LXR REGULATION AND FUNCTION IN AIRWAY SMOOTH MUSCLE

LXR REGULATION AND FUNCTION IN HUMAN AIRWAY SMOOTH MUSCLE

By

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ABSTRACT

The liver X receptors (LXRs) are members of the nuclear hormone receptor (NHR) superfamily of transcription factors and are activated by oxysterols. As such, LXRs act as "cholesterol sensors" and play an integral role in cholesterol homeostasis by modulating the expression of genes involved in lipid transport and metabolism as well as inflammation.

This thesis begins by describing the modulation of LXR transactivation by PKC. Specifically, transactivation by LXRα is decreased upon activation of PKC signalling pathways as assessed by LXR reporter gene analysis and endogenous target gene expression. These findings reveal a mode of regulation of LXRα that may be relevant to disease conditions where aberrant PKC signalling is observed.

The second and third part of the thesis turns the attention to the role of LXR in human airway smooth muscle (hASM), a crucial effector cell in asthma progression. For the first time, research described here indicates that primary human ASM cells express functional LXRs. Moreover, LXR target genes ABCA1 and ABCG1 were highly induced upon the addition of LXR agonists leading to enhanced cholesterol efflux to apoAI and HDL, a process dependant entirely on ABCA1. Furthermore, activation of LXR inhibited the expression of multiple cytokines in response to inflammatory mediators and inhibited the proliferation and migration of hASM cells, two important processes that contribute to the airway remodelling observed in the asthmatic lung.

This body of work suggests that modulation of LXR offers prospects for new therapeutic approaches in the treatment of asthma. Furthermore, it establishes a critical role for ABCA1 in lipid transport in ASM cells and suggests that dysregulation of cholesterol homeostasis in these cells may be important. These findings have broad implications in the association of hypercholesterolemia and AHR and places LXR at the forefront of novel therapeutic avenues to treat inflammatory lung disease.

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LIST OF ABBREVIATIONS

ABCA1/G1	Adenosine Triphosphate Binding Cassette Transporter A1/G1
ACAT	Acyl-coenzyme A:cholesterol Acyltransferase
AF-1	Activation Function-1
AHR	Airway Hyperresponsiveness
ApoAI	Apolipoprotein AI
AR	Androgen Receptor
ASC-1	Activating Signal Co-integrator-1
BAL	Bronchoalveolar Lavage
Bis	Bisindolylmaleimide
C/EBP2	CCAAT/Enhancer Binding Protein 2
CCL24	Chemokine Ligand 24 (also known as Eotaxin 2)
COPD	Chronic Obstructive Pulmonary Disorder
COUP-TFII	Chicken Ovalbumin Upstream Promoter Transcription Factor II
Cox-2	Cyclooxygenase-2
CYP27	Cytochrome Protein 27
DAG	Diacylglycerol
DBD	DNA Binding Domain
ECM	Extracellular Matrix
ER	Estrogen Receptor
ERK	Extracellular Signal Regular Protein Kinase
FAK	Focal Adhesion Kinase
FGF	Fibroblast Growth Factor
FXR	Farsenoid X Receptor
G-CSF	Granulocyte-Colony Stimulating Factor
GLUT4	Glucose Transporter 4
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
GR	Glucocorticoid Receptor
hASM	Human Airway Smooth Muscle
HDL	High Density Lipoprotein
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
Inr	Initiator Sequence
IP3	Inositol Triphosphate
LBD	Ligand Binding Domain
LBP	Ligand Binding Pocket
LDL	Low Density Lipoprotein
LPL	Lipoprotein Lipase
LPS	Lipopolysaccharide

LRH-1	Liver Receptor Homologue-1
LXR	Liver X Receptor
LXRE	Liver X Receptor Response Element
MCP-1	Monocyte Chemotactic Protein-1
mDia	Mammalian homologue of Diaphenous
mmLDL	Minimally Modified Low Density Lipoprotein
MMP-9	Matrix Metalloproteinase-9
MR	Mineralocorticoid Receptor
NCoR	Nuclear Co-Repressor
NF-κB	Nuclear Factor-κB
NHR	Nuclear Hormone Receptor
oxLDL	Oxidized Low Density Lipoprotein
PAK	p21-activated Kinase
PDGF	Platelet Derived Growth Factor
PI3K	Phosphoinositide Kinase-3
PIC	Pre-initiation Complex
PIP2	Phosphatidylinositol 4,5-Bisphosphate
PKC	Protein Kinase C
PLC	Phospholipase C
PMA	Phorbol 12-myristate-13-acetate
PPAR	Peroxisome Proliferator-Activated Receptor
PR	Progesterone Receptor
RANTES	Regulated upon Activation, Normal T cell Expressed and Secreted
RAR	Retinoid Acid Receptor
Rb	Retinoblastoma
RNApolII	RNA Polymerase II
RXR	Retinoic X Receptor
SHP	Small Heterodimer Partner
SIRT1	Sirtuin-1
SRC	Steroid Receptor Co-activator
SREBP1c	Sterol Response Element Binding Protein 1c
TGFβ	Transforming Growth Factor β
VASP	Vasodilator-stimulated Phosphoprotein
VCAM-1	Vascular Cell Adhesion Molecule-1
VDR	Vitamin D Receptor

CHAPTER ONE
INTRODUCTION

1.1) General Transcription

1.1.1) Background

Higher eukaryotes must control the spatial and temporal expression of genes for development, survival and maintenance of homeostasis (Rosenfeld et al 2006). To accomplish this, a set of regulatory DNA binding proteins termed transcription factors have evolved to positively or negatively regulate the cellular transcriptome in response to extra- or intracellular stimuli. By interacting with specific DNA elements located in the promoter and enhancer regions of their target genes, transcription factors recruit co-factor complexes with multiple enzymatic activities that ultimately regulate the binding and activation of RNA polymerase for a particular target gene or locus (Chen & Rajewsky 2007). Multiple layers of complexity such as histone modifications (histone code), genomic DNA modifications, chromatin remodelling, microRNA regulation of gene expression, ubiquitin-mediated protein degradation pathways and the role of DNA repair enzymes in transcription initiation allows the cell to fine tune transcript levels (Chen & Rajewsky 2007). Overall, the diversity of factors involved in altering levels of gene expression and the specificity by which they can be recruited to particular promoter regions exemplifies the complexity and elegance of gene expression.

1.1.2) Currents Views on Basal and Enhanced Transcription

The transcription of protein coding mRNA transcripts requires the recruitment of RNA polymerase II (RNApolII) to the core promoter of target genes. Classically, the core promoter consists of multiple sequence elements (eg. TATA box, Inr elements, etc.)

that serve as a platform for the assembly of the pre-initiation complex (PIC) (consisting of general transcription factors TFIIA, B, D, E, F, H and RNApolII) (Thomas & Chiang 2006). Two models exist by which RNApolII assembles onto the core promoter region: 1) sequential assembly and 2) two-component pathway (Thomas & Chiang 2006). In both models, PIC nucleation is initiated by TFIID binding to the core promoter followed by TFIIA recruitment which acts to stabilize the interaction. In the sequential assembly model, each general transcription factor then follows a sequential and ordered recruitment to the promoter region (TFIID—>TFIIA—>TFIIB—>TFIIF—>RNApolII—>TFIIE—>TFIIH). In the two component model, RNApolII exists as a holoenzyme and is recruited as a whole to form the pre-initiation complex (Fig. 1). The model that best describes the *in vivo* regulation of PIC formation remains to be conclusively determined and may involve both processes in a gene-specific manner. Overall, the formation of PIC under appropriate conditions and its maturation to the elongation phase of transcription is sufficient to drive basal expression of genes *in vitro*, however, to achieve regulated and enhanced levels of gene expression, these core promoters must crosstalk with enhancer regions occupied by gene-specific transcription factors.

Enhancer regions are distal DNA sequence elements located upstream or downstream from the transcription start site that act in *cis* to its cognate core promoter to influence the level of RNA polymerase II recruitment and PIC formation. These elements are orientation-independent and communication with core promoters can result in increased or decreased gene expression and, as such, these enhancers are referred to as 'activators' when levels of their target genes are increased and 'repressors', when

decreased (Sipos & Gyurkovics 2005). Transcription factors work in *trans* by binding to enhancer regions and recruiting various ancillary factors to alter the surrounding chromatin environment. These co-factors can be gene- and cell-type specific and act as platforms for the recruitment of histone modifying enzymes, chromatin remodelling complexes and the mediator complex which bridges and transmit signals from the enhancer to the core promoter for PIC assembly (Fig. 1).

The above models and conclusions were elegantly elucidated using purified RNApolII complexes and *in vitro* reconstitution experiments with GTFs along with the innovative reporter gene co-transfection assays developed over 20 years ago (Giguere et al 1986; Kornberg 2007). What is now known concerning gene expression is that there are a multitude of additional factors involved in the *in vivo* coordination of transcription such as the nuclear membrane itself as well as the nuclear architecture, once thought of as a stagnant skeleton. The spatial positioning of genes relative to the nuclear membrane can positively or negatively alter levels of gene expression, depending on the specific genes and cell type, indicating that sub-nuclear locales can function as regulatory domains (Ragoczy et al 2006). Additionally, specific *inter*-chromosomal interactions in unique sub-nuclear domains enriched for RNApolII, transcriptional elongation factors and chromatin remodelers are required for maximal responses from estrogen receptor-mediated gene expression (Hu et al 2008). These responses depend on molecular motors such as dynein that move along actin networks to regulate chromosomal movement and crosstalk. Future work to elucidate the molecular mechanisms and precise machinery

involved may allow for certain predictions to be made on individual genes and increase our understanding of how cells co-ordinate the transcription of gene networks.

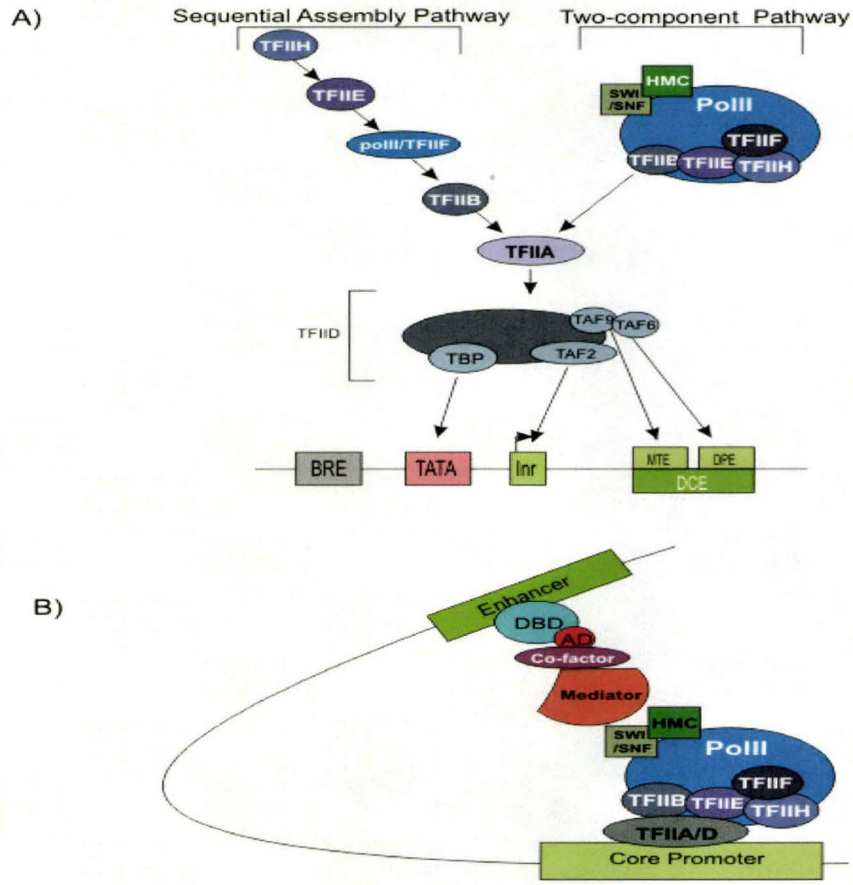


Figure 1. A) Sequential and Two-component Pathways of PIC assembly. PIC assembly occurs on the core promoters of target genes and may occur in a sequential ordered recruitment (left panel) or in a two component pathway where RNA polymerase II (polII) exists as a holoenzyme in complex with other general transcription factors (GTFs) and chromatin remodelling enzymes. PIC assembly is nucleated by TFIID which recognizes and binds to core promoter elements as shown (lower panel). (Figure adapted from Thomas et al., 2006) **B) Enhancer-mediated PIC formation involves co-factors and the mediator complex**. For proper regulation of gene expression in response to intra- or extracellular cues, gene-specific transcription factors bound to enhancers of target genes recruit ancillary factors to alter the local chromatin environment. The factors include nucleosome remodelling complexes and histone modifying enzymes which results in Mediator recruitment. In turn, the Mediator is believed to make crucial protein-protein contacts with both co-factors, transcription factors and the basal transcription machinery to nucleate PIC formation. For enhancers located many kilobases from the transcriptional start site, chromatin is believed to form loops to physically bridge transcriptional mediators to basal machinery via protein-protein interactions. (Adapted from Thomas and Chang, 2006). Inr – Initiator; BRE – TFIIB Response Element; DCE – Downstream Core Element; MTE – Motif Ten Element; DPE – Downstream Promoter Element; HMC – Histone Modifying Complex; DBD – DNA binding domain; AD – Activation Domain; TAF – Transcription Associated Factor

Overall, the emerging picture of general transcription is beginning to appear less general than previously thought. The varying composition of DNA elements in promoter regions, the vast numbers of co-factors that display opposing roles depending on the particular gene, a dizzying array of combinatorial post-translational modifications occurring at multiple protein levels and environmentally-induced epigenetic changes to chromatin all contribute to specialized activation of gene transcription. With the stochastic nature of gene expression and extensive array of factors involved, the level of precision at the organismal level is remarkable.

1.2) The Nuclear Hormone Receptor Superfamily

1.2.1) Background

The nuclear hormone receptor (NHR) superfamily represents a family of ligand-activated transcription factors pertinent to development, metabolic homeostasis and inflammation (Mangelsdorf et al 1995). NHRs play a role in virtually every aspect of physiology and serve to translate signals of endocrine or metabolic origin into complex transcriptional outputs in a tissue- , cell- and temporally-specific manner. To date, 48 related members have been identified in the human genome along with greater than 75 protein isoforms. Despite high structural homology, the NHR superfamily have evolved distinct functions and can be subdivided into three broad classes: type I steroid receptors (ex. glucocorticoid receptor (GR)), type II thyroid/retinoid receptor family (ex. retinoid X receptor (RXR)) and type III orphan receptors (ex. COUP-TFII).

1.2.2) Nuclear Receptor Subfamilies

Type I receptors include the classical steroid receptors such as the estrogen receptor (ER), progesterone receptor (PR) and the androgen receptor (AR), among others (Fig. 2A). Traditional models posit that type I receptors, in the absence of ligand, are located mainly in the cytoplasm in complex with heat shock proteins such as HSP90 (Ribeiro et al 1995). With the advent of more modern biophotonics, more recent models suggest that a small percentage of receptors may continuously cycle between the cytoplasm and nucleus (Hager et al 2000). Upon ligand binding, these receptors release heat shock proteins, homodimerize and translocate to the nucleus whereupon they bind to their respective DNA response elements usually characterized by a palindromic inverted repeat sequence located in the promoter regions of target genes (Hager et al 2004; Nagaich et al 2004) (Fig. 2A). Depending on the particular gene, response element and co-factors recruited, steroid receptor can activate or repress gene expression.

Type II receptors include members such as the peroxisome proliferator-activated receptors (PPAR; subtypes α,β/δ, and γ), the retinoic acid receptors (RAR) as well as the liver X receptors described in this thesis (LXR; subtypes α and β). These receptors all heterodimerize with the obligate partner RXRα and are mainly localized to the nucleus (Fig. 2A) (Gronemeyer et al 2004). As with type I receptors, a small proportion of the receptors are also reported to localize in the cytoplasm (Hozoji et al 2008). In the absence of ligands, type II receptors are thought to remain bound to their response elements, characteristically direct repeat elements separated by a number of nucleotides

specific to each sub-type, in complex with co-repressor proteins such as nuclear co-repressor (NCoR) (Fig. 2A) (Glass & Rosenfeld 2000). Upon ligand binding, a conformational change in the ligand binding domain (LBD) results in decreased affinity for co-repressors and increased affinity for co-activator proteins ultimately resulting in activation of downstream target genes (Glass & Rosenfeld 2000).

Type III receptors, including COUP-TFII and NR4 receptors, are termed orphan receptors due to their high amino acid sequence similarity to known NHRs but for which an endogenous ligand has not been identified (Mohan & Heyman 2003). Type III receptors show varying cellular localization, can homodimerize or heterodimerize with RXR or function as monomers (Fig. 2A). Due to the fact that murine models with targeted deletions of some orphan receptors display phenotypes of human disease (eg. Retinoic acid-related Orphan Receptor and Spinocerebellar ataxia type 1 disease (Serra et al 2006)), intense research interest has focused on identifying the physiological ligands of orphan receptors. In some cases, such as for the NR4 group of orphan receptors involved in multiple physiological processes including gluconeogenesis, it is likely that no physiological ligands exists since the ligand binding domain is occupied by large hydrophobic residues that would theoretically stearically interfere with ligand docking (Martinez-Gonzalez & Badimon 2005). As an alternative mode of regulation, these receptors are regulated at the transcriptional and post-translational levels thereby altering their expression, localization and transcriptional activity (Fig. 2A) (Wingate et al 2006).

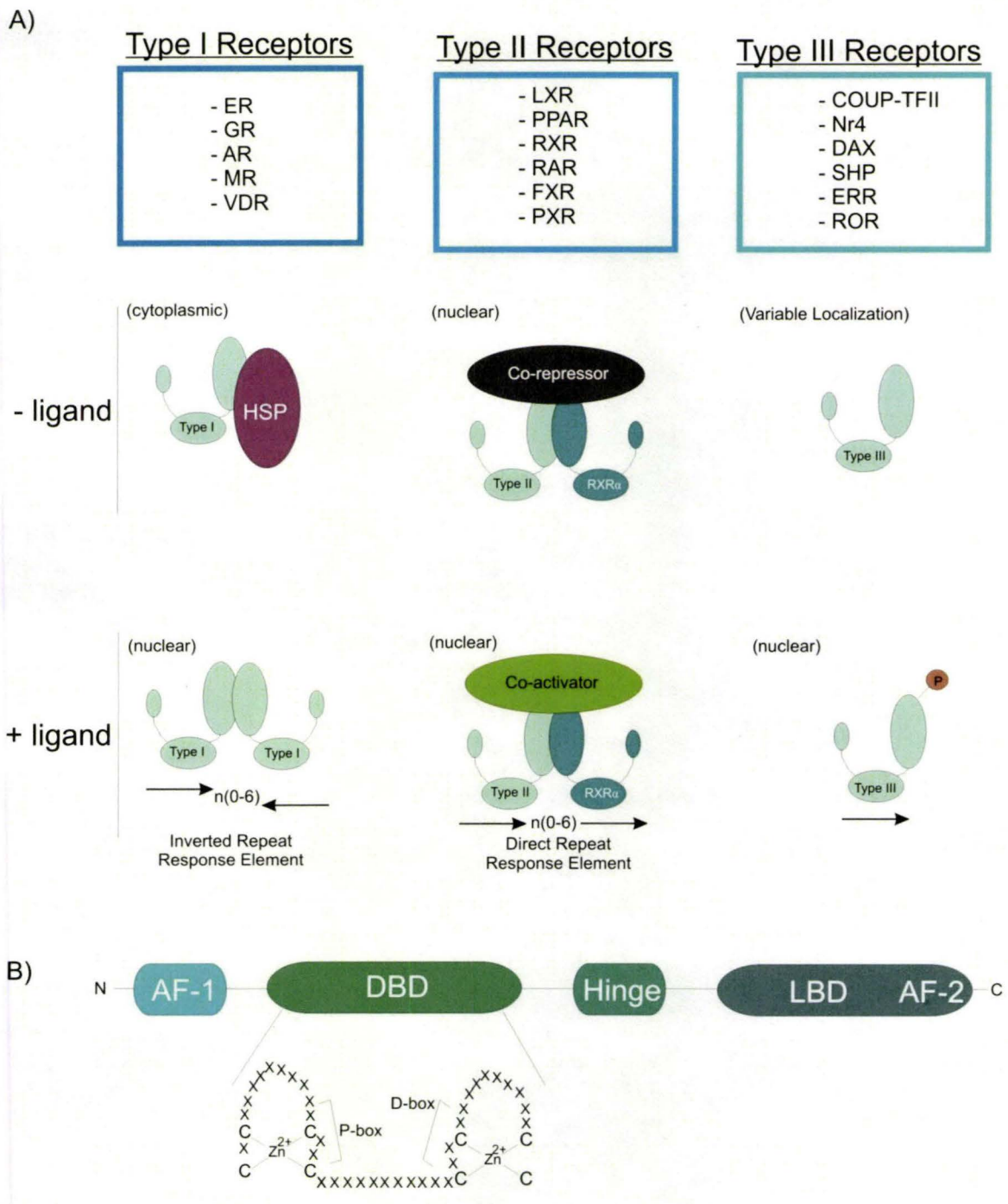


Figure 2. Domain structure and classification of common NHRs. A) Classification of schematic representation of the various NHR groupings and their mode of action. B) General schematic representation of the functional domains of nuclear hormone receptors. Sub-domains of the DNA binding domain (DBD) are identified.

1.2.3) Domain Structure and Mechanisms of Transcription

Nuclear hormone receptors share a canonical structure characterized by modular domains that can be subdivided into 5 regions: an amino-terminal activation domain (AF-1), a centrally located DNA binding domain (DBD), the hinge region, a unique ligand binding domain (LBD) and a carboxy-terminal, ligand-dependent activation domain (AF-2) (Fig. 2B). The AF-1 region is the least conserved domain within the nuclear receptor superfamily showing less than 15% amino acid similarity. In the absence of ligand, the AF-1 region can act as a ligand-independent activation domain in addition to synergizing with the AF-2 region in the presence of activating molecules. It is hypothesized that the small degree of conservation may account for cell-type and promoter-specific functions observed even within nuclear receptor sub-families (Gronemeyer et al 2004; Lu & Cidlowski 2005). For example, multiple isoforms of the glucocorticoid receptor are expressed in a tissue-specific manner, each with its own unique N-terminal sequence. The resulting effect is a non-overlapping regulated gene set for each isoform however, the physiological relevance remains to be determined (Lu & Cidlowski 2005).

The DBD is the most conserved region in the NHR family and is characterized by two zinc fingers that coordinate their zinc atoms using 4 highly conserved cysteine residues. This domain contains two subdomains known as the P-box and D-box (Fig. 2B) (Bain et al 2007; Gronemeyer & Moras 1995). The P-box located in the first zinc finger from the amino terminus confers sequence specificity to each nuclear receptor. Interestingly, single amino acid substitutions in the glucocorticoid receptor DBD can

convert the receptor to an estrogen response element binding protein (Khorasanizadeh & Rastinejad 2001). The D-box, located in the second zinc finger, dictates the preferred spacing between response elements, with each receptor class preferentially binding to elements spaced by 0-6 nucleotides (Khorasanizadeh & Rastinejad 2001). Thus, the sequence elements, the spacing of the sequences and the orientation all confer specificity of nuclear receptor binding to chromatin. Additionally, the DBD not only interacts with chromatin but also plays a role in homo- or heterodimerization and is the target of post-translational modifications that alter its affinity for binding partners and DNA itself (Chandra et al 2008; Rochette-Egly 2003).

The hinge region is located between the DBD and LBD and, as its name suggests, allows for conformational and rotational changes in the DBD and LBD. The nuclear localization signals are often present in the hinge region and, in some cases, may be important for dimerization with the NRs binding partner and heat shock proteins in the cytoplasm (Claessens et al 2008). Recent elucidation of PPARγ/RXRα full length proteins bound to DNA revealed that the PPARγ N-terminal segment of the hinge region can also make a significant DNA contact while the RXRα hinge region formed a dimer contact (Chandra et al 2008). The RXRα hinge region was notably less structured than the corresponding PPAR region and was proposed to allow for the promiscuity observed in RXRs interactome (Chandra et al 2008). The question remains whether these findings are unique to this complex or whether this is a general phenomenon indicating a more prominent role for the hinge region.

The LBD was first crystallized and resolved for the unliganded RXRα and subsequently shown in multiple other nuclear receptors to have highly conserved overall structure (Bourguet et al 1995). These studies revealed that the LBD is composed of 12 α-helices that fold into a globular domain formed of an antiparallel α-helical sandwich (Bourguet et al 1995; Folkertsma et al 2004). The ligand binding pocket (LBP) is positioned in the interior of the LBD and is unique for each receptor conferring ligand specificity based on the size of the pocket and the overall shape. Each receptor has specific affinity for a subset of ligands where some, such as the xenobiotic receptors, have a large cavity to accommodate a wide range of ligands while others, such as the retinoic acid receptors, have a smaller cavity to accommodate only retinoic acid and other synthetic molecules with similar structure (Folkertsma et al 2004). Specific hydrophobic interactions between highly conserved amino acids that line the LBP and the ligand also control specificity, as well as stereospecificity, of the ligands (Gronemeyer et al 2004).

Located at the carboxy terminus of the LBD is the second activation domain (AF-2). The AF-2 activity is localized to the important helix 12 which undergoes large repositioning events upon ligand binding (Warnmark et al 2003). In the absence of ligand, the AF-2 domain assumes a position with high affinity to a co-repressor interaction domain (CoRID) that has as a consensus sequence LxxxIxxxI/L found in most co-repressor proteins such as Nuclear Co-Repressor (NCoR). When bound to ligand, the conformational change in helix 12 creates a new binding surface that stabilizes interactions with co-activators with a consensus LxxLL motif (such as ASC-1, SRC-1, p300) while also decreasing affinity for co-repressor proteins. Partial agonists can trap

the receptors in either an activated or repressed state depending on factors such as response element sequence and co-factor concentrations, which can be cell-type specific (Warnmark et al 2003).

1.2.4) Post-translational Modifications of NHRs

The NHR superfamily, in addition to ligand stimulation, can also integrate signalling pathways and respond to extracellular cues (Weigel & Moore 2007). Multiple post-translational modifications have been identified on NHRs such as acetylation, ubiquitination, SUMOylation and phosphorylation with the resulting effect on transcriptional activity being receptor and context dependent (Duma et al 2006; Li et al 2007; Wang et al 2008a; Weigel & Moore 2007; Yang et al 2009). The most highly studied modification to date is phosphorylation due to the ease of manipulating signalling cascades, the ability to recapitulate phosphorylation *in vitro* and to detect *in vivo* phosphorylation status using radiolabelled ATP or phospho-specific antibodies. Phosphorylation of nuclear receptors provides a reversible level of regulation resulting in activation or repression depending on the specific phosphorylation site, cell type and particular nuclear receptor (Rochette-Egly, 2003). Most NHRs are phosphorylated in the AF-1, AF-2 or the DNA binding domain which results in altered ligand affinity, DNA-binding affinity or co-factor interactions (Rochette-Egly, 2003). One family of kinases reported to alter the activity of multiple nuclear receptors is the protein kinase C family.

1.2.5) Protein Kinase C and Nuclear Hormone Receptor Regulation

1.2.5.1) Protein Kinase C Superfamily

Protein Kinase C consists of a family of kinases that mediate responses from extracellular stimuli, usually for proliferation, survival and the regulation of neurotransmitter release (Newton, 2001). The accepted model of PKC activation results from membrane bound phospholipase C (PLC) cleaving its substrate phosphoinositol 4,5 bisphosphate (PIP_2) producing the products inositol triphosphate (IP_3) and diacylglycerol (DAG). DAG, the effector molecule in PKC activation, binds to the C1 domain in conventional and novel PKCs (see below) resulting in membrane recruitment and increased kinase activity. To date, there are 10 known isoforms of PKC, encoded by different genes that are classified according to their activation requirements. Conventional PKCs (cPKC), comprised of members α, βI, βII, and γ, are activated by DAG, phosphatidylserine and Ca^{2+} (βI and βII are alternatively spliced transcripts). The novel PKCs (nPKC), specifically isoforms δ, ε, η, and θ, do not require Ca^{2+} for activation; however, phosphatidylserine and diacylglycerol activate these isoforms as well. A third classification, atypical PKCs (aPKC; ζ and λ), require only phosphatidylserine for activation (Newton 2008). Groupings and activators are summarized in Fig. 3A.

A)

Isoform	Family	Ca++	Phosphatidylserine	Diacylglycerol
α	Conventional	+	+	+
β	Conventional	+	+	+
γ	Conventional	+	+	+
δ	Novel		+	+
ε	Novel		+	+
η	Novel		+	+
θ	Novel		+	+
ξ	Atypical		+	
λ	Atypical		+	

B)

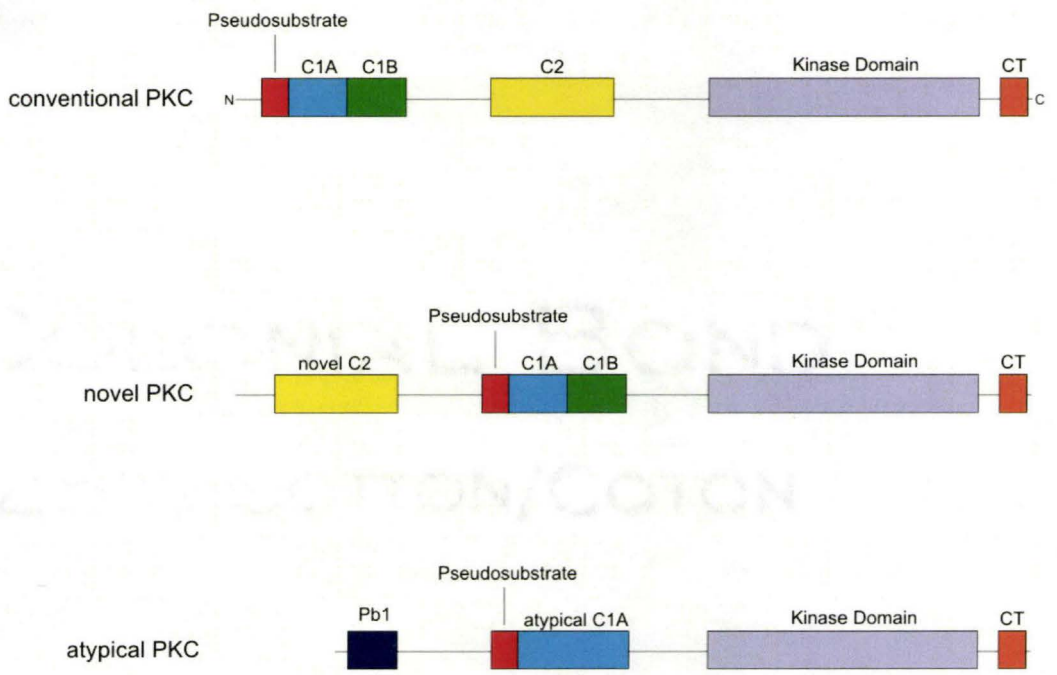


Figure 3. Schematic representation of PKC domains and summary of isoform-specific activation requirements. (Adapted from Newton et al., 2009)

1.2.5.2) PKC Domain Structure

PKC members are multidomain proteins that functions as monomers (Fig. 3B). The N-terminal end of the protein contains regulatory domains whereas the C-terminal region contains the kinase or catalytic domain (Fig. 3B). The regulatory domain is subdivided into the membrane-targeting modules C1, C2 and a pseudosubstrate. The pseudosubstrate resembles target substrate sequences recognized by the catalytic core, however, the phosphoacceptor is substituted for an alanine thereby locking the protein in an inactive basal state (Newton 2003). The C1 domain, a cysteine-rich domain of approximately 50 amino acids, is found as a tandem repeat in conventional and novel PKC and as a single copy in atypical PKC and has high affinity for membrane DAG. The C2 domain, only present in cPKC and nPKC, is a membrane targeting module that binds to negatively charged phosphoryl groups of lipids. Interestingly, the cPKC C2 domain requires Ca^{2+} to bind tightly to its substrate whereas the C2 domain in nPKC can function without Ca^{2+} indicating that cPKCs would require a signal that not only forms DAG but also increases intracellular calcium.

The catalytic region of PKC functions to phosphorylate serine or threonine residues in substrate proteins. Unique optimal peptide sequences have been determined for each isoform and therefore, the diverse array of PKCs is speculated to not only have overlapping functions but also unique roles in a cell and substrate specific manner. For example, the PKCθ isoforms play a specific role in T-cell activation where it acts as a

necessary downstream effector for proper maturation and responses to inflammatory stimuli (Chaudhary & Kasaian 2006).

1.2.5.3) *PKC AND NHR Crosstalk*

PKCs both positively and negatively regulate NHR transactivation through direct phosphorylation of sub-domains. While the transactivation capabilities of certain receptors, such as the farsenoid X receptor (FXR), are enhanced upon PKC-mediated phosphorylation, inhibition of NHR transcriptional activity has also been reported for the vitamin D and retinoic acid receptors (VDR; RAR) (Delmotte et al 1999; Gineste et al 2008; Hsieh et al 1991; Hsieh et al 1993). Despite phosphorylating similar domains, the resulting outcomes can vary depending on receptor type. Interestingly, phosphorylation of FXR in the DBD increases co-activator recruitment and transactivation while for RARα, phosphorylation at Ser198 in the DBD results in reduced DNA binding and thus, reduced activity. In both cases, the phosphorylation effects can be mimicked by mutation to a constitutively negatively charged amino acid (Delmotte et al 1999; Gineste et al 2008). It is hypothesized for RAR that PKC-mediated phosphorylation blocks the expression of pro-differentiation genes in leukocytes normally regulated by retinoic acid (Launay et al 2003). Lastly, the vitamin D receptor is also phosphorylated in its DBD by PKC *in vitro* and *in vivo*, resulting in diminished activation in a transient transfection assay due to a decrease in DNA binding (Hsieh et al 1991; Hsieh et al 1993). Overall, PKC signaling represents an important pathway that can modulate the response of NHR to ligand.

