no defect in their ability to copulate.

5.2.2 Cyb5 Testicular Null Mice have no Changes in Body Weight or Sex Organ Weight

To determine if the cyb5 knockdown had an impact on sexual maturation, I measured the weight of the epididymis, vas deferens, seminal vesicles, preputial glands and testis from $cyb5^{flox/flox} * CRE^{SF1}$ mice and wild type littermates (Figure 5.2C). No discernible differences were seen between the two groups. Additionally, no changes in body weight were observed (Figure 5.2B). If $Cyb5^{-/-}$ mice had a decrease in steroid production, one would expect an increase in serum LH and FSH levels to maintain normal serum steroid levels. However, no changes in FSH and LH levels were observed in $cyb5^{-/-}$ mice relative to wild type controls.

5.2.3 Testicular Homogenates from $Cyb5^{-/-}$ Mice have an Increased CYP17A1 Hydroxylase: Lyase Ratio

Cyb5 is essential for the lyase activity of CYP17A1 but has no effect on the hydroxylase activity. Thus we expect that the homogenates from B5 K.O.'s would have an increased hydroxylase:lyase ratio and impaired formation of androstenedione from 17-OHP but normal conversion of progesterone into 17-OHP. Testicular homogenates from $cyb5^{-/-}$ and wild type mice were incubated with radiolabeled progesterone or 17-OHP,

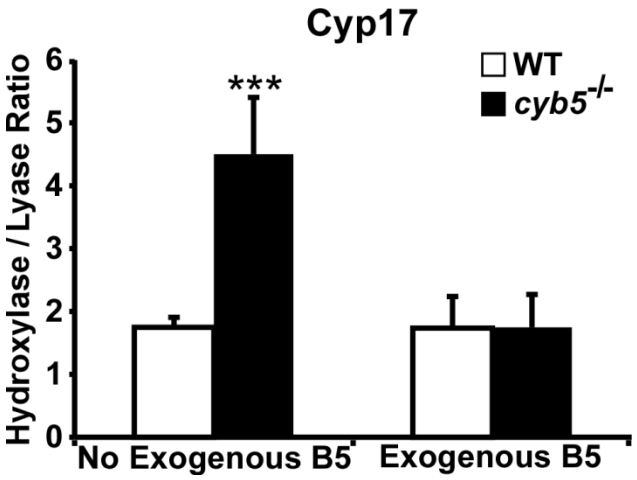


Figure 5.3 Testicular homogenates from cyb5$^{-/-}$ mice have an increased CYP17A1 hydroxylase:lyase ratio Two month old cyb5$^{-/-}$ mice and wild type littermates (n=10 per group) were sacrificed and testes collected. Testicular homogenates were incubated with either radiolabeled progesterone and 17-OHP levels measured, (hydroxylase activity) OR with radiolabeled 17-OHP and androstenedione and testosterone levels measured (lyase activity). Recombinant cyb5 was added to the testicular homogenates to rescue the decrease in lyase activity observed in cyb5$^{-/-}$. Values are means ± SEM. Statistics by two-tailed *t* test. ****P*<0.0005

and conversion into androstenedione and testosterone was measured at 30, 60 and 90 minute time points by HPLC with an attached radiolabel detector. I observed that homogenates from the cyb5$^{-/-}$ mice had approximately 30% of the CYP17A1 lyase activity of a wild type mouse. The hydroxylase to lyase ratio increased from 1.7 in the wild type to 4.7 in the knockout (Figure 5.3). Addition of exogenous B5 to the cyb5$^{-/-}$ homogenates restored the hydroxylase:lyase ratio to that of the wild type, confirming that lack of B5 was responsible for the observed decrease in lyase activity.

5.2.4 No Changes in Basal Plasma Steroid Levels Observed in Cyb5$^{-/-}$ Mice

As cyb5 is required for the CYP17A1 lyase reaction and testicular homogenates from cyb5$^{-/-}$ mice had a profound reduction in testosterone production from 17-OHP, I expected that plasma levels of testosterone would be decreased in CYP17A1 mice. If testosterone levels are maintained, an increase in serum levels of the precursor 17-OHP might be seen. With this in mind, I collected plasma from cyb5$^{-/-}$ mice and wild type littermates. Steroid levels were measured by Dr. Richard Auchus using high performance liquid chromatography (HPLC) coupled to tandem mass spectrometry. Plasma testosterone, 17-OHP and progesterone were not significantly different between wild type and cyb5$^{-/-}$ mice (Figure 5.4). Interestingly, 17-OHP levels were found to be low to undetectable. This indicated that the residual lyase activity, in the absence of cyb5, was perhaps sufficient to maintain plasma testosterone levels. It is also possible that extra testicular sites of androgen synthesis, where b5 was present, could compensate for the lack of androgen production by the testis.

5.2.5 Stimulation of Steroid Production in Cyb5$^{-/-}$ mice leads to Accumulation of 17-OHP

Unlike humans, mice have low basal levels of plasma testosterone that increase in the presence of a female mouse or female bedding. To simulate this effect, I administered 10 mIU of human chorionic gonadotropin (hCG) to cyb5$^{-/-}$ mice and wild

