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Regulation of Secretory Phospholipase A2 by Thyroid Hormone

Pragya Sharma

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**REGULATION OF SECRETORY PHOSPHOLIPASE A2 BY THYROID
HORMONE**

A Dissertation
Presented for
The Graduate Studies Council
The University of Tennessee
Health Science Center

In Partial Fulfillment
Of the Requirements for the Degree
Doctor of Philosophy
From The University of Tennessee

By
Pragya Sharma
December 2013

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DEDICATION

This dissertation is dedicated to my beloved grandmother and parents.

ACKNOWLEDGEMENTS

First of all I want to thank the almighty for everything I have today. I would like to express my deepest gratitude to my advisor Dr. Edwards A. Park, for his intellectual guidance, patience and support throughout my graduate studies. I will like to thank Dr. Park for having faith in me and allowing me the freedom to pursue the project.

I want to express my sincere gratitude my committee members Dr. George A. Cook, Dr. Marshall B. Elam, Dr. Rao N. Gadiparthi and Dr. Roderick T. Hori for their valuable advice and suggestions.

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Finally, I would like to thank to my parents for everything. I owe a lot to them and words could not do justice for their unconditional love and support. I would like to thank my mother for believing in me more than myself. I would like to thank my husband Horrick, for being my inspiration and strength.

ABSTRACT

Rationale: Low grade inflammation has been correlated with elevated risk of hepatic steatosis and atherosclerosis. Secretory phospholipase A2 group IIA (PLA2g2a) enhances the progression of several chronic inflammatory diseases including arthritis and atherosclerosis. The potential linkage of hypothyroidism with inflammation led us to examine the modulation of sPLA2 expression by thyroid hormone (T₃) in liver.

Objective: Most of the studies of phospholipase A2 group IIA (PLA2g2a) have been conducted with macrophages and vascular smooth muscle cells with regard to atherosclerosis. The liver is one of the major contributors to the total pool of extracellular PLA2g2a. The aim of the present study was to characterize the regulation of PLA2g2a and other sPLA2 genes by T₃ in liver. The second aim was to identify the mechanism by which T₃ inhibits the expression of PLA2g2a gene.

Methods and Results: In the first part of my project, I assessed the effect of T₃ on sPLA2 gene expression in liver. I found that T₃ inhibited the expression of the PLA2g2a in human and rat hepatocytes. Thyroid hormone status regulated the expression of endogenous PLA2g2a in both rats and mice. Other sPLA2 isoforms including PLA2g1b, PLA2g3 and PLA2g5 were also suppressed by T₃ in liver. PLA2g2a was induced by cytokines and high fat diet. Both the cytokine and fatty acid mediated induction of PLA2g2a gene was blocked by T₃. In an effort to dissect the mechanism of repression by T₃, I cloned the PLA2g2a gene and identified a negative T₃ response element in the promoter. This thyroid receptor (TRβ) binding site differs considerably from consensus T₃ stimulatory elements. Using *in vitro* and *in vivo* binding assays, I demonstrated that TRβ bound directly to the PLA2g2a promoter. In present studies, I found that disruption of the corepressor binding site in TRβ decreased the T₃ inhibition of PLA2g2a. Conversely a mutation in coactivator binding site had no effect on the T₃ mediated inhibition of PLA2g2a. Knockdown of nuclear corepressor (NCoR1) or silencing mediator for retinoid and thyroid receptors (SMRT) by siRNA blocked the T₃ inhibition of PLA2g2a. Using chromatin immunoprecipitation assays, I showed that NCoR1 and SMRT were associated with the PLA2g2a gene in the presence of T₃.

Conclusion: The thyroid hormone status modulates the expression of PLA2g2a and other sPLA2 isoforms. Both hyperthyroid rats and mice had significantly lower levels of PLA2g2a as compared to hypothyroid rats and mice. My data suggest that the thyroid status modulates aspects of the inflammatory response. In contrast with the established role of T₃ to recruit coactivators to TRβ, my experiments demonstrate a novel inverse recruitment mechanism in which unliganded TRβ is stimulatory while liganded TRβ recruits corepressors to inhibit PLA2g2a expression. In conclusion, present study provides a cellular mechanism by which T₃ inhibits PLA2g2a expression.