1.3) The Liver X Receptors

1.3.1) Overview

The Liver X Receptors (LXR; subtypes α and β) are lipid-sensing members of the type II class of nuclear hormone receptors originally cloned from a liver cDNA library (Willy et al 1995). LXRβ (NR1H2) is ubiquitously expressed whereas LXRα (NR1H3) is highly expressed in the liver, macrophage, kidney, lung and adipose tissue and at lower levels in other tissues (Tontonoz & Mangelsdorf 2003). Both receptors display the typical NHR domain structure described above and share approximately 75% amino acid sequence identity with greatest variations occurring in the AF-1 and hinge domains (Fig. 4). LXRs were initially classed as orphan receptors and were later 'adopted' when oxidized cholesterol metabolites, or oxysterols, were identified as their endogenous ligand (described in detail below) (Janowski et al 1996). In addition, cholesterol biosynthetic metabolites (eg. desmosterol), plant sterols and even glucose are also reported to act as LXR agonists (Mitro et al 2007; Plat et al 2005; Plosch et al 2004; Yang et al 2006). In concordance with this, knockout mouse models have confirmed LXRs as 'cholesterol sensors' that regulate multiple genes variously involved in lipid transport and metabolism (see table 1 for list of genes).

LXRs form obligate heterodimers with the 9-*cis* retinoic acid receptor (RXRα) and LXR/RXR is a so-called "permissive heterodimer" where agonists for either receptor are able to activate the complex (Fujita & Mitsuhashi 1999; Zhang et al 2002). LXR/RXR heterodimers bind to liver X response elements (LXREs) with the half-site

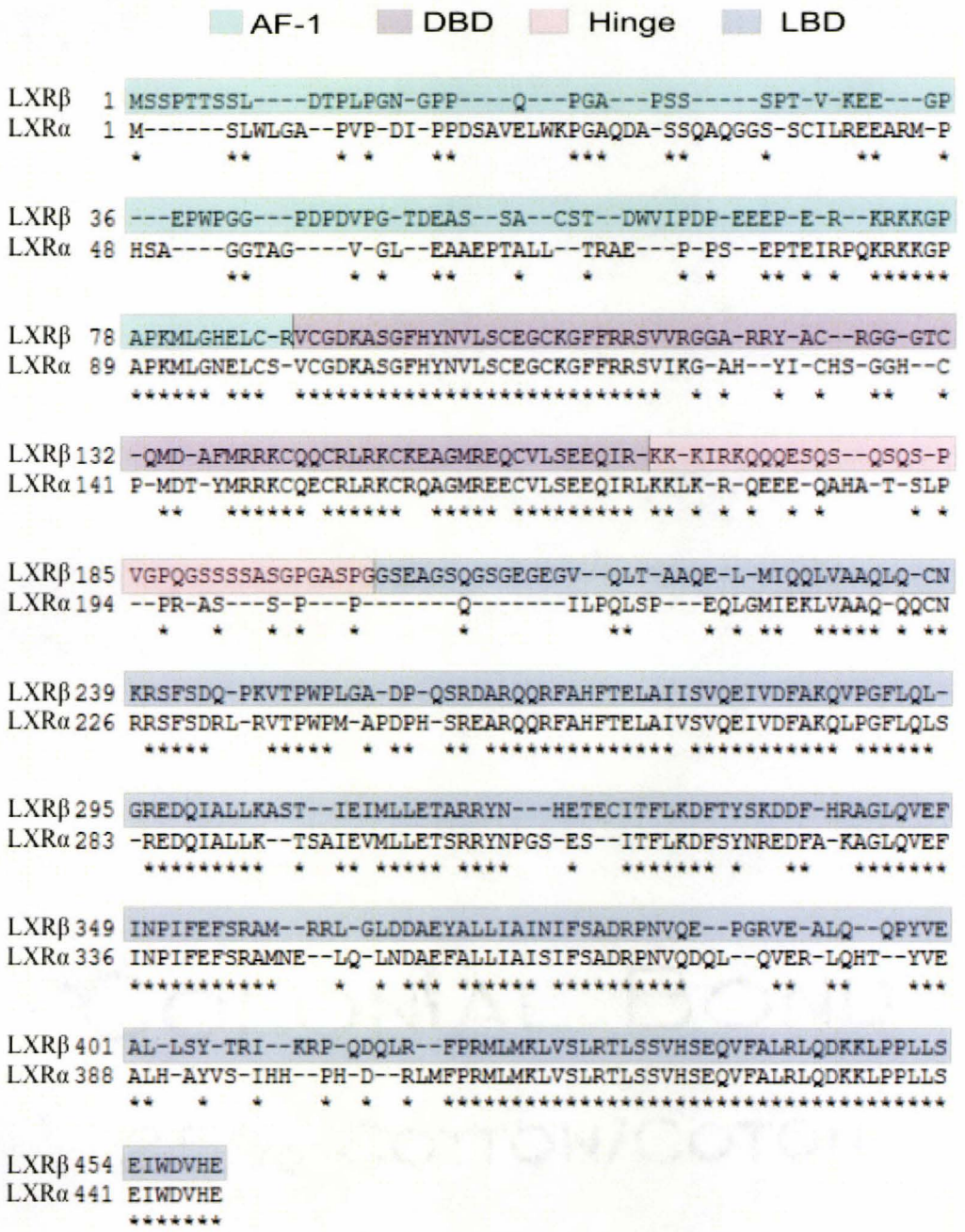


Figure 4. Amino Acid Sequence Alignment of Human LXRα and LXRβ. Amino acid sequences for human LXRα (NR1H3; gene - human Chromosome 11) and human LXRβ (NR1H2; gene - human chromosome 19) are shown and the indicated domain is colour coded. LXRα and LXRβ share approximately 75% amino acid similarities and differ mostly in the AF-1 and hinge regions. * indicates identical amino acids in both sequences

Table 1 – LXR-regulated genes (adapted from Kalaany et al., 2006)

Group	Gene	Positive/Negative Regulation	Direct/Indirect
Cholesterol Metabolism	ABCA1	P	D
	ABCG1	P	D
	ABCG5	P	D
	ABCG8	P	D
	ApoA5	N	I
	ApoCI/IV/II	P	D
	ApoD	P	D
	ApoE	P	D
	hCETP	P	D
	mCYP7A1	P	D
	LPL	P	D
	hLXRα	P	D
	PLTP	P	D
	hSR-BI	P	D
Fat Metabolism	Angptl3	P	D
	FAS	P	D
	SCD-1	P	N/D
	SPOT14	P	N/D
	SREBP1c	P	D
Carbohydrate Metabolism	G6Pase	N	N/D
	GK	N	N/D
	GLUT4	P	D
	PEPCK	N	N/D
Immune Response/Inflammation	COX-2	N	N/D
	G-CSF	N	N/D
	Il-1β	N	N/D
	Il-6	N	N/D
	iNOS	N	N/D
	IP-10	N	N/D
	MCP-1	N	N/D
	MCP-3	N	N/D
	MIP-1β	N	N/D
	MMP-9	N	N/D
	SPα	P	D
Miscellaneous	Fascin-1	P	I
	FPPS	P	N/D
	Fra-1	P	N/D
	IBABP	P	N/D
	Involucrin	P	I
	Loricrin	P	N/D
	L-UrdPase	P	N/D
	Profilaggrin	P	N/D
	Renin	P	D
	Transglutaminase-1	P	N/D
	VEGF	P	D

consensus sequence AGGTCA separated by four nucleotides (DR4 element). In the absence of ligand, LXR/RXR typically recruits co-repressors such as nuclear co-repressor (NCoR) resulting in suppression of target genes (Wagner et al 2003). In agreement with this, LXR$\alpha^{-/-}\beta^{-/-}$ mice have elevated mRNA levels of certain target genes compared to wild-type (de-repression) and, as expected, these genes are no longer enhanced in the presence of LXR ligands (Wagner et al 2003). Upon ligand stimulation, LXR undergoes a conformation change that results in re-organization of helices in the ligand binding domain and dissociation of co-repressors (Zelcer & Tontonoz 2006). This conformational change also forms a new interaction surface with increased affinity for co-factors with a characterized LXXLL (Antonson et al 2008; Son et al 2008). Co-factors, such as the steroid receptor co-activator (SRC) and Receptor Interacting Protein 250 (RIP250; ASC-2) then act to recruit various other ancillary factors that ultimately results in enhanced expression of LXR target genes (Fig. 5A).

1.3.2) LXR agonists

LXR isoforms were originally classified as 'orphan receptors' prior to the identification of their natural ligand. Screening natural ligand extracts identified oxysterols with relatively high affinity and specificity for LXR isoforms over other nuclear hormone receptors (Fig. 5B) (Janowski et al 1996). Oxysterols that can act as LXR agonists are generally formed enzymatically within the cell and require proper enantiomeric configuration to display any potency (Deng et al 2005). Ligand binding studies indicate that oxysterols such as 22*(R)*-hydroxycholesterol, 27-hydroxycholesterol

and 24*(S)*,25-epoxycholesterol display the highest potency in transactivation assays. Moreover, oxysterol ligands do not display any LXR isoform selectivity indicating that both receptors can respond *in vivo* to elevated levels of these modified sterols. Importantly, oxysterols have also been identified in human plasma, atherosclerotic plaques and in association with oxidized LDL (oxLDL) (Kaul 2001; Schroepfer 2000). In addition to oxysterols, synthetic LXR agonists have been created that show essentially no preferential activation of either LXR isoform. These include the molecules GW3965 and TO091317 (T1317) which are extensively used here (Fig. 5B).

22*(R)*-hydroxycholesterol is generated as an intermediate in the reactions converting cholesterol to pregnenolone (Lala et al 1997). This reaction occurs mainly in steroidogenic tissues such as the adrenal glands, testes and ovaries. Related to this, LXR positively regulates the important steroidogenic protein StAR involved in mitochondrial cholesterol transport and steroid synthesis (Cummins et al 2006). 24*(S)*,25-epoxycholesterol is synthesized from dioxidosqualene in a shunt pathway of the cholesterol biosynthetic pathway. It is believe that 24*(S)*, 25-epoxycholesterol is important for negative feedback control of cholesterol synthesis and may represent an underestimated product in the liver (Brown 2008; Javitt 2008; Wong et al 2008). Furthermore, 27-hydroxycholesterol is generated enzymatically by a mitochondrial enzyme of the cytochrome P450 superfamily called CYP27 (D'Ambra et al 2000; Wassif et al 2003). CYP27 is expressed in many cell types including macrophage and smooth muscle cells and functions to convert cholesterol to 27-hydroxycholesterol, a reaction important in the regulation of cholesterol efflux. As cholesterol levels increase, levels of

27-HC are proportionally elevated and act as potent LXR activators resulting in LXR target gene expression and efflux of excess cholesterol (Escher et al 2003). Lastly, 24*(S)*-hydroxycholesterol (24*(S)*-HC), also a potent LXR agonist, represents the major brain-derived oxysterol. Because cholesterol does not cross the blood-brain barrier, cells of the central nervous system synthesize their required sterols and eliminate excess by conversion to 24*(S)*-HC, which is able to cross the blood brain barrier. These pathways are regulated by LXR and also play key roles in neurological diseases such as Alzheimer's disease (Abildayeva et al 2006; Adighibe et al 2005; Scott Kim et al 2009).

Work by Janowski et al. elucidated the structural requirements of an LXR ligand and concluded that cholesterol metabolites with a single oxygen on the sterol side chain likely functions as a hydrogen bond acceptor (Janowski et al 1999). Importantly, this oxygen moiety required specific stereochemistry that was identical to the stereochemistry of oxysterols detected *in vivo*. Later, crystallography studies confirmed that tryptophan 443 (Trp^{443}) and Histadine 421 (His^{421}) of LXRα were the crucial amino acids required for docking and hydrogen bonding to the oxysterol (Svensson et al 2003). Although oxysterols showed high specificity *in vitro*, it still remained to be definitively proven that LXRs are regulated by oxysterols *in vivo*. Elegant studies by Chen et al. addressed this question by using triple knockouts of CYP hydroxylase enzymes (Chen et al 2007). In these experiments, it was shown that upon cholesterol loading in the triple knockouts, there was a decreased fold activation of ABCA1 transporter mRNA expression mediated by LXR compared to wild-type (Chen et al 2007). However, synthetic agonists retained full potency in the triple knockout cell lines thus establishing that cholesterol is likely

converted to an oxysterol that subsequently activates LXR/RXR heterodimers to regulate transcription of cholesterol transport/metabolism genes.

Lastly, work by Saez and colleagues has shown that glucose and glucose-6-phosphate can also act as LXR agonists with slightly higher affinity for LXRβ (Mitro et al 2007). Interestingly, glucose did not bind to the LBD as demonstrated by the inability of glucose to displace oxysterols or vice versa in ligand binding assays (Mitro et al 2007). Furthermore, the authors demonstrated that glucose and oxysterols could bind LXR simultaneously. The physiological consequences of LXR as a glucose sensor remain to be determined however, LXR has indeed been previously implicated in glucose metabolism. GW3965 and T0901317, synthetic LXR agonists, reduce the expression of gluconeogenesis enzymes in the liver such as PEPCK and enhanced expression of glucose-6-phosphate dehydrogenase thereby increasing glucose uptake and metabolism of glucose in the liver (Laffitte et al 2003). Furthermore, LXR agonists regulate the insulin-stimulated glucose uptake transporter 4 (GLUT4) in white adipose tissue and airway smooth muscle (described in this thesis) (Dalen et al 2003; Delvecchio et al 2007). Lastly, insulin-stimulated transcription of SREBP1c in liver is dependent on LXR expression and furthermore, insulin stimulated signaling can activate LXR reporter genes (Cagen et al 2005); whether this is dependent on intracellular oxysterol formation or post-translational modifications of LXR or LXR accessory factors remains to be elucidated.

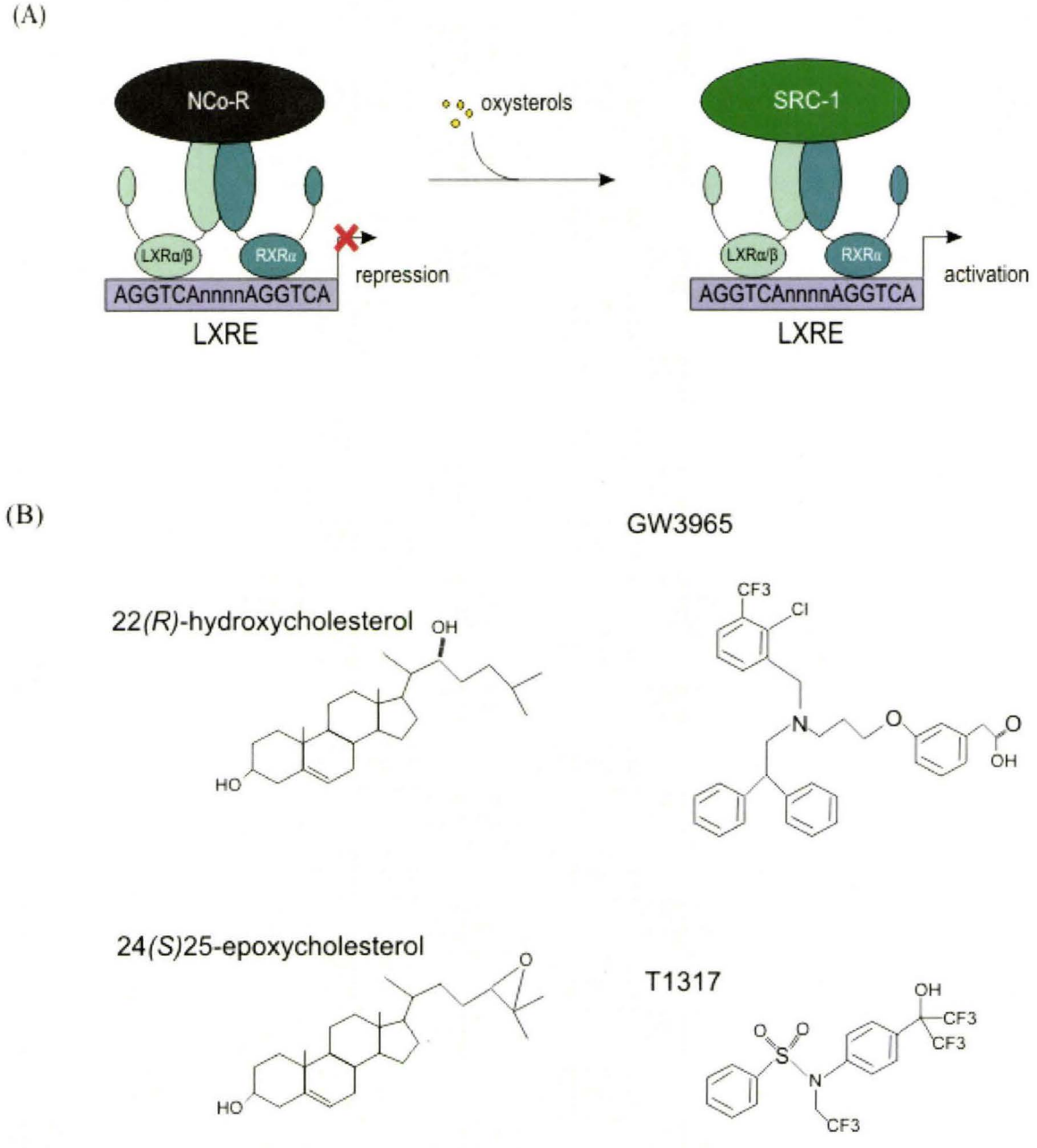


Figure 5. Schematic representation of LXR domain structure and LXR ligands. A) In the absence of ligand, LXRs recruit co-repressor proteins and repress gene activation. In the presence of ligands such as oxysterol, LXRs undergo a conformational change releasing co-repressors and recruiting co-activators such as SRC-1 to activate gene transcription. B) Structure of natural LXR agonists (left side) and synthetic LXR agonists (right side). (Adapted from Janowski et al., 1999; Zelcer et al., 2006; Willy et al., 1995)

1.3.3) LXR antagonists

Few reports have studied the effects of LXR antagonists however, there are some that indicate that enantiomers of LXR ligands, such as 22*(S)*-hydroxycholesterol, can act as LXR antagonists by occupying LXR's ligand binding pocket but preventing proper conformational changes that allow co-repressor release and activator recruitment (Kase et al 2006). Polyunsaturated fatty acids such as arachidonic acid, but not saturated or monounsaturated fatty acids, also inhibit LXR's transcriptional activity even in the presence of LXR agonists (Murthy et al 2004; Ou et al 2001; Yoshikawa et al 2002). Additionally, geranylgeranylpyrophosphate (GGPP) represses LXR transactivation ability by blocking co-factor recruitment and decreasing DNA binding (Argmann et al 2005; Denis et al 2003). A *bona fide* specific synthetic antagonist useful for studying LXR biology in a reversible manner, however, remains to be synthesized.

1.3.4) Post-translational Regulation of LXRs

The majority of work on LXR transactivation has focused on ligand-stimulated mechanisms, however, very little is known with regards to regulation of LXR isoforms themselves. As described above, NHRs are highly regulated by post-translational modifications such as phosphorylation. LXRα has been shown to be constitutively phosphorylated in a MAPK conserved consensus sequence (Ser198), a site also conserved in LXRβ (Chen et al 2006). However, mutation of Ser198 to alanine showed no apparent effect on transactivation of target genes such as ABCA1 (Chen et al 2006). Later, Torra et al. demonstrated that phosphorylation at Ser198 is enhanced by LXR ligands and

reduced by casein kinase 2 inhibitors as well as the RXR agonist 9-cis retinoic acid (Torra et al 2008). In the murine macrophage, the regulation of a subset of LXR target genes such as lipoprotein lipase (LPL), but not ABCA1 or SREBP1c, was increased following overexpression of $LXR\alpha^{S198A}$. Furthermore, $LXR\alpha^{S198A}$ expression resulted in transcriptional activation of CCL24 and thus, it was concluded that LXR phosphorylation may prevent the expression of a specific subset of LXR target genes (Torra et al 2008). The physiological consequences, contexts and the particular upstream pathways involved in determining the selectivity remain to be uncovered.

Other signaling pathways reported to alter LXR activity include the Protein Kinase A (PKA) pathway, stimulated by cyclic AMP. Activation of PKA by 8Br-cAMP, a stable cyclic AMP analogue, results in enhanced expression of LXR-responsive reporter genes as well as endogenous LXR targets such as *renin* in mouse renal As4.1 cells (Tamura et al 2000). *In vitro* analysis indicated that murine LXRα was phosphorylated in the AF-2 region (Ser431) resulting in increased transcriptional activity (Tamura et al 2000; Tamura et al 2004). Contrary to this, analysis in rat primary hepatocytes and whole mouse liver extracts revealed that PKA activators decreased the expression of SREBP1c, an LXR target gene, as a result of LXRα phosphorylation at Ser195 and Ser290. The resulting phosphorylation decreased heterodimerization with RXRα and thus, reduced DNA binding to LXREs (Yamamoto et al 2007). The discrepancy in PKA/LXR signaling may result from cell- and promoter- specific effects and requires more thorough analysis *in vivo*.

In addition to phosphorylation, LXRs are acetylated and regulated by deacetylases such as SIRT1 (Li et al 2007). Acetylation of LXRα at lysine residue 432 (K432; K433 in LXRβ) in the activation domain decreases LXR target gene expression. Furthermore, interaction with SIRT1, a deacetylase variously involved in metabolism and life span, removes acetyl groups from LXR and increases target gene expression (Feige & Auwerx 2007; Li et al 2007). Confirming these finding *in vivo*, SIRT1$^{-/-}$ murine macrophages display decreased LXR target gene expression following administration of an LXR agonist providing further evidence that acetylation plays a role in LXR regulation (Li et al 2007).

To date, numerous studies indicate that post-translational modifications play a key role in LXR regulation. Evidence also presented in this thesis further shows that LXR is also regulated by PKC resulting in decreased transactivation (see chapter 2) (Delvecchio & Capone 2008). However, in all cases of post-translational LXR regulation described above, physiological relevance is lacking. The correct scenarios and settings for proper activation of these pathways, which play roles in a plethora of physiological processes, and the downstream regulation of LXRs remains to be determined *in vivo*. Transgenic mouse models with LXR point mutations in key kinase consensus sites (or other sites) may help to elucidate the role of post-translational modifications and the regulation of LXR itself.

1.3.5) LXR knockout mouse models

The generation of LXR knockout mice has been instrumental in elucidating the role of LXR in lipid metabolism. Moreover, isoform-specific knockout mouse models have revealed non-overlapping roles for LXRα and LXRβ. LXRα$^{-/-}$ mice fed a standard chow diet do not display many gross phenotypes when compared to wild type mice. They do, however, display slightly reduced fat mass and decreased adipocyte size (Peet et al 1998). Total cholesterol levels remain normal with a tendency toward increased LDL levels and decreased HDL levels. However, when LXRα$^{-/-}$ mice are fed a diet containing 2% cholesterol, dramatic phenotypes are observed compared to wild type mice. LXRα$^{-/-}$ mice accumulate large amounts of cholesterol ester in the liver because they are unable to catabolize cholesterol to bile acids. This is due to a lack of CYP7A induction, the rate-limiting bile acid synthesis enzyme and first identified LXR target gene (Peet et al 1998). On a 2% cholesterol diet, serum and LDL-cholesterol are also elevated and HDL levels are significantly reduced.

LXRβ$^{-/-}$ mice interestingly do not display similar phenotypes to LXRα$^{-/-}$ mice on a high cholesterol diet and the above also indicate that LXRβ cannot compensate for the loss of LXRα in the liver. On a standard diet, LXRβ$^{-/-}$ mice have reduced fat mass and decreased triglyceride levels both in the serum and liver as well as decreased insulin levels (Alberti et al 2001). On a diet supplemented with 2% cholesterol, LXRβ$^{-/-}$ mice are capable of eliminating excess cholesterol and do not show altered liver phenotypes like those observed in LXRα$^{-/-}$ mice. Furthermore, LXRβ$^{-/-}$ mice are resistant to diet-induced

obesity displaying a leaner phenotype when compared to wild-type counterparts. Overall, LXRβ may play a crucial role in extrahepatic tissue and fat metabolism where LXRα is either not expressed or incapable of compensating due to cell specific factors (Gerin et al 2005).

Double knockout LXRα$^{-/-}$β$^{-/-}$ display somewhat of an additive phenotype compared to the single knockout models. On a standard diet, LXRα$^{-/-}$β$^{-/-}$ have reduced body weight compared to wild type. The levels of triglycerides are lower both in the liver and serum and total cholesterol is increased. LXRα$^{-/-}$β$^{-/-}$ mice are resistant to weight gain on a 0.2% cholesterol diet but interestingly, gain weight when fed a high fat diet without cholesterol (discussed further below) (Kalaany et al 2005). Overall, the above findings point to a role for LXR in the post-prandial state where they promote storage of lipids and increase cholesterol excretion, when in excess.

1.3.6) LXR and Reverse Cholesterol Transport Pathways

1.3.6.1) Background

Cholesterol is crucial for cellular membranes and proper signaling, however, cells cannot break down cholesterol for use as an energy source and excess levels of cholesterol can be cytotoxic. To circumvent cholesterol toxicity, cells downregulate cholesterol biosynthesis and LDL receptor expression and activate LXR signaling. Activation and translation of LXR's transcriptional network results in efflux of cholesterol from peripheral cells, transport of cholesterol back to the liver and conversion

or incorporation of cholesterol into bile acids for excretion from the body in a process called reverse cholesterol transport (RCT) (Fig. 6). Most genes involved in RCT are direct targets of LXRs (see table 1) (Kalaany & Mangelsdorf 2006).

The majority of cholesterol efflux pathways and the process of reverse cholesterol transport have been elucidated in the macrophage due to their prominent role in atherosclerotic plaque formation, however, based on results presented in this thesis and elsewhere, the findings in these cells and the generalizations below should not be taken as universal (also see chapter 4).

1.3.6.2) Atherosclerosis, LXR and Reverse Cholesterol Transport

In susceptible regions of arteries, circulating low density lipoprotein (LDL) in the plasma can enter the arterial intima and become trapped in the extracellular matrix (ECM) (Lusis 2000). Long term retention of LDL results in oxidation of its lipid components to form minimally oxidized or modified LDL (mmLDL). These mmLDL particles are pro-inflammatory in nature and results in the expression of adhesion molecules such as VCAM-1 on the luminal surface of endothelial cells that are recognized by circulating leukocytes. Monocytes, via cell surface receptors, bind to the adhesion molecules and migrate into the sub-endothelial space whereupon they differentiate into macrophages and begin to internalize mmLDL. The increase in intracellular lipid such as free fatty acids is believed to act as an agonist for the peroxisome proliferator-activated receptor γ (PPARγ) (Chawla et al 2001). PPARγ, in turn, activates scavenger receptors such as CD36 that are expressed in mature macrophages. These scavenger receptors recognize and internalize

enhanced oxidized lipoprotein particles (oxLDL) and unlike the LDL receptor (LDLR), scavenger receptors are not under a negative feedback control (Pluddemann et al 2007). Therefore, the macrophages in the sub-endothelial space continuously scavenge excess extracellular lipid. Excess internalized cholesterol is esterified by acyl-coenzyme A:cholesterol acyltransferase (ACAT) and stored in lipid droplets in the cytoplasm giving the macrophage a "foamy" appearance and thus, these cells are referred to colloquially as "foam cells" (Daub et al 2007). Foam cell formation is recognized as one of the initial components of plaque formation known as a primitive fatty streak. The fatty streak is pathologically silent but can further develop into a necrotic lipid core with fibrous cap able to rupture and cause thrombosis leading to myocardial infarction (MI) (Bobryshev 2006).

The accumulation of lipid in the macrophage is also believed to result in increased oxysterol concentrations that activate LXR/RXR heterodimers. LXRs then drive the expression of genes involved in reverse cholesterol transport including lipoproteins, transporters and lipoprotein remodeling enzymes (Fig. 6) (Pelton et al 2005). Among these target genes, the ATP binding cassette (ABC) transporters have been highly investigated.

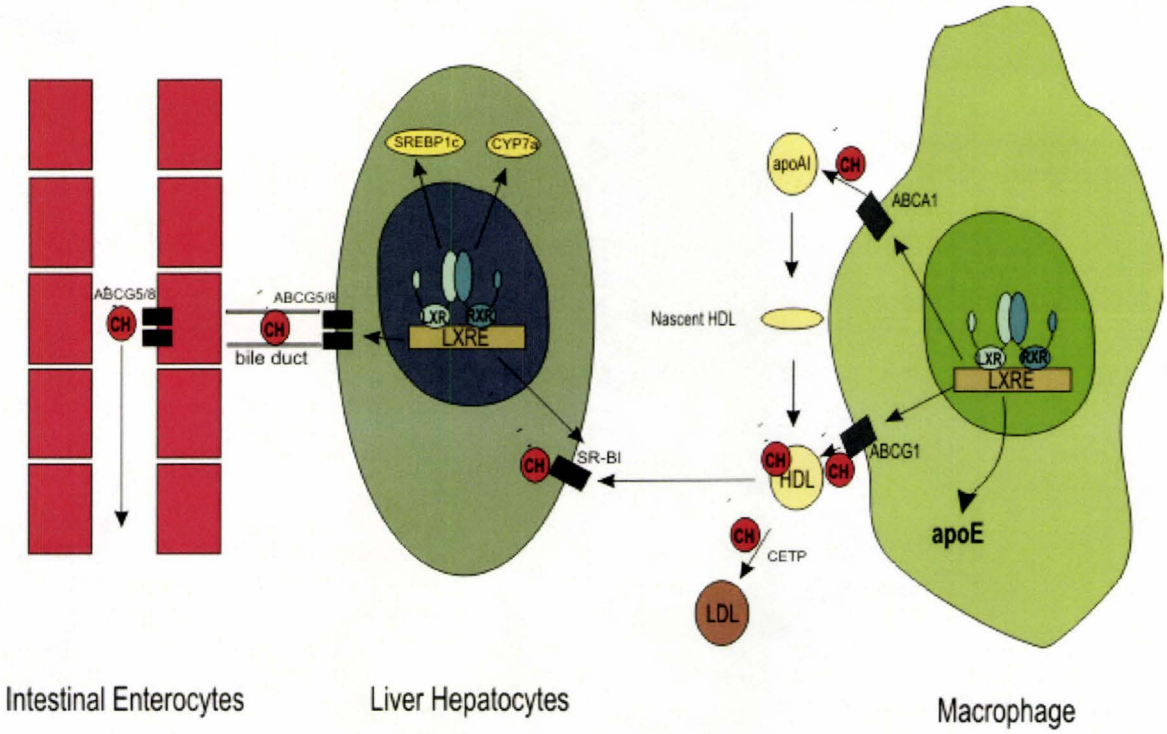


Figure 6. LXR regulates multiple genes involved in reverse cholesterol transport (RCT). When peripheral cells accumulate excess intracellular cholesterol, such as when macrophages scavenge lipoproteins in the arterial intima, levels of oxysterols are proportionately elevated which activate LXR/RXR heterodimers. In peripheral cells (macrophage shown as an example), LXRs regulate the expression of ABC transporters ABCA1 and ABCG1 as well as apolipoproteins apoE, among other genes. ABCA1 and ABCG1 efflux cholesterol to plasmatic acceptors such as apoAI or HDL particles, respectively. However, ABCA1 may also stimulate transport of cholesterol to HDL as well (as described in this thesis). Moreover, LXRs regulate lipoprotein remodeling enzymes such as CETP and PLTP which modify circulating lipoproteins. HDL particles formed in peripheral tissues are eventually circulated back to the liver. In the liver, LXRs activate the expression of the HDL receptor SR-BI as well as LDL receptors (LDLR) resulting in increase cholesterol uptake in hepatocytes via separate mechanisms. Additionally, the rate-limiting enzyme in the bile acid synthesis pathway, CYP7A, is regulated by LXRs (not conserved in humans) and controls conversion or incorporation of cholesterol into bile. Excess cholesterol and bile is then secreted into the bile duct and eventually into the intestinal lumen by LXR target genes ABCG5 and ABCG8. Important to note, LXRs also activate a *de novo* lipogenesis program in the liver by positively regulating the SREBP1c transcription factor involved in lipogenesis, among other genes. Overall, activation of LXR increases transport of peripheral cholesterol to the liver for eventual excretion from the body. (Adapted from Zelcer et al., 2006)

1.4) LXR target genes and non-lipid related roles of LXR

1.4.1) ABC Transporters

The ATP-binding cassette (ABC) transporters represent one of the largest protein families and spans all phyla with over 140 members discovered thus far (Dean et al 2001). The human genome encodes 49 ABC transporters subdivided into seven families (A to G) based on sequence similarity and number of transmembrane domains (Seeger & van Veen 2008). An unconventional nomenclature refers to transporters as either "full transporters" (containing 12 membrane spanning regions) or "half transporters" (6 membrane spanning domains). Half transporters are believed to function as homo- or heterodimers to form a fully functional transporter. ABC transporters mediate the movement of a wide range of substrate from ions to peptides and are characterized by the highly conserved nucleotide binding domain (NBD) (Dean et al 2001). The NBD consists of three motifs: the Walker A and B motifs and the ABC signature motif (also known as Walker C) (Fig. 7). This domain is required for nucleotide binding and hydrolysis which provides the energy required for substrate transport across membranes. The importance of ABC transporters in human biology is exemplified by the large number of diseases or resistance to cancer treatments associated with ABC transporter function (or lack thereof). These include resistance to anticancer agents (ABCC2 (also known as multidrug resistance protein)) as well as multiple lipid and other disorders such as sitosterolemia (ABCG5/G8), progressive familial intrahepatic cholestasis (ABCB11), Stargardt's disease (ABCA4), cystic fibrosis (ABCC7) and Tangier's disease (ABCA1) (Takahashi et al 2005).

1.4.2) ABCA1 – Background

ABCA1 is a full transporter encoded by 2261 amino acids (210kD) that contains two NBDs and 12 membrane-spanning helices (Zarubica et al 2007). The protein is highly expressed in the lung, brain, heart, bladder, liver, kidney and macrophage and at lower levels in various other tissues. The topology of the protein is predicted to have a cytosolic amino and carboxy terminus with large extracellular loops that are highly glycosylated (Fig. 7A) (Zarubica et al 2007). Mutations in *abca1* were identified as the cause of Tangier's disease, characterized by abnormally low levels of HDL and increased risk of cardiovascular disease (CVD) (Oram 2002). Subsequent work based on these findings identified ABCA1 as a cholesterol and phospholipid transporter although the exact mechanisms were unknown. $ABCA1^{-/-}$ mice recapitulated the observations in humans displaying dramatically low levels of HDL. Moreover, targeted deletion of ABCA1 has confirmed tissue-specific roles for ABCA1 in HDL physiology. Specifically, deletion of ABCA1 in the liver dramatically reduced HDL (>80%) levels in the mouse pointing to a crucial role for hepatic ABCA1 (Singaraja et al 2006). Additionally, intestinal ABCA1 also contributes significantly to total body HDL levels as tissue-specific deletion of ABCA1 in enterocytes reduces HDL levels greater than 20%. Lastly, in bone-marrow transplantation studies, macrophage-specific deletion of ABCA1 exacerbated atherosclerotic plaque formation in $LDLR^{-/-}$ and $apoE^{-/-}$ mouse models of atherosclerosis which points to a crucial role for ABCA1 in reverse cholesterol transport and foam cell formation (Aiello et al 2002).