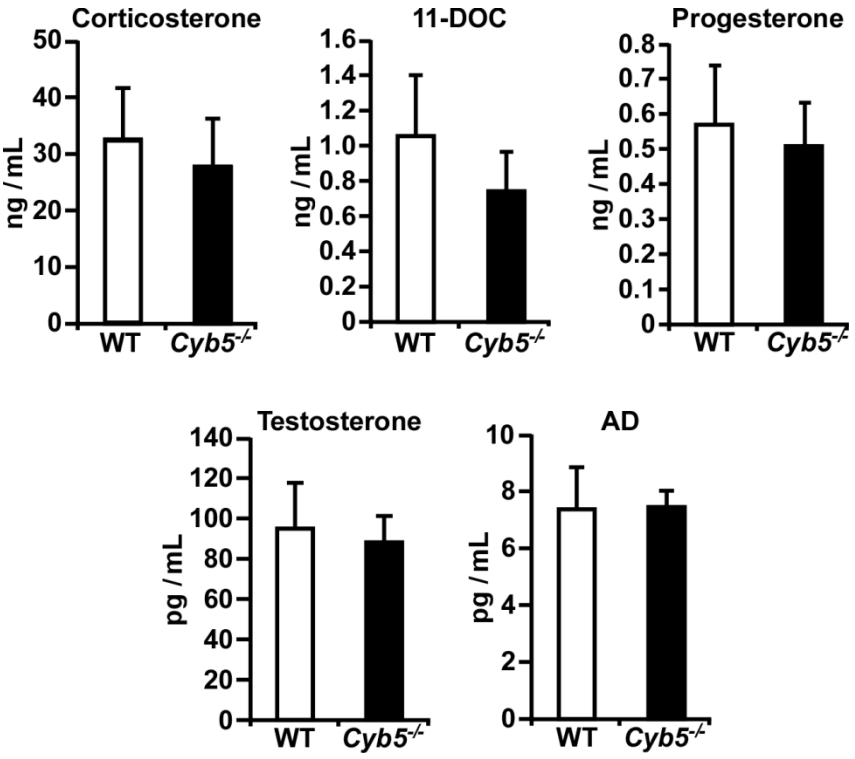


Figure 5.4 Plasma levels of steroids are unchanged in the cyb5$^{-/-}$ mice. Two month old cyb5$^{-/-}$ mice and wild type littermates (n=10 per group) were sacrificed and plasma collected. Plasma levels of corticosterone, 11-deoxycorticosterone (11-DOC), progesterone, testosterone and androstenedione (AD) were measured by HPLC-MS/MS. Values are means ± SEM. Statistics by two-tailed *t* test.

type mice (n=10 per group). Plasma steroid levels in mice have been shown to peak two hours post hCG injection (Jean-Faucher et al. 1985) and that time point was chosen for plasma collection. Plasma steroid levels were assayed using HPLC MS/MS as previously described. We observed a 100 fold increase in testosterone levels between the saline and hCG injected wild type animals indicating that the hCG was successful in stimulating steroid production (Figure 5.5). Serum testosterone and androstenedione levels in the

cyb5$^{-/-}$ mice were roughly half those of the wild type in the hCG injected cohort. A doubling of progesterone levels was also observed in the knockout relative to the wild type control. While hCG injection caused a modest increase in 17-OHP levels in the hCG injected wild type animals, there was marked serum accumulation of 17-OHP observed in the cyb5$^{-/-}$. This dramatic increase in 17-OHP in the cyb5$^{-/-}$ mice helps explains the relatively modest differences in testosterone and androstenedione levels between the untreated knockout and wild type animals. The high 17-OHP levels suggest that the mouse is capable of pushing through with the lyase reaction by increasing the concentration of substrate, thus coming close to matching the testosterone and androstenedione levels of the hCG stimulated wild type animals. We also observed an increase in plasma cortisol levels in the hCG treated knockouts relative to wild type controls, presumably caused by spillover into the cortisol pathway due the relatively high levels of 17-OHP observed in the cyb5$^{-/-}$ animals.