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LIST OF ABBREVIATIONS

AD	Activation domain
AF-1	Activation function-1
ANGPLT3	Angiopoitin like-3
AP-1	Activator protein-1
CBP	CREB binding protein
C/EBPs	CCAAT enhancer binding protein
ChIP	Chromatin immunoprecipitation
cPLA2	Cytosolic phospholipase A2
CPT-1a	Carnitine palmitoyltransferase-1a
DBD	DNA binding domain
DR4	Direct repeat separated by four nucleotides
ELISA	Enzyme- linked immunosorbent assay
FAS	Fatty acid synthetase
GR	Glucocorticoid receptor
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDL	High density lipoprotein
HFD	High fat diet
IL-6	Interleukin-6
IPs	Inverted palindrome
LBD	Ligand binding domain
LDL	Low density lipoprotein
LPS	Lipopolysaccharides
Luc	Luciferase
LXR	Liver X receptor
Mut	Mutant
NCoR1	Nuclear receptor corepressors
NF-KB	Nuclear factor kappa beta
NR	Nuclear receptor
nTRE	Negative thyroid response element
p/CAF	p300/CBP-associated factor
PDK4	Pyruvate dehydrogenase kinase 4
PEPCK	Phosphoenolpyruvate carboxykinase
PGC-1	Peroxisome proliferator-activated receptor gamma coactivator 1
PLA2	Phospholipase A2
PLA2g1b	Phospholipase A2 group IB
PLA2g2a	Phospholipase A2 group IIA
PLA2g3	Phospholipase A2 group III
PLA2g5	Phospholipase A2 group V
PPAR	Peroxisome proliferator-activated receptor
pTRE	Positive thyroid response element
PTU	Propylthiouracil
RAR	Retinoic acid receptor

RLNE	Rat liver nuclear extract
RXR	Retinoid X receptor
siRNA	Small interference RNA
SMRT	Silencing mediator of retinoid or thyroid hormone receptor
sPLA2	Secretory phospholipase A2
SP-1	Specificity protein 1
SRC-1	Steroid receptor coactivator-1
SREBP-1c	Steroid receptor element binding protein -1c
T ₃	Triiodothyronine
TNF	Tumor necrosis factor
TR	Thyroid receptor
TRAP	Thyroid hormone receptor associated protein
TRE	Thyroid response element
TSH- α	Thyroid stimulating hormone α
TSH- β	Thyroid stimulating hormone β
VDR	Vitamin D receptor
VDRIP	Vitamin D receptor interacting protein
WT	Wild type

CHAPTER 1. INTRODUCTION

General Biology

The purpose of this chapter is to review the background information relevant to the research reported in the dissertation. These topics include thyroid hormone biology, thyroid hormone receptors types, coregulator proteins and the mechanisms of positive and negative gene regulation by thyroid hormone. Finally, a general overview of secretory phospholipases is provided.

Thyroid hormone (T_3) is secreted by the follicular cells of the thyroid gland. T_3 (L-3, 5, 3-tri-iodothyronine) is the derivative of the amino acid tyrosine, consisting of two tyrosine moieties covalently attached to iodine. There are two major forms of the thyroid hormone thyroxine (T_4) and T_3 . T_3 is the active form of thyroid hormone and is formed by the deiodination of T_4 by type I and type II deiodinase enzymes (Oetting & Yen, 2007; Yen, 2001). Recent studies have found that rT_3 , T_2 and other deiodinated thyroid derivatives have biologic activity (Moreno et al 2008). The structures of T_3 and T_4 are shown in **Figure 1-1**.

Modular Structure of Thyroid Hormone Receptor

The thyroid hormone receptor (TR) belongs to a family of nuclear receptors (NR). NRs are ligand dependent transcription factors which control gene transcription in response to hormone binding. The NR family is the largest family of transcriptional regulators consisting of 48 members in human. This superfamily serves as receptors for various lipophilic molecules including steroid hormones (estrogen, testosterone, glucocorticoids, mineralocorticoid, and vitamin D), thyroid hormone, retinoic acid, fatty acids and others. The TR and other nuclear receptors share similar functional domains (Steinmetz et al, 2001). As shown in **Figure 1-2**, the TR consists of four main domains:

N-Terminal A/B Domain

A/B is the most variable region among the TR isoforms in terms of the amino acid sequence and length. A constitutively active domain (AF-1) resides in A/B domain. AF-1 functions in a gene and cell specific manner. The role of the A/B domain in transcriptional activation by TRs is controversial.