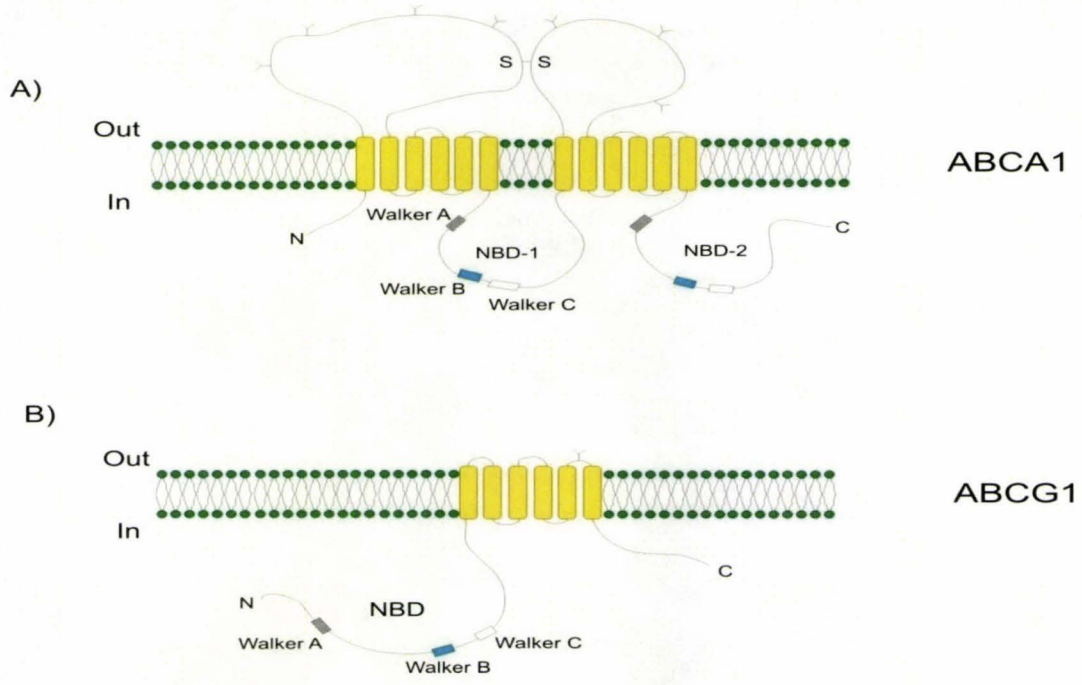


Figure 7. Topology of ABC transporter ABCA1 and ABCG1. A) ABCA1 predicted topology with glycosylation sites indicated with Y and a predicted di-sulfide bond (S-S). Two nucleotide binding domains (NBD-1 and NBD-2) are shown with Walker A, B and C motifs indicated. **B)** ABCG1 predicted topology with indicated domains as described in (A). (Adapted from Velamakanni et al., 2007 and Oram and Heinecke, 2005)

1.4.3) ABCA1 efflux mechanisms

The extracellular acceptors involved in ABCA1 mediated transport are typically lipid-poor apolipoproteins although, as presented here (chapter 4) and elsewhere (O'Connell et al 2004; Out et al 2008), HDL particles themselves can also act as acceptors in an ABCA1-dependent pathway. The main apolipoprotein associated with HDL is apolipoprotein AI which has been shown to interact directly with ABCA1 and act as an acceptor for phospholipid and cholesterol transport. Other apolipoproteins, such as

ApoE, among others, can also function as lipid acceptors for ABCA1-mediated efflux (Kypreos 2008; Kypreos & Zannis 2007; Zannis et al 2008).

Despite intense investigation into the role of ABCA1 in lipid transport, the mechanisms of action remain to be clearly defined. It is still unclear whether ABCA1 physically transports phospholipids or cholesterol through an internal pore similar to other ABC transporters. Two models have been proposed where 1) cholesterol and phospholipid efflux mediated by ABCA1 to apoAI occurs at the plasma membrane and 2) retroendocytosis of apoAI/ABCA1 is required for lipidation and efflux. The latter model was proposed based on studies using small molecule inhibitors of endocytosis which resulted in decreased lipid efflux (Lorenzi et al 2008). Furthermore, apoAI was identified in endosomes and other intracellular compartments. However, due to the non-specific effects of the endocytosis inhibitors, this model remains to be definitively proven (Denis et al 2008). Recently, the former model of efflux, occurring at the plasma membrane, has garnered greater attention. A mechanism was proposed by Vedhachalam et al. that incorporates findings from previous models and attempts to reconcile disparate observations from multiple labs (Vedhachalam et al 2007). They proposed the following model: 1) lipid-free apoAI binds to the ABCA1 at the plasma membrane which results in increased phospholipid translocase activity where ABCA1 transports PLs from the inner leaflet to the exofacial leaflet 2) The resulting increase in PLs in the outer leaflet causes strain resulting in membrane bending. This membrane curvature was shown to have high affinity for apoAI 3) Lastly, apoAI spontaneously solubilizes membrane phospholipids and cholesterol in the curved membrane portion to create discoidal HDL with two to four

apoAI molecules (Vedhachalam et al 2007) (Fig. 8). In support of these findings, Denis et al. showed that ABCA1 mediated cholesterol efflux occurs at the plasma membrane and that internalized apoAI contributes less than 2% of discoidal HDL particles (Denis et al 2008). Their findings also show that the majority of internalized apoAI is degraded in lysosomal compartments (Denis et al 2008). Lastly, a separate group confirmed these findings and indicated that the small percentage of apoAI that was recycled to the plasma membrane and secreted was degraded and did not contribute significantly to ABCA1-mediated lipid efflux, confirming the above results (Faulkner et al 2008).

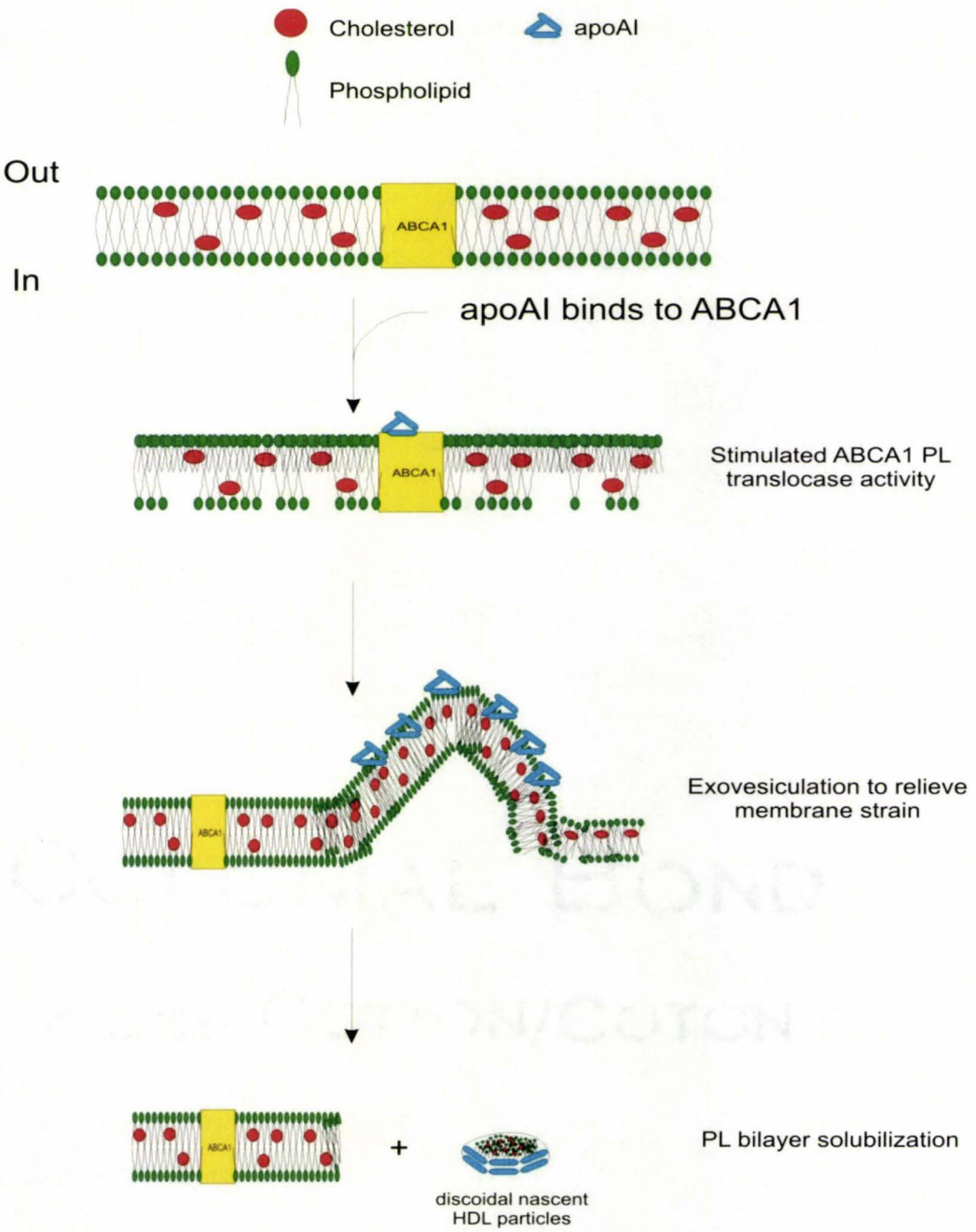


Figure 8. Mechanisms of ABCA1-mediated cholesterol efflux as proposed by Vedhachalam et al., 2008. Step 1: ApoAI bind to ABCA1 stimulating its phospholipid (PL) translocase activity leading to membrane compression in the exofacial leaflet. **Step 2**: Membranes bend to relieve strain creating a high affinity binding site for apoAI. **Step 3**: ApoAI spontaneously solubilizes the bound PLs and cholesterol creating discoidal nascent HDL particles (see text for further details). (Adapted from Vedhachalam et al., 2008)

1.4.4) Transcriptional and Translational Regulation of ABCA1

Levels of ABCA1 transcripts are highly induced by cholesterol loading in multiple cell types (Denis et al 2003). This marked induction is mediated by LXRs as cholesterol loading in macrophages with targeted deletion of LXR α and β fail to activate ABCA1 transcription. It is believed that elevated cholesterol levels raise the concentration of intracellular oxysterols via hydroxylase enzymes such as CYP27, as described above. This in turn activates the LXR/RXR heterodimers bound to the promoter regions of target genes resulting in increased transcriptional activity. The ABCA1 gene contains a highly characterized LXRE located 933bps upstream of the major transcriptional start site (Costet et al 2000). In addition to oxysterols, cAMP signalling, cytokines, PKC (described in this thesis), retinoids, PPAR agonists have all been shown to activate or repress ABCA1 transcription (Schmitz & Langmann 2005). Indeed, multiple transcription factor binding sites (eg. Glucocorticoid response elements, NF-κB elements) have been identified in the ABCA1 promoter that can influence transcription under various conditions.

ABCA1 protein levels are also highly regulated. ABCA1 displays rapid turn-over with a half-life of 1-2hrs in the absence of extracellular acceptors and low cholesterol concentrations (Hozoji et al 2008). This rapid turnover is mediated by the PEST motif (proline-glutamate-serine-threonine) that, when phosphorylated, is recognized by the proteosomal degradation pathways. Interestingly, a recent report has shown that LXR itself interacts with ABCA1 at the plasma membrane in the human monocyte cell line

THP-1 through an ABCA1 sequence LTSFL (Hozoji et al 2008). This sequence resembles the well characterized LXXLL motif found in multiple co-factors that interact with the LBD of NRs. The functional consequence of this interaction is suppression of ABCA1 activity in the presence of basal or low cholesterol. In the presence of high cholesterol levels or synthetic LXR agonists, the LXR-ABCA1 complex dissociates resulting in increased ABCA1 activity, longer half-life of the protein and enhanced transcription of ABCA1 mRNA. Whether this phenomenon occurs in all cell types, or in murine cells where atherosclerotic mouse models can better characterize the relevance of the interaction, remains to be determined.

1.4.5) ABCG1 – Background

ABCG1 is a 1000 amino acid protein characterized as a "half transporter" with 6 membrane spanning domains (Baldan et al 2006b) (Fig. 7B). ABCG1 is mainly expressed in the macrophage and neuronal tissues with high levels in the eye and brain. The topology of ABCG1 is predicted to have a cytosolic amino- and carboxy-terminus and ABCG1 is proposed to function as a homodimer (Fig. 7B). Similar to ABCA1, ABCG1 is highly induced by cholesterol loading in multiple cell types and ABCG1 mRNA and protein levels are increased when cells or animals are treated with LXR agonists. Moreover, ABCG1 is upregulated upon monocyte differentiation to mature macrophage and is highly expressed in atherosclerotic "foam cells".

A definitive role in lipid metabolism for ABCG1 was established with the creation of $ABCG1^{-/-}$ knockout mouse models which have provided valuable information on the physiological function of ABCG1 (Kennedy et al 2005). $ABCG1^{-/-}$ mice display massive

lipid accumulation in alveolar macrophages and develop progressive lipid accumulation in various tissues, particularly the lung, as the mice age (Baldan et al 2006c). These findings point to a role for ABCG1 in cholesterol and lipid transport which has been confirmed in multiple reports (Gelissen et al 2006; Ito 2003; Karten et al 2006; Kennedy et al 2005; Kim et al 2007b; Klucken et al 2000; Nakamura et al 2004). It should be noted that, as described in this thesis and elsewhere, the role of ABCG1 in lipid efflux has been highly characterized in the macrophage and neuronal cells and may not function similarly in other cell types such as smooth muscle or epithelial cells (Delvecchio et al 2008; O'Connell et al 2004).

1.4.6) ABCG1 efflux mechanisms

ABCG1 is believed to homodimerize to create a fully functional transporter and reports indicate that ABCG1 enhances cholesterol efflux to HDL particles but not to lipid poor apolipoproteins (Kennedy et al 2001). However, unlike ABCA1 with apoAI, ABCG1 does not bind directly to HDL particles (Wang et al 2008b). Additionally, the cellular localization of ABCG1 remains controversial. While some reports indicate an intracellular localization of ABCG1 in the endoplasmic reticulum and Golgi network, others have reported exclusive plasma membrane localization (Baldan et al 2006b; Xie et al 2006). Moreover, Wang et al. found that LXR agonists cause redistribution of ABCG1 from intracellular domains to the plasma membrane where it functions as a cholesterol transporter to HDL, a finding refuted by others (Wang et al 2006a; Xie et al 2006). The exact mechanisms by which ABCG1 exports cholesterol to HDL is unclear but may

involve redistribution of membrane domains and cholesterol to sites accessible to HDL. In support of this, ABCG1 overexpression increases access of cholesterol oxidase to cholesterol (Vaughan & Oram 2005). Therefore, similarly to ABCA1, ABCG1 may not directly efflux its substrate like other characterized ABC transporters but rather, alters the microenvironment of the plasma membrane allowing access of acceptor particles to remove cholesterol and other lipid.

1.4.7) Transcriptional and Translational Regulation of ABCG1

Two LXREs, LXRE-A and LXRE-B, have been identified in the human ABCG1 gene and are located in the first and second intron, respectively. These response elements are highly conserved across multiple species and play an established regulatory role in the macrophage and liver (Sabol et al 2005). Multiple alternative transcripts have also been identified in the murine macrophage which encode proteins that differ at the amino terminus (Nakamura et al 2004). Analysis of the protein isoforms revealed no differences in their ability to efflux cholesterol to HDL particles and therefore, the physiological significance remains to be determined (Nakamura et al 2004). Moreover, very little is known regarding ABCG1 post-translational modifications. Nagelin et al. report that 12/15 lipoxygenase increases serine phosphorylation of ABCG1 resulting in increased degradation although the exact mechanisms remain to be elucidated (Nagelin et al 2008).

1.4.8) ABCA1$^{-/-}$ ABCG1$^{-/-}$ double knockout mouse models

Recently, the creation of ABCA1$^{-/-}$ and ABCG1$^{-/-}$ double knockout mouse models has provided interesting observations in the macrophage (Out et al 2008; Terasaka et al 2008). The ABCA1$^{-/-}$ ABCG1$^{-/-}$ double knockouts display severe plasma

hypocholesterolemia yet accumulate large amounts of lipid in tissue macrophage (Out et al 2008). Moreover, there is complete abrogation of cholesterol efflux from peritoneal macrophages. The authors concluded that despite low cholesterol levels in the plasma, macrophages can still develop into foam cells characteristic of atherosclerotic plaques (Out et al 2008). It should be noted however, that no cholesterol accumulation was observed in the arterial intima in plaque-prone sites likely due to the low plasma cholesterol levels. Rather, the lipid engorged macrophages were observed in tissues where they are naturally resident such as the lung, spleen and liver.

1.4.9) Other LXR target genes involved in lipid metabolism

In addition to ABCA1 and ABCG1, LXR also regulates the expression of multiple other genes variously involved in lipid homeostasis, lipoprotein remodelling and fatty acid synthesis (Table 1). In addition to ABCA1 and ABCG1, two other members of the ABC superfamily, namely ABCG5 and ABCG8, are direct LXR target genes (Repa et al 2002). ABCG5 and ABCG8 function as heterodimers in hepatocytes and enterocytes to promote cholesterol and bile efflux into the bile duct and intestinal lumen. Other direct LXR targets include lipoprotein remodelling enzymes such as lipoprotein lipase (LPL), cholesterol ester transfer protein (CETP) and phospholipid transfer protein (PLTP). CETP and PLTP function to transfer cholesterol esters and phospholipids, respectively, between lipoprotein particles. CETP has received greater attention as this protein transfers cholesterol from HDL to LDL particles thereby increasing so called 'bad cholesterol'. Torcetrapib, a CETP inhibitor, however, actually increased mortality due to cardiovascular events in human trials despite elevating HDL levels (Barter et al 2007;

Tall 2007). This may be due to off target effects of the drug or inhibition of CETP itself and may also indicate that LDL represents an underestimated route of cholesterol elimination from the human body via hepatic LDL receptors. Additionally, LXRs also regulate the expression of apolipoproteins such as ApoE, ApoC-I, ApoC-II, ApoC-IV and ApoD (Hummasti et al 2004; Mak et al 2002). All of these apolipoproteins can act as cholesterol acceptors for ABCA1-dependent cholesterol efflux with apoE and apoAI receiving the greatest attention.

Lastly, LXRs regulate lipogenic programs in the liver as administration of LXR agonists to rodents markedly elevates plasma triglyceride levels. LXR response elements have been identified in multiple genes such as SREBP1c, acetyl-CoA carboxylase (ACC), stearoyl-CoA desaturase 1 (SCD-1) and fatty acid synthase (FAS) (DeBose-Boyd et al 2001; Joseph et al 2002; Talukdar & Hillgartner 2006; Wang et al 2004b). SREBP1c is particularly important as this 'master' transcription factor regulates the expression of multiple enzymes involved in lipogenesis and as such, the current therapeutic potential of the present class of LXR agonists is hampered by undesirable triglyceride elevation. Tissue selective LXR agonists, like those developed for the estrogen receptors (SERMS), that may function only in the macrophage to regulate cholesterol efflux are under intense investigation.

1.4.10) Repression of Inflammatory Genes by LXR

In addition to its role in cholesterol metabolism/transport, LXR also inhibits the inflammatory response in the macrophage and other cell types (Joseph et al 2003). LXR

reduces the expression of pro-inflammatory genes, many of which have been shown to be the direct targets of the NF-κB transcription factors. These include the down-regulation of MCP-1, iNOS and Cox-2, among others, following LPS challenge in the macrophage (Joseph et al 2003). The mechanism by which LXR blocks expression is not completely understood. NF-κB transcription factors retain their ability to enter the nucleus and bind to DNA and it has therefore been proposed that LXR may modulate the levels of co-activators or the release of co-repressors from NF-κB (Joseph et al 2003). A recently described mechanism that involves ligand-dependent SUMOylation of LXR and recruitment to pro-inflammatory target genes has been proposed (Ghisletti et al 2007). This post-translational event blocks the release of repressor co-factor NCoR from NF-kB transcription factors on the target promoters of pro-inflammatory genes. However, not all NF-kB target genes are blocked by LXR and there appears to be promoter and signal-specific regulation. Furthermore, it has been shown that LXR functions in an overlapping yet distinct manner to the glucocorticoid receptor (GR) in anti-inflammatory programs (Ogawa et al 2005). While GR has been shown to interact directly with the p65 NF-κB transcription factor preventing translocation to the nucleus, no direct binding of LXR to NF-κB has been reported (Joseph et al 2003). Therefore, it is possible that combination therapies of glucocorticoids and LXR agonists to treat inflammation may be synergistic thereby requiring lower levels of administered steroids.

1.4.11) Non-lipid related roles of LXR

Recent work has shown that LXR plays a role in multiple physiological processes in addition to lipid metabolism. These include extracellular matrix (ECM) remodeling, anti-proliferative effects in multiple cell types, migration of dendritic and neutrophil cells, hypertension and vasculogenesis (Blaschke et al 2004; Castrillo et al 2003; Geyeregger et al 2007b; Leik et al 2007b; Walczak et al 2004).

LXR activation decreases expression of the zinc endopeptidase matrix metalloproteinase-9 (MMP-9) in the macrophage (Castrillo et al 2003). MMP-9 plays a crucial role in the degradation of ECM during pathological tissue remodeling in atherosclerosis as well as asthma (Han et al 2003). The degradation of ECM promotes the migration of vascular smooth muscle cells and can also weaken the fibrous cap of an atherosclerotic lesion rendering it more prone to rupture. The effect of LXR agonists on MMP expression is limited to MMP-9 as other MMPs were not repressed upon challenge with TNFα, an inducer of MMP expression.

LXR also abrogates dendritic cell migration by decreasing Fascin-1 protein levels (Geyeregger et al 2007a). Fascin-1 functions as an actin bundling protein that serves to stabilize actin filaments in the protruding lamellapodia of migrating cells. It is currently unclear how LXR reduces Fascin-1 protein levels as transcript levels are unaffected. Further evidence to support a role for LXR in migratory regulation comes from studies of neutrophil migration into the lung (Smoak et al 2008). LXR agonists dose-dependently decreased neutrophil migration in *in vitro* migration assays and $LXR^{-/-}$ mice displayed

reduced neutrophilia in the lung following allergic challenge, an effect attributed to decreased migration into the site of inflammation (Smoak et al 2008).

Lastly, LXR also inhibits the proliferation of vascular smooth muscle and other cell types (Blaschke et al 2004). LXR agonists inhibited G_1 exit, dose-dependently blocked retinoblastoma (Rb) protein phosphorylation and prevented degradation of $p27^{kip}$, a cell cycle inhibitory protein. Moreover, LXR agonists also inhibit proliferation of cancer cell lines of the prostate, breast, lung, cervix and liver in a similar manner (Chuu et al 2006; Chuu et al 2007b; Fukuchi et al 2004; Vedin et al 2009). Interestingly, a recent report indicated that T-cells decrease the expression of LXR and LXR target genes such as ABCA1 and activate cholesterol synthesis when stimulated to proliferate (Bensinger et al 2008). Furthermore, T-cells derived from $LXR^{-/-}$ mice are hyperproliferative indicating that LXR signaling pathways are crucial in maintaining T-cell homeostasis (Bensinger et al 2008).

1.4.12) Other Lipid Sensing Nuclear Hormone Receptors

In addition to LXR, other members of the NHR superfamily have evolved to sense and respond to elevated levels of intracellular lipids and are variously involved in metabolic homeostasis. These include the fatty acid receptors peroxisome proliferator-activated receptors (PPAR; subtypes α, β/δ, γ) and the bile acid receptor farsenoid X receptor (FXR). These receptors, along with LXR, allow for rapid adaptation to nutritional changes by activating the expression of appropriate transcriptional networks leading to energy production, storage or elimination.

Of particular interest are the PPARs due to their functional and mechanistic similarities to LXRs. PPARs are endogenous sensors of fatty acids, eicosinoids as well as other lipids, and play fundamental roles in lipid homeostasis and insulin sensitivity. PPARα is expressed highly in the liver where it regulates fatty acid oxidation during fasting states by upregulating transcription of genes involved in the peroxisomal β-oxidation pathways. These include ACC, CPT1 and ACO, among others (Berger et al 2005). In support of this, PPARα-deficient mice show an impaired response to starvation and severe hepatic steatosis. Moreover, PPARα plays a crucial role in the macrophage where it displays anti-atherosclerotic and anti-inflammatory effects; patients taking fibrate drugs that target PPARα show decreased CV mortality and morbidity (Sonoda et al 2008).

PPARγ, a master regulator of adipogenesis, is the target of thiazolidinediones (TZDs), the most prescribed treatment to combat insulin resistance. Not only is PPARγ required for adipocyte differentiation, it also has a fundamental anti-atherosclerotic function in the macrophage where it decreases inflammation and works in conjunction with LXR to regulate lipid efflux to apoAI and HDL. For example, PPARγ agonists up-regulate ABCA1 partially by directly activating PPARγ on the ABCA1 promoter but also by increasing the expression of LXRα itself. Addtionally, PPARγ regulates macrophage CD36 scavenger receptor expression in the arterial intima which internalizes oxidized lipoproteins that in turn, activate LXR signaling and cholesterol transporters (Chawla et al 2001). This feed-forward loop results in net efflux of cholesterol from peripheral cells or the sub-epithelial region of the vasculature for transporter back to the liver.

Lastly, PPARβ/δ is less understood than other PPAR members. It is believed that PPARβ/δ is also involved in insulin sensitivity, inflammatory gene expression and lipoprotein metabolism playing a prominent role in fatty acid oxidation in muscle fibers (Evans et al 2004). Overall, PPARs regulates pathways interlinked with LXR to modulate and coordinate lipid homeostasis. In addition to the above roles, PPARs also function prominently in the lung (see Function of PPARs in Asthma).

Expanding on the PPAR-LXR crosstalk, important and seminal findings from the Capone lab indicated that PPARα and LXRα directly interact (Miyata et al 1996). Interestingly, this interaction antagonized PPARα signaling raising the intriguing possibility that PPAR/LXR crosstalk *in vivo* synchronizes the transcriptional output in response to varying levels of intracellular lipids and the fed state of the organism. Indeed, fatty acid oxidation would be undesired when cholesterol levels are elevated since cholesterol esterification to fatty acids is believed to prevent cellular toxicity. Thus, in different fed states, this interaction may provide certain regulation on each receptor's function. Further *in vivo* tests to elucidate the role of PPAR/LXR interactions are warranted in light of more recent findings.

Finally, the farsenoid X receptor (FXR) serves as a bile acid sensor. Since LXR regulates CYP7A, the rate limiting enzyme in bile acid synthesis, it is expected that FXR would act downstream of these pathways. Indeed, FXR regulates multiple genes that transport bile into the bile duct. Moreover, FXR induces the expression of hepatic Small

Heterodimer Partner (SHP) which inhibits LXRs and reduces CYP7A expression thus acting as a negative feedback loop. Interestingly, in enterocytes specifically in the ileum, when bile acids levels are elevated, FXR induces the expression of fibroblast growth factor 15 (FGF15) which then circulates to the liver and activates signaling pathways that further reduce bile acid synthesis in conjunction with SHP. Recent findings also indicate that PPARα regulates hepatic FGF21 that stimulates lipolysis in white adipose tissue during starvation (Inagaki et al 2007). These findings point to a crucial role for hormonal FGFs in regulating metabolism. Future work to identify other circulating signaling molecules regulated by NHRs, specifically LXR, may provide additional information on how these receptors display such pleiotropic effects *in vivo*.

1.4.13) The PPAR-LXR-FXR Trio In Lipid Metabolism

The above indicates that each arm of PPAR-LXR-FXR triangle plays a role in lipid metabolism during the starvation, feeding and fed states, respectively. Briefly, in the starved state, when glycogen stores have been exhausted and other fuel sources are required, PPARα mediates the adaptive response by inducing lipolysis in adipocytes and fatty acid oxidation in hepatocytes. As described above, lipolysis is triggered by PPARα signaling originating from the liver where PPARα activates the fasting-induced protein FGF21. This hormone, in turn, signals to adipocytes to increase expression of lipases and release fatty acids into the circulation, although the exact pathways have not been described. The high levels of fatty acids are mobilized to hepatocytes where they are then oxidized and synthesized to ketone bodies used as an energy source by peripheral tissues.

In the post-prandial state, for example following a high fat diet containing sterols, LXR controls the expression of cholesterol transport genes and regulates the balance between storing fat in adipose tissue and its oxidation in peripheral tissue. This is exemplified by the fact that $LXR^{-/-}$ mice fed a high-fat diet containing cholesterol are resistant to diet-induced obesity as a result of deregulated expression of upcoupling proteins (UCPs) in muscle and adipose and abnormal energy dissipation (Kalaany et al 2005). Thus, as the authors indicate, LXR activation eliminates excess cholesterol while promoting the storage of an essential energy source thereby eliminating the potential toxic hazard of high cholesterol levels while preserving the conservation of a rich energy-source (Kalaany et al 2005).

Following the digestion, metabolism and conversion of cholesterol to bile acids for elimination from the body, negative feedback loops controlled by FXR return the body to a basal metabolic state. FXR senses and responds to excess bile acids both in hepatocytes and enterocytes of the ileum stimulating expression of SHP and FGF15 (FGF19 in humans) in respective tissue. SHP and FGF15 coordinate to inhibit LXR and Liver Receptor Homologue-1 (LRH-1)- mediated activation of CYP7A as well as bile importers from portal circulation. Furthermore, FXR stimulates the expression of multidrug resistance-associated protein MRP-2, ileal bile acid transporter (IBAT) and apical sodium-dependent bile salt transporter (ASBT), among others, involved in excretion of bile acids into the bile duct and intestinal lumen. For the reason above, it has thus been proposed that LXR and FXR represent the "yin and yang of cholesterol and fat metabolism" (Kalaany & Mangelsdorf 2006).

While the role of PPAR-LXR-FXR in lipid metabolism is highly studied by several groups, their role in lung pathophysiological conditions such as asthma, especially that of LXR, remains under-investigated. Recently, however, the role of PPARs in lung function (discussed further below) have attracted intense interest due to the fundamental importance of these receptors in cell growth and their potential as therapeutic targets in inflammatory airway disease (Becker et al 2006; Belvisi et al 2006; Grenningloh et al 2006; Honda et al 2004; Sin & Man 2006; Stapleton et al 2005). Thus, due to the functional interplay of LXR and PPAR as described above, this work extends the role of LXRs beyond the macrophage, liver and intestine and studies the function of these receptors in airway smooth muscle, a crucial effector cell of airway disease.

1.5) Asthma and the Role of Airway Smooth Muscle in Disease Progression

1.5.1) Background

Asthma is a chronic disease of the lung characterized by bronchial hyper-responsiveness, airway obstruction, and chronic inflammation (Busse & Lemanske 2001). Airway hyperresponsiveness (AHR) occurs in sensitized individuals and is typically in response to normal non-hazardous substances. These environmental factors trigger an IgE immune response resulting in infiltration of leukocytes into the airway and increased lung remodeling which, over time, can lead to non-reversible airflow obstruction (Lemanske & Busse 2006). The genetic association for AHR susceptibility in certain individuals is unknown and no one single gene has been identified that displays any dominant features that link its function to airway disease (Willis-Owen et al 2009). This

is similar to the genetic complexity of cardiovascular disease and type II diabetes. The increased prevalence of asthma over the past 20 years has prompted researchers to suggest that new environmental factors influence the progression of the disease state. This has led to the so called 'hygiene hypothesis' which has garnered much attention in recent studies. This hypothesis states that the decreased exposure to environmental immunological stimuli of newborn infants, for example, due to increased antibiotic use and decreased exposure to other children, skews the immune system towards Th_2-type T-cells. (Tse & Horner 2008) This imbalance between Th_1 and Th_2 T-cell populations may actually begin *in utero* and is associated with allergic reactivity to non-hazardous substances.

Disease initiation is believed to occur when dendritic cells that line the airway encounter an inhaled allergen (Henderson et al 2009). For reasons that are still unclear, in susceptible individuals, the dentritic cells phagocytose the allergen and migrate to the lymph nodes and present the antigen to T and B cells (Lemanske & Busse 2003). With the appropriate co-stimuli, B-cells are triggered to synthesize and release IgE antibodies into the circulation. These antibodies are recognized by receptors expressed on the surface of mast cells. These mast cells, resident in submucosal areas in the lung, are now sensitized for allergen recognition. Upon a second encounter with an allergen, IgE molecules bind to their allergen epitope and activate downstream intracellular signaling in mast cells. These activated cells degranulate and secrete histamine and other contractile agents that cause constriction of the airway smooth muscle and reduced airflow (Busse & Rosenwasser 2003; Lemanske & Busse 2003). This acute response is followed by a

prolonged late-phase inflammatory response. In addition to contractile agents, mast cells secrete various cytokines and chemokines, such as GM-CSF and TNFα, that results in the infiltration of eosinophils, monocytes, and T-cells into the airway and sub-epithelial space of the bronchi.

Further disease progression is associated with airway smooth muscle (ASM) cell hyperplasia and hypertrophy, and secretion of inflammatory cytokines, growth factors, and various co-stimulatory molecules which promote activation, recruitment, and survival of inflammatory cells that in turn lead to airway narrowing, microvascular damage, and tissue re-modeling (Busse & Lemanske 2001). While a plethora of cell types and factors are involved in the sequence of events associated with the development and progression of asthma, human airway smooth muscle (hASM) cells are emerging as the pivotal effector cells of this disease (Hershenson et al 2007).

1.5.2) Airway Smooth Muscle

ASM represents the effector cells in the asthmatic airway playing a multifaceted role in disease progression (Fig. 9). In the non-disease state, ASM interestingly plays an unknown role and has even been proposed to be a vestigial tissue (Cox et al 2004). In the disease state, ASM cells contribute to exaggerated broncho-constriction (hyper-responsiveness) during an asthmatic attack and an increase in smooth muscle mass due to hyperplasia and hypertrophy in the asthmatic airway leads to airway constriction and eventual irreversible tissue remodeling . ASM cells also mediate immune modulation in the airway and are increasingly seen to be central in orchestrating and perpetuating

airway inflammation by promoting the recruitment, activation, and migration of inflammatory cells.