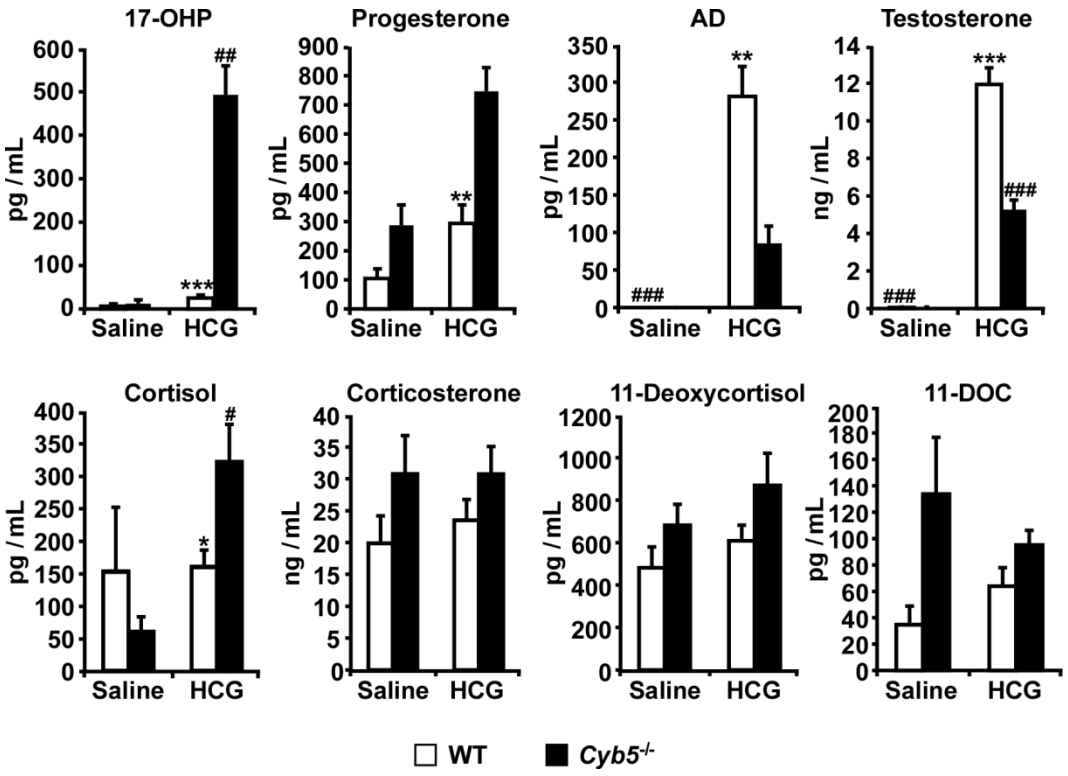


Figure 5.5 Accumulation of 17-OHP is observed in cyb5$^{-/-}$ mice post hCG injection. Two month old cyb5$^{-/-}$ mice and wild type littermates (n=10 per group) were IP injected with saline or 10 mIU of hCG and sacrificed two hours later. Plasma levels of corticosterone, 11-deoxycorticosterone (11-DOC), progesterone, testosterone ,androstenedione (AD), 17-hydroxyprogesterone (17-OHP), 11-deoxycortisol and cortisol were measured by HPLC-MS/MS. Values are means ± SEM. Statistics by two-tailed *t* test. (#) refers to differences between saline and hCG injected wild type/wild type or cyb5$^{-/-}$/cyb5$^{-/-}$ groups. (*) refers to differences between hCG injected wild type/cyb5$^{-/-}$ groups. **P*<0.05, ***P*<0.005, ****P*<0.0005. #*P*<0.05, ##*P*<0.005, ###*P*<0.0005.

5.2 Summary

Cytochrome b5 is an important modulator of the CYP17A1 lyase reaction. Cyb5 mutations are a known cause of isolated 17, 20 lyase deficiency. In addition, increased expression of cyb5 may play a role in various disease pathologies and androgen excess syndromes (Sakai et al. 1993). Here I show that in a testicular specific cyb5 knockout mouse, plasma testosterone levels and male sexual development and function are unchanged. However, testicular homogenates from $cyb5^{-/-}$ mice have a profound reduction in their ability to convert 17-OHP to androstenedione and testosterone. The mechanism by which $cyb5^{-/-}$ mice maintain normal testosterone levels with a block in the 17,20 lyase reaction is not entirely clear, but evidence from hCG administration indicates that the animals may compensate for deficient lyase activity by dramatically but transiently increasing the concentration of the substrate 17-OHP, thus pushing through with the lyase reaction.

CHAPTER 6

Discussion

6.1 Testicular Cyb5$^{-/-}$ Mice Have No Overt Phenotype

Cyb5 causes a profound increase in the CYP17A1 lyase activity, either through direct electron transfer or through allosteric regulation of the CYP17A1-POR interaction. In human patients, cyb5 mutations present with classic isolated lyase deficiency, with decreased androgen levels and ambiguous external genitalia (Idkowiak et al. 2012). Thus we expect that testicular cyb5$^{-/-}$ mice will have similar deficiencies in androgen production and sexual development. However, our cyb5$^{-/-}$ mice had no overt phenotypic differences compared to wild type mice. The mice bred normally and produced offspring in a normal mendelian ratio. No changes in sex organ weight or serum FSH/LH levels were observed and basal serum androgen levels were surprisingly normal.

When we incubated testicular homogenates with progesterone or 17-OHP, a profound reduction in 17,20-lyase activity and increase in the hydroxylase:lyase ratio was observed in the cyb5$^{-/-}$ mice. These mice clearly exhibit a clear isolated 17, 20-lyase deficiency but are able to compensate and maintain testosterone levels.

6.2 Stimulation of Steroidogenesis in Cyb5-/- mice Leads to Accumulation of Serum 17-OHP

To investigate the mechanism of compensation of 17, 20-lyase deficiency in mice, I injected the cyb5$^{-/-}$ and wild type mice with hCG. hCG is a LH receptor agonist and causes a rapid increase in sex steroid synthesis (Dalterio et al. 1983). Two hours after hCG injection I observed a large increase in testosterone and androstenedione level in the wild type and cyb5$^{-/-}$ mice. Upon hCG administration, the cyb5$^{-/-}$ mice were able to increase circulating testosterone levels to roughly half those observed in wild type mice. The mechanism of this increase, despite the 17, 20-lyase deficiency, appears to be an increase in serum 17-OHP levels, which rises a hundred fold higher in the cyb5$^{-/-}$ mice relative to wild type controls. It thus appears that mice compensate for the lyase deficiency by dramatically increasing the available substrate concentrations, allowing residual CYP17A1 lyase activity to maintain testosterone levels.

6.3 The Backdoor Pathway to Steroidogenesis

An important alternate pathways to steroidogenesis has recently been shown to be important in both rodents and humans (Ghayee and Auchus 2007). Progesterone and 17-OHP are substrates for 5α-reductases. These 5α-reduced pregnanes are then converted through a series of steps, including the CYP17A1 lyase and hydroxylase reactions, into androstanediol. Androstanediol can subsequently be converted into DHT by 3α-HSD. In tammar wallaby this backdoor pathways is the major pathways of steroidogenesis and crucial for normal male sex organ development (Wilson et al. 2002). The backdoor pathway has also been shown to be important in mice and its activation can be observed in mice at day 25-26.

As the cyb5$^{-/-}$ mice have no defects in sexual development, it is possible that the alternate pathways is being activated sooner in mice, providing DHT that can compensate for low testosterone during development. With this in mind, our ongoing studies involved measuring DHT and steroid levels in neonates along with crossing the cyb5$^{-/-}$ mice with SRD5A1$^{-/-}$ mice to abolish the alternate pathway of steroidogenesis.