DNA-Binding Domain (DBD)

The DBD is the most conserved domain of TRs. It is involved in identification and binding of TR to the thyroid response element. This region is located in the central portion of TR and is 66 amino acids long. It consists of two zinc fingers each containing

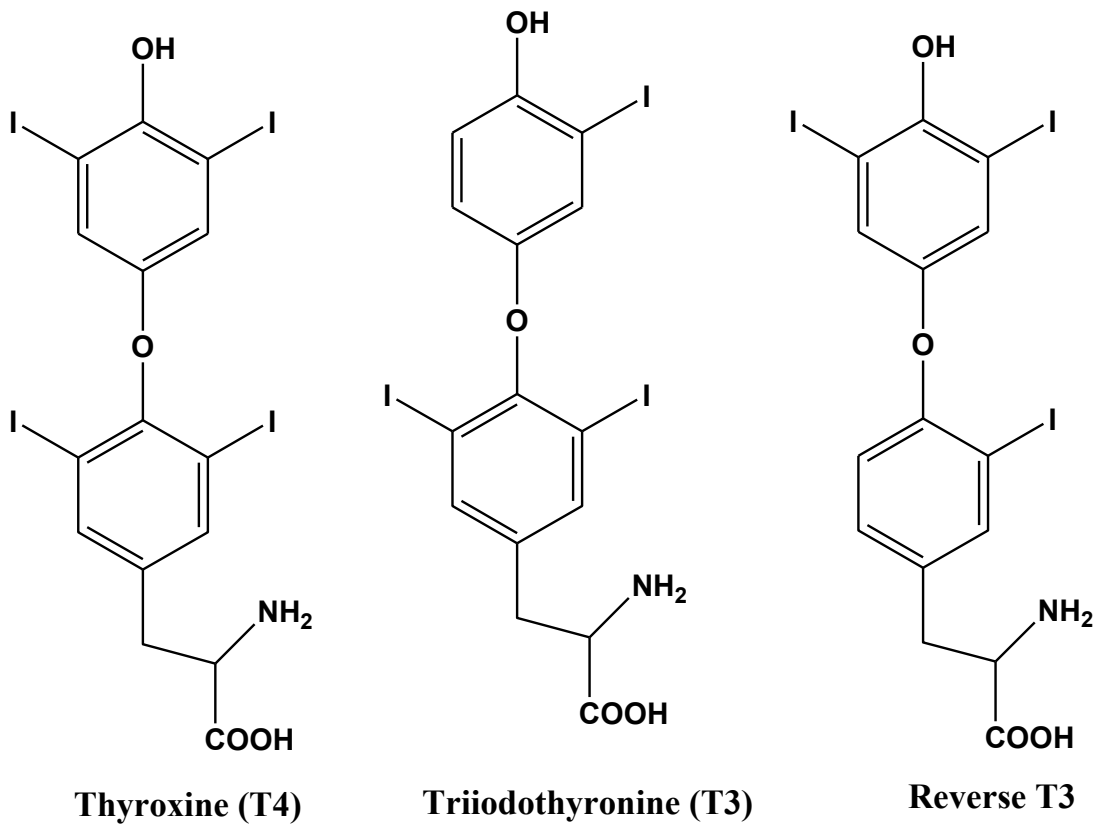


Figure 1-1. Structure of thyroid hormone

The thyroid hormone are basically two tyrosine moieties linked together with the addition of iodine at three or four positions. Different forms of thyroid hormones including T₄, T₃, and reverse T₃ are generated based on the number and position of the iodine group.

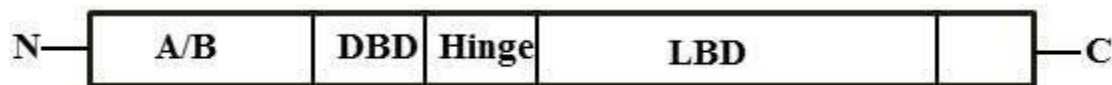


Figure 1-2. Modular structure of thyroid hormone receptor

TR is divided into four major region Activation function -1(A/B) domain, DNA binding domain (DBD), hinge region and Ligand binding domain (LBD).

four cysteines coordinated with Zn^{2+} ion. Within the first Zn finger there is P box, which is responsible for the identification of the thyroid response element. The D box located in the second Zn finger contributes to the dimerization of TR. TR generally binds as heterodimer with retinoic acid X receptor (RXR).

Hinge Region

Hinge region lies between the DBD and the ligand binding domain and provides flexibility to TR. This region is rich in the amino acid lysine. The Hinge region interacts with various corepressors including the nuclear corepressor (NCoR1) and the silencing mediator of retinoid and TR (SMRT). This region also has nuclear localization signals for TR.