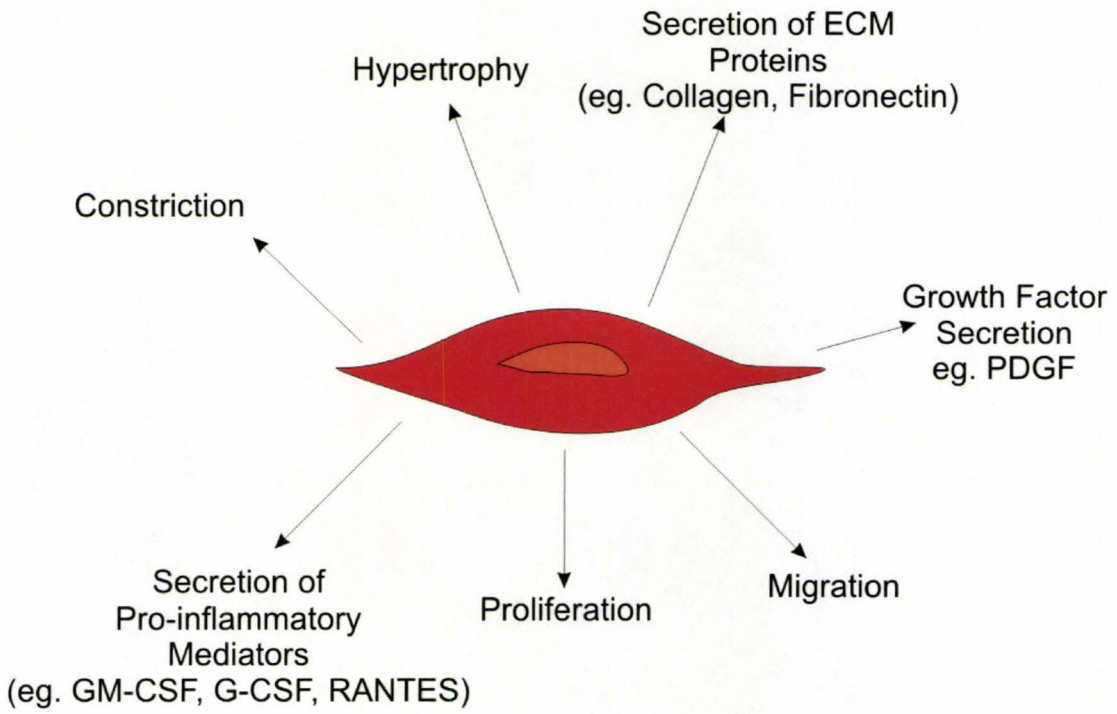


Figure 9. Roles of airway smooth muscle in asthma progression. Beyond the classical role of constriction of the bronchioles, ASM plays a significant function in multiple processes known to be hallmarks of airway disease.

1.5.2.1) Migration of ASM

Increased smooth muscle mass is partially the result of migrated myocytes into the submucosa of asthmatic airways (Hasaneen et al 2005; Krymskaya et al 2005). Multiple inflammatory mediators such as cytokines (eg. Il-1β), intracellular eicosanoids (eg. leukotriene E4) and small proteins (eg. PDGF) have been demonstrated to promote chemotactic migration of ASM (Parameswaran et al 2002). Furthermore, extracellular

matrix proteins such as collagen-I also induce migration of hASM (Parameswaran et al 2004). These effects are dependent on intracellular signaling via focal adhesion kinase (FAK), Src, Akt, PI3K, Rho, PAK and MAPK pathways among others (Goncharova et al 2002; Krymskaya et al 2005; Parameswaran et al 2004). These pathways converge and cross-talk to regulate effector proteins such as VASP, mDia, cofilin and myosin motor proteins that modulate actin nucleation, stability, degradation and contraction to coordinate movement at the leading edge of the smooth muscle cell (Gerthoffer 2008).

1.5.2.2) Proliferation of ASM

In addition to migration, proliferation represents the major mechanism that results in increased smooth muscle mass in the asthmatic airway (Hirst et al 2004). ASM derived from asthmatic patients proliferates faster than non-asthmatic ASM (Johnson et al 2001). Multiple stimuli that induce migration also induce smooth muscle proliferation including PDGF, histamine and cytokines (Bentley & Hershenson 2008). Furthermore, multiple signaling pathways regulating migration overlap to regulate proliferation as well. Src kinase activation has been shown to be necessary and sufficient for hASM proliferation and other signaling pathways also play a strong role including ERK, p38 MAPK and PI3K (Hershenson et al 2007; Krymskaya et al 2005). While corticosteroids can inhibit smooth muscle proliferation from normal subjects, smooth muscle derived from asthmatic patients are occasionally insensitive to corticosteroids due to dysfunctional C/EBPα (Bentley & Hershenson 2008). Therefore, additional therapies that target these pathways are needed.

1.5.2.3) ASM, Extracellular Matrix (ECM) Remodeling, and Airway Disease

Airway smooth muscle is a source of extracellular matrix proteins induced by TGFβ signaling (Zhang & Gunst 2008). The observation that greater deposition of collagen I, III and V, fibronectin and laminin in the airways of asthmatic patients has led to increased investigation of ECM effects on smooth muscle (Gueders et al 2006). These extracellular matrix proteins found in the airways are able to promote survival, proliferation and migration of airway smooth muscle (Parameswaran et al 2006). Current treatments for asthma however, do not prevent tissue remodeling and eventually, airway hyperresponsiveness becomes more severe as the remodeling progresses (Parameswaran et al 2006).

1.5.2.4) Immunomodulatory effects of ASM

ASM is now recognized as an important immunomodulator in the airways of asthmatic lungs and a source of pro-asthmatic factors (Amrani & Panettieri 2003; Chung 2000; Crimi et al 2001; Tliba & Amrani 2008). hASM secretes multiple cytokines with established roles in recruiting and promoting survival of infiltrating leukocytes. Furthermore, these secreted cytokines also display autocrine and paracrine effects on neighbouring smooth muscle cells which alter proliferation and migration as described above (Hershenson et al 2007). Crucial inflammatory mediators known to be expressed in hASM include Il-13, Il-8 (recruitment of neutrophils), eotaxin (recruitment of eosinophils), RANTES (recruitment of T-cells), Il-1β, MCP-1α (recruitment of monocytes), G-CSF, GM-CSF (recruitment of leukocytes), Il-5 (increases contractile

response in hASM), among many others (Hershenson et al 2007). Although the precise relevance of hASM derived immunomodulators in the lung is not clear, *in situ* hybridization has confirmed the expression of multiple genes in ASM from asthmatic tissue sections. Current corticosteroid treatments are initially effective at reducing inflammation however, many patients become resistant after long-term administration (Panettieri 2004).

1.5.3) The Role of Nuclear Hormones Receptors in Asthma

The role of many nuclear hormone receptors in the lung has been established and, recently, has received increased attention due to their therapeutic potential. Corticosteroid and other synthetic glucocorticoid receptor agonists have long been used in decreasing the inflammatory reactions that occur after an asthmatic attack. In addition, other classical steroid receptors also play an important role in the lung. Vitamin D receptor knockout mouse models interestingly do not develop allergic inflammation despite the fact that VDR is proposed to be anti-inflammatory. One finding, however, indicates that vitamin D administration in patients with airway disease may re-establish responsiveness to glucocorticoids (Farrow 2008). Of growing interest however are the non-steroidal NHRs (type II) and their potentially beneficial effects in treating lung diseases.

1.5.4) Function Of PPARs in Asthma

Of the non-steroidal hormone receptors, or Type II receptors, the peroxisome proliferator-activated receptors (PPARs; subtypes α, β/δ, γ) and their role in lung physiology and pathophysiology have been extensively studied and are emerging as *bona fide* targets in the treatment of COPD, asthma as well as lung cancer (Belvisi & Hele 2008; Belvisi et al 2006; Denning & Stoll 2006; Han & Roman 2006; Huang et al 2005). PPARs are activated by endogenous fatty acids and are the target of anti-diabetic and lipid-lowering drugs such as thiazolidines and fibrates.

Due primarily to its anti-inflammatory actions, PPARα receptor activation can ameliorate several features of AHR such as T-cell proliferation, leukocyte infiltration into the bronchoalveolar lavage (BAL) and pro-inflammatory cytokine levels (Honda et al 2004; Woerly et al 2003). Moreover, PPARα$^{-/-}$ mouse models display increased inflammation and AHR in ovalbumin challenged mice (Delayre-Orthez et al 2005). The role of PPARγ has also been extensively studied in a variety of cell types associated with disease progression in the lung. PPARγ agonists display anti-inflammatory, anti-proliferative and anti-migratory effects in multiple lung cells types and in murine models of asthma. For example, the PPARγ agonist ciglitazone reduced eosinophil degranulation and migration. Furthermore, potent *in vivo* anti-inflammatory effects were observed following treatment with rosiglitazone. A murine model of asthma administered rosiglitazone showed reduced G-CSF, GM-CSF and neutrophilia in BAL fluid following an inflammatory challenge (Standiford et al 2005). The above have placed PPARs in the

spotlight for future clinical treatments of lung disease. Since the bioavailability, safety, and mode of action of several PPAR agonists are known and in use clinically to treat other illnesses, large scale studies analyzing the potentially beneficial effects in humans with lung conditions will provided further information into their efficacy in lung disease (Farrow 2008). Indeed, clinical trials analyzing the effects of PPAR agonists in smokers with COPD or asthma are completed or underway (www.clinicaltrials.gov).

1.5.4) Role of LXR and LXR target genes in Various Lung Cell Types

We, and others, have demonstrated that LXR is expressed and functional in multiple lung cell types including airway smooth muscle, alveolar macrophages and type II pneumocytes (Bortnick et al 2003; Delvecchio et al 2007). Multiple LXR target genes including ABCA1 and ABCG1, and therefore LXR itself, play fundamental roles in lung development and lung lipid homeostasis (Baldan et al 2006c). ABCA1 knockout mice display shallow breathing, reduced lung development and severe lipid accumulation in alveolar macrophages and lung parenchyma (Bates et al 2005; van der Deen et al 2005). Furthermore, ABCA1 activation in type II pneumocytes results in specific basolateral directional movement of cholesterol by specifically localizing to the basal membrane (Zhou et al 2004). ABCG1$^{-/-}$ mouse models also display a severe lung phenotype. ABCG1-null mice have massive lipid accumulation in type II pneumocytes and alveolar macrophages (Kennedy et al 2005). ABCG1 mRNA and protein levels are also reduced in the alveolar macrophages of patients with alveolar proteinosis, a surfactant metabolizing disease (Thomassen et al 2007). Accordingly, ABCG1 has been proposed

to play a fundamental role in the recycling and transport of surfactant, a substance composed of 90% lipid (Baldan et al 2006b). As described in this thesis, we have recently demonstrated that ABCA1 and ABCG1 also play a role in hASM (Chapter 4) (Delvecchio et al 2007). Treatment of hASM with specific LXR agonists results in a large induction of ABCA1 and ABCG1 expression resulting in increased cholesterol efflux to both ApoAI and HDL cholesterol acceptors (Delvecchio et al 2007). These works described above establish LXR and LXR target genes in the function of normal and disease states of the lung (see Chapter 5 for an expansion on the role of LXR in the lung).

1.6) Overview of Findings and Organization of Thesis

In this thesis, the role of LXR, and the regulation of LXR itself, is extensively studied and furthers our knowledge of the role of nuclear hormone receptors in lung physiology. It is shown here that LXR is repressed by PKCα activation as assessed by reporter gene analysis in monkey kidney cells (Cos-1) (Chapter 2). These findings are recapitulated in human embryonic kidney cells (HEK293) on endogenous genes (ABCA1 and SREBP1c) using PKC activators/inhibitors and real-time PCR analysis. Due to the emerging role of LXR's sister receptors PPARs in lung function, as described above, and due to our unique opportunity to receive primary human tissue, the focus of this thesis shifted to the role of LXR in human airway smooth muscle cells (Chapter 3). The expression and functional translation of LXR is demonstrated along with the expression of multiple LXR target genes including ABCA1 and ABCG1. Moreover, the repression of pro-inflammatory cytokines upon challenge with an inflammatory cocktail by LXR is

also shown. The therapeutic potential of LXR agonists is further demonstrated by the fact that LXR agonists decrease the proliferative and migratory capacity of ASM cells. Lastly, the role of ABCA1 and ABCG1 in ASM is further analyzed (Chapter 4). Despite intense research into the role of ABC transporters in the macrophage, little is known about their role in airway or vascular smooth muscle. Using a siRNA knockdown approach, we show that LXR-induced cholesterol efflux is mediated exclusively by ABCA1 while ABCG1 does not appear to play a role. Finally, hypotheses of the findings are discussed in Chapter 5. Overall, the findings presented here further our knowledge of the regulation of LXR itself as well as LXR-regulated processes in airway smooth muscle and pave the way for future work using *in vivo* models of asthma to determine the physiological consequences of these findings. Please note that due to the previous publication of this material, there may be some degree of repetition between chapters. Related to this, introductions in the following sections have been edited from their originally published formats to reduce redundancy.

CHAPTER TWO PREFACE

Protein Kinase C α Modulates Transactivation of the Liver X Receptor α

This work has been previously published in:

Delvecchio, C.J. and Capone, J.P. (2008) Protein Kinase Cα Modulates Transactivation of the Liver X Receptor α. *Journal of Endocrinology*. 197(1):121-130.

I conducted all experiments described in this chapter.

2.1) Introduction

The function of nuclear hormone receptors can be regulated at multiple levels, including transcriptional regulation and post-transcriptional modification such as phosphorylation. One such signalling pathway is the Protein Kinase C (PKC) pathway activated by membrane lipids. As described in preceding chapters, PKC signalling has been previously implicated in NHR regulation where PKC inhibits the activity of RAR and VDR but also activates transcriptional activity of other receptors such as GR and FXR. Despite increasing knowledge of the physiological function and mechanisms of action of LXR, little is known about the mechanisms by which LXR itself is regulated. This chapter focuses on the crosstalk between LXR and PKC pathways and aims to descrive the novel observation that PKC activators can modulate the transactivation potential of LXR. Research describing post-translational regulation of LXR is emerging rapidly and the findings described in this work contribute significantly to this field.

In order to explore the potential role of PKC signaling on LXR function, the activity of LXR in was assessed in the presence of modulators of PKC signaling pathways. It is shown here that activation of PKC signaling with phorbol 12-myristate 13-acetate (PMA) repressed LXR-dependent transactivation of LXRE-reporter plasmids as determined by transient transfection assays as well as qPCR of endogenous LXR target genes. The effect of PMA was both dose- and time-dependent and could be mimicked by constitutively active PKCα. The inhibitory effects were abrogated upon co-incubation with the PKC inhibitor bisindolylmaleimide. Finally, PKCα was shown to phosphorylate

LXRα *in vitro*. These findings reveal that PKC activation can regulate LXR-mediated gene expression and may have implications in diseases where PKC signaling is altered (Rask-Madsen & King 2005).

2.2) Materials and Methods

Reagents and plasmids

22R-hydroxycholesterol (22R-HC), 9-*cis*-retinoic acid (9-cisRA), PMA, and GW3965 were purchased from Sigma. Bisindolylmaleimide was purchased from Calbiochem (San Diego, CA, USA). Rabbit antibody to human RXRα was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and mouse antibody to human LXRα was purchased from R&D Systems (Minneapolis, MN, USA). The rabbit LXRα/β antibody (sc-1000) used for chromatin immunoprecipitation (ChIP) was purchased from Santa Cruz Biotechnology. Mammalian expression vectors expressing human LXRα, LXRβ, PPARα, and RXRα (pRC-CMV-LXRα, pRC-CMV-LXRβ, pSG5-PPARα, and pSG5-RXRα, respectively) and luciferase reporter plasmids harboring response elements for LXR and PPAR have been described previously (Landis et al 2002; Meertens et al 1998; Miyata et al 1998; Willy et al 1995). PKC7, expressing constitutively active PKCα, was a generous gift from Dr J L Staudinger (University of Kansas, Lawrence, KS, USA). Human ABCA1 promoter (–928/+107) luciferase reporter plasmid was a generous gift from Dr A Tall (Columbia University, New York, NY, USA).

Cell lines

Cos-1, HEK293, and HepG2 cells were obtained from American Type Tissue Collection (ATCC, Manassas, VA, USA). Cos-1 and HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% v/v fetal bovine serum, 1% v/v penicillin/streptomycin, and 1% v/v L-glutamine. HepG2 cells were cultured in MEM-F15 supplemented with 10% v/v fetal bovine serum, 1% v/v sodium pyruvate, 1% v/v L-glutamine, and 1% penicillin/streptomycin.

Transfection and reporter gene assays

Transient transfections of cells were carried out using FuGene 6 reagent (Roche) according to manufacturer's instructions. Briefly, cells ($3.5x10^5$ cells/well in six-well plates) were transfected using 3 µl Fugene to 0.5 µg reporter plasmid, 0.25 µg pRC-CMV-LXRα, 0.25 µg pSG5-RXR, and 0.1 µg pCMV-lacZ (encoding β-galactosidase) for normalization of transfection efficiency. The total amount of DNA was kept constant using pRC-CMV and pSG5 empty vectors. Following transfection, the media was aspirated and replaced with complete DMEM supplemented with 10% charcoal-stripped FBS and appropriate ligand as indicated in the figures. LXR agonist 22(R)-hydroxycholesterol was dissolved in 95% ethanol, and 9-cisRA and GW3965 were dissolved in Me_2SO (DMSO). Control cells received the equivalent amount of vehicle. Cells were harvested for 24 or 48 h post-transfection as indicated and lysed in reporter lysis buffer (Promega), and luciferase, β-galactosidase and Bradford assays were carried out as described previously (Landis et al 2002).

Electrophoretic mobility shift assays

The Cos-1 cells were transfected with 0.5 μg LXRα and/or RXRα expression plasmids and incubated overnight, as described previously. The cells were then treated with PMA for an additional 24 h and Cos-1 nuclear extracts were prepared, as described previously (Andrews & Faller 1991). Ten micrograms of nuclear extract were used in gel retardation assays, as previously described (Landis et al 2002). Briefly, double-stranded oligonucleotide probes (sequence 5'-GATCCCAGGTCACAGGAGTCAGAA-3'; 5'-GATCTTCTGACCTCCTGTGACCTGG-3') were annealed and radiolabeled with [^{32}P] ATP using Klenow enzyme. Binding reactions consisted of 5 μl Buffer C (20 mM HEPES (pH 7.9); 25% Glycerol; 0.42 M NaCl; 0.2 mM EDTA (pH 8.0); 1.5 mM $MgCl_2$; 0.5 mM dithiothreitol), 1 μl BSA (4 mg/ml), 1 μl PolydI/dC (8 mg/ml), 1 μl salmon sperm DNA (4 mg/ml), 4 μl radiolabeled oligo (20 pmol/reaction), 10 μg nuclear extract, and H_2O to 30 μl total volume. Reactions were incubated at 30 °C for 1 h and stopped by the addition of 2 μl loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, and 30% glycerol). Samples were separated on a 4% polyacrylamide gel (pre-run for 2 h) in 0.25x Tris-borate-EDTA (TBE) running buffer at 240 V at 4 °C. The gel was dried and probes were detected by autoradiography.

Chromatin immunoprecipitation assay

ChIP assays were performed in HEK293 cells using the ChIP-IT Express Enzymatic Kit according to manufacturer's instructions (Active Motif, Carlsbad, CA, USA). Briefly, HEK293 cells were grown to 85% confluency in 10 cm dishes and treated

with GW3965 (2 μM) and/or PMA (80 nM) for 2 h. Cells were fixed with 1% formaldehyde for 10 min at room temperature. Nuclei were isolated and chromatin was digested enzymatically for 10 min at 37 °C resulting in chromatin fragments of ≈ 200–1500 bps. LXRα/β was immunoprecipitated overnight at 4 °C with 3 μg antibody and ≈ 8 μg chromatin, and rabbit pre-immune immunoglobulin served as a negative control. Bound chromatin was washed and the recovered DNA was assayed for enrichment of the human ABCA1 promoter by PCR using the following primers: forward 5'-CCC AAC TCC CTA GAT GTG TC-3'; reverse 5'-CCA CTC ACT CTC GCT CGC A-3'. The PCR contained 5 μl eluted chromatin, 2.5 μl PCR buffer, 10 pmol/μl primers (forward and reverse), 1 U/reaction platinum *Taq* polymerase (Invitrogen) and H_2O to 25 μl. The PCR program consisted of 34 cycles of 94 °C for 20 s, 58 °C for 30 s, and 72 °C for 30 s. Reactions were separated on a 1.5% agarose gel and stained with SYBR-green. Gels were imaged on a Typhoon 9200 Variable Mode Imager (Molecular Dynamics, Amersham Biosciences, Baie D'urfée, QC, Canada).

In vitro kinase assay

In vitro kinase assays were performed by mixing 0. 4 μg purified LXRα (ProteinOne, Bethesda, MD, USA) in 20 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 0.2 mM $CaCl_2$, 5 μg/ml phosphatidylserine, 0.5 μg/ml diolein, 5 μM ATP, and 5 μCi/μl [^{32}P]ATP along with 50 ng purified PKCα (Calbiochem) in a final reaction volume of 20 μl. The reaction was incubated at 30 °C for 30 min and products were analyzed by sodium dodecyl sulfate PAGE and detected by autoradiography.

Western blot analysis

Western blot analysis was carried out with commercially available kits (Amersham) according to manufacturer's instructions. Briefly, 25 μg protein isolated from the Cos-1 cells were subjected to PAGE and transferred to a nitrocellulose membrane. Blots were incubated with mouse anti-LXRα (1:1000) or rabbit anti-RXRα (1:200) antibodies for 1 h, followed by 2° goat HRP-conjugated antibody (1:5000) for an additional hour and visualized by enhanced chemiluminescence. Anti-β-actin was used as a loading control.

Real-time PCR

Total RNA was isolated using RNeasy mini kits (Qiagen), and cDNA was prepared from 1 μg RNA using the quantitect reverse transcription kit (Qiagen) according to the manufacturer's instructions. The real-time PCR was performed using platinum SYBR green supermix-UDG with ROX PCR mix (Invitrogen) according to manufacturer's instructions and as described previously (Bookout & Mangelsdorf 2003). Briefly, 5 μl SYBR-green Supermix, 2.5 μl H_2O, 1.25 μl primer sets (1.25 μM each forward and reverse primer; specific for human ABCA1, ABCG1, and SREBP1c respectively), and 1.25 μl cDNA was mixed with a final reaction volume of 10 μl. The PCR amplification was carried out in 384-well plates in an Applied Biosystems 7900HT real-time PCR machine (Applied Biosystems, Foster City, CA, USA). Values were normalized to β-actin levels and relative abundance of transcripts was calculated using the $\Delta\Delta C_t$ method, as described previously (Bookout & Mangelsdorf 2003).

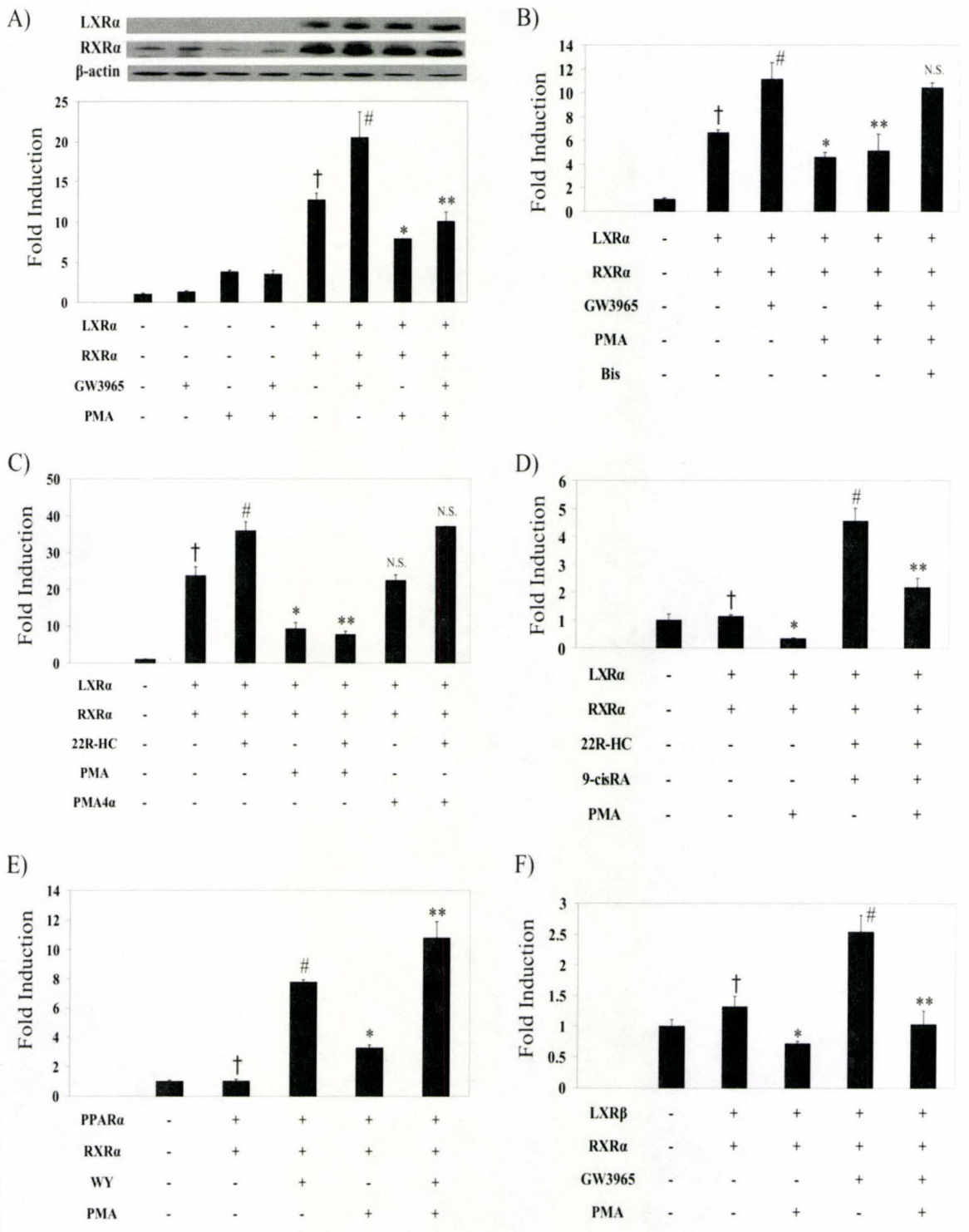
Statistical analysis

Unpaired *t*-tests were used for comparison of groups. *P* values <0.05 were considered significant. Samples were compared with the corresponding samples treated without PMA.

2.3) Results

PMA treatment downregulates LXRα transactivation

To determine whether PKC signaling alters LXRα transactivation, transient transfection assays were carried out with LXR-responsive luciferase reporter genes. As expected, cells co-transfected with expression plasmids for human LXRα and RXRα showed a 12- and 20-fold increase in luciferase activity over basal activity of the reporter gene alone when treated in the absence or presence of the LXR agonist GW3965 respectively (Fig. 10A). Addition of PMA, a PKC activator that mimics DAG in the cellular membrane, inhibited the ligand-independent effect by ≈30–50% and completely eliminated the ligand-dependent effect (Fig. 10A). The PMA-mediated decrease in transactivation was not due to increased degradation or decreased expression of LXRα or RXRα as determined by western blot analysis (Fig. 10A). The repressive effect of PMA was blocked by co-treatment with bisindolylmaleimide, a PKC inhibitor (Fig. 10B). Similar effects on LXR transactivation were observed, when 22R-HC was used in place of GW3965 (Fig. 10C). Furthermore, the inactive enantiomer of PMA, 4α-PMA, did not decrease LXR-mediated transactivation (Fig. 10C).



(figure legend p.73)

Figure 10. PMA modulates LXR transactivation. Cos-1 cells were transfected with luciferase reporter genes containing a consensus LXRE **(panels A–C)** or **(D)** an ABCA1 promoter, in the presence or absence of expression vectors for human LXRα and RXRα and treated with GW3965 (1 μM), 22R-hydroxycholesterol (22R-HC; 10 μM), 9-cis-retinoic acid (9-cisRA; 10 μM), Bisindolylmaleimide (Bis; 50 nM), PMA (1 nM) or 4α-PMA (PMA4α; 1 nM) for 48 h as indicated, and luciferase activity was measured. In panel **A**, total protein extracts (25 μg) from the corresponding transfections were analyzed by western blot with antibodies specific for human LXRα and RXRα. β-actin levels served as a loading control. **(E)** Cos-1 cells were transfected with a PPRE-luciferase construct in the presence or absence of expression vectors for human PPARα and RXRα in the presence of WY14,643 (WY; 5 μM) and PMA (100 nM) as indicated, and luciferase activity was measured. **(F)** Cos-1 cells were transfected with an ABCA1 promoter reporter vector in the presence or absence of expression vectors for human LXRβ and RXRα and treated with GW3965 (1 μM) and/or PMA (1 nM) for 48 h as indicated, and luciferase activity was measured. The values presented represent the average (± S.D.) relative to untreated cells (taken as 1) from three independent transfections carried out in triplicate and normalized for β-galactosidase and protein levels. *P<0.02 versus corresponding sample minus PMA indicated by †; **P<0.02 versus corresponding sample minus PMA indicated by #; In panel B, not significant (N.S.) difference versus sample treated with GW3965 alone indicated by #. In panel C, not significant (N.S.) difference versus corresponding samples minus 4α-PMA indicated by † and #.

The PMA-mediated repressive effects on the LXR activity were also observed on a natural LXR target promoter, the human ABCA1 promoter. As shown in Fig. 10D, the PMA treatment inhibited 22R-HC and 9-cisRA mediated transactivation of an ABCA1-linked reporter gene by 50–70%.

The effects of PMA on PPARα transactivation were then analyzed to determine whether PKC activation resulted in a general decrease in reporter activity in Cos-1 cells. In contrast to LXRα, transactivation by the related nuclear receptor PPARα is increased under similar conditions. As shown in Fig. 10E, PPARα-mediated induction of a PPRE-reporter gene in the presence of the PPAR ligand WY14-643 was increased by co-treatment with PMA in concordance with previous findings (Gray et al 2005).

Finally, the effects of PKC signaling were tested on LXRβ-mediated transactivation of the ABCA1 luciferase reporter construct. LXRβ activity was also decreased by PMA treatment similar to LXRα (Fig. 10F). Our results indicate that under

our experimental conditions, PKC activation represses LXR activity in contrast to the related nuclear hormone receptor PPARα.

To determine whether the repressive effects of PMA on LXR activity was a general phenomenon of LXRα or specific to Cos-1 cells, similar transfection experiments were performed in HEK293T and HepG2 cells. PMA treatment decreased LXRα transactivation in HEK293T (Fig. 11A) as well as HepG2 cell lines (Fig. 11B).

The repressive effects of PMA on LXR activity was dose-dependent and saturated at 1–2 nM, a concentration that is within the physiological range that activates PKC (Fig. 12A and B). This PMA dose–response profile was similar when LXR agonist 22R-HC was used in place of GW3965 (not shown). Extended treatment of PMA (>24 h) can cause depletion of PKC and, therefore, the response to PMA may not involve signaling via PKC but rather a lack of PKC kinase activity (Ohno et al 1990). To explore this, we examined the time course of PMA-mediated inhibition. As shown in Fig. 12C, the inhibitory effects on LXR activity were observed between 3 and 4 h following PMA addition, implying that PKC depletion is likely not the cause of the decrease in LXR function (Fig. 12C). No decrease in protein expression of LXR or RXR was observed throughout the time trial (Fig. 12D), as determined by western blot analysis.

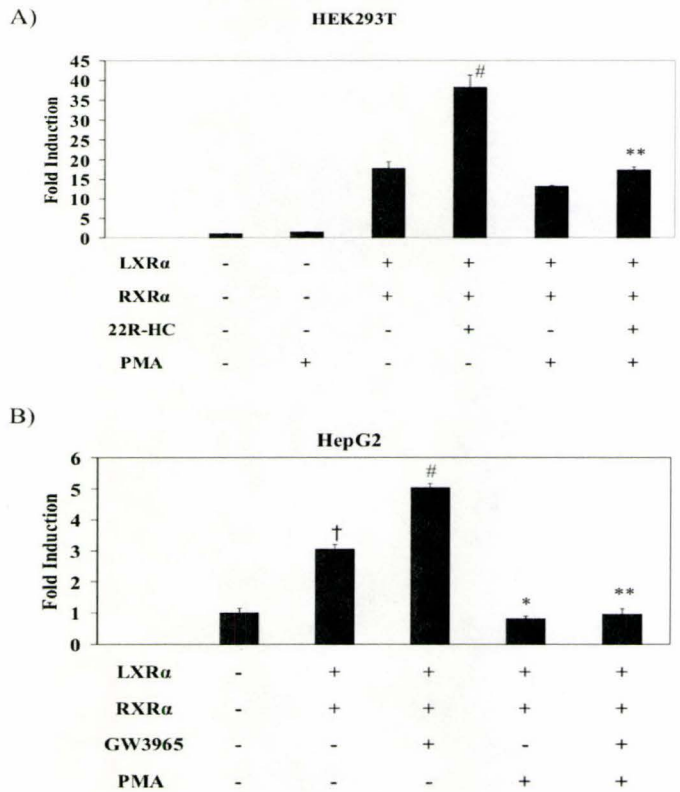


Figure 11. PMA decreases LXRα transactivation in HEK293T cells and HepG2 cell lines. (A) HEK293T cells or (B) HepG2 cells were transfected with ABCA1-luciferase plasmid in the presence or absence of expression vectors for human LXRα and RXR and treated as indicated. Following transfection, cells were treated with 22R-hydroxycholesterol (22R-HC; 10 μM), 9-cis-retinoic acid (9-cisRA; 10 μM) or GW3965 (2 μM) and PMA (1 nM for HEK293 cells; 80 nM for HepG2 cells) for 48 h as indicated and luciferase activity was measured. The values presented represent the average (±S.D.) relative to untreated cells (taken as 1) from three independent transfections carried out in triplicate and normalized to β-galactosidase and protein levels. *P<0.02 versus corresponding sample minus PMA indicated by†; **P<0.02 versus corresponding sample minus PMA indicated by #.