CHAPTER 7

Materials and Methods

7.1 Cell Culture

HEK293, HepG2, EL4 and Jurkat cells were obtained from the ATCC. HEK293 cells were maintained in DMEM high glucose + 110 mg/L sodium pyruvate (Invitrogen 11995-065) + 10% FBS (Gemini 900-108) + 1x penicillin/streptomycin. HepG2 cells were maintained in MEM + 10% FBS + 1x penicillin/streptomycin. EL4 cells were maintained in DMEM (Invitrogen 11995-065,) + 10% Horse Serum + 1x penicillin/streptomycin. Jurkat cells were maintained in RPMI 1640 (ATCC) + 10% FBS + 2μL β-mercaptoethanol / 500 mL + 1x penicillin/streptomycin. For transfection of HEK293 cells, cells were harvested at 80-100% confluence and plated at a density of a 20,000 cells per well of a 96 well plate. The following day, transfections were performed with XtremeGene DNA transfection reagent (Roche) according to the manufacturer's protocol. HepG2 cells were harvested and plated at a density of 20,000 cells per well of a 96 well plate and transfected using XtremeGene. For EL4 cells, $2*10^6$ cells were placed in a cuvette along with 30 μg of DNA. Electroporation was performed using the Amaxa Nucleofector kit L (Lonza) according to manufacturer's instructions. All cell lines for transfection and assays were split into growth media containing 10% charcoal stripped FBS (Gemini 100-119), lacking antibiotics. Ligands were added 24 hours after

transfection. Cells were harvested 24 hours after treatment and luciferase activity assayed. Luciferase values in all transfection assays were normalized to β-galactosidase transfected as an internal control.

CMX-RORγ-Gal4 and RORγ-full length vectors were obtained from Dr. Dan Littman (NYU). The RORE-Luciferase vector (RORE2P) was constructed by inserting 4X ROR response elements (GGTAAGTAGGTCAT) (Austin et al. 1998) into the pGL4.32 Luc2P vector (Promega). LXRβ-Gal4 and Daf12-Gal4 vectors have been previously described (Janowski et al. 1996; Motola et al. 2006).

7.2 Lipid Extraction from Bovine Calf Thymus

Twenty-eight kilograms of bovine calf thymus was put through a meat grinder and combined with 280 liters of 2:1 chloroform: methanol followed by homogenization. Addition of 0.8 volumes of 0.9% NaCl led to separation into two phases. The bottom organic phase was isolated and dried. Lipids were separated on 10g silica columns (Sep-Pak, Waters). 10 grams of lipids were resuspended in 20 mL chloroform and adsorbed onto a silica column pre-equilibrated with 100 mL of chloroform. Three phases were eluted from column a) 240 mL chloroform b) 360 mL acetone c) 240 mL methanol (Figure 4.3). Lipids from the same fraction from multiple separations on silica columns were pooled and dried using a rotary evaporator.

7.3 HPLC Separation of Lipid Fractions

Each of the lipid fractions, chloroform, acetone and methanol, were further separated using an Agilent 1100 HPLC system. 200 mg of each fraction was dissolved in 10 mL of methanol with 900 μL injected per HPLC run. Fractions were separated on a Xorbax C18 9.8*250 mm column (Agilent) with a water: methanol gradient from 30% water to 0% water over 30 minutes, followed by 100% methanol for 30 minutes. The flow rate was set at 3 mL/min and fractions were collected every 2 minutes. Fractions were dried using a gentle nitrogen stream, dissolved in 3 mL of 1:1 chloroform: methanol and stored at -20C. Active methanol fractions were further purified using a series of HPLC columns (Figure 4.4). The pholipidec column (Astec) was used to separate phospholipids by head group. The pholipidec method utilized a four mobile phases, A: 80/19/1, CHCl3/CH3OH/NH4OH (v/v/v) B: 60/39/1, CHCl3/CH3OH/NH4OH (v/v/v) C: 50/48/1/1, CHCl3/CH3OH/H2O/NH4OH (v/v/v/v) D: 100, CH3OH with a the following program : 100% A to 100% B over 25 minutes, gradient from 100% B to 100% C at 30 minutes, hold in 100% C until 45 minutes, gradient from 100% C to 100% D at 48 minutes, hold in 100% D until 55 minutes, gradient to 100% A at 56 minutes, hold in 100% A until 65 minutes. Final purification was performed on a SB-C18 4.6*250 mm (Agilent) column utilizing a water: methanol gradient of 15% water to 0% water over 30 minutes, followed by 100% methanol for 30 minutes (flow rate 0.7 mL/min).

7.4 Mass Spectrometric Analysis of Lipids

HPLC runs previously described were coupled with a single quadrupole mass spectrometer (G1956B, Agilent). Lipids were analyzed by scanning from mass range m/z

100-1000 in both positive and negative ion mode. Both atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) modes were employed. For ESI the conditions used were, drying gas flow = 12 L/min, nebulizer pressure = 35 psi, drying gas temperature = 300C. MS parameters for APCI were as follows: gas temperature = 250°C, nebulizer pressure = 35 psig, drying gas (nitrogen) = 12 L/min, Capillary voltage =4000V, fragmentor voltage= 150V (positive ions) or 200V (negative ions).

Phospholipid analysis was performed by Dr. Pavlina Ivanova (Dr. Alex Brown Laboratory, Vanderbilt) using methods previously described (Ivanova et al. 2007). Briefly, lipids were separated on a Luna silica column 2*250 mm (Phenomenex) with a gradient program with the following mobile phases A: IPA:Hexane:100 mM NH_4CO_2H 58:40:2 and B: IPA:Hexane:100 mM NH_4CO_2H 50:40:10. The LC gradient was as follows, 50% B from 0 to 5 minutes, 50-100% B from 5 to 30 minutes, 100% B from 30 to 40 minutes, 100-50% B from 40-41 minutes and 50% B from 41-50 minutes. MS analysis was performed on an Applied Biosystems/MDS SCIEX 4000 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer.