Ligand-Binding Domain (LBD)

The LBD is located in the C terminal region and consists of approximately 250 amino acids. This domain is critical for T_3 binding. It is responsible for the unliganded TR mediated repression of gene expression as well as T_3 dependent gene activation. The LBD is comprised of 12 alpha helices which form a pocket for T_3 binding. Binding of T_3 to TR causes a conformational change in helix 12 which contains the activation function-2 (AF-2) domain. The AF-2 domain interacts with co-activators in a ligand dependent manner.

Thyroid Hormone Receptor Types

Thyroid receptor has two major types $TR\alpha$ and $TR\beta$. These two isoforms of TR are encoded by two different genes located on chromosomes 3 and 17 respectively (Cheng et al, 2010a).

$TR\alpha$ Receptor

The $TR\alpha$ gene encodes for T_3 receptor $\alpha 1$ and a C terminal splice variant $TR\alpha 2$. $TR\alpha 2$ differs from $TR\alpha 1$ in length and the sequence of amino acids in the C terminal region. The $TR\alpha 2$ variant cannot bind T_3 but it retains its DNA binding ability. It antagonizes the T_3 mediated action of $TR\alpha$, $TR\beta 1$ and $TR\beta 2$ (Koenig et al, 1989; Plateroti et al, 2001). The $TR\alpha 1$ and $TR\alpha 2$ are expressed in brain, kidney, skeletal muscles, lungs, heart. The actions of T_3 in heart are mediated, primarily by the $TR\alpha 1$ isoform.

TR β Receptor

TR β has 3 isoforms TR β 1, TR β 2 and TR β 3. The TR β 1 is highly expressed in brain, liver, and kidney. TR β 2 is expressed in pituitary, hypothalamus, and inner ear while TR β 3 is predominantly expressed in kidney, liver and lungs. The actions of T₃ in liver are mediated by TR β 1 isoform. All three isoforms of TR β are functionally active.

Coregulators

Thyroid hormone regulates gene transcription by the interaction of TR with other regulatory proteins. Coregulator proteins often act as a docking scaffold for other proteins like histone deacetylases (HDACs) and histone acetyl transferases (HATs). Coregulator proteins depending up on whether they promote or inhibit transcription are divided into coactivator and corepressors respectively.

Corepressors

In absence of T₃ unliganded TR binds DNA and represses the transcription of positively regulated target genes. This basal repression by unliganded TR is mediated by NCoR1 and SMRT along with histone deacetylases (HDACs).

SMRT and NCoR1. SMRT and NCoR1 were the first two corepressors identified for nuclear receptors. SMRT and NCoR1 are structurally and functionally related. As shown in **Figure 1-3**, these corepressors can be divided into an N terminal region having 3-4 transcriptional repression (RD) domains and a C terminal region having 2-3 nuclear receptor interaction domains (Li et al). NCoR1 and SMRT serve as an adapter between the corepressor complex and nuclear receptors.

The repression domain (RD). The RD serves as a docking site for the various other components of the corepressors complex, including HDACs, transducing like protein 1 (TBL-1) and mSin3 and sirtuin 1 (SIRT1). Antibodies blocking of each of the component of NCoR1-SMRT-Sin3-HDAC complex relieve the repression by unliganded TR suggesting that each component is crucial for repression.

Switching-defective protein 3 (Swi3), adaptor 2 (Ada2), nuclear receptor co-repressor (N-CoR), transcription factor (SANT) like domain. The SANT domain is a 50 amino acid motif present in nuclear corepressors. Both NCoR1 and SMRT contain a pair of closely related SANT domains. This domain along with RD synergistically promotes histone decetylation. The binding of NCoR1 and SMRT to HDAC3 is necessary to form active repressor complexes. SANT 1 domains comprise a part of the deacetylases interacting domain (DAD) responsible for interaction of SMRT and NCoR1