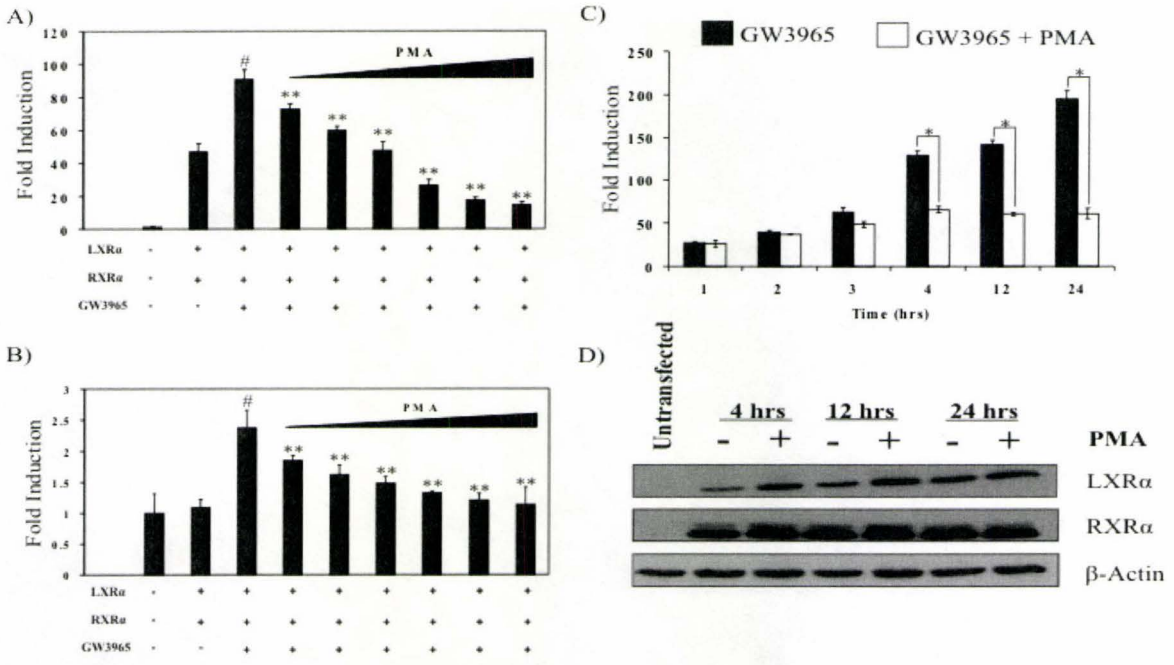


Figure 12. PMA exhibits a dose- and time-dependent effect on LXR transactivation. Cos-1 cells were transfected with (A) an LXRE-luciferase reporter gene or (B) an ABCA1-luciferase reporter gene and human LXRα and RXRα expression plasmids in the presence of GW3965 (1 μM) and increasing levels of PMA (0.25–2 nM) as indicated. Cells were lysed 24 h post-transfection and luciferase activity was measured. (C) Cos-1 cells were transfected with an LXRE-luciferase reporter gene along with expression plasmids for human LXRα and RXRα. GW3965 (1 μM) or both GW3965 and PMA (1 nM) were added to the cells 24 h post-transfection and luciferase activity was measured at the indicated times. The values presented represent the average (±S.D.) relative to untreated cells (taken as 1) from three independent transfections carried out in triplicate and normalized for β-galactosidase and protein levels. (D) Extracts (25 μg) from samples in (C) were analyzed by western blot with antibodies specific for LXRα and RXRα. Antibodies to β-actin served as a loading control. *P<0.05; **P<0.05 versus corresponding sample minus PMA indicated by #.

Constitutively active PKCα mimics the effects of PMA in Cos-1 cells

To further confirm the role of PKC signaling in LXR function, we carried out transfection experiments with a constitutively active PKCα expression vector. Constitutively active PKCα decreased ligand-dependent and ligand-independent LXR transactivation with the LXRE-reporter plasmid (Fig. 13A) and with the ABCA1-luciferase reporter construct (not shown). This finding further confirms that PMA does not act by depleting PKCα. In contrast to the effects observed with LXR, the expression of constitutively active PKCα increased PPARα ligand-independent transactivation but had minimal effect on the ligand-dependent activation (Fig. 13B).

PMA treatment does not decrease LXRα/RXRα DNA binding

PKC-mediated phosphorylation of the VDR causes decreased DNA binding (Hsieh et al 1993) to target sites. To determine whether this is also the case for LXR, Cos-1 cells were transfected with LXRα and RXRα expression plasmids and treated with PMA. Nuclear extracts were prepared and used in gel retardation assays with a radiolabeled LXRE probe. As shown in Fig. 14A, nuclear extracts prepared from cells transfected with LXRα and RXRα treated in the presence or absence of PMA formed a protein/DNA complex of similar intensity, and with migration similar to that formed with *in vitro*-synthesized LXR/RXR translated proteins.

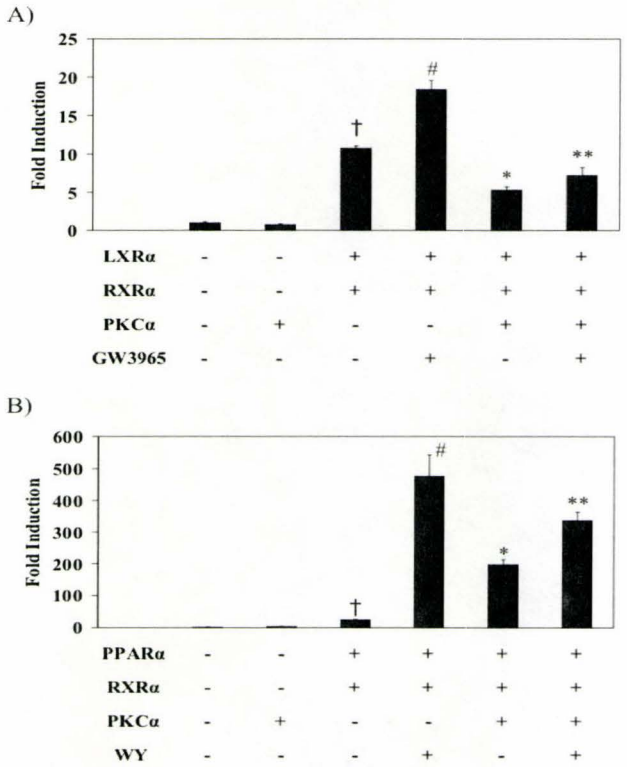


Figure 13. Constitutively active PKCα mimics the effects of PMA. (A) Cos-1 cells were transfected with an LXRE-luciferase reporter plasmid and expression vectors for human LXRα and RXRα and constitutively active PKCα as indicated, in the presence of GW3965 (1 μM). Cells were lysed 24 h post-transfection and luciferase activity was measured. (B) Cos-1 cells were transfected with a PPRE-luciferase plasmid and expression vectors for PPARα, RXRα and constitutively active PKCα in the presence of Wy14,643 (WY; 100 μM) as indicated, and luciferase activity was measured as above. The values presented represent the average (±S.D.) relative to untreated cells (taken as 1) from three independent transfections carried out in triplicate and normalized for β-galactosidase and protein levels. *P<0.02 versus corresponding sample minus PKCα indicated by†; **P<0.02 versus corresponding sample minus PKCα indicated by #.analyzed by western blot with antibodies specific for LXRα and RXRα. Antibodies to β-actin served as a loading control. *P<0.05; **P<0.05 versus corresponding sample minus PMA indicated by #.

We also performed ChIP analysis of endogenous LXR protein bound to the ABCA1 promoter in HEK293 cells. As shown in Fig. 14B, HEK293 cells treated with PMA+GW3965 show similar enrichment of the ABCA1 promoter following LXR immunoprecipitation when compared with GW3965 alone. These findings indicate that PMA-treated cells do not decrease the binding of LXR/RXR heterodimers to DNA.

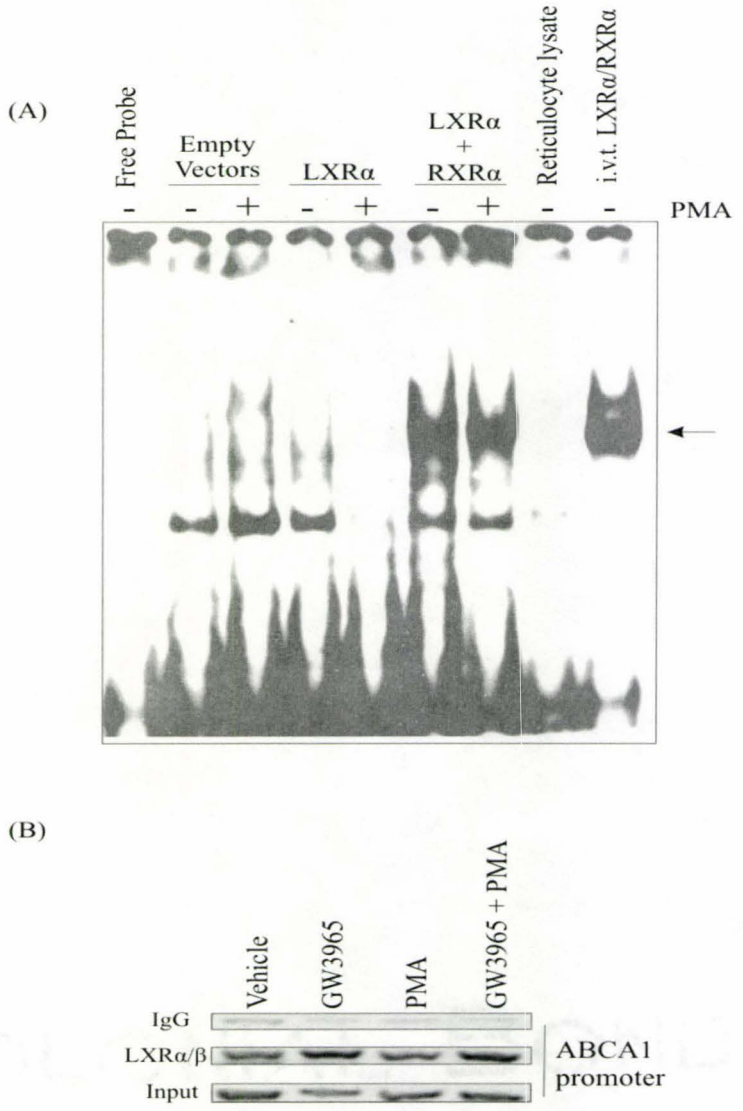


Figure 14. PMA does not block LXR/RXR heterodimer DNA binding. (A) Gel retardation analysis. Cos-1 cells were transfected with expression plasmids for human LXRα and RXRα and treated with PMA (1 nM) for an additional 24 h. Nuclear extracts (NE) were prepared and 10 μg were incubated with a P32 labeled LXRE DNA probe. Complexes were resolved by native PAGE and detected by autoradiography. Arrow indicates the LXR/RXR protein DNA complex. In vitro translated (ivt) LXRα and RXRα served as a positive control. (B) Chromatin immunoprecipitation (ChIP) analysis of LXRα/β binding to the ABCA1 promoter following PMA treatment. HEK293 cells were treated with GW3965 (2 μM) and/or PMA (80 nM) for 2 h prior to ChIP analysis, as described in Materials and Methods. Rabbit pre-immune immunoglobulin G (IgG) or antibodies specific for LXRα/β were used to immunoprecipitate the ABCA1 promoter region. Precipitated DNA was used as template in PCR with primers specific for the human ABCA1 promoter region containing the putative LXRE. SYBR-green-stained bands representing ABCA1 are shown after 36 cycles. Lanes marked 'input' represent DNA isolated prior to immunoprecipitation.

PKCα phosphorylates LXRα in vitro

To determine whether PKC could indeed phosphorylate LXR, purified LXRα was added to an *in vitro* kinase assay with PKCα. As shown in Fig. 15A, PKCα was able to phosphorylate LXRα *in vitro* indicating a possible direct modulation of LXR activity *in vivo*.

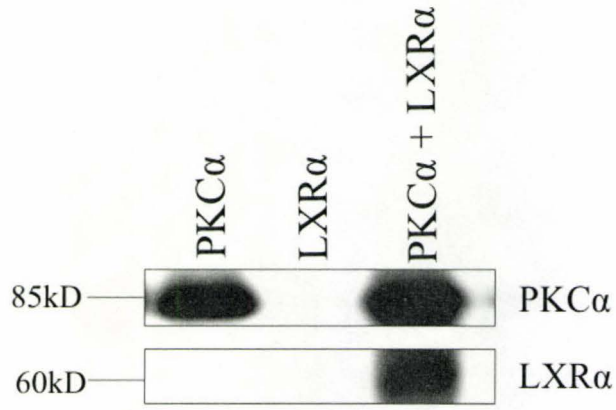


Figure 15. PKCα phosphorylates LXRα in vitro. Recombinant PKCα and purified LXRα were incubated alone or in combination in PKC buffer for 30 min as described in experimental procedures in the presence of P32-labeled ATP. Proteins were separated by SDS-PAGE and visualized by autoradiography.

LXR transactivation of endogenous target genes is repressed by PKC signaling

The above experiments were carried out using transient transfection assays. To determine whether PKC activation downregulates endogenous LXR target genes, we treated HEK293 cells with LXR agonist±PMA for 24 h and analyzed expression of bona fide LXR target genes by real-time PCR. As shown in Fig. 16A and B, PMA treatment repressed ligand-induced expression of endogenous LXR target genes ABCA1 and

SREBP1c. The repressive effects of PMA were blocked by the PKC inhibitor bisindolylmaleimide (Fig. 16A and B). The results indicate that PKC signaling can downregulate LXR activation of endogenous target genes.

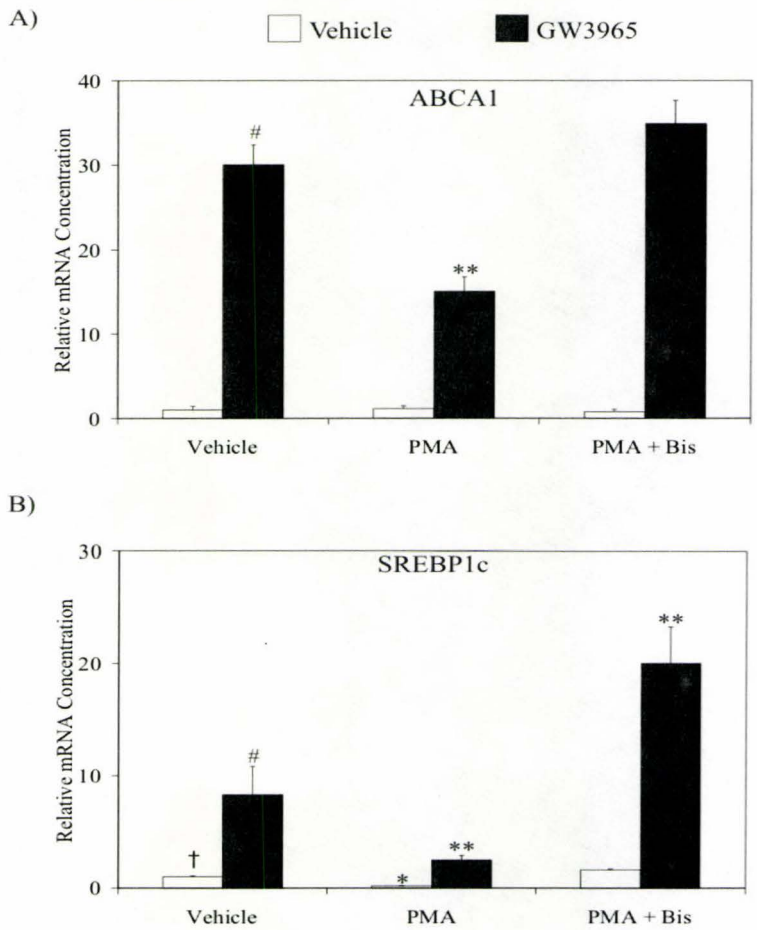


Figure 16. PMA downregulates LXR target genes in HEK293 cells. HEK293 cells were treated with GW3965 (2 μM), PMA (80 nM), and/or bisindolylmaleimide (Bis; 50 nM) as indicated for 24 h and RNA was isolated. One microgram RNA was reverse transcribed and levels of (A) ABCA1 and (B) SREBP1c were determined by real-time PCR, as described in the material and methods. Relative expression was normalized to β-actin levels. Values represent the average of triplicates from two independent experiments ±S.D. *P<0.01 versus corresponding sample minus PMA indicated by†; **P<0.01 versus corresponding sample minus PMA indicated by #.

2.4) Discussion

Post-translational modification of nuclear hormone receptors provides the cell with important modes of regulation and allows for rapid regulation of receptor function in response to intra- and extracellular stimuli. In particular, phosphorylation of nuclear receptors has been shown to affect their sub-cellular localization, stability, ability to bind DNA, affinity for co-activator or co-repressor proteins, and/or alter their affinity for ligands (Diradourian et al 2005; Khan et al 2006; Tahayato et al 1993; Xu & Koenig 2005).

The findings reported here show that PKC activation, as shown through activation of endogenous PKC isoforms by PMA as well as by over-expression of constitutively active PKCα and the use of specific PKC inhibitors, downregulates the activity of human LXRα in transient transfection assays. The effect was observed on a consensus LXRE-luciferase construct as well as a luciferase construct harboring the human ABCA1 promoter region that contains the reported LXRE in multiple cell types (Costet et al 2000). Moreover, in contrast to LXR, experiments done under similar conditions with PPARα and PPRE-reporter plasmids indicate that PKC signaling does not decrease PPARα activity, a result reported by others as well (Gray et al 2005).

The mechanisms by which the PKC signaling pathway alters LXR function *in vivo* remains to be determined. We demonstrate here that PKCα can phosphorylate LXRα *in vitro*; however, it is not yet clear whether LXR is directly phosphorylated by PKC *in vivo* or whether this in fact correlates with the observed attenuation of receptor function.

Chen et al. 2006, using over-expression studies with FLAG-tagged LXR, have recently shown that LXR is constitutively phosphorylated in HEK293 cells and further demonstrated that S198, which is part of a consensus mitogen-activated protein kinase phosphorylation sequence, is the major phosphorylated residue. However, the physiological consequence of this modification is not apparent. Mutation of S198 did not affect the ability of LXR to bind to DNA, its ability to activate target genes, its sub-cellular distribution, nor its response to ligand (Chen et al 2006).

Similarly, while PKA signaling in liver cells has been reported to decrease LXR/RXR heterodimer binding to DNA (Yamamoto et al 2007), we did not observe any changes in LXR/RXR DNA complex formation using nuclear extracts or ChIP analysis from cells stimulated with the PKC activator PMA. Therefore, a potential conformational modification of LXR/RXR heterodimers induced by PKC signaling may alter LXR function. LXR is known to undergo 'heterodimerization-induced activation' when heterodimerized to RXR, a mechanism not reported for other nuclear receptors (Son et al 2008; Wiebel & Gustafsson 1997; Wiebel et al 1999). This response is dependent on the activation domain-2 (AF-2) of LXR and is the result of a conformational change in LXR induced allosterically by RXR (Wiebel & Gustafsson 1997).

Indeed, we observe high basal activity when LXRα and RXRα are co-expressed in Cos-1 cells in the absence of ligand (see Fig. 10A). Our findings show that PKC signaling reduces both basal and ligand-induced activation on both a consensus LXRE-reporter plasmid as well as the natural ABCA1-reporter construct, while maintaining the ability of

LXR/RXR heterodimers to bind DNA (see Fig. 14). Therefore, PKC activation could potentially prevent the allosteric modulation of LXR induced by RXR or alternatively, mask the transcriptionally active structure via post-translational modification. At present, it cannot be ruled out that RXR itself is post-translationally modified thereby blocking its ability to allosterically modulate LXR. Ongoing studies to determine the phosphorylation status of LXR and RXR *in vivo*, specific residues phosphorylated by PKC and domains responsible for activation/repression in response to PKC may potentially uncover a direct link between PKC, LXR and RXR phosphorylation and function.

Alternatively, it is also possible that the PKC pathway modulates LXR activity through indirect mechanisms in response to extracellular cues. For instance, it is possible that PKC signaling modulates the recruitment, function, and/or affinity of co-repressor/co-activator complexes to LXR. Altered co-factor recruitment has been reported for the related nuclear receptor PXR in response to PKC activation (Ding & Staudinger 2005), and with LXR in liver cells through PKA activation (Yamamoto et al 2007). At present, it cannot be ruled out that co-activator or co-repressor molecules are themselves post-translationally modified by PKC, thereby blocking LXR-mediated transactivation of reporter genes (Rochette-Egly 2003). Indeed, co-activators specifically modulating the function of LXR have been identified such as activating signal cointegrator-2 (Lee et al 2001). More recent findings have demonstrated that glucose signaling alters the sub-cellular distribution of LXR, although there is no evidence that this is phosphorylation dependent (Helleboid-Chapman et al 2006).

Tranheim Kase *et al.* (2006) have also demonstrated that the synthetic LXR agonist T0901317 can induce DAG formation in cultured myoblasts. However, it has yet to be tested whether GW3965 induces similar changes in DAG levels and, furthermore, physiological LXR ligands such as 22R-HC did not alter DAG levels. In the studies presented here, we show that PKC activation has similar effects on LXRα transactivation in the presence of GW3965 or 22R-HC, and thus the physiological relevance of DAG formation by T0901317 remains to be determined.

Our findings also show that attenuation of LXR activity by PKC occurs in multiple cells types. In addition to HepG2 cells, PMA decreased LXRα transactivation in Cos-1 cells and HEK293T cells, two kidney-derived cell lines. This points to a possible role for PKC modulation of LXR activity in the kidney. Indeed, LXR plays an important role in regulating cholesterol efflux in the kidney as well as controlling the expression of renin in juxtaglomerular cells (Morello et al 2005; Wang et al 2005; Wu et al 2004; Zhang et al 2005). Interestingly, renin promoter activity is increased by cAMP/PKA signaling, a response dependent on LXRα expression. Consistent with this, we also observed an increase in LXRα activity when Cos-1 cells are treated with 8Br-cAMP, which activates the PKA pathway, on an LXRE-luciferase construct (not shown). Other reports indicate that angiotensin II, a product of renin-induced cleavage in the blood plasma, decreases renin expression via a PKC-dependent pathway (Muller et al 2002). A possible negative feedback loop may therefore exist to regulate blood pressure by modulating LXRα activity. Indeed, LXR has recently been reported to play a role in regulating blood

pressure in male Sprague–Dawley rats by modulating the angiotensin-II receptor gene in vasculature (Leik et al 2007a).

In summary, we demonstrate that PKC signaling can attenuate LXRα transactivation in Cos-1, HEK293T, and HepG2 cells. The findings reveal a potentially important mechanism of regulation of LXR that warrants further study as abnormal PKC signaling has been observed in diabetes, atherosclerosis, renin expression and glucose metabolism in the liver, all conditions in which LXR is known to play an important role (Aiello et al 2006; Collins 2004; Dey et al 2006; Glass et al 1988; Grefhorst et al 2005).

CHAPTER THREE PREFACE

LXR Stimulates Cholesterol Efflux and Inhibits Expression of Inflammatory Mediators in ASM Cells

This work has been previously described in:

Delvecchio, C.J., Bilan, P., Radford, K., Stephen, J., Trigatti, B.L., Cox, G.P., Parameswaran, K. and Capone, J.P. (2007) LXR Stimulates Cholesterol Efflux and Inhibits Expression of Inflammatory Mediators in ASM Cells. *Molecular Endocrinology.* 21(6): 1324-1334.

Permission has been granted by the publisher to reproduce the material here.

I conducted all experiments described in this chapter with the exception of the airway smooth muscle migration assays (Fig. 22A – performed by Jancy Stephen). ASM cell cultures were established from lung tissue by K. Radford at St. Joseph's Hospital.

3.1) Introduction

The preceding chapter describes the regulation of LXR activity. Concurrently during the investigations of PKC-LXR crosstalk described in this thesis, the role of LXR in primary human airway smooth muscle (hASM) biology was also assessed. Findings prior to conducting this work pointed to a role for the PPARs in hASM (Patel et al 2003). PPAR agonists effectively decreased inflammatory gene expression as well as cellular proliferation. Thus, with a unique opportunity to obtain primary human tissue through collaborations with Dr. P. Nair (St. Joseph's Healthcare) and the emerging role of the functionally related PPARs in hASM and lung biology we shifted directions away from our work with PKC-LXR to pursue the function of LXRs in hASM cells.

Human airway smooth muscle (hASM) cells are established modulators of the inflammatory process observed in airway diseases such as asthma and chronic obstructive pulmonary disease (COPD) (Lazaar & Panettieri 2005; Patel et al 2003). Airway hyper-responsiveness and inflammation characteristic of asthma is associated with increased proliferation of hASM cells and secretion of inflammatory cytokines, growth factors, and various co-stimulatory molecules which promote activation, recruitment, and survival of leukocytes, lymphocytes, eosinophils, neutrophils, and monocytes that in turn promote airway narrowing and re-modeling (Anderson 1996). Current therapeutic protocols, in particular the use of corticosteroids that inhibit expression of pro-inflammatory factors through the actions of the glucocorticoid receptor (GR), are limited in their effectiveness and can have severe side effects. Identification of novel anti-inflammatory agents and therapeutic targets in the airway would thus potentially be of significant clinical value.

At the time this research was conducted, the role of LXR in lung biology was unexplored. Given the established roles of LXR in inflammation described in Chapter 1, this work sought to determine if LXRs were expressed and functional in primary cultures of hASM cells. It is described in this chapter here that both LXRα and LXRβ are abundantly expressed in hASM cells. Moreover, LXR agonists stimulated expression of exogenously introduced LXR-responsive reporter genes, as well as endogenous LXR target genes including ABCA1 and ABCG1. In addition, LXR agonists blocked the release of pro-inflammatory cytokines granulocyte macrophage colony stimulating factor (GM-CSF), and granulocyte colony stimulating factor (G-CSF), from cells that were challenged with pro-inflammatory cytokines. LXR activation attenuated cell migration towards platelet-derived growth factor (PDGF) and inhibited proliferation induced by PDGF. These observations establish a role for LXR and hASM cells in modulating expression of proteins involved in the reverse cholesterol transport process, and point to the existence of a novel LXR-modulated anti-inflammatory pathway in these cells. LXR may thus represent a potentially new target for therapeutic intervention in inflammatory respiratory diseases such as asthma and COPD.

3.2) Materials and Methods

Reagents and Plasmids

The synthetic LXR agonist TO901317 (herein after referred to as T1317), the synthetic LXR agonist GW3965, 9-*cis* retinoic acid (9-cisRA), interferon (INF)γ, tumor necrosis factor (TNF)α, interleukin(Il)-1β and human ApoAI were purchased from Sigma-Aldrich (Oakville, ON, CA). PDGF was purchased from Invitrogen Canada (Burlington, ON, CA). Rabbit antibodies to human ABCA1 and ABCG1 were purchased from Novus Biologicals (Littleton, CO, USA). Mouse antibody to human β-actin was purchased from MP Biomedicals (Irvine, CA, USA). ^{3}H-cholesterol was obtained from PerkinElmer (Boston, MA, USA).

Human Airway Smooth Muscle cells

hASM cells were obtained as described previously (Parameswaran et al 2004) from human lungs that were resected at St. Joseph's Healthcare (Hamilton, Canada), following approval from the Institutional Review Board and the consent of patients undergoing resection. Smooth muscle tissue was isolated from disease-free areas of the bronchi. Airway smooth muscle cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin/streptomycin. All experiments were done with cells at passage 5 or earlier.

Analysis of LXR mRNA expression

Total RNA was isolated from hASM cells using the RNeasy QIAGEN kit (QIAGEN, Chatsworth, CA, USA) according to manufacturer's instructions. cDNA was prepared from 1μg of RNA by reverse-transcription using a commercially available kit (QIAGEN, Chatsworth, CA, USA) according to the manufacturer's instructions and amplified by PCR with primers specific for human LXRα (forward - 5'-GGAGGTACAACCCTGGGAGT

-3'; reverse - 5' – AGCAATGAGCAAGGCAAACT-3') and LXRβ – (forward - 5'-TCACCTACAGCAAGGACGAC -3'; reverse – 5'- AGAAGATGTTGATGGCGATG -3').

Briefly, reactions contained 12.5μl of SYBR-supermix (Invitrogen), 10.5μl of H_2O, 1μl of primer sets (10μM each) and 1μl of cDNA. PCR amplification was carried out for 30 cycles with the following parameters: denaturation at 95°C for 15 minutes; 30 cycles at 95°C for 30 sec and 60°C for 1 minute. Products were separated on 2% agarose gels, stained with SYBR green and imaged on a Typhoon 9200 Variable Mode Imager (Molecular Dynamics, Amersham Biosciences, Baie D'Urfe, QU, CA).

Transfections and Reporter Gene Assay

hASM cells were transfected with 0.4 μg of pLXRE*luc* in 6-well dishes at approximately 75% confluency using Effectene reagent (QIAGEN) according to the manufacturer's instructions. pLXRE*luc* is an LXR-responsive luciferase reporter plasmid

that contains three tandem copies of the LXRE from the mouse mammary tumor virus long terminal repeat and has been described (Willy et al 1995). Following transfection, plates were incubated overnight in DMEM lacking phenol red and supplemented with 10% charcoal-stripped FBS, 1% L-glutamine and 1% penicillin/streptomycin with 10μM T1317 and/or 10μM 9-cisRA (from stock solutions prepared in dimethyl sulphoxide) for an additional 48hrs (fresh ligand was added after 24hrs) as described in the figure legends. Control cells received an equivalent amount of vehicle. Luciferase activity was assayed as described previously (Marcus et al 1993).

Real-time PCR

hASM cells were incubated in the presence or absence of T1317 and/or 9-cisRA and cytomix (TNFα – 30ng/ml; INFγ – 100ng/ml; Il-1β – 5ng/ml) as indicated in figure legends. Total RNA was isolated and cDNA was prepared as described above. Real-time PCR was performed using Platinum SYBR Green Supermix-UDG with ROX PCR mix (Invitrogen) according to the manufacturer's instructions. Briefly, 12.5μl of SYBR-green Supermix, 10.5μl of H_2O, 1μl of primer sets (10μM each forward and reverse primer; specific for human ABC1, ABCG1, LPL, FAS, GLUT4, and SR-BI, respectively) and 1μl of cDNA was mixed with a final reaction volume of 25μl. PCR amplification was carried out in 96-well plates in an Applied Biosystems 7900HT real-time PCR machine (Applied Biosystems, Foster City, CA, USA). Relative expression was determined using the standard curve method (Rajeevan et al 2001) and by normalizing to β-actin expression levels.

siRNA transfection

hASM cells were transfected with 5nM of siRNA oligos against the gene of interest using HiPerfect reagent (QIAGEN) according to manufacturer's instructions (ratio of 3μl HiPerfect to 5nM siRNA). Double-knockdown experiments were performed with a mixture of individual siRNAs. The transfection complex was added to the cells for 48hrs and knockdown and off-target specificity was assessed by real-time PCR. Following 48hrs, assays were conducted as described for real-time PCR and cholesterol efflux assays. All siRNA oligos were purchased from QIAGEN. LXRα – Cat # SI00080416; LXRβ – Cat # SI00094787; Luciferase negative control – Cat # - 1022070.

Western Blot Analysis

hASM cells were grown to confluency in 100mm plates and incubated in the presence of T1317 (10μM) and/or 9-cisRA (10μM) for 24hrs as indicated in the figure legends. Western blot analysis was carried out with 25μg total protein for each sample using a commercially available kit (Amersham) according the manufacturer's instructions. Following transfer to nitrocellulose, blots were incubated with rabbit anti-ABCA1 or rabbit anti-ABCG1 polyclonal antibody (1:2000) for 1hr followed by goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:5000) (Amersham) for an additional hour and visualized by enhanced chemiluminescence. Blots were probed with rabbit anti-β-actin as a loading control.

Cholesterol Efflux

Cholesterol efflux was performed as described (Kennedy et al 2005). HDL (density between 1.063 and 1.215 g/ml) was purified from human plasma by sequential KBr density gradient centrifugation (Chapman et al 1981). hASM cells were grown to 90% confluency in 6-well dishes and incubated for 48hrs in the presence of ^{3}H-cholesterol (5μCi/ml). Cells were then washed and incubated for an additional 18hrs with equilibration medium (DMEM + 2% BSA) supplemented with T1317 (10μM) and 9-cisRA (10μM) where indicated. Efflux was initiated by the addition of efflux medium (DMEM) plus either BSA (0.2%), ApoAI (50μg/ml) or HDL (50μg/ml) where indicated. Supernatants were collected at various times up to 7.5hrs and the cells were washed and lysed with 0.1M NaOH for 15 minutes. Radioactivity in the media and cell extracts was measured by scintillation counting. Cholesterol efflux was calculated as the amount of ^{3}H-cholesterol in the media at the time indicated / total ^{3}H-Cholesterol associated with the cells.

ELISA

Confluent hASM cells in 100mm plates were incubated in DMEM supplemented with 0.3% bovine serum albumin for 48hrs. Cytomix was added with or without T1317 (10μM) for an additional 24hrs. Supernatants were collected and GM-CSF and G-CSF concentrations were measured using commercially available ELISA kits according to manufacturer's instructions (R & D Systems - Minneapolis, MN).

Migration Assay

Migration experiments were performed using a 6.5mm Transwell culture plate with a 8.0μM pore, collagen-I coated, polycarbonate membrane separating the inner and the outer chambers (Fisher Scientific Limited, Nepean, ON) as previously described (Parameswaran et al 2004). Briefly, confluent smooth cells were maintained in growth-factor free medium for 24 hours prior to the experiments. Cells (100μl) were treated with varying concentrations of T1317 (1, 10, 50μM) and plated on the upper side the membrane and PDGF (1 ng/ml, 600μl) was added to the lower wells. After 5 hours, the membranes were peeled off and the cells on the upper face of the membranes were scraped using a cotton swab. Cells that migrated to the lower face of the membrane were fixed with 3.7% formaldehyde and stained with Diff-Quik (VWR International, Mississauga, ON, CA). The number of migrated cells on the lower face of the filter was counted in four random fields under 20x magnification (Olympus BX40 microscope; Sony 3CCD Power HAD video camera, and Northern Eclipse Software from Empix Imaging, Mississauga, ON, CA). Assays were done in duplicate using tissues from four different lung specimens.