7.5 Synthesis of Lysophospholipids by Phospholipase A_2

Lysophospholipids were synthesized using three methods. For lysophospholipids for which the corresponding phospholipid was available, the A_2 chain was removed utilizing phospholipase A_2, followed by HPLC purification and removal of the fatty acid and enzyme. Diacyl-L-α-phosphatidylcholines were purchased from Avanti and dissolved in methanol to give a final concentration of 10 mg/mL. The substrate solution

was added to a solution of 500 μM $CaCl_2$ and 50 μM EDTA (pH 8.0) to give a final ratio of 3:1 buffer: methanol (Cottrell 1981). 2 μg of Bee venom phospholipase A_2 (Sigma P9279) was added to the reaction and the reaction allowed to stir overnight. The following day chloroform and methanol were added to the reaction to bring the ratio of chloroform: methanol: water to 2:1:0.8. Following phase separation, the organic phase was isolated, dried, the lysophosphatidylcholine (LPC) purified by HPLC and mass confirmed by MS.

7.6 Tin Mediated Acylation of Glycerophosphorylcholine

LPCs for which the corresponding PC was not commercially available were synthesized by tin-mediated acylation of glycerophorylcholine as previously described (Fasoli et al. 2006). Fifty milligrams of L-α-glycerophosphorylcholine (Sigma G5291) was combined with dibutyltin oxide in 2-propanol (200 mL) and heated under refluxing conditions for 6 hours. The reaction was cooled to room temperature and 50 mg of acid chloride in triethylamine added. The reaction was stirred for 1 hour, followed by filtration to remove the tin, rotary evaporation of the solvent, HPLC purification of the LPC and mass confirmation by MS.

7.7 Transphosphatidylation Reaction: LPC to LPE Conversion

Lysophosphatidylethanolamines (LPE) were synthesized from LPCs by a transphosphatidylation reaction (Wolf and Gross 1985). Fifty milligrams of LPC was

dried down at the bottom of 20 mL glass scintillation vial. 20 mL of methanol was added and the vial sonicated in a bath sonicator for 15 minutes. The reaction solution consisted of 61.5 mM Tris HCL, 6.1 mM $CaCl_2$, 0.38% Triton X and 0.077% BSA. 65 mL of reaction solution was added to 15 mL of ethanolamine and pH adjusted to 8.5. This was followed by addition of the substrate in methanol (20mL) and 5 μL of phospholipase D from *Streptomyces chromofuscus* (Sigma P0065) (50,000 units/mL). Reaction was stirred and pH monitored and maintained at 8.5 by drop wise addition of NaOH. Once pH had stabilized, reaction was stopped byaddition of 10 mL of 0.5 M EDTA. Lipids were extracted using the Blight and Dyer protocol. Chloroform and methanol were added to bring the ratio of chloroform: methanol: water to 2:2:1.8. Following phase separation, organic phase was isolated and washed several times with water to remove all traces of ethanolamine. Lipids were dried using a rotary evaporator, LPE purified by HPLC and mass confirmed by MS.

7.8 Synthesis of 18:1p LPE

The 18:1 LPE plasmalogen was synthesized by Dr. Kamlesh Sharma in our laboratory using established protocols (Van den Bossche et al. 2007).

7.9 Synthesis of Platelet Activating Factor

Platelet Activating Factor analogues of 18:0 LPC, 15:0 LPC, 18:0p LPE, 16:0 LPC, 19:0 LPC 18:1p LPC and 18:1 LPC were synthesized by acetylation of 100mg of

the LPC/LPE with acetic anhydride (700 mg) and DMAP (25 mg) in 10 mL of chloroform. The reactions were stirred for 3 hours at room temperature following which 5 mL of methanol and 4 mL of water were added and phases allowed to separate. The lower organic phase was isolated, dried under a gentle stream of nitrogen, purified by HPLC and mass confirmed by MS.

7.10 Testing of Fractions and Phospholipids

Fractions, commercially available and synthesized phospholipids were tested for RORγ activity in HepG2 and HEK293 cells. Cells were transfected with RORγ-Gal4 and UAS-Luc vectors as previously described. Fractions were reconstituted in 3 mL of 1:1 chloroform: methanol. 200 μL of each fraction was dried down at the bottom of a glass scintillation vial. DMEM + 10% C.S. FBS was added to the vial followed by vortexing and sonication in a bath sonicator for 15 minutes (Fisher Scientific 15-335-20). Media containing ligand was applied to cells and luciferase activity assayed 24 hours later.

7.11 ALPHA Screen

The binding of ligands to RORγ was determined by an ALPHA screen assay (Perklin Elmer). pET24-hRORγ-HIS6GST was obtained from Dr. Eric Xu (Van Andel Institute, Michigan). BL21 cells transformed with RORγ vector were added to 500 mL LB with Kanamycin and placed in shaker at 180 rpm and 37C overnight. Next day 150 mL aliquots of overnight cultures were added to 1 Liter aliquots of LB (+Kanamycin)

followed by incubation for 3 hours in shaker at 30 C and 180rpm until an OD of 0.8-1.0 was achieved. Protein expression was induced with 100 μM of IPTG and cultures left in shaker overnight at 16 C. Cells were harvested by spinning at 3500 g for 30 minutes. Cell pellet was resuspended in 20 mL of GST buffer A (10 mM Tris / 150 mM NaCl / 10% glycerol/pH 8.0) containing protease inhibitors and PMSF (to a final concentration 1 mM). The cell suspension was sonicated followed by centrifugation at 20,000 g for 1 hour. Supernatant containing RORγ-HIS-GST was filtered using a 0.45 micron syringe filter followed by purification on an AKTA Design (GE) FPLC system. Supernatant was loaded onto a GST column and column washed with 20 mL of GST buffer A. RORγ-HIS-GST was eluted from the column with 20 mL of GST Buffer B (20 mM Tris / 150 mM NaCl / 10% Glycerol/ 20 μM reduced L-glutathione /pH 8.0). Protein was further purified using a Nickel column.

ALPHA screen was performed using 100 nM of RORγ protein and 20 nM bSRC3-1b (GHKKLLQLLTS-COOH) in the presence of 5 μg/mL donor and acceptor beads. The 10X ALPHA screen buffer contained 500 mM MOPS / 0.5 mM CHAPS /500 mM NaF / 1% BSA/ pH 7.4. Reaction mix was incubated with ligand for 90 minutes and read using a FluoStar Omega plate reader (BMG Corporation). Assays were repeated with no protein or no peptide to check for ligand induced nonspecific bead interactions.