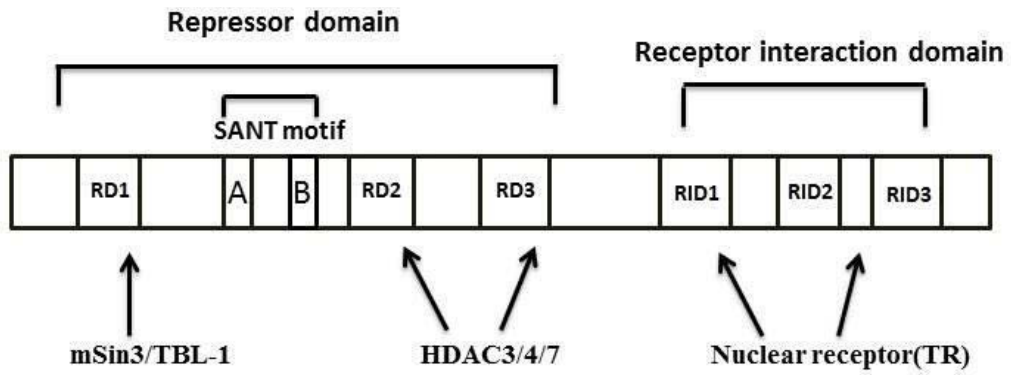


Figure 1-3. Structural organization of NCoR1

The modular structure of NCoR1 is depicted in the figure. It consists of three repression domains (RD) and three nuclear receptor interaction domains (RID). The sites of interaction of other components of the corepressor complex are shown.

with HDAC3. A single amino acid change in SANT A domain disrupts the binding of NCoR1 and SMRT with HDAC3. The SANT B domain is known to function as histone interaction domain.

Receptor-interaction domain (RID). The RID consists of signature motif L/I-X-X-I/V-I known as CoR-nuclear receptor interaction box (CORNR) through which NCoR1 and SMRT interact with nuclear receptors. SMRT contains two while NCoR1 contains three RID. TR interacts with RID3 and RID2 domains of NCoR1 with high affinity, and with low affinity to the RID2 and RID1 of SMRT. The CORNR box forms an extended alpha helical domain that interacts with the helices 4/5/6 of the nuclear receptor ligand binding domain.

Histone deacetylases (HDACs). Transcriptional regulation is dependent on the structure of the chromatin and the binding of the transcription factors and transcriptional machinery to the promoter of genes. DNA is wrapped around histones H2A, H2B, H3 and H4 to form nucleosomes which are the fundamental unit of chromatin. The histones in the nucleosomes undergo various post- translational modifications and thereby modify the chromatin structure. In general deacetylation of lysine residues of histone tails is associated with condensed chromatin and gene silencing (Kuo & Allis, 1998; Ng & Bird, 2000).

HDACs are enzymes which deacetylate (**Figure 1-4**) the lysine residues of histone tails. Removal of the acetyl group from the histone tails results in tighter binding of the negatively charged DNA around the positively charged lysine residues of histones leading to the formation of heterochromatin. Tighter wrapping of DNA diminishes the accessibility of transcription factors leading to gene silencing. Other than histone tails, HDACs can deacetylate various transcription factors like p53, GATA-1 etc.

HDACs are recruited to TR in a complex with NCoR1 and SMRT. For its deacetylase activity, HDAC3 needs to interact with NCoR1 and SMRT. HDAC3 is one of the major enzymes associated with NCoR1 and SMRT. The HDAC family consists of 18 genes which are further divided into four major categories (de Ruijter et al, 2003; Witt et al, 2009).

Class I HDACs. This class includes HDACs 1, 2, 3 and 8. Members of this class are found exclusively in nucleus. They are involved in transcriptional repression by nuclear receptors. HDAC 1, 2 and 3 by themselves are not active. They require other proteins which are necessary for their deacetylase activity and DNA association. For example HDAC1 and HDAC2 are found in complex with Sin 3 and NuRD. Similarly HDAC3 is found in complex with NCoR1/SMRT.