Proliferation Assay

hASM cell proliferation was determined using an ELISA-based bromodeoxyuridine (BrdU) incorporation assay according to manufacturer's instructions (Roche). Briefly, hASM cells were plated in 96-well dishes at approximately 60% confluency and serum-starved for 24hrs. Proliferation was induced by the addition of

PDGF-BB (50ng/ml) with varying concentrations of T1317 as indicated in the figure legends and incubated for 24hrs. BrdU (final concentration 10μM) was then added to each well for an additional 24hrs. Cells were then fixed and incubated with an anti-BrdU antibody followed by incubation with substrate solution for 30 minutes according to manufacturer's instructions. The colorimetric absorbance readings were performed at 370nm and corrected for background at 492nm on a SpectraMax Plus plate reader (Molecular Devices).

Adherence Assay

Adherence assays were done as described (Girard & Springer 1996). Briefly, 96-well plates were coated with collagen Type I (100μg/ul solution) and incubated for 2 hours at 37°C. 100ul of cells at a concentration of 3×10^5 cells/ml was added per well in combination with PDGF alone or increasing concentration of T1317 as indicated in the figure legend. Cells were allowed to adhere for 1 hour at 37°C. Cells were then fixed with 4% paraformaldehyde containing 0.5% crystal violet stain for 30 minutes at 4°C. The plates were washed extensively in PBS to remove excess stain and read at 595nm on a SpectraMax Plus plate reader.

Statistical Analysis

Unpaired *t* tests were used for comparison of groups. p Values less than 0.05 were considered significant.

3.3) Results and Discussion

LXRα and LXRβ are functionally expressed in hASM cells

To determine if LXR α and β mRNAs are expressed in hASM cells, total RNA was isolated from primary cells and analyzed by reverse-transcriptase PCR using isoform-specific primers. mRNA for both LXR isoforms was detected in primary hASM cells isolated from 3 individuals (Fig. 17A). To determine if hASM cells produce functional LXR protein, we undertook transient transfection assays of hASM cells using a LXRE-*luciferase* reporter gene.

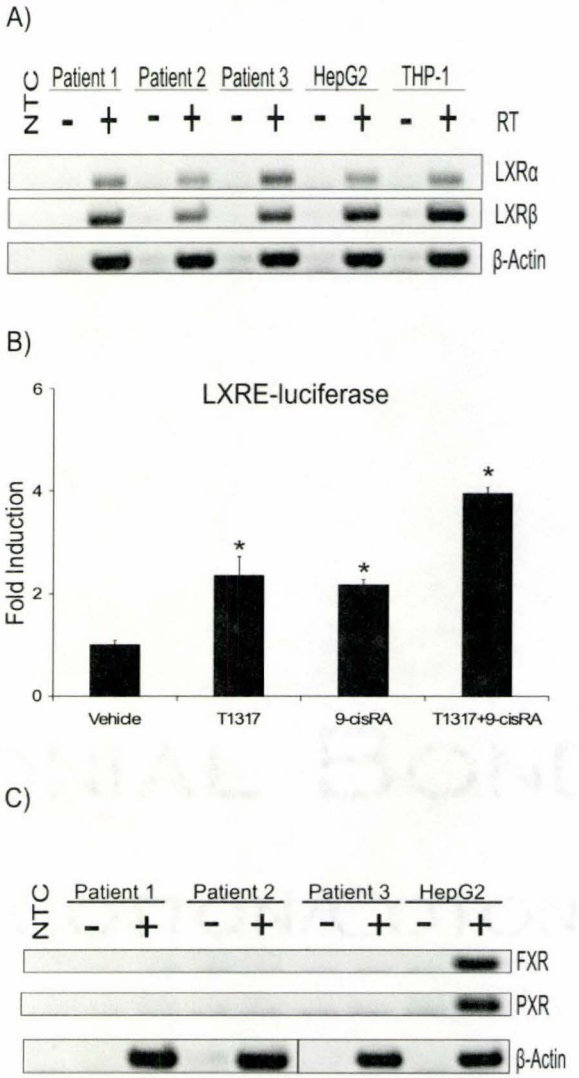


Figure 17. LXRα and LXRβ mRNAs are functionally expressed in hASM cells. (A) cDNA was prepared from total hASM RNA from three patients or RNA isolated from HepG2 and THP-1 cells (positive controls), incubated with primers specific for LXR or LXR, and subjected to PCR as indicated. Amplification products were of the expected sizes (173 base pairs and 141 base pairs for LXR and LXR, respectively). Controls included reactions carried out in the absence of template (NTC) or reverse transcriptase (RT). (B) LXR ligands activate expression of LXR responsive reporter genes. hASM cells were transfected with pLXREluc in the presence of T1317 (10μM) and/or 9-cisRA (10μM) as indicated, incubated for 48 hrs and luciferase activity was measured. The values presented represent the average (+/- S.E.M.) from three independent transfections (each done with cells isolated from different patients) done in triplicate and normalized to protein levels, and to the value obtained from control cells treated with vehicle (taken as 1). *$p < 0.05$ as compared to vehicle treated samples. (C) cDNA was prepared as in (A) and subjected to PCR with primers specific for FXR or PXR. HepG2 cDNA served as a positive control while β-actin levels were used as a loading control.

As shown in Fig. 17B, reporter gene activity was increased over control levels in the presence of the synthetic LXR ligand T1317 or the RXR ligand 9-cisRA 2-fold whereas activity was increased 3-4 fold in cells treated with both ligands (results in the figure represent pooled data from independent transfections carried out with hASM cells prepared from three patients). These findings are consistent with the observations that permissive LXR/RXR heterodimers can be activated by ligands for either LXR or RXR, and that activity is synergistically enhanced in the presence of ligands for both partners (Joseph & Tontonoz 2003; Luo & Tall 2000). The synthetic agonist T1317 is also reported to activate pregnane X Receptor (PXR) and farsenoid X Receptor (FXR) (Deng et al 2006; Shenoy et al 2004), however, these nuclear receptors are not detectably expressed in hASM cells (Fig. 17C). The specificity of our findings to LXR was further confirmed with transfections carried out with GW3965, a highly selective synthetic LXR agonist (not shown).

LXR agonists activate expression of endogenous LXR-target genes in hASM

The foregoing indicates that endogenous LXR is functional as assessed by transient assays. To determine if LXR agonists modulate expression of *bona-fide* endogenous LXR-target genes, cells were treated with T1317 and/or 9-cisRA and the expression of known LXR target genes was monitored by real-time PCR. As shown in Fig. 18A and B, exposure of hASM cells to LXR/RXR agonists stimulated expression of endogenous genes for ABCA1, ABCG1, FAS, LPL, GLUT4, and SR-BI. Induction ranged from 2-fold for SR-BI to approximately 14-fold for ABCA1, ABCG1, and GLUT4. To further confirm that the above findings were mediated by LXR, we examined

the expression of representative LXR-target genes ABCA1, ABCG1, and LPL in cells treated with GW3695, a highly specific synthetic LXR agonist. As shown in Fig 18C, treatment of cells with GW3965 led to the induction of these genes as expected, thereby confirming that the effects were mediated by LXR.

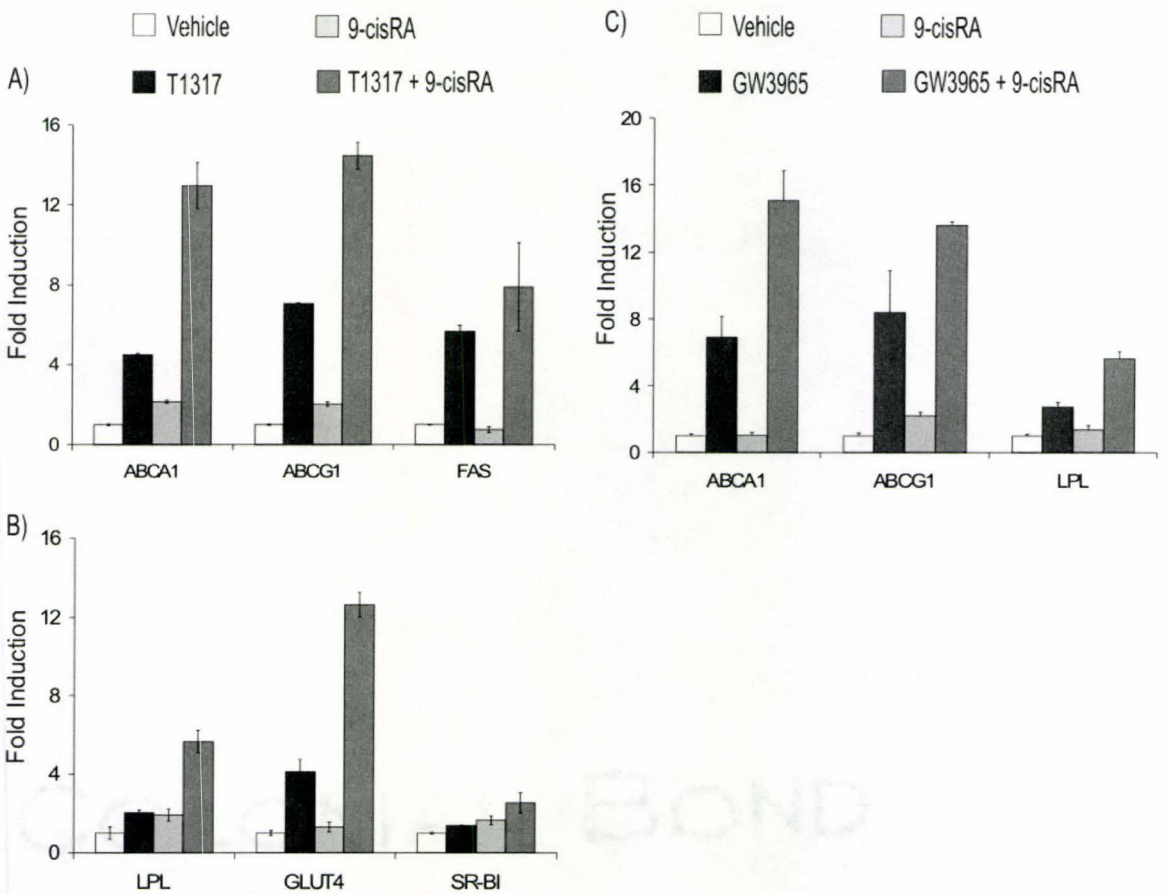


Figure 18 – LXR activation induces expression of endogenous LXR-target genes. cDNA was prepared from RNA isolated from three independent preparations of hASM cells incubated in the presence or absence of T1317 and/or 9-cisRA (panels A and C) or GW3965 (2μM) and/or 9-cisRA, (panel C) where indicated. The resulting cDNA was pooled and subjected to real-time PCR amplification using primers specific to the indicated genes. The values represent the fold-induction above vehicle treated cells (taken as 1) done in duplicate and normalized using β-actin as an internal standard.

Induction of ABCA1 and ABCG1 was also shown at the protein level by western blot analysis with specific antibodies to human ABCA1 and ABCG1 (Fig. 19A), consistent with the real time PCR analysis. While not an exhaustive survey, our findings indicate that LXR activation induces a spectrum of genes in hASM cells that are variously involved in lipogenesis, lipoprotein re-modeling, glucose uptake, and cholesterol homeostasis.

The expression and LXR-dependent induction of ABCA1 and ABCG1 are of particular interest. ABCA1 and ABCG1 regulate cholesterol homeostasis by controlling cholesterol and phospholipid efflux from tissues to extracellular lipid-poor acceptors apolipoprotein AI (ApoAI) and high-density lipoprotein (HDL), respectively, for transport to the liver and subsequent catabolism (Wang et al 2004a; Wang & Tall 2003). Both ABCA1 and ABCG1 are highly expressed in lung tissue and have been implicated in normal lung physiology, although the roles of these transporters in the lung are poorly understood (Kennedy et al 2005; van der Deen et al 2005). ABCA1 has been shown to regulate reverse transport of cholesterol and phospholipids to ApoA1 acceptors in alveolar type II cells, and to enhance oxysterol-dependent basolateral surfactant efflux from alveolar epithelia (Agassandian et al 2004). ABCA1 null mice die from respiratory failure due to pulmonary edema (Bates et al 2005). ABCG1-null mice display profound changes in lung morphology and histology and altered plasma lipid levels and lipid accumulation in subpleural regions (Kennedy et al 2005).

To determine if hASM cells transport cholesterol in response to LXR activation, hASM cells were incubated with 3[H]-cholesterol and efflux to ApoA1 and HDL was

measured following stimulation with LXR/LXR ligands. As shown in Fig. 19B, activation of LXR/RXR led to increased cholesterol efflux to both HDL and to ApoA1. The ligand-induced efflux was shown to be dependent on the expression of LXRα/β isoforms as determined by siRNA-mediated inhibition of LXR expression (Fig. 19C). Figure 19D confirms that the siRNAs used effectively knocked down expression of the LXR isoforms in hASM cells.

The role of cholesterol in asthma and other inflammatory lung diseases is not fully resolved. There is indirect evidence that cholesterol accumulation may be associated with airway hyperresponsiveness and airway inflammation as seen in asthma. Male C57BL6 mice fed on a diet supplemented with 2% cholesterol showed higher numbers of eosinophils and elevated levels of IL-5, PGE2, and MCP-1 in BAL fluid following sensitization and inhalation exposure to ovalbumin (Yeh & Huang 2004). In addition, dietary cholesterol also resulted in elevated production of IL-4 and IFN-gamma by lymphocytes isolated from the lungs. These inflammatory indicators were all significantly correlated with serum cholesterol levels. Cholesterol constitutes approximately 10% of the composition of lung surfactant and is the major neutral lipid of surfactant. Surfactant deficiency can lead to airway closure which has been postulated to be one of the mechanisms of increased airway hyperresponsiveness. A retrospective analysis demonstrated that serum cholesterol was higher in children with asthma compared to non-asthmatic children (Al-Shawwa et al 2006). However, there is little direct evidence at present that raised serum cholesterol or accumulation of cholesterol in the lung or airways worsens airflow obstruction or airway inflammation.

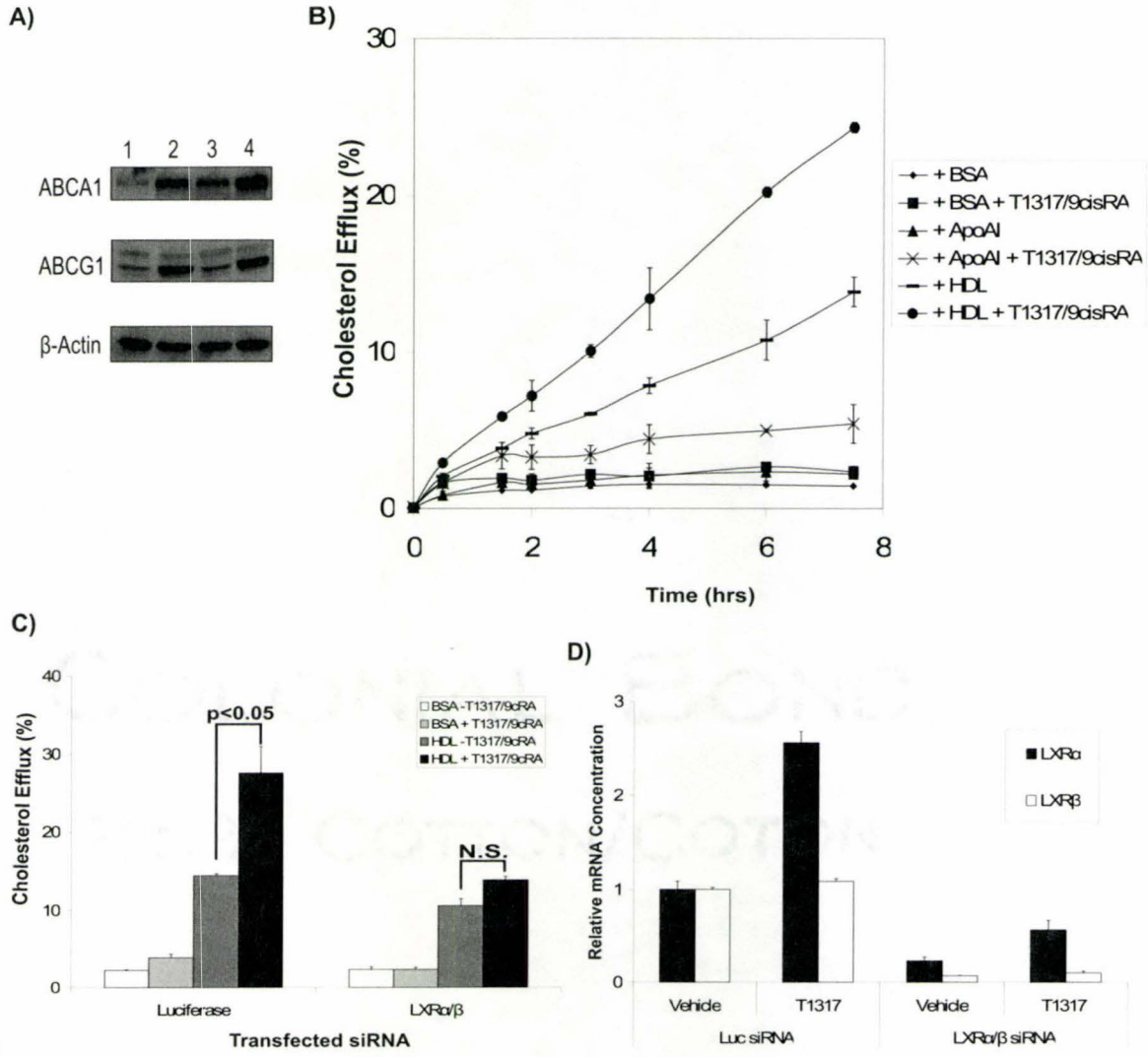


Figure 19 – LXR activation increases ABCA1 and ABCG1 protein levels and stimulates reverse cholesterol transport. (A) LXR activation induces expression of ABCA1 and ABCG1 proteins. hASM cells were treated for 24hrs with T1317 and/or 9-cisRA and protein extracts were analyzed by Western blot with antibodies specific for human ABCA1, ABCG1 and β-actin as shown. 1, untreated; 2, T1317; 3, 9-cisRA; 4, T1317 + 9-cisRA. (B) LXR activation increases cholesterol efflux to HDL and ApoAI. hASM cells were incubated with 3H -cholesterol in the presence or absence of LXR/RXR ligands T1317 (10μM) and 9-cisRA (10μM). Cholesterol efflux was then measured in the presence of BSA (0.2%), ApoAI (50μg/ml) or HDL (50μg/ml) as described in the Materials and Methods. (C) hASM cells were transfected with siRNA targeting Luciferase (negative control) or LXRα and β isoforms as described in the Materials and Methods. Cells were then incubated with 3H-cholesterol as in (B) and cholesterol efflux was measured in the presence of BSA (0.2%) or HDL (50ug/ml) in supernatants collected after 5hrs. N.S. – not significant (D) hASM cells were transfected with siRNA specific to luciferase (negative control) or with a mixture of two siRNAs targeting LXRα and LXRβ isoforms and incubated for 48 hours. . Cells were treated with T1317 for an additional 24 hrs and RNA was isolated and quantified by real-time PCR. The data represents the average of duplicate experiments done in triplicate (+/- S.D.) and normalized using β-actin as an internal standard.

The most convincing evidence of the role of cholesterol in airway pathophysiology is the accumulation of cholesterol-rich protein in the alveolar spaces in patients with pulmonary alveolar proteinosis. Activity of ABCA1 is important for the maintenance of normal lung lipid composition, structure, and function (Bates et al 2005). While it is thought that cholesterol homeostasis is largely maintained by the alveolar macrophages, our findings that both ABCA1 and ABCG1 are expressed in hASM cells and promote efflux of cholesterol following induction by LXR activation suggests that airway smooth muscle cells may also participate in this process.

LXR agonists have anti-inflammatory effects on hASM cells

Asthma, and other respiratory diseases such as COPD, is characterized by narrowing of the airways and chronic inflammation (Panettieri 2003; 2004). ASM cells secret a variety of cytokines and chemokines in response to inflammatory signals. These mediators include GM-CSF and G-CSF which promote recruitment and survival of infiltrating eosinophils and neutrophils, leading to further propagation of the inflammatory response and to airway constriction and remodeling.

Since LXR is known to inhibit inflammatory signaling in macrophages and other cell types, we wished to determine if LXR also displays anti-inflammatory properties in hASM cells. hASM cells were incubated in the presence of a mixture of TNFα, INFγ and Il-1β (cytomix), and expression of multiple cytokine/chemokines was determined at the transcriptional level by real-time PCR. As shown in Fig. 20A, treatment of hASM cells with cytomix resulted in the increased expression of MCP-1α, Cox-2, Il-6,

RANTES, G-CSF and GM-CSF as expected. Co-incubation of cells with T1317 inhibited cytomix-mediated induction of mRNA expression of all cytokines tested. Inhibition of cytomix- mediated expression by T1317 was similar to that observed with the steroid dexamethasone, an agonist for GR. The only exception was MCP-1α where inhibition T1317 was not statistically significant. The levels of cytomix-induced expression of individual cytokines differed slightly between THP-1 cells and hASM cells as determined by comparison of Ct values however, the level of LXR-mediated repression was similar between both cell types (not shown). To determine if reduction of mRNA expression corresponded to reduced protein levels, we examined release of GM-CSF and G-CSF by ELISA. As shown in Fig 20B, cytomix-stimulated release of G-CSF and GM-CSF was inhibited 30% and 70%, respectively, by co-incubation with T1317. To confirm that the above results were dependent upon LXR, the expression of two candidate genes, Cox-2 and Il-6, were analyzed after knockdown of LXR isoforms with transfected siRNA (Fig. 21C). As shown in Fig 21A and 21B, transfection of cells with LXRα/β siRNAs attenuated T1317-mediated repression of Cox-2 and Il-6 following cytomix treatment (Fig. 21A,B). The foregoing indicates that LXR agonists have anti-inflammatory properties in hASM cells.

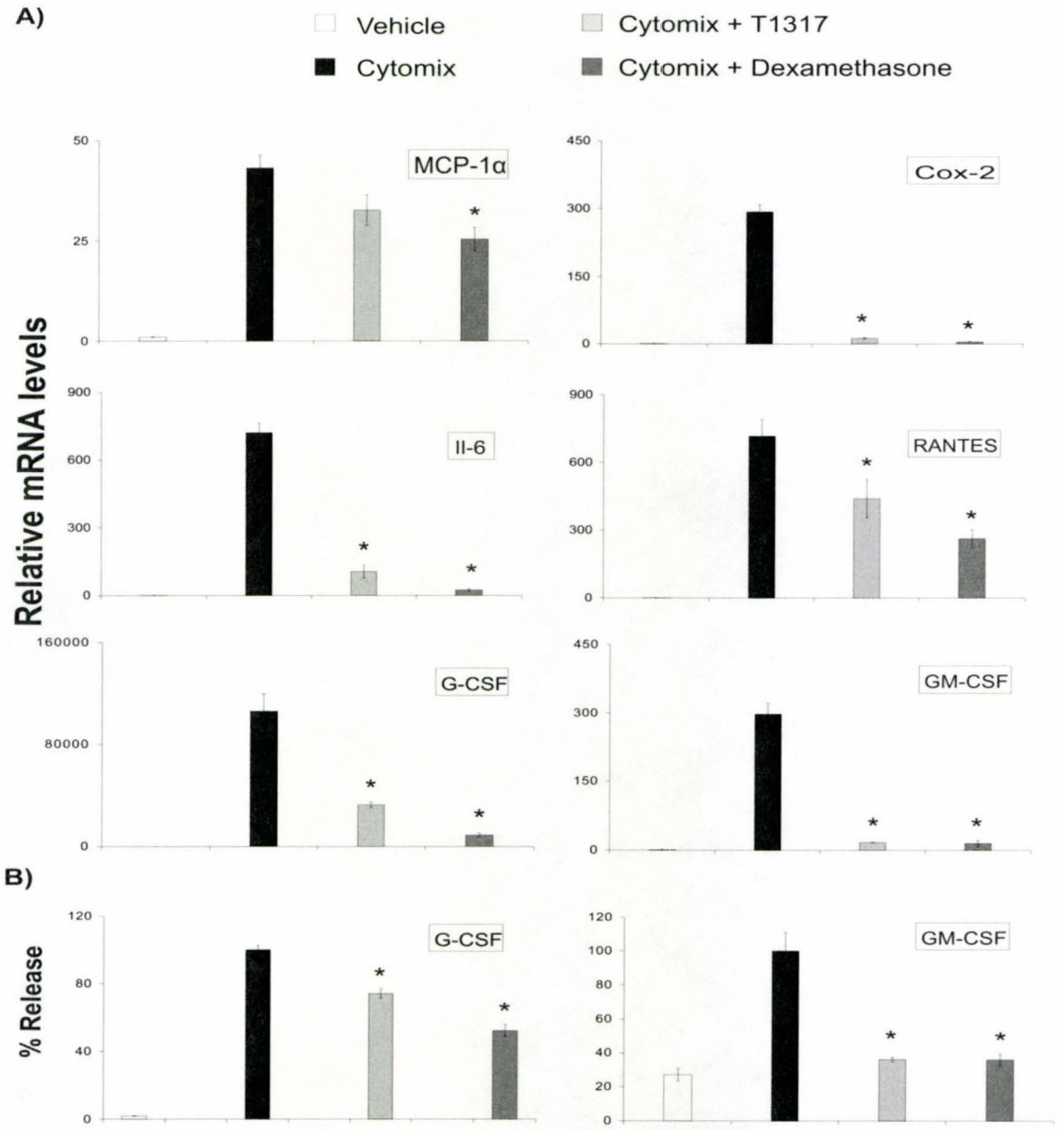


Figure 20 - LXR agonists inhibit cytokine release and cytokine mRNA expression in hASM cells. (A) hASM cells were serum-starved for 48hrs and incubated with cytomix (TNFα , INFγ, and Il-1β) (Ctmx) for an additional 24 hrs. Where indicated, cells were also co-treated with cytomix and T1317 (10μM) or dexamethasone (Dex) (10μM). cDNA prepared from RNA isolated from three independent biological samples for each experimental condition was pooled and subjected to real-time PCR with primers specific for the indicated cytokines. The values represent the fold-induction above vehicle treated cells (taken as 1) from duplicate experiments and normalized using β-actin as an internal standard. *$p < 0.05$ as compared to cytomix treated samples. **B)** Supernatants were collected and the levels of extracellular GM-CSF and G-CSF were assessed by ELISA. Values shown represent the percent release relative to cytomix alone (+/- S.D.) which was taken as 100%.*$p < 0.05$ as compared to cytomix treated samples.

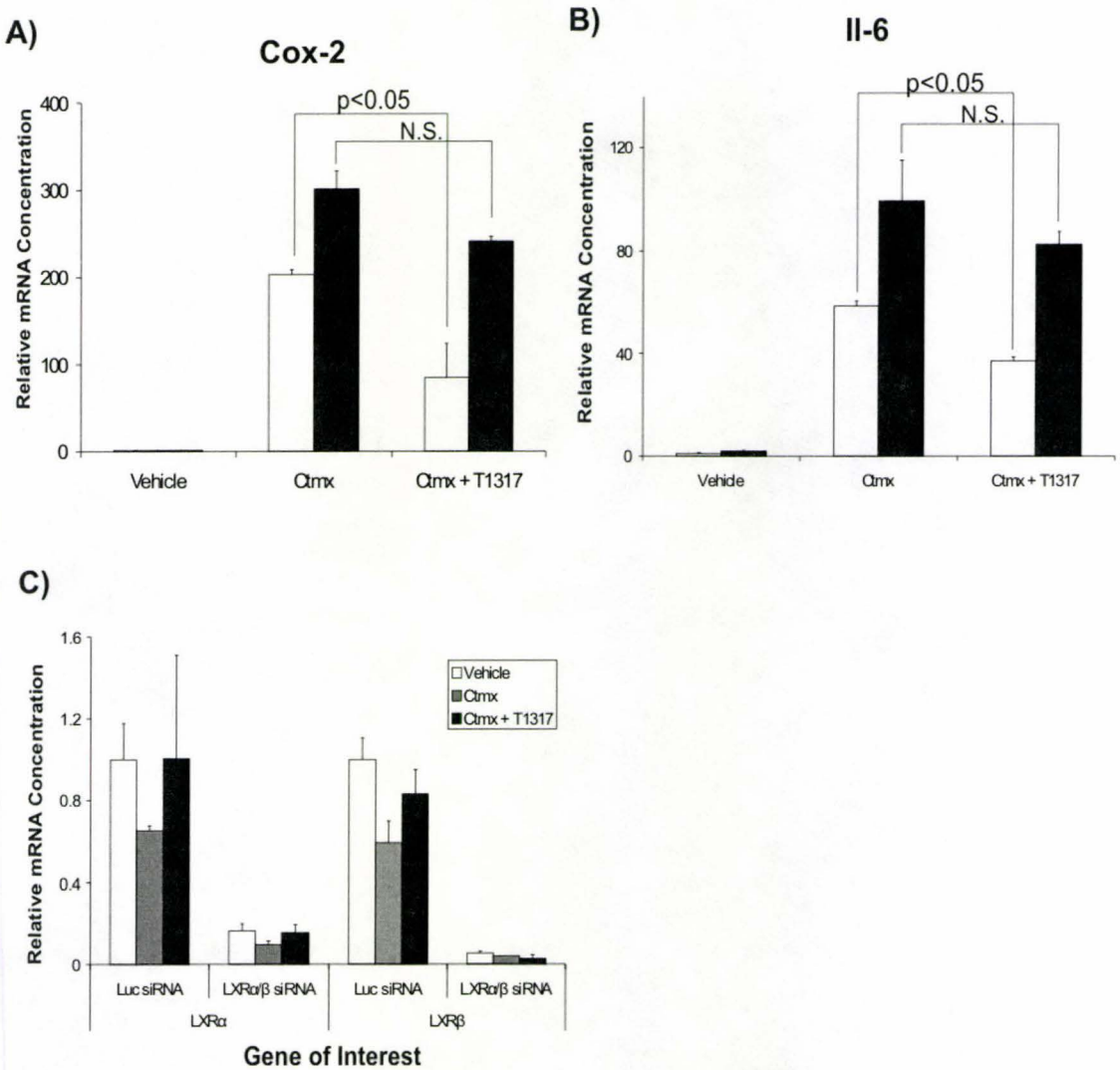


Figure 21 – LXRα/β expression is required for the anti-inflammatory effects of T1317. hASM cells were transfected with siRNA for 48hrs with sequences specific to luciferase (negative control; white bars) or with a mixture of two siRNAs targeting LXRα and LXRβ isoforms (black bars). Cells were serum-starved for 48hrs and incubated with cytomix (TNFα, INFγ and Il-1β) (Ctmx) for an additional 24hrs. Where indicated, cells were also co-treated with cytomix and T1317 (10μM). RNA samples were subjected to real-time PCR with primers specific for (A) Cox-2 and (B) Il-6. (C) LXR isoform knockdown was confirmed by real-time PCR. The data represents the average of duplicate experiments done in triplicate (+/- S.D.) and normalized using β-actin as an internal standard. N.S. – not significant

LXR activation attenuates hASM migration and proliferation

Smooth cell migration is thought to contribute to smooth muscle accumulation in the sub-mucosa and promote airway re-modeling in patients with chronic asthma, analogous to vascular smooth muscle migration in atherosclerosis (Lazaar & Panettieri 2005). ASM cells migrate towards chemotactic gradients initiated by a variety of cytokines and growth factors. To determine if LXR agonists modulate migration of hASM cells, we examined PDGF-induced chemotaxis using transwell migration assays. As shown in Fig. 22A, hASM cells showed a 3.5-fold increased chemotaxis toward 1ηg/ml PDGF. Migration was inhibited in a concentration-dependent manner by 70-80% in the presence of T1317. LXR did not decrease the adherence of ASM cells to collagen type I coated wells at concentrations tested in the migration assay indicating cell viability was not compromised (Fig. 22B). To begin to explore possible mechanisms involved in inhibition of migration, we examined the phosphorylation status of several signaling molecules known to be involved in migration such as Src kinase, Akt and p38 MAPK (Day et al 2006; Hedges et al 1999; Krymskaya et al 2005). In this preliminary analysis, we did not observe any modulation of the phosphorylation status of the above signaling molecules when cells were treated with LXR agonists before stimulation with PDGF, indicating that these particular factors may not be involved in the LXR-mediated inhibition of migration that we observed (not shown). We are currently investigating the role of other signaling pathways.

In addition to migration, hASM cell proliferation is also an important parameter in airway remodeling (Hirst et al 2004). We therefore examined the role of LXR activation

on cell proliferation by measuring bromodeoxyuridine incorporation. As shown in Fig 22C, LXR agonists decreased proliferation of ASM cells in a dose-dependent manner (Fig. 22C).