7.12 RT-qPCR for cell lines

EL4 cells were split into 6 well plates with 2 mL per well (density 2*10,000 cells/mL). The following day, 22:4 LPE, 20:3 LPE or vehicle were added to cells. 24

hours post treatment cells were harvested and spun down at 700g for 3 minutes. Supernatant was removed and cells lysed with 500 μL of RNA-STAT60 (Isotex diagnostics). 100 μL of chloroform was added and phases separated by centrifugation. Upper phase was removed and RNA precipitated with 500 μL of ice cold n-propanol. RNA pellet was precipitated by centrifugation and washed with 70% ethanol followed by DNase treatment and reverse transcription using random hexamers. cDNA was analyzed for IL17a mRNA by RT-qPCR. 25 ng of cDNA and 150 nmol of primer were mixed with SYBR GreenER PCR Master Mix (Invitrogen). Reactions were performed on an ABI PRISM 7900 HT (Applied Biosystems). mRNA levels were calculated by normalization to cyclophilin using the comparative CT method. mIL17a primers were designed using Primer Express (Applied Biosystems): 5'-AGGACGCGCAAACATGAGT-3', 5'-GCAGCAACAGCATCAGAGACA-3'

7.13 IL17a Elisa

EL4 cells were split into 12 well plates with 1 mL per well (density 2*10,000 cells/mL). The following day cells were treated with 22:4 LPE, 20:3 LPE, 22-hydroxycholesterol, SR1001, Tcompound or vehicle. 24 hours post treatment cells were precipitated by centrifugation at 600g for 3 minutes. Media was removed and diluted fivefold before assaying IL17 protein levels by ELISA according to the instructions of the manufacturer (Abcam ab100702).

7.14 Mice

Cyb5$^{fl/fl}$ mice were maintained by random breeding on a 129P2 * C57BL/6 genetic background (Finn et al. 2008). Cyb5$^{fl/fl}$ mice were crossed with a transgenic mouse line expressing Cre recombinase under the control of the testis and adrenal specific SF1 promoter (Dhillon et al. 2006). Cyb5$^{fl/fl}$Cre^{SF1} mice were backcrossed to cyb5$^{fl/fl}$ mice to generate testis and adrenal specific cyb5 knockouts. The presence of the flox cyb5 and CRE^{SF1} transgene was determined as previously described (Dhillon et al. 2006; Finn et al. 2008).

All animal protocols were approved by the Institutional Animal Care and Research Advisory Committee of the University of Texas Southwestern Medical Center. All mice used in in experiments were fed a standard irradiated chow diet and housed in a temperature controlled environment with a 6am-6pm light/dark cycle. Unless specified, all mice used in experiments were 6-8 weeks old.

For hCG injections, hCG from Sigma was dissolved in 0.9% saline solution for a final concentration of 100 mIU/mL. Mice received either 100 μL of hCG solution or 0.9% saline administered via an intraperitoneal injection. Mice were sacrificed 2 hours post injection, exsanguinated and tissues collected.

7.15 RT-qPCR for Tissues

Frozen liver or testis samples were homogenized in 500 μL of RNA-STAT60 (Isotex diagnostics). 100 μL of chloroform was added, and phases were separated by

centrifugation. Upper phase was removed, and RNA was precipitated with 500 μL of ice cold n-propanol. RNA pellet was precipitated by centrifugation and washed with 70% ethanol followed by DNase treatment and reverse transcription using random hexamers. cDNA was analyzed for mRNA levels by RT-qPCR. 25 ng of cDNA and 150 nmol of primer were mixed with SYBR GreenER PCR Master Mix (Invitrogen). Reactions were performed on an ABI PRISM 7900 HT (Applied Biosystems). mRNA levels were calculated by normalization to cyclophilin using the comparative C_t method. Cyb5 primers were designed using Primer Express (Applied Biosystems): 5'-CGATCTGACCAAGTTTCTCGAA-3', 5'-CCCCAGCTTGCTCTCTTAGG-3'

7.16 Western Blotting

Frozen livers and testis were homogenized in protein lysis buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% NP40, 10% glycerol, 5 mM EDTA and 1 complete mini protease inhibitor tablet / 10 mL (Roche 11836153001). Samples were homogenized and centrifuged at 13,000g for 20 minutes. Supernatant was assayed for protein concentration using a Lowry assay. 40 μg of protein was loaded onto a 15% SDS-PAGE gel. Protein was transferred to a nitrocellulose membrane at 75 V for 1 hour. Primary mouse anti-cyb5 antibody (Abcam) incubation was performed in TBST containing 0.05% Tween and 3% BSA. Secondary HRP conjugated antibody incubation was in TBS-T containing 5% milk. SuperSignal West Pico western blotting substrate (Pierce) was used to visualize results. β-Actin antibody was used as a loading control.

7.17 Testicular Homogenates

Testes (80-130 mg) were homogenized in 0.30 mL of 0.25 M sucrose/10 mM TrisHCl pH 7.0/1 mM EDTA with 25 strokes of a dounce homogenizer. The debris was pelleted by centrifugation for 5 min at 5,000g and discarded. The decanted suspension (crude homogenate) was stored frozen at -20C. 0.2 M monobasic potassium phosphate (19%) and 0.2 M dibasic potassium phosphate (81%) were combined to get a 0.2 M potassium phosphate buffer of pH 7.4. Aliquots (20 μL) of homogenates were incubated with 10 μM [^{3}H]-labeled progesterone or 17-hydroxyprogesterone, 100,000 CPM per incubation, and 1 mM NADPH in 50 mM potassium phosphate buffer at 37C in a total volume of 0.2 mL. Aliquots were removed every 30, 60 and 90 minutes. Steroids were extracted by adding an ethyl acetate: isooctane (1:1) solution into each tube followed by centrifugation at 8000g for 1 minute. Top layer was isolated, dried under a gentle stream of nitrogen and analyzed by HPLC coupled to a radiochemical detector.