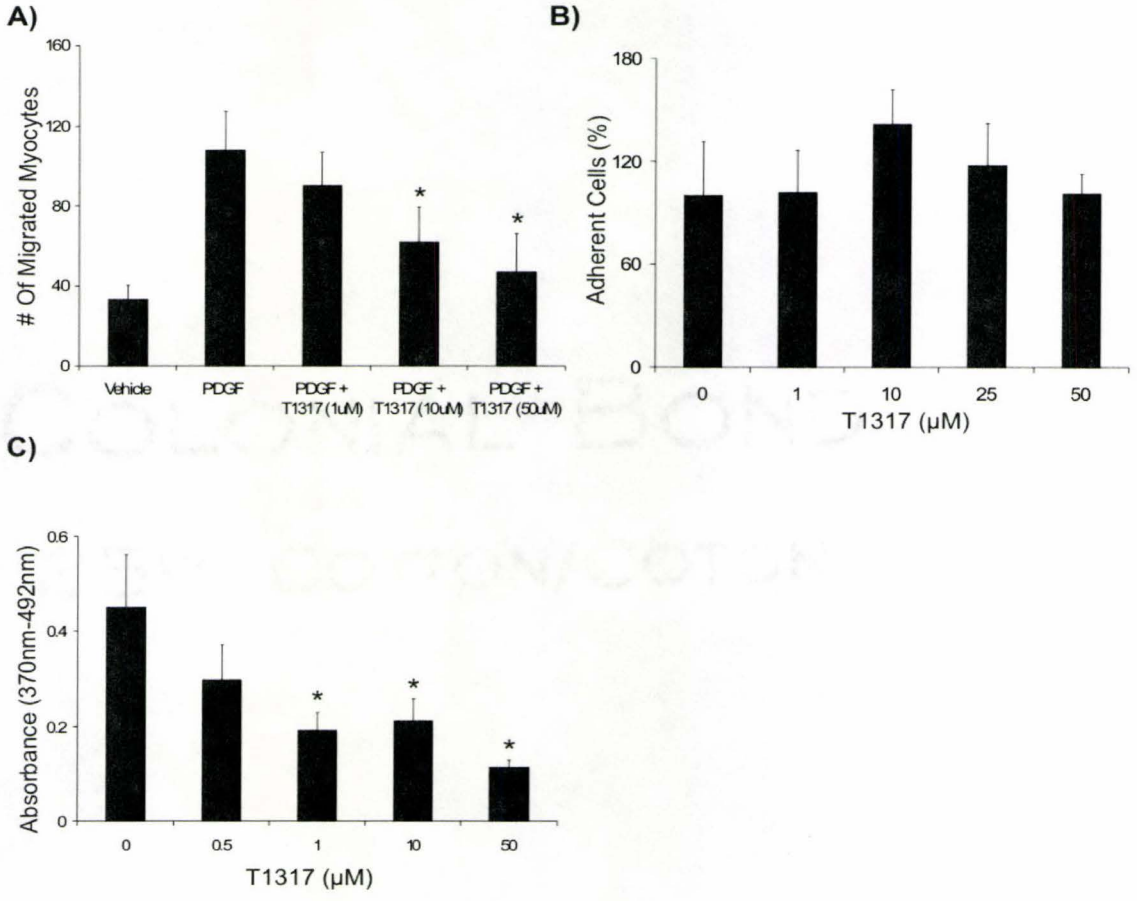


Figure 22 – LXR activation attenuates migration and proliferation of hASM cells. (A) hASM cells were incubated in transwell plates in the presence or absence of the indicated concentration of T1317 and migration towards PDGF (1 ηg/ml) was measured as described in Materials and Methods. The Y-axis shows the number of migrated myocytes (mean from 4 experiments in duplicate +/- S.D.). (B) hASM cells were treated with PDGF (1 ng/ml) alone or in the presence of increasing amounts of T1317, as indicated, and adherence to collagen type I coated plates was measured as described in Material and Methods. (C) LXR agonists decrease hASM proliferation. hASM cells were treated with PDGF (50 ng/ml) alone or in the presence of increasing amounts of T1317, as indicated, for 48hrs and proliferation was assayed by BrdU incorporation as described in Material and Methods. *$p < 0.05$ as compared to PDGF alone.

In summary, it is demonstrated in this chapter that hASM cells functionally express both LXRα and β isoforms and that activation leads to the regulation of a variety of genes and cellular processes that are important in normal lung cell function and in pulmonary diseases such as asthma. Recent findings showing that arginase II, a gene that has been implicated in asthma, is a direct LXR target, support a role for LXR in pulmonary inflammation (Marathe et al 2006). Ongoing studies of the mechanisms of action and biological functions of LXR in airway, including studies using animal models of airway inflammation, will provide insights into the importance of LXR in normal airway smooth muscle function and in airway disease.

CHAPTER FOUR PREFACE

LXR-Stimulated Cholesterol Efflux in Human Airway Smooth Muscle is Mediated Exclusively by ABCA1

This work has been previously published in:

Delvecchio, C.J., Bilan, P., Nair, P. and Capone, J.P. (2008) LXR-Stimulated Cholesterol Efflux in Human Airway Smooth Muscle is Mediated Exclusively by ABCA1. *American Journal of Physiology Lung Cellular and Molecular Physiology.* 295(5):L949-57.

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I conducted all experiments described in this chapter.

4.1) Introduction

The preceding chapter characterizes the expression and function of LXR as well as LXR target genes in hASM. Ligand-activation of LXR resulted in robust cholesterol efflux in ASM cells, presumably through the actions of ABC transporters. This chapter extends on these findings to investigate the contribution of individual ABC transporters ABCA1 and ABCG1 to these processes. The rationale is based on previous work described in Chapter 1 indicating that metabolic defects in lipid and cholesterol homeostasis may contribute to AHR and lung disease and furthermore, that ABCA1 and ABCG1 knockout mouse models develop massive lipid accumulation in the lung.

Many factors are proposed to contribute to the initiation and progression of asthma including genetic predisposition and environmental factors such as pollutants (Brisbon et al 2005). The increased co-incidence of obesity and asthma, as observed with recent longitudinal and cross-sectional studies (Gennuso et al 1998; von Mutius et al 2001), further suggests that metabolic dysregulation may also contribute to AHR. As well, hypercholesterolemia, which is often observed in obese patients with asthma, has been associated with AHR in animal models of lung disease (Al-Shawwa et al 2006; Plumb et al 2007; Yeh & Huang 2004). Consistent with the latter, statin therapy has been shown to reduce pulmonary inflammation and proliferation of airway smooth muscle cells (Takeda et al 2006; Yeh & Huang 2004).

While ABC transport proteins have been highly characterized in the macrophage, their role in other lung cell types, such as human airway smooth muscle (hASM), that are crucial in diseases such as asthma, had not been investigated. Thus, given the emerging

importance of cholesterol and lipid metabolism in the lung, this chapter describes findings that characterize the function of ABCA1 and ABCG1 in hASM cells using siRNA knockdown approaches and small molecule inhibitors of these transporters. It is shown here that LXR-ligand induced reverse cholesterol and phospholipid transport to both apoAI and HDL is mediated exclusively by ABCA1 whereas ABCG1 appears to play no observable role in this process. Moreover, cholesterol-loading of hASM cells specifically increases the expression of ABCA1 and ABCG1, and that this induction is dependent on LXRα/β expression. The findings indicate that cholesterol and lipid homeostasis are of importance in normal hASM function and suggest that dysregulation of these pathways may contribute to the pathogenesis of respiratory diseases such as asthma.

4.2) Materials and Methods

Reagents

Human apolipoprotein A-I (apoAI), TO901317 (T1317), GW3965, 9-cis retinoic acid (9-cisRA), Interleukin 1-β (Il-1β) and probucol were purchased from Sigma-Aldrich (Oakville, ON, CA). Human high density lipoprotein (HDL) purified by ultracentrifugation (d=1.063-1.21g/cc) was purchased from Biomedical Technologies (Stoughton, MA, USA). ^{3}H-cholesterol and ^{3}H-choline were purchased from Perkin Elmer (Boston, MA, USA). Gene-specific siRNA oligonucleotides were purchased from QIAGEN (Chatsworth, CA, USA). All other chemicals were purchased from Sigma-Aldrich unless stated otherwise.

Human airway smooth muscle

hASM cells were obtained as described previously (Parameswaran et al 2004) from human lungs that were resected at St. Joseph's Healthcare (Hamilton, Canada), following approval from the Institutional Review Board and the consent of patients undergoing resection. Smooth muscle tissue was isolated from disease-free areas of the bronchi. Airway smooth muscle cells were grown in RPMI media supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin/streptomycin. All experiments were done with cells at passage 5 or earlier. All experiments described herein include a 24-48hrs serum deprivation step. As has been shown previously, serum removal results in the expression of contractile proteins and other differentiation markers and increases the number of caveolae which is thought to represent a more physiological relevant state since cells are not normally exposed to high levels of serum (Halayko et al 2008). This step also synchronizes hASM cell cultures resulting in decreased heterogeneity and more uniform responses.

Small interfering RNA (siRNA) transfections

hASM cells were transfected with siRNA target-specific oligonucleotides using HiPerfect reagent and reverse-transfection protocols according to manufacturer's instructions (QIAGEN, Chatsworth, CA, USA). Briefly, siRNA (19ng-285ng) was incubated with 1.5µl HiPerfect reagent in 100µl of serum-free media at a final concentration of 5nM-75nM and incubated for 10 minutes at room temperature. During the incubation period, hASM cells were harvested and split into 48-well dishes ($5x10^5$

cells/well in a volume of 250ul) and 100µl of siRNA complex was added to each well. Cells were incubated for 24hrs or longer as (indicated in the figure legends) and analyzed by real-time (RT)-PCR as described below.

Real-time PCR

Total RNA was isolated from hASM cells using the RNeasy QIAGEN kit (QIAGEN) and cDNA was prepared from 1µg of RNA by reverse-transcription using the Quantitect Reverse Transcription kit (QIAGEN) according to the manufacturer's instructions. Real-time PCR was performed using Platinum SYBR Green Supermix-UDG with ROX PCR mix (Invitrogen, Burlington, ON, CA). Briefly, 5µl of SYBR-green Supermix, 2.55µl of H_2O, 1.25µl of primer sets and 1.25µl of cDNA was mixed with a final reaction volume of 10µl and PCR amplification was carried out in 384-well plates in an Applied Biosystems 7900HT real-time PCR machine (Applied Biosystems, Foster City, CA, USA). Relative expression was determined using the $2^{(-\Delta\Delta Ct)}$ method (Bookout & Mangelsdorf 2003) and by normalizing to β-actin expression levels. Primer sequences used are: hABCA1 Forward 5'-CAGGTGAAAGGCTGGAAACT-3'; hABCA1 Reverse 5'- GGCAAGACAATCTGAGCAAA-3'; hABCG1 Forward 5'-GGTGTCCTGCTACATCATGC-3'; hABCG1 Reverse 5'-TCTGCCTTCATCCTTCTCCT-3'; hβ-actin Forward 5'-CACTCTTCCAGCCTTCCTTC-3'; hβ-actin Reverse 5'-GGATGTCCACGTCACACTTC-3'; hSR-BI Forward 5'-

GTACAGGGAGTTCAGGCACA-3'; hSR-BI Reverse 5'-GAACTGGAAGGTGCGGTACT-3'.

Cholesterol Efflux

Cholesterol efflux assays were carried out as previously described (Delvecchio et al 2007) with minor modifications. Briefly, hASM cells were split into 48-well dishes at a concentration of $5x10^5$ cells/well and allowed to adhere overnight followed by incubation for 48hrs in DMEM + 10%FBS + ^{3}H-cholesterol (5μCi/ml). Cells were then washed and incubated for an additional 18hrs with equilibration medium (DMEM + 2% BSA) supplemented with T1317 (1μM), 9-cisRA (1μM) and probucol (10μM) where indicated. Efflux was initiated by the addition of efflux medium (DMEM + 0.2% BSA) plus either, vehicle, apoAI (50μg/ml) or HDL (50μg/ml) where indicated. Radioactivity was measured by scintillation counting and cholesterol efflux was calculated by dividing the amount of ^{3}H-cholesterol in the media by the total ^{3}H-cholesterol associated with the cells plus media. For efflux experiments using cells that were transfected with siRNA, cell plating and transfections were done on the same day and the siRNA complex was incubated with the cells overnight. The following day, the media was removed, and replaced with labeling media and incubated for 48hrs as described above.

Western Blot Analysis

hASM cells were transfected with siRNA (50nM) as indicated in the figure legends, and cholesterol efflux assays were carried out as described above. Total protein was then isolated using 1% triton X-100 detergent and Western blot analysis was carried

out (25μg total protein for each sample) using a commercially available kit (Amersham, Baie D'urfe, QC, CA) according the manufacturer's instructions. Following transfer to nitrocellulose, blots were incubated with rabbit anti-ABCA1 (1:1000) (Novus Biologicals, Littleton, CO, USA) or rabbit anti-ABCG1 polyclonal antibody (1:2000) (Novus Biologicals) for 1hr followed by goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:5000) (Amersham) for an additional hour and visualized by enhanced chemiluminescence. Blots were probed with rabbit anti-β-actin as a loading control.

Cytoxicity Assay

hASM cells (2.0 x 10^5 cells/well) were seeded into 96-well dishes and allowed to adhere overnight. Cells were either untreated or treated with probucol (10μM) in the absence or presence of T1317/9-cisRA (1μM each) for 18hrs in 2% BSA-RPMI. Cells also received equivalent levels of vehicle (DMSO). Cell viability was measured using the Cell Counting-8 kit according to manufacturer's instruction (Dojindo, Rockville, MD, USA). Colorimetric absorbance readings were performed at 450nm on a SpectraMax Plus plate reader (Molecular Devices, Amersham Biosciences, Baie D'Urfe, QU, CA).

Phospholipid Efflux

Phospholipid efflux assays were carried out as previously described (Waddington et al 2005). Briefly, hASM cells were split into 48-well dishes at a concentration of $5x10^5$ cells/well and allowed to adhere overnight. The media was then replaced by DMEM + 2% BSA + ^{3}H-choline (5μCi/ml) and cells incubated for 48hrs. Cells were

washed and incubated for an additional 18hrs in DMEM + 2% BSA supplemented with T1317 (1μM) and 9-cisRA (1μM) where indicated. Phospholipid efflux was initiated by the addition of efflux medium (DMEM + 0.2% BSA) plus either, vehicle, apoAI (50μg/ml) or HDL (50μg/ml) where indicated in a total volume of 250μl. Supernatants (200μl) were collected after 5hrs and lipid was extracted using Folch mixture (chloroform:methanol (2:1)) and measured by scintillation counting. Lipids were extracted from cell monolayers using the hexane:isopropanol method as described (Waddington et al 2005). Phospholipid efflux was calculated as the amount of extracellular ^{3}H-choline / total ^{3}H-choline associated with the lipid fraction of the cells plus media.

Cholesterol loading and unloading with methyl-β-cyclodextrin

hASM cells were plated in 6-well dishes and allowed to adhere overnight, transfected with the indicated siRNA as described above and serum starved for 24hrs in 0.2% BSA-RPMI. Cells were then incubated with methyl-β-cyclodextrin (MβCD, 5μg/ml) or methyl-β-cyclodextrin in complex with cholesterol (5μg/ml) (Sigma) in 0.2% BSA-RPMI for an additional 24hrs where indicated in the figure legends. RNA was isolated and quantified by RT-PCR as described above.

Analysis of HDL and ApoAI preparations by FPLC

Purified HDL and pure apoAI described above were separated by gel filtration chromatography using an AKTA FPLC with a Superose 6 HR 10/30 column and eluted into eighty 250μl fractions (Rigotti et al 1997). Fractions were analyzed for total

cholesterol using the Infinity Cholesterol Liquid Stable Reagen Kit according to manufacturer's instructions (Thermo Electron, Pittsburgh, PA, USA). HDL and free apoAI fractions were also analyzed for apoAI content by western blot as described above. Briefly, 10µls of each fraction was separated by SDS-PAGE and transferred to nitrocellulose. Blots were incubated with goat anti-apoAI (1:10000) (Midland Bioproducts, Boone, IA, USA) for 1hr followed by rabbit anti-goat horseradish peroxidase-conjugated secondary antibody (1:10000) (Amersham) for an additional hour and visualized by enhanced chemiluminescence.

Statistical Analysis

Unpaired *t* tests were used for comparison of groups. $P < 0.05$ was considered significant.

4.3) Results

ABCA1, but not ABCG1, mediates LXR-stimulated cholesterol efflux to apoAI and HDL

The previous chapter describes that activation of LXR in hASM cells induces expression of ABCA1 and ABCG1 and stimulates cholesterol efflux to apoAI and HDL. To determine if reverse cholesterol transport in hASM cells requires ABCA1 and/or ABCG1, and, if so, to assess the contribution of each receptor in this process, knockdown strategies were employed using small interfering RNAs (siRNAs) to specifically reduce the expression of ABCA1 or ABCG1 and tested the effects on cholesterol efflux. As previously demonstrated, cholesterol efflux in ASM cells, using apoAI as an acceptor,

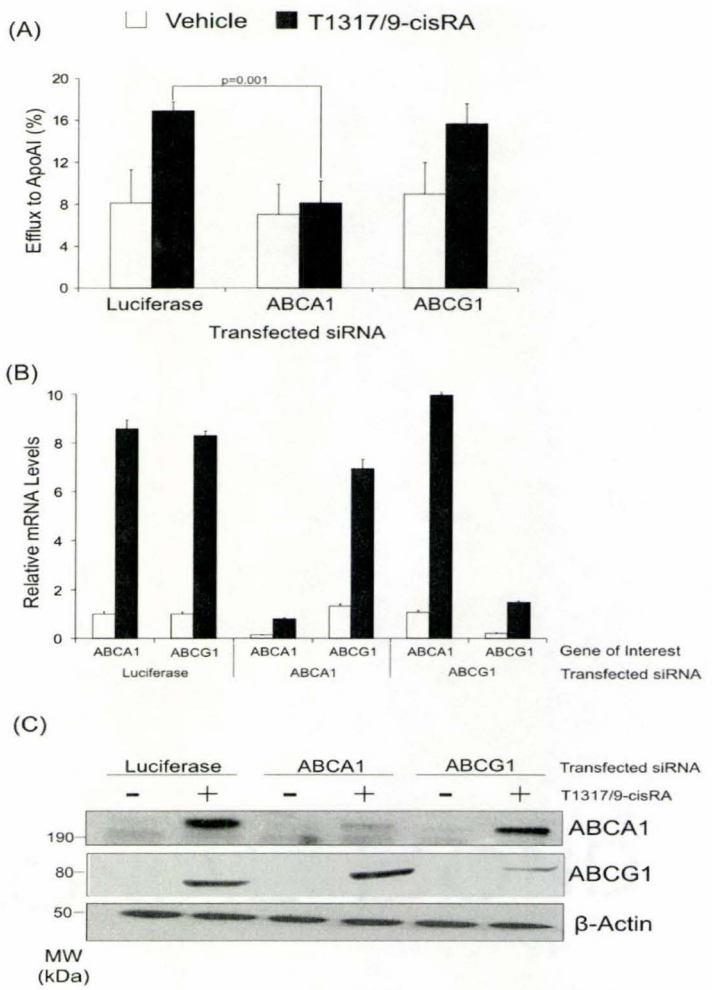

Figure 23 – ABCA1 expression, but not ABCG1, is required for LXR-induced cholesterol efflux to apoAI. (A) hASM cells were transfected with the indicated siRNA (50nM) and subsequently labeled with 3H-cholesterol in the presence or absence of LXR/RXR ligands T1317 (1μM) and 9-cisRA (1μM). Cholesterol efflux was initiated by the addition of apoAI (50μg/ml) and following incubation for 5hrs, supernatants were collected and extracellular cholesterol was measured by scintillation counting as described in Material and Methods. Luciferase siRNA was used as the negative control. The values are expressed as the percentage of cholesterol in supernatants relative to total cholesterol associated with the cells plus supernatants and are the average of three experiments done in triplicate (+/- S.D.). (B) Cells were transfected with the indicated siRNA and treated as described in (A) and total RNA was isolated and quantified by real-time PCR. The data represent the average of triplicates (+/- S.D.) normalized using β-actin as an internal standard and taking untreated control luciferase siRNA as 1. (C) hASM cells were transfected with the indicated siRNA and treated as described in (A). Protein extracts were prepared and analyzed by Western blot with antibodies specific for human ABCA1, ABCG1 and β-actin as shown.

was increased 2-fold over control levels in the presence of the LXR ligand T1317 and 9-cisRA, a ligand for the LXR obligate heterodimerization partner retinoic-X-receptor (RXR) (Fig. 23A). Knockdown of ABCA1, but not ABCG1, blocked all LXR-ligand induced cholesterol efflux to apoAI acceptor. To determine the efficiency and specificity of knockdown of ABC transporters, RNA and protein was isolated from cells treated in parallel during the cholesterol efflux assays and analyzed expression levels by RT-PCR and western blot analysis. As shown in Fig.23, siRNAs targeted to ABCA1 or ABCG1 showed specific and efficient knockdown of each respective transporter at both the RNA (Fig. 23B) and protein levels (Fig. 23C). In each case, LXR-dependent induction over control levels was reduced 85-95%. These above findings establish that ABCA1 is necessary and sufficient for LXR-mediated cholesterol efflux to apoAI.

Previous reports have suggested that in macrophages, ABCG1 is responsible for preferential cholesterol efflux to the lipid-rich acceptor HDL. To determine if this is also the case in hASM cells, the above experiment was repeated with HDL in place of apoAI. As shown in Fig. 24A, LXR activation increased cholesterol efflux to HDL by 2-fold over control levels. Surprisingly, knockdown of ABCG1 did not alter the ability of hASM cells to efflux cholesterol to HDL (Fig. 24A). Thus, in contrast to what has been reported in the macrophages (Ito 2003; Out et al 2006; Vaughan & Oram 2005; Wang et al 2006b), HDL does not serve as a cholesterol acceptor for ABCG1-mediated cholesterol efflux in hASM cells. In order to identify which transporters were responsible for cholesterol efflux to HDL, the role of the scavenger receptor B1 (SR-BI) and ABCA1, both of which have also been reported to efflux cholesterol to HDL in macrophages, was investigated.

(Delvecchio et al 2007; Duong et al 2006; Out et al 2008). Knockdown of SR-BI (>80%) had no effect on efflux to HDL acceptors (Fig. 24B and C) whereas knockdown of ABCA1 blocked all LXR ligand-induced efflux to HDL (Fig. 24A). To ensure that the observed efflux was not due to contaminating free apoAI in the HDL preparations, HDL and pure apoAI protein preparations were fractionated by FPLC. ApoAI in the HDL preparations eluted in fractions rich in cholesterol and as a distinct and separate peak, with only minor overlap, compared to pure apoAI (not shown). These findings indicate that the majority of the apoAI in the HDL preparations was associated with HDL particles and not in free form. Furthermore, the observation that the raw percentage of cholesterol efflux observed with HDL preparations was greater compared to free apoAI (compare Fig. 23A vs Fig. 24A) despite using equivalent protein amounts (50μg/ml), indicates that ABCA1-dependent cholesterol efflux to HDL was not a result of contaminating free apoAI in the HDL preparations. The finding that ABCA1 mediated efflux to HDL was further confirmed by using an independent siRNA construct to reduce expression of ABCA1 (Fig. 24D) and by undertaking dose-response and time-course experiments.

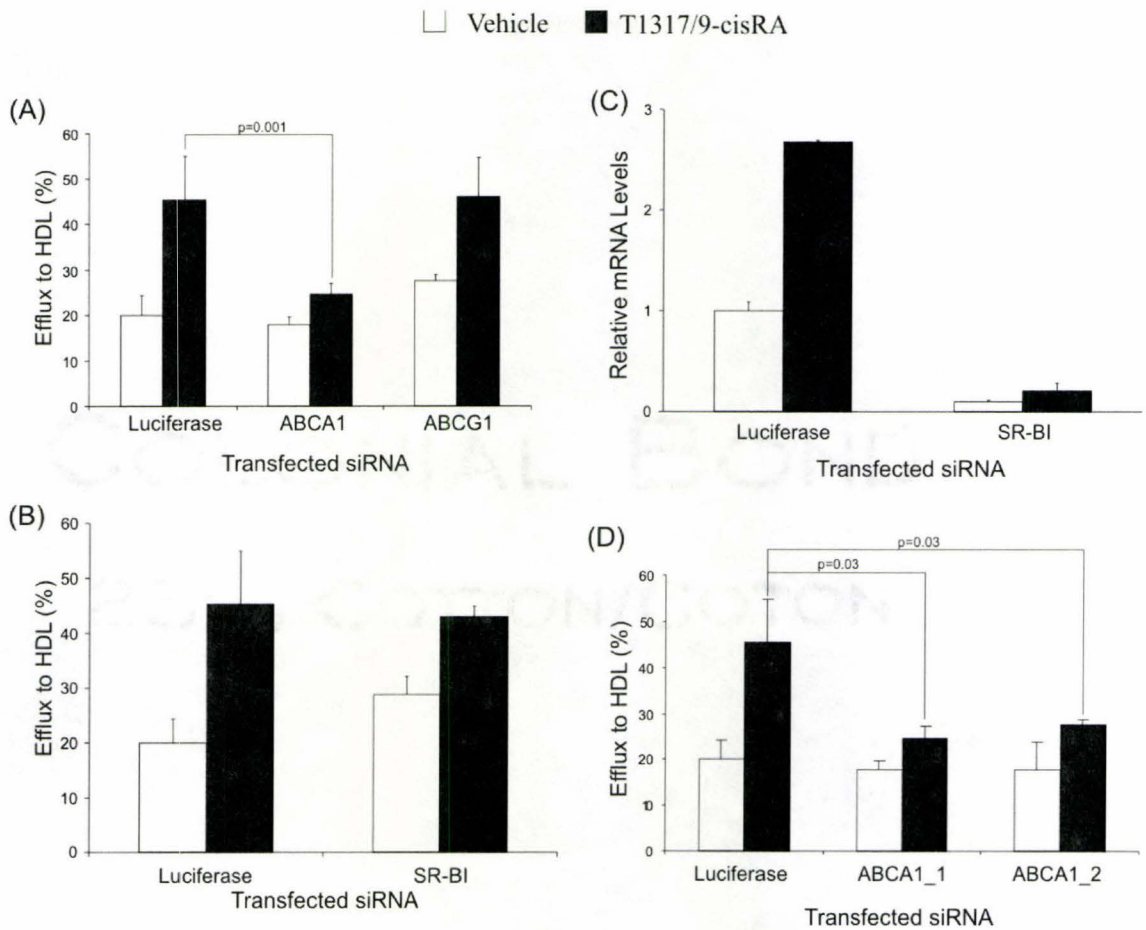

Figure 24 – ABCA1 expression, but not ABCG1, is required for LXR-induced cholesterol efflux to HDL. (A,B) hASM cells were transfected with the siRNA (50nM) targeting ABCA1, ABCG1, or SR-BI, as indicated, and subsequently labeled with 3H-cholesterol in the presence or absence of LXR/RXR ligands T1317 (1μM) and 9-cisRA (1μM). Cells were then incubated in the presence of HDL (50μg/ml) for 5hrs after which supernatants were collected and extracellular cholesterol was measured by scintillation counting as described in Material and Methods. (C) Cells were transfected with the indicated siRNA and treated as in (B) and total RNA was isolated and SR-BI levels were quantified by real-time PCR. The data represent the average of triplicates (+/- S.D.) normalized using β-actin as an internal standard and taking untreated control luciferase siRNA as 1. (D) Cells were transfected with either luciferase siRNA (negative control) or one of two siRNA constructs targeting non-overlapping sequences in ABCA1 mRNA as indicated and cholesterol efflux was measured as above. The values are expressed as the percentage of cholesterol in the supernatants relative to total cholesterol associated with the cells plus supernatants and are the average of three experiments done in triplicate (+/- S.D.).

As shown in Fig. 25, increasing amounts of ABCA1 siRNA blocked LXR-stimulated efflux in a dose-dependent manner, an effect that was evident within 1-2 hours (Fig. 26A and 26B). To independently confirm that ABCA1 is involved in cholesterol efflux to both HDL and apoAI, cells were incubated with probucol, a small molecule that has been shown to specifically block ABCA1-dependent cholesterol efflux, while having no effect on ABCG1 (Favari et al 2004). As shown in Fig. 27A, probucol, inhibited all LXR-mediated cholesterol efflux to both HDL and apoAI while having no affect on cell viability (Fig. 27B). The foregoing establishes that ABCA1 mediates all LXR ligand-induced cholesterol efflux in hASM cells to both apoAI and HDL acceptors and moreover, that ABCG1 is not involved in this process.

(A)

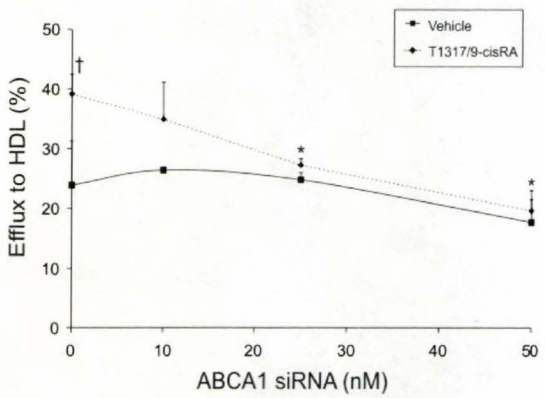

Figure 25 – LXR-induced cholesterol efflux to HDL correlates with ABCA1 expression in hASM cells. (A) hASM cells were transfected with the indicated concentration of ABCA1 siRNA and subsequently labeled with 3H-cholesterol for 48hrs. Cells were then treated with T1317 (1μM) and 9-cisRA (1μM) for an additional 18-24hrs in 2% BSA. Cholesterol efflux was initiated by the addition of HDL (50μg/ul) in 0.2% BSA, and following incubation for 5hrs, supernatants were collected and extracellular cholesterol was measured by scintillation counting as described in Materials and Methods. The values are expressed as the percentage of cholesterol in supernatants relative to total cholesterol associated with the cells plus supernatants. The data is representative of at least two experiments done in triplicate (+/- S.D.). *$p<0.05$ vs control indicated by †

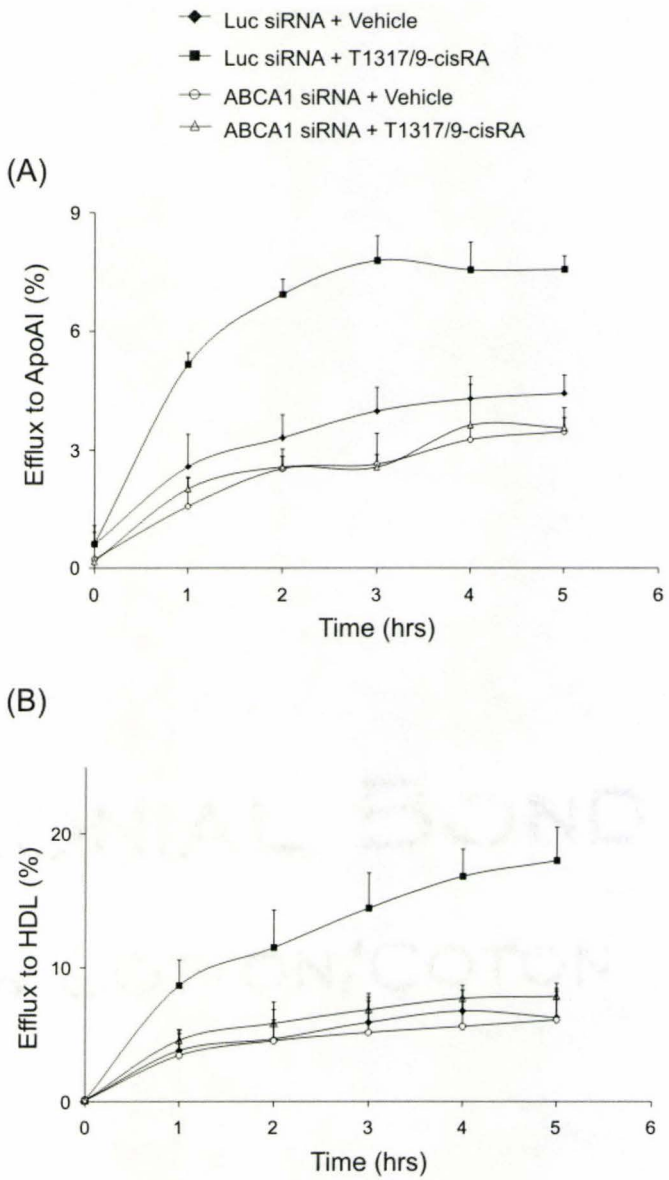

Figure 26 - Effects of ABCA1 knockdown on kinetics of cholesterol efflux in hASM cells. hASM cells were transfected with Luciferase (negative control) siRNA or ABCA1 siRNA, as indicated, and subsequently labeled with 3H-cholesterol for 48hrs. Cells were then treated with T1317 (1μM) and 9-cisRA (1μM) for an additional 24hrs in 2% BSA and cholesterol efflux was initiated by the addition of (A) apoAI (50μg/ml) or (B) HDL (50μg/ml) in 0.2% BSA and extracellular cholesterol was measured as described in Materials and Methods. The values are expressed as the percentage of cholesterol in supernatants relative to total cholesterol associated with the cells plus supernatants. The data for (A) is representative of 4 independent experiments done in triplicate and the data for (B) is representative of two experiments done in triplicate (+/- S.D.).

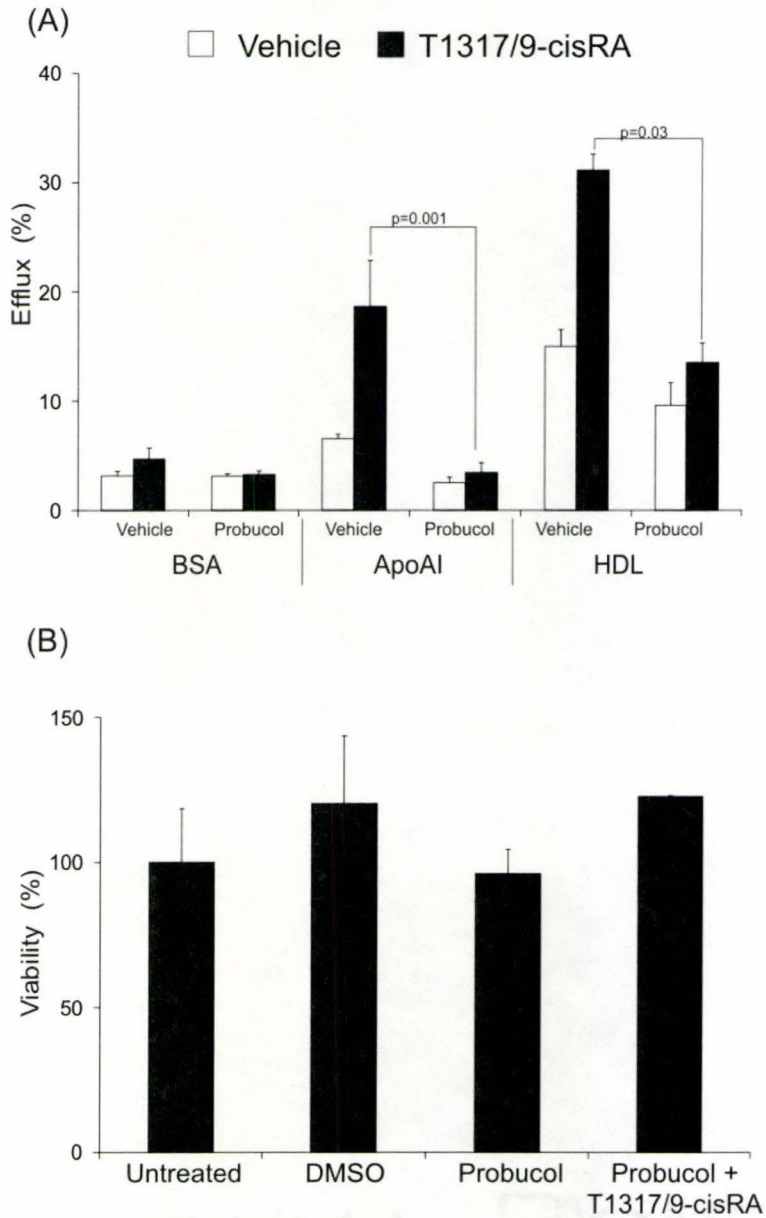

Figure 27 – Probucol treatment inhibits cholesterol efflux to apoAI and HDL in hASM. (A) hASM cells were plated in 48-well dishes and labeled with 3H-cholesterol for 48hrs. Cells were then treated with vehicle, T1317/9-cisRA (1μM each) and/or probucol (10μM), as indicated, for an additional 18hrs. Cholesterol efflux was initiated by the addition of 0.2% BSA, apoAI (50μg/ml) or HDL (50μg/ml), as indicated and extracellular cholesterol was measured as above. The values are expressed as the percentage of cholesterol in supernatants relative to total cholesterol associated with the cells plus supernatants. The data represents the average of three experiments done in triplicate (+/- S.D.). (B) hASM cells were plated into 96-well dishes and treated as described in materials and methods. Cell viability was measured in the presence of the indicated compounds and compared to untreated cells taken as 100%.

Phospholipid efflux in hASM is mediated by ABCA1

Phospholipids constitute approximately 80-90% of the lipid found in surfactant and moreover, play a crucial role in lipid-mediated signal transduction pathways in airway smooth muscle cells (Ryan & Spiegel 2008; Thomassen et al 2007). Since ABCA1 and ABCG1 knockout mouse models each display significant accumulation in phospholipid levels in the lung (Baldan et al 2006c; Bates et al 2005), we sought next to determine which transporter regulated PL efflux in airway smooth muscle cells. ABCG1 has been previously reported to promote phospholipid efflux, specifically sphingomyelin and phosphatidylcholine, to apoAI when over-expressed in human embryonic kidney (HEK293) cells, although others have shown no change in type II pneumocytes (Baldan et al 2006c; Kobayashi et al 2006). Additionally, ABCG1 is proposed to be the major phospholipid transporter in alveolar macrophages since elevated levels of ABCA1 cannot compensate for the loss of ABCG1 in alveolar macrophages isolated from *abcg1*$^{-/-}$ mouse models (Thomassen et al 2007). To determine if these pathways are conserved and to possibly assign a function for ABCG1 in hASM cells, we knocked down ABCA1 or ABCG1 and measured phospholipid efflux following incubation of cells with ^{3}H-choline. As shown in Fig. 28A and 28B, LXR agonists increased phospholipid efflux to apoAI and HDL by 2-fold. LXR-dependent efflux was inhibited in the absence of ABCA1 but not in the absence of ABCG1. These findings indicate that ABCA1, but not ABCG1, regulates phospholipid efflux in hASM cells.

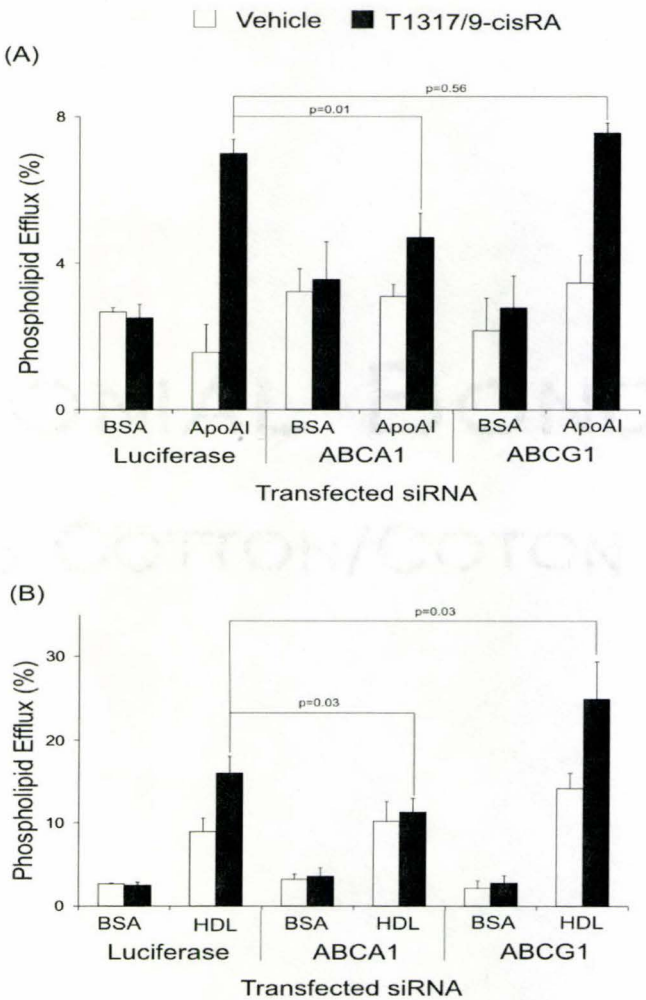

Figure 28 – ABCA1 expression is required for phospholipid efflux in hASM cells. (A,B) hASM cells were plated in 48-well dishes and transfected with the indicated siRNA. Cells were subsequently labeled with 3H-choline in the presence or absence of T1317 (1μM) and 9-cisRA (1μM). Phospholipid efflux was initiated by the addition of (A) apoAI (50μg/ml) or (B) HDL (50μg/ml), where indicated. Following incubation for 5hrs, lipids were extracted from supernatants and cell monolayers as described in material and methods and radioactivity was measured by scintillation counting. Values are expressed as the percentage of phospholipids in the supernatants relative to total phospholipids associated with the cells plus supernatants. The data is representative of at least two trials done in triplicate (+/- S.D.).

ABCA1 and ABCG1 expression is elevated upon cholesterol loading

Cells sense cholesterol and respond in part by inducing expression of ABCA1 and G1 transporters (Llaverias et al 2005). To determine if this is the case in hASM cells, cells were treated with cholesterol in complex with methyl-β-cyclodextran (MβCD), to load cells with cholesterol, or with MβCD alone, to deplete cells of cholesterol. As shown in Table 2, ABCA1 and ABCG1 expression levels were induced 11-fold and 18-fold, respectively, by cholesterol loading, while expression levels were decreased by 70-90% by cholesterol depletion, relative to controls. As expected, knockdown of LXRα/β isoforms blocked the induction of ABCA1 and ABCG1 following cholesterol loading (Table 2). Thus, both ABCA1 and ABCG1 mRNA levels are sensitive to cellular cholesterol levels. These findings imply that ABCG1 does in fact respond to changes in cholesterol levels in hASM cells; however, its precise physiological role remains to be determined.

Table 2 – ABCA1 and ABCG1 mRNA levels are increased by cholesterol loading and decreased by cholesterol depletion. hASM cells were transfected with the indicated siRNA and serum-starved for 24hrs followed by treatment with vehicle (0.2% BSA), methyl-β-cyclodextrin (MβCD) (5μg/ml) alone or MβCD in complex with cholesterol (MβCD:Chol) (5μg/ml) for an additional 24hrs. RNA was then isolated and levels of ABCA1 and ABCG1 were quantified by real-time PCR. The data represents relative fold changes and are the average of triplicates (+/- S.D.) normalized using β-actin as an internal standard with control vehicle treated taken as 1. *p<0.05 vs control indicated by †

Gene of Interest	*0.2% BSA*		*MβCD*		*MβCD:Chol*	
siRNA	**Luciferase**	**LXRα/β**	**Luciferase**	**LXRα/β**	**Luciferase**	**LXRα/β**
ABCA1	**1**† (+/- 0.03)	**0.70** (+/- 0.09)	**0.14*** (+/- 0.02)	**0.15*** (+/- 0.03)	**11.00*** (+/- 0.62)	**1.03** (+/- 0.01)
ABCG1	**1**† (+/- 0.11)	**0.13*** (+/- 0.02)	**0.07*** (+/- 0.01)	**0.04*** (+/- 0.67)	**18.00*** (+/- 0.68)	**1.57** (+/- 0.19)

4.4) Discussion

A correlation between high cholesterol levels and AHR has raised many questions in regards to the molecular mechanisms mediating this response and the physiological relevance. Our recent findings that LXR agonists reciprocally regulate the expression of multiple inflammatory mediators in ASM cells *in vitro* as well as promote reverse cholesterol transport through the induction of ABC transporters suggests a possible molecular link between airway inflammation and cholesterol homeostasis. However, the role of lipid and cholesterol homeostasis in airway smooth muscle function is not understood and further studies are warranted. In contrast, lipid homeostasis plays a well established role in *vascular* smooth muscle biology, where elevated levels of intracellular cholesterol and oxidized phospholipids, derived from uptake of oxidized (ox) LDL through scavenger receptors such as SR-BI and CD36 (Boullier et al 2001), induces a pro-inflammatory reaction by vascular cells that can eventually lead to atherosclerotic lesion development (Pidkovka et al 2007; Rong et al 2003). It is interesting to note that asthma is in many ways a disease analogous to atherosclerosis in that both diseases are characterized by chronic inflammation and involve increased smooth muscle hyperplasia and hypertrophy, pro-inflammatory gene expression, lesion development, extracellular matrix remodeling, and infiltration of inflammatory leukocytes. Analogous to atherosclerotic lesions, the airways of asthmatic patients display endothelial and microvascular damage which is thought to expose the lung to blood plasma and its constituents including lipoprotein particles such as LDL (Reynolds et al 2002). Thus, the

asthmatic airway may be subject to a similar inflammatory microenvironment as vascular cells following endothelial damage.

Using siRNA knockdown approaches, we have shown that ABCA1 is necessary and sufficient for all LXR-ligand induced cholesterol and phospholipid efflux in hASM cells whereas ABCG1 appears not to be required. We further demonstrate that ABCA1 effluxes cholesterol to both apoAI and HDL acceptors, in concordance with others who have shown, in endothelial cells, that ABCA1 but not ABCG1 mediates cholesterol efflux to HDL (Lin et al 2007; O'Connell et al 2004). We confirmed these results using probucol, a small molecule inhibitor specific for ABCA1 as demonstrated by the ability of this compound to inhibit cholesterol efflux in normal fibroblasts but not in fibroblasts derived from Tangier's patients which lack ABCA1 (Favari et al 2004). The raw percentages of efflux observed from hASM to both apoAI (4-14%) and HDL (30-40%) are also highly consistent with previous reports in other cell types including macrophages (Favari et al 2004; Lin et al 2007). Recently, Mukhamedova et al. (Mukhamedova et al 2008) using *in vitro* labeled cells injected into mouse models have shown a correlation between *in vitro* cholesterol efflux percentages and *in vivo* reverse cholesterol transport providing evidence that hASM cells potentially function *in vivo* to regulate reverse cholesterol transport.

Recently, cholesterol mass efflux to HDL was reported to be dramatically reduced in peritoneal macrophages isolated from *abca1*$^{-/-}$ mice however, *abcg1*$^{-/-}$ mice also displayed reduced capacity to efflux cholesterol to HDL, suggesting that in macrophages, both transporters contribute to efflux to HDL (Out et al 2008). These findings point to a

possible cell-type specific role for ABCG1 and cholesterol efflux in macrophages compared to smooth muscle cells. Why airway smooth muscle cells differ from the macrophage in mechanisms of cholesterol efflux to HDL is currently unclear. The macrophage is a highly specialized cell able to phagocytose extracellular materials including apoptotic cells and surfactant (Cui et al 2007). Both processes dramatically increase intracellular sterol content and thus, alveolar macrophages have possibly evolved separate mechanisms to handle increased intracellular lipids (Cui et al 2007). Additionally, it is possible that macrophage cell types may express additional co-factor proteins that work in conjunction with ABCG1 to mediate efflux of lipids that are not present in hASM. Thus, for hASM cells, it is perhaps sufficient and efficient that ABCA1 can both lipidate apoAI particles to form nascent HDL and in addition, further lipidate HDL itself.

While ABCG1 is robustly induced in hASM cells by activation of LXR, its function in these cells remains unclear. Recent findings indicate that ABCG1$^{-/-}$ mice have elevated levels of multiple pro-inflammatory mediators in the lungs (Baldan et al 2008) and it was suggested that this was likely due to local elevated cholesterol levels. However, the progressive lipid accumulation observed in *abcg1*$^{-/-}$ mice can be reversed by bone-marrow transplantation of wild-type cells suggesting that hematopoietic ABCG1 is required for proper lipid homeostasis in the lung (Wojcik et al 2008). In agreement with these findings, we did not observe an effect on LXR-induced cholesterol transport after ABCG1 knockdown in ASM, a non-hematopoietic cell. Whether ABCG1 is linked to roles unrelated to direct lipid efflux in hASM cells (eg. intracellular lipid trafficking)

remains to be tested. Recently, transforming growth factor β (TGFβ) signaling has been shown to specifically increase the expression of ABCG1 and not ABCA1 (Antonson et al 2008). Since TGFβ plays a fundamental role in ECM production in hASM cells, it is possible that ABCG1 has a function in this context (Makinde et al 2007; Parameswaran et al 2006).

These findings raise several questions as to the role of cholesterol efflux and metabolism to AHR *in vivo*. While we, and others, have shown that LXR agonists reduce the inflammatory response in multiple lung cell types (Delvecchio et al 2007; Smoak et al 2008), a direct correlation between reverse cholesterol transport and AHR remains to be determined *in vivo*. Baldan et al. have proposed that cholesterol itself is pro-inflammatory (Baldan et al 2008) and thus, elevated levels may exacerbate an inflammatory milieu thereby accelerating the remodeling process occurring in the asthmatic lung. LXR's beneficial effects may thus be partially due to efflux of excess cholesterol for transport back to the liver and eventually excretion via an ABCA1-dependent mechanism. In support of this, statin therapy, a cholesterol lowering drug, is recently reported as a potential therapeutic avenue for the treatment of lung diseases (Chiba et al 2008; Kim et al 2007a; Paraskevas et al 2007).

In summary, this chapter aims to demonstrate that LXR-ligand induced cholesterol and phospholipid efflux in airway smooth muscle cells is mediated exclusively by ABCA1 and that elevated levels of cholesterol affect biological processes that are highly relevant to the pathophysiology of asthma. The findings may thus have relevance to understanding the molecular mechanisms that link obesity and

hypercholesterolemia to airway diseases such as asthma. Future studies investigating the mechanisms of high cholesterol diet on AHR and the effects of LXR-activation *in vivo* will provide evidence to support cholesterol lowering as a potential therapeutic avenue in treating asthma in hypercholesterolemic and obese patients.

CHAPTER FIVE

Extension of this work and concluding remarks

5.1) Contribution of thesis to the field of nuclear hormone receptor biology

The studies described in this thesis add to the growing knowledge of LXR biology. Given the central role of post-translational modifications such as ubiquination, sumoylation and phosphorylation on protein function, work at the onset of this thesis focused on signalling pathways that impinge on LXR function. Activation of PKC signalling pathways resulted in an attenuation of LXR transactivation both on canonical LXR reporter genes and endogenous target genes ABCA1 and SREBP1c. The discovery adds to the short but growing list of signal transduction pathways that modulate LXR transcriptional activity. Moreover, this finding has strong implications in diseases where aberrant PKC activation is observed including diabetes and atherosclerosis (discussed further below).

While pursuing LXR protein regulation, a growing body of evidence suggested that non-steroidal NHRs, such as PPARs, play fundamental roles in lung pathophysiology. PPARs exerted anti-inflammatory effects *in vitro* and in animal models of asthma. Furthermore, PPARs decreased ASM proliferation and migration and increased phagocytosis in alveolar macrophages. Due to the functional and mechanistic similarities of PPARs and LXRs, we logically pursued the role of LXR in ASM, an important effector cell in lung diseases such as asthma. As a result, our focus shifted from LXR protein regulation to LXR's anti-inflammatory role in ASM. For the first time, LXR expression and function was assessed in human airway smooth muscle. Moreover, many features in the asthmatic lung, such as inflammation, migration and proliferation,

considered important for disease progression, are reduced in cultured ASM in the presence of LXR agonists. These findings provide strong support for future *in vivo* investigations of LXR function.

Lastly, analysis of LXR target gene function, namely ABCA1 and ABCG1 was assessed for the first time in hASM. This work is among the first to analyze the contribution of different cholesterol efflux pathways in smooth muscle, both airway or vascular. The unique discoveries described here indicate that ABCA1 and ABCG1 may play cell-type specific roles (discussed further below). Furthermore, the findings have strong implications with regards to metabolic disorders and lung lipid homeostasis. Based on findings described above, work derived from this thesis adds LXR as an emerging player in lung homeostasis and may pave the way for synergistic drug treatments to ameliorate lung pathophysiological conditions with an emphasis on asthma.

5.2) Post-translational regulation of LXR and physiological hypotheses

The findings in Chapter 2 lead to the conclusion that activation of PKC signalling pathways decrease LXR's transcriptional activity. While this work provides a valuable contribution to our understanding of LXR regulation, much work remains to determine the *in vivo* relevance. The physiological roles of PKC isoforms are vast and include, among others, the survival, growth and proliferation of cells (Battaini & Mochly-Rosen 2007). Moreover, multiple diseases, such as diabetes mellitus and its associated vasculature complications, also display aberrant PKC signalling which is linked to disease progression (Das Evcimen & King 2007; Noh & King 2007). Certainly, LXRs play a key role in all of these processes where they regulate hypertension, glucose metabolism,

atherosclerosis plaque progression as well as cell proliferation (Kim et al 2009; Meng et al 2009; Morello et al 2005; Vedin et al 2009). Thus, signalling cross-talk between PKC and LXR may function in normal physiology as well as in the pathophysiological state.

As discussed in Chapter 2, PKA-LXR-PKC may form a feedback loop in the kidney to regulate the expression of *renin*, an important enzyme involved in hypertension. However, the opposing roles of PKC and LXR in cell cycle regulation may also warrant further investigation. In this respect, recent work in T-cell proliferation showed that cells decrease LXRs transcriptional activity, down-regulate protein and mRNA levels of cholesterol efflux proteins (eg. ABCA1 and ABCG1) and increase the expression of SREBP2, a master regulator of transcription of cholesterol biosynthetic genes. The authors propose that this may be a general phenomenon for all cell types since, as expected, cells would require sufficient cholesterol levels for proper membrane integrity (Bensinger et al 2008). Therefore, under normal and appropriate physiological conditions, PKC may partially function to regulate LXR activity resulting in sufficient intracellular cholesterol accumulation that allows for cell cycle progression.

It should also be noted that PKC activation can also increase the half-life of ABCA1 protein via direct phosphorylation and increase cholesterol efflux which is counterintuitive to our findings (Kiss et al 2005; Yamauchi et al 2003). In both cases, the cells used (fibroblasts and macrophages) were either grown to confluency or terminally differentiated and therefore, were not proceeding through the cell cycle. Furthermore, Kiss et al. treated cells with PKC inhibitors in the absence of any PKC activators and therefore, only basal PKC activity was analyzed (Kiss et al 2005). Thus, the precise

timing and level of PKC activation, as well as co-stimulatory cues and cell type, may influence the outcomes of PKC activation on LXRs transcriptional activity.

Under a disease state where aberrant PKC signalling is observed, such as in diabetes mellitus, different roles of PKC-LXR crosstalk may emerge. Diabetic patients are more susceptible to cardiovascular disease partially as a result of advanced glycation end-products (AGEs). AGEs activate cell surface receptors such as RAGE expressed in the macrophage that, in turn, increase DAG formation and PKC activation (Beauchamp et al 2004). Downstream effects include increased extracellular matrix production, increased inflammatory gene expression as a result of NF-κB activation and increased oxidative stress that exacerbates the inflammatory response. More specifically related to LXR, AGEs decrease cholesterol efflux in the macrophage (Isoda et al 2007). Thus, in a disease state, AGE products may produce a unique signalling signature that may be transduced via PKC proteins to decrease LXRs transcriptional activity thereby reducing cholesterol efflux in the arterial intima and increasing plaque formation and progression. *In vivo* models of atherosclerosis or diabetes administered PKC inhibitors and their effects on LXR target gene expression may provide further evidence for a role for PKC-LXR crosstalk.

Although the role of PKC in ASM cells and the potential impact on LXR signalling was not tested, it is interesting to note that PKC also plays a major role in lung disease such as asthma (Dempsey et al 2007). Human ASM express multiple PKC isoforms, including PKCα and β (Pang et al 2002), that are activated in the presence of inflammatory stimuli. PKCα, in particular, is thought to be important for human airway

smooth muscle proliferation (Dempsey et al 2007). Thus, our findings in Cos-1 and HEK293 cells may potentially be transferred to ASM smooth muscle where LXR is anti-proliferative in nature (Chapter 3).

5.3) The role of LXR in lung biology and potential as a therapeutic target for inflammatory lung disease

The non-contractile roles of ASM are increasingly recognized as important modulators of the inflammatory and remodelling process that occurs in asthmatic airways. As such, ASM cells are one of many targets for the anti-inflammatory corticosteroids used as treatment during asthmatic attacks. However, continued use of steroids has severe side effects and long term complications such as growth inhibition and osteoporosis and thus, novel therapeutic targets are needed to treat lung disease. The work described in this thesis establishes, for the first time, a role for LXR signalling in ASM cells. It is shown that LXR agonists significantly reduce three hallmarks of asthma: 1) proliferation 2) inflammatory gene expression and 3) migration. Importantly, the efficacy of LXR agonists was comparable to dexamethasone, a corticosteroid used as a positive control in our experiments.

It is noteworthy that multiple studies are now reporting a role for LXR in multiple lung cells types and has been proposed as a novel therapeutic target in numerous reviews (Birrell et al 2007; Farrow 2008; Nomiyama & Bruemmer 2008; Smoak et al 2008; Wojcicka et al 2007). One report however, indicates that *in vivo* administration of LXR agonists increases the severity of asthma by *increasing* smooth muscle mass *in vivo* and

stimulating hASM proliferation (Birrell et al 2008). This is in direct contrast to our findings as well as multiple other groups (Bensinger et al 2008; Blaschke et al 2004; Chuu et al 2007a; Chuu et al 2006; Chuu et al 2007b; Delvecchio et al 2007; Kim et al 2009; Meng et al 2009; Vedin et al 2009). In the paper by Birell et al., the authors suggest that GW3965 and T1317 dose-dependently increases human airway smooth muscle proliferation and increases smooth muscle mass in the airways of allergic asthma mouse models. However, different from the analysis described here, they do not assess proliferation in the presence of a mitogen (eg. PDGF) nor do the authors analyze cell cycle directly, but rather by counting total cell numbers, an indirect measure of cell cycle progression. Our analysis, like that of many others, uses bromodeoxyuridine (BrdU) incorporation assays which indicates that the cells have passed through S-phase, a hallmark of the cell cycle. We observe a decrease in BrdU incorporation in the presence of LXR agonists following challenge of ASM cells with PDGF. Based on previous research, LXR agonists inhibit vascular smooth muscle cell cycle progression in the G_1 phase, consistent with our findings (Blaschke et al 2004). During the course of this research, LXR agonists alone were never observed to increase proliferation of ASM cells. It is possible that, due to the very small increase in cell number induced by GW3965 described by Birell et al., LXR agonists induce proliferation to a degree that was below the BrdU assay detection limit however, again, this is contrary to numerous reports. Nevertheless, an increase in smooth muscle mass *in vivo* may be due to other phenomenon such as decreased apoptosis. Indeed, LXR agonists have been shown to be anti-apoptotic (Arai et al 2005; Valledor et al 2004). Since ASM cells are continually

undergoing a balancing act between cell death and survival, LXR agonists may promote the latter *in vivo*. More detailed analysis of LXR's role in asthmatic airway is thus warranted to delineate LXR function *in vivo*. The advent of aerosolized synthetic LXR agonists may provide a better assessment of their potential role in inflammatory lung disease and limit other systematic factors that may influence smooth muscle biology. Overall, despite the report by Birell et al. 2008, others analyzing LXR's function in asthmatic mouse models have reported reduced inflammatory cytokine levels (see below) and decreased lymphocyte infiltration following allergic challenge warranting greater focus on LXR's function *in vivo* (Smoak et al 2008).

A hallmark of asthma, inflammation influences the lung remodelling process that occurs over time in asthmatic patients. With this, it is increasingly recognized that ASM plays a major role in responding to, and secreting, cytokines that in turn, recruit leukocytes to the airway and exacerbate the inflammatory process. The work described here also provides crucial evidence that LXR agonists are anti-inflammatory in hASM. Moreover, other groups have found similar results in alveolar macrophages and lung neutrophils indicating a more broad anti-inflammatory effect of LXR agonists in the lung (Birrell et al 2007; Smoak et al 2008).

The anti-inflammatory effects of LXR have been highly characterized and compared to other NHRs such as GR and PPAR in the macrophage (Ogawa et al 2005). Findings from these studies suggest that each receptor represses inflammatory gene expression in both an overlapping and distinct manner. Thus, work described here imply that combination therapy in the treatment of asthma or other inflammatory lung diseases

warrants further investigation as dual activation of GR and LXR may synergistically reduce inflammation at lower doses. This may prove beneficial in reducing the side effects observed with long term corticosteroid administration. Interestingly, a unique LXR agonist that maintains its anti-inflammatory properties but does not increase LXR target gene expression has been synthesized (Chao et al 2008). This molecule has great potential in reducing the expression of inflammatory mediators while preventing the potentially unpredictable systemic consequences of LXR target gene expression (ie. *de novo* lipogenesis). Further work to characterize this molecule will provide novel and exciting possibilities for LXR biology.

Lastly, migration of smooth muscle, a contributing factor to the increased muscle mass observed in the airways of asthmatic patients, was investigated. Work described in Chapter 3 indicates that LXR agonists dose-dependently decrease ASM migration. Recent findings in support of this show that LXR plays a crucial role in dendritic, neutrophil and even neuronal migration (Fan et al 2008; Geyeregger et al 2007a; Smoak et al 2008). The mechanisms remain to be elucidated but may involve the actin bundling protein Fascin-1 required for dendritic cell migration or decreased activation of RhoA (Geyeregger et al 2007a; Smoak et al 2008). Interestingly, Fascin-1 protein levels were reduced in a manner dependent on LXR expression, however, a modulation of Fascin-1 mRNA levels was not observed (Geyeregger et al 2007a). Therefore, LXRs role in abrogating migration may not be related to transcriptional mechanisms but rather post-translational regulation of Fascin-1. While the mechanisms remain elusive, a role for LXR in the cytoplasm, where LXR influenced the half-life of ABCA1 protein, has been

previously reported and represents an under investigated area of study (Hozoji et al 2008; Mo et al 2002). Alternatively, LXR may increase transcription of anti-migratory proteins that counteract the function of Fascin-1. Focused microarray analysis of migratory pathways comparing untreated ASM with ASM cells treated with LXR agonists may uncover novel LXR target genes that will explain the observed phenomenon.

5.4) Cholesterol efflux in human airway smooth muscle (ASM)

After preliminary analysis of cholesterol efflux in ASM (Chapter 3), studies were conducted to determine the contribution of ABCA1 and ABCG1 to this process. As described in Chapter 4, LXR-mediated cholesterol and phospholipid efflux is mediated exclusively by ABCA1. These studies have numerous implications in the association of asthma and obesity, where hypercholesterolemia is a feature. Multiple epidemiological studies have linked metabolic syndrome with increased risk of airway disease, however, the molecular mechanism remains poorly understood. A direct correlation with cholesterol levels and airway hyperresponsiveness has been observed both in animal models and human association studies however, more pertinent to the motivation behind this work, mouse models with genetic deletion of ABCA1 and ABCG1 show dramatic lung phenotypes (Baldan et al 2006b; Baldan et al 2006c; Bates et al 2005). Furthermore, these knockout animal models have increased expression of inflammatory cytokines and chemokines and it has even been proposed that cholesterol itself, when in excess, is pro-inflammatory in the lung (Baldan et al 2008). Thus, the question remained as to whether ASM cells contribute to cholesterol transport in the lung and whether they can respond to

elevated levels of cholesterol in a manner analogous to the macrophage. Indeed, the work described here indicates that ASM cells respond to elevated levels of cholesterol by upregulating ABCA1 and ABCG1 expression in a process dependent on LXR expression and may contribute to cholesterol elimination in the lung. However, ASM cells utilized unique pathways to efflux cholesterol to HDL in an ABCA1-dependent manner.

The exact mechanisms of ABCA1-mediated efflux in hASM, however, remain unclear. This controversy also extends to the macrophage where ABC-mediated transport of lipids is extensively studied. Based on findings here and elsewhere, a reassessment of the ABCA1/lipid poor apolipoprotein efflux model may be warranted. Currently, theories state that ABCA1 mediates efflux only to lipid-poor apolipoproteins such as apoAI and apoE while ABCG1 and SR-BI can promote efflux to lipid-rich particles such as HDL. Recent studies analyzing cholesterol efflux from macrophages derived from ABCA1/ABCG1 single and double knockout models also raise some doubt with this model. Out et al. show reduced capacity of *abca1*$^{-/-}$ knockout macrophages to efflux cholesterol directly to HDL (Out et al 2008). Furthermore, O'Connell et al. report no change in cholesterol efflux after ABCG1 overexpression in epithelial cells. Similar to findings described in this thesis, fibroblasts derived from Tangier's patients showed reduced efflux to both apoAI and HDL compared to normal fibroblasts (O'Connell et al 2004). These discrepancies differ from conclusions drawn from multiple studies, some of which were based on the function of ABC transporters in overexpression studies in commonly used cell lines such as 3T3 fibroblasts and HEK293 cells.

Two possibilities may therefore exist to explain these discrepancies: either cell-type specific effects alter the contribution of different efflux pathways or HDL preparations are varied from each study which influences the contribution of each transporter, thus indicating sub-classes of HDL are substrates of different receptors. The latter is proposed to be unlikely as we observed total repression of LXR-mediated efflux to HDL following ABCA1 knockdown. If a specific sub-class was a substrate for ABCG1, we would continue to observe a small response in cholesterol efflux levels as our HDL preparations should theoretically contain all HDL subclasses. Therefore, cell-specific roles for ABCA1 and ABCG1 transporters may occur. The pathways may involve specific intracellular co-factors or cell-specific membrane proteins that function with ABC transporters to influence the availability of cholesterol to plasmatic acceptors or binding of acceptors to the cell membrane. Overall, findings for ABCA1/G1 in specific cell-types cannot be translated to universal models and until we have a thorough understanding of the molecular mechanisms of ABCA1- and ABCG1-mediated transport, these questions will remain difficult to answer.

In a broader sense, the role of ABCA1 transporters, based on knockout murine models, is essential for proper lipid homeostasis in the lung. Thorough literature searches failed to uncover studies of lung function in patients who suffer from Tangier's disease and based on the findings described in this thesis, and elsewhere, this may warrant an assessment. This work hopefully also provides a basis for future molecular studies in the nascent and novel field investigating the role of cholesterol and lipid metabolism in the context of the lung.

Lastly, the role of ABCG1 remains speculative in hASM cells. Interestingly, this also extends to the macrophage where perplexing results involving ABCG1 knockouts and atherosclerotic plaque formation have arisen (Curtiss 2006). Using bone-marrow transplant studies, ABCG1$^{-/-}$ macrophages have reported to both increase and, unexpectedly, decrease the size of atherosclerotic plaques in LDLR$^{-/-}$ mouse models (Baldan et al 2006a; Out et al 2006). This unexpected decrease in plaque size has led to varying conclusions that support the findings including a role for ABCG1 in apoE secretion and apoptosis (Baldan et al 2006a). Others have proposed that disparate results may partially be due to the technical differences in the studies and the complex nature of plaque progression (Curtiss 2006). Furthermore, reports on whether ABCG1 can promote phospholipid efflux are also conflicting; while Kobayashi et al. show enhanced PC efflux, Baldan et al. report no change in PL transport mediated by ABCG1 (Baldan et al 2006a; Kobayashi et al 2006). Overall, the findings above, in addition to the contradicting studies on the localization of ABCG1 and lipid substrates, call for further biochemical analysis of ABCG1 function.

While it is was observed in hASM cells that ABCG1 is responsive to increased levels of cholesterol, it is unclear what function it plays in cholesterol transport, if any. As discussed in chapter 4, ABCG1 may be involved in intracellular transport however, a second possibility remains that ABCG1 may transport cholesterol derived from different sources than does ABCA1. Indeed, it has been suggested that cholesterol derived from oxLDL sources is not a substrate for ABCA1-mediated efflux (Favari et al 2005). Thus, it cannot be completely excluded that, under conditions where cholesterol is derived from

oxidized LDL via scavenger receptors or when cholesterol is overloaded, ABCG1 can act as a transporter to HDL particles. Based on the lung phenotypes in ABCG1$^{-/-}$ mice, further studies to delineate a role for ABCG1 in hASM cells are warranted.

5.5) Is LXR Really a Target to Treat Lung Disease?

The work described in this thesis outlines the regulation and roles of the nuclear hormone receptor LXR and constitutes a considerable advance in the field of NHR and lung biology. The framework for many future studies using *in vivo* animal models of asthma and lung disease is established here and provides a promising future for LXR as a therapeutic target in pulmonary pathophysiology. Thus, the question remains, does LXR truly represent an innovative pathway to treat inflammatory diseases such as asthma? It is interesting to note that asthma is in many ways analogous to atherosclerosis. Both diseases are characterized by increased leukocyte infiltration, tissue remodelling, smooth muscle migration and proliferation and inflammation. Due to the highly beneficial effects of LXR activation in reducing atherosclerotic plaque progression, LXR agonists may also prove to be a valuable therapeutic option for asthma in patients with or without metabolic disorders. Certainly, this thesis describes, in primary human airway smooth muscle, many advantageous actions of LXR agonists against hallmarks of asthma progression including proliferation, migration and inflammation. Additionally, other groups have analyzed the function of LXR in other lung cell types such as alveolar macrophages, neutrophiles, pneumocytes and T-cells in addition to mouse models of AHR reaching similar conclusions regarding LXR's potential use in inflammatory lung disease (Baldan

et al 2006c; Birrell et al 2007; Smoak et al 2008). Since NHR ligands are currently used to target other receptors in the lung, specifically in ASM, it is conceivable that LXR agonists in an aerosolized form could also target these cells as well as other cell types. Furthermore, an inhaled agonist with a short half-life could prevent secondary unwanted side effects that occur in the liver such as SREBP1c activation.

In conclusion, with growing resistance and adverse reactions in patients following prolonged administration of steroids, there is a call for novel therapies to work synergistically with, or independently of, glucocorticoids. With the body of evidence described in this thesis and elsewhere, LXR agonists are positioned to be the vanguard of future inflammatory lung disease treatments.

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