7.17 Plasma Steroid Measurement

Plasma steroids were measured by Dr. Susan Mathews in the laboratory of Dr. Richard Auchus (University of Michigan) utilizing HPLC-MS/MS. The internal standard mix (IS) was made from the following deuterated sterols dissolved in ACN at a final concentration of 1ng/mL: cortisol-d4, 11-deoxycortisol-d5, 17-OHP-d8, 11-DOC-d8, testosterone-d3 and progesterone-d9. 100 μl of plasma was combined with 100 μl methanol, a 100 μL IS mix and 200uL ACN. The mixture was vortexed every 10 minutes

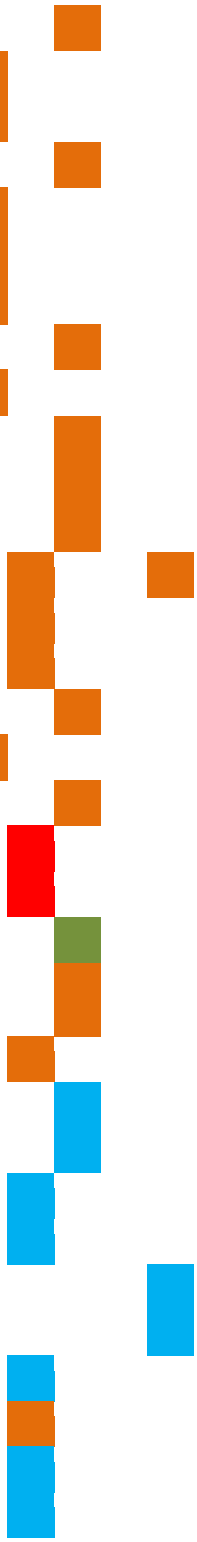
for 30 minutes followed by centrifugation at 15,000g for 5 minutes. Supernatant was transferred to a 2 mL glass tube, and 1 mL of MTBE (*Methyl Tertiary Butyl Ether)* was added. Sample was vortexed, and 500 μL of water was added for phase separation. The top later was transferred to a new glass tube and dried under a gentle stream of nitrogen. Samples were reconstituted in 100 μL of methanol, transferred to autosampler vials and injected into the HPLC. Steroid levels were analyzed using a 6490QQQ LC-MS system (Agilent) using positive electrospray ionization mode. Quantification against a 10-point external calibration curve was performed using multiple reaction monitoring (MRM).

Appendix A: List of Sterols Tested

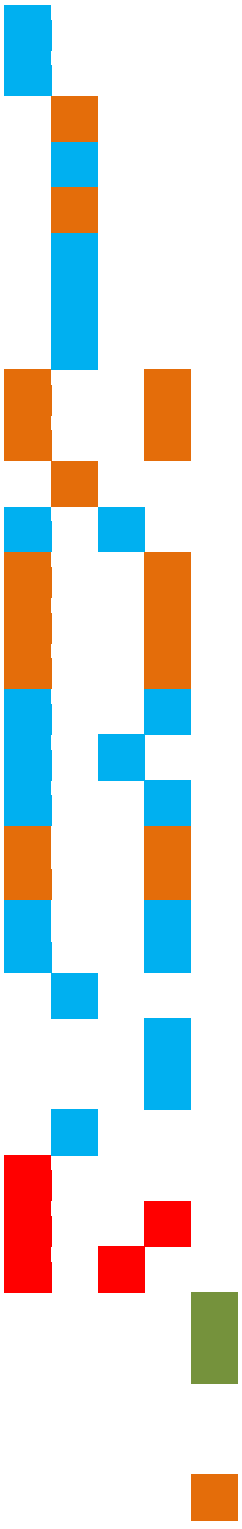
Red = Active in HEK293 and S2
Green = Active in HEK293
Blue = Active in S2
Brown = Not active
4,5,6,7,8 indicate position of double bond on A or B ring

Compound	HEK293T	S2	N	4	5	6	7	8
Desmosterol	+	-						
7-dehydrocholesterol	++	++						
Lathosterol	-	-						
Ursodeoxycholic acid	-	-						
Cholesteryl oleate	-	-						
Cholesteryl palmitate	-	-						
Cholesteryl linoleate	-	-						
Lanosterol	-	-						
7a-Hydroxycholesterol	-	-						
5a-epoxy-cholesterol	-	-						
5b-epoxy	-	-						
5-CHOLESTEN-3-ONE	-	-						
5-CHOLESTEN-3B,4B-DIOL	-	-						
5B-CHOLANIC ACID-3A-OL	-	-						
SITOSTEROL	-	-						
Glucuronate_Chol	-	-						
9(11)_cholenic acid	-	-						
Lithocholenic acid	-	-						
Ketocholanic acid	-	-						
Coprostenol	-	-						
5-cholenic-acid-3b-ol	++	-						
11,5b-cholenic acid-3a-ol	-	-						

8,14,5b,-cholenic acid 3a,12a-diol	-	-
5b-cholanic-acid-3a,6a-diol	-	-
5b-cholanic acid 3-one	-	-
Cholesten-3a,7a,12a triol	-	-
Cholestanol	-	-
5a-hydroxycholestanol	-	-
6a-hydroxycholestanol	-	-
27-dehydrocholesterol	-	-
5-b-cholanic acid 3, 7,12 trione	-	-
5-cholesten-3b-ol-22-one	-	-
3-keto-5-cholestene	-	-
7,5a-cholesten-3b-ol	-	-
4,7-androstenadien-19-ol-3,17 dione	-	-
4,9,11-estratrien-17b-ol-3-one	-	-
16α-Hydroxyestradiol	-	-
Cholest-5-ene-3b,4b-diol	-	-
7β-Hydroxylithocholic acid	-	-
Campesterol	-	-
Cholest-4-en-3,6-dione	++	++
Cholesta4,6-dien-3-one	+	+
Oxo-cholenic acid	+	-
3a-hydroxy-12-keto-delta9(11) cholenic acid	-	-
Methyl-stradiol	-	-
Daf5S	-	+
Daf5R	-	++
Daf4S	-	+++
Daf4R	-	++++
Daf7S	-	+++
Daf7R	-	++++
4-Cholesten-3-one 20aol	-	++++
4-Cholesten-3-one 22S	-	+/-
4-Cholesten-3-one 22R	-	++++
4-Cholesten-3-one 24S	-	++



4-Cholesten-3-one 25,26olS	-	++	
4-cholesten-3-one 25,26olR	-	++	
19a_Cholesterol	-	-	
20a-hydroxycholesterol	-	+++	
22S-hydroxycholesterol	-	-	
22R-hydroxycholesterol	-	+++	
24(R/S) hydroxycholesterol	-	++	
25-hydroxycholesterol	-	++	
Ccdysone	-	-	
20a-ecdysone	-	-	
7a,22SOH	-	+/-	
4,7-cholestadien-3-one	-	++	
4,6,8(14) cholestatrien-3-one	-	+/-	
4,7-cholestadien-3,6-dione	-	-	
4,7-cholestadien 3,6 dione, 14-ol	-	-	
4,7-cholestadien-3-one,25-ol	-	++	
4,6,8(14)-cholestatrien-3-one,25-ol	-	++	
4,7-cholestadien 3,6 dione, 25 ol	-	+	
4,7-cholestadien 3,6 dione, 14,25 diol	-	+/-	
4,7-cholestadien 3,6 dione, 11,14 diol	-	+	
7aOH	-	++	
7DHC,25ol	-	++	
7b,25 hydroxycholesterol	-	++	
24/25 epoxycholesterol	-	++	
4-Cholesten-3-one	+	++	
4,7-Cholesten-3-one	+	++	
4,6-cholestadien-3b-ol	+	++	
FF-mas	++		
Zymosterol	+	+ +	
Squalene	-	-	
Farnesol	-	-	
T-mas	-	?	



Rdi244 (FF-mas derivatives)	+	-
Rdi-93 (FF-mas derivative)	++	-
24,25 dehydrocholesterol	-	-
5b-Pregnan-3a-ol-20-one	-	-
Deoxycholate	-	-
7-ketocholesterol	-	-
11 (5b) cholanic acid	-	-
Cholesterol sulfate	-	-
4b-hydroxycholesterol	-	-
Cycloartenol	-	-
6-dehydrochol	-	-
Coprostanol	-	-
5-keto-cholestene	-	-
Cholestanone	-	-
Daf_Delta -	-	-
Lathosterone	-	-
Lophenol	-	-
Delta_5_ cholesten 17,20,3 triol	-	-
4-cholesten-3-keto,7b-ol	-	-
4-cholesten-3-keto,6b-ol	-	-
Vitamin D3 Cholecalciferol	-	-
Delta-7 Lanosterol Aldehyde	-	++++
Delta-7 Lanosterol Aldehyde Mono F	-	+++
Delta-7 Lanosterol Carboxy DiF	-	++
Delta-8 Lanosterol carboxy DiF	-	++
Delta-7 Lanosterol C14 cyclopropane	-	-
4-methyl-zymosterol	++	++
3-keto-4-methyl-zymosterol	-	++
3-keto-zymosterol	-	++++++

Appendix B : List of Lysophospholipids Tested

Red = Synthesized	
Compound	**Concentration at Maximum Activity**
Lysophosphatidyl Choline (LPC)	
16:0	**X**
16:1	**X**
18:1	300 uM
18:2	100 uM
18:3	**X**
19:0	**X**
20:1	300 uM
22:1	**X**
20:4	**X**
20:2	12 uM
20:5	100 uM
22:4	12 uM
22:6	50 uM
18:0p	**X**
18:0e	**X**
18:1e	**X**
Lysophosphatidyl Ethanolamine (LPE)	
16:1	125 uM
16:0	**X**
18:0	**X**
18:1	300 uM
18:2	25 uM
20:1	**X**
20:3 DGLA	**X**
20:3 Mead	**X**
20:4	**X**
20:2	12 uM
20:5	50 uM

22:4	12 uM
22:6	100 uM
16:0p	150 uM
18:0p	**X**
18:1p	**X**
Lysophosphatidyl Inositol (LPI)	
18:0	**X**
18:1	**X**
Lysophosphatidyl Serine (LPS)	
18:0	**X**
18:1	**X**
Lysophosphatidic Acid (LPA)	
18:0	**X**
18:1	**X**
20:4	**X**
20:2	**X**
20:5	**X**
22:4	**X**
22:6	**X**
Sphingolipids	
C24 Dihydroceramide	**X**
C24:1 Dihydroceramide	**X**
20:0 2-OH Ceramide	**X**
24:0 2-OH Ceramide	**X**
18:0 S1P	**X**
18:1 S1P	**X**
20:1 S1P	**X**
Platelet Activating Factor (PAF)	
18:0e	**X**
18:1e	**X**
15:0	**X**
16:0	**X**
18:0	**X**

18:0p	**X**
18:0p (PE head group)	**X**
Head Groups Alone	
Choline	**X**
Ethanolamine	**X**
Phosphocholine	**X**
Phosphate	**X**
Phosphoglycerol	**X**
Fatty Acids	
Oleic Acid	**X**
Linoleic Acid	**X**
Conjugated Linoleic Acid	**X**
20:2	**X**
22:4	**X**

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