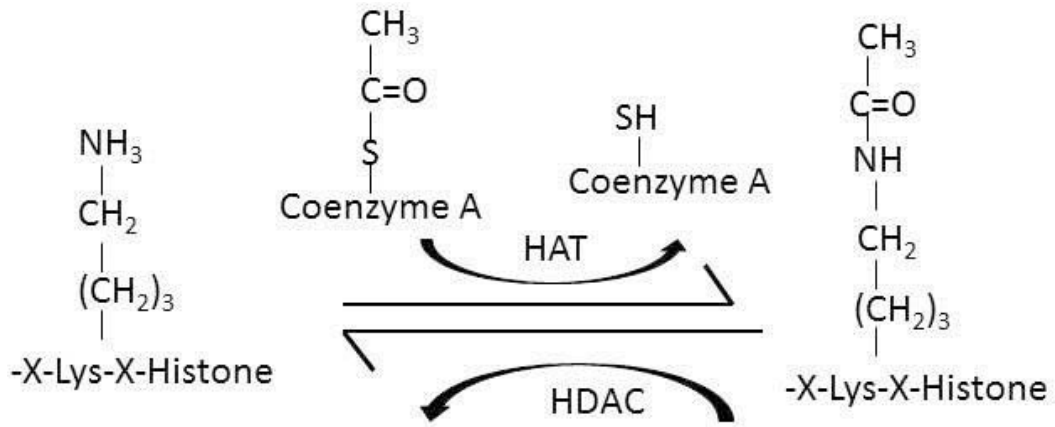


Figure 1-4. Histone tail modification catalyzed by histone acetylases and deacetylases

Class II HDACs. This class is further subdivided into type IIa and IIb. Class II HDACs are located in both nucleus and cytoplasm. Class IIa includes HDAC 4, 5, 7 and 9 while class IIb includes HDAC 6. Class IIa HDACs interact with myosin interacting factor, Ca²⁺/ calmodulin dependent kinases and protein kinase D. On the other hand HDAC 6 interacts with α -tubulin and HSP 90 dependent kinases.

Class III HDACs. Sirtuins belongs to class III HDACs and there are seven members of sirtuins (Sirtuin1-7) in this class. These are NAD⁺ -dependent histone deacetylases. Sirtuin 1, 6 and 7 are found in nucleus, sirtuin 3, 4 and 5 are located in mitochondria while sirtuin 2 is located in cytoplasm (Kim & Bae, 2011).

Class IV HDACs. HDAC 11 is the sole member of this group and is closely related to HDAC 3 and 8. The function and expression patterns of HDAC 11 had not been investigated extensively.

Other corepressors. Other proteins in the corepressors complex include Sin3, transducing like protein 1 (TBL-1) and TBL-1 related protein (TBL-R). These proteins make additional contacts with HDACs and stabilize the corepressor complex formation.

Coactivators

Coactivators are usually associated with the ligand bound nuclear receptors and are responsible for enhancing the gene transcription. Coactivators can stimulate gene transcription by various methods. In order to facilitate the binding of basal transcription machinery and transcription factors to chromosomal DNA, coactivators mediate multiple chromatin modifications. Coactivators are broadly classified into following categories (McKenna & O'Malley, 2002; Rachez et al, 1998):

ATP-dependent chromatin remodeling complexes. In order to facilitate transcription, the binding of basal transcription machinery and general transcription factors to the promoter is necessary. This class of ATP-dependent chromatin remodeling complex utilizes the energy derived from the hydrolysis of ATP to destabilize and displace histone DNA interactions. Examples of this class include SWI/SNF complexes (Laurent et al, 1993).

Histone acetyl transferases (HATs). This class containing the Steroid receptor coactivator (SRC) /p160 family includes SRC-1, glucocorticoid receptor interacting protein (GRIP/SRC-2), P300/ cAMP response element binding protein and the P300 associated factor (PCAF). Members of this family have intrinsic histone acetylase

activity. These coactivators acetylate lysine residues of histones which in turn serve as a signal for transcriptional activation (Naar et al, 2001).

Mediator complex. The mediator complex was simultaneously identified as thyroid receptor associated proteins (TRAPs) and vitamin D receptor interacting protein (DRIP). These complexes were initially purified as proteins recruited by thyroid hormone receptor and vitamin D receptor (Fondell et al, 1996; Rachez et al, 1998). This complex is known to promote and stabilize the formation of pre-initiation complex. It helps in recruiting the basal transcription machinery and RNA polymerase II to the promoter (Roeder, 2005).

Regulation of Gene Expression by Thyroid Hormone

Thyroid hormone regulates expression of genes involved in various processes like gluconeogenesis, fatty acid oxidation, lipogenesis cell proliferation and apoptosis. By binding to the thyroid response element (TRE), the TR can either activate or repress the transcriptional activity (Lazar, 2003). Examples of both positively and negatively regulated genes by T₃ are mentioned in **Table 1-1**.

Thyroid Response Elements (TRE)

TR regulates gene expression by directly binding to specific DNA sequence known as thyroid response elements (TRE) present in the promoter of genes regulated by T₃. TR binds to TRE either as a monomer, homodimer or as a heterodimer with retinoic X receptor (RXR). RXR is the dimerization partner for retinoic acid receptor (RAR), vitamin D receptor (VDR) and peroxisome proliferator-activated receptor (PPAR). RXR has a ligand but activation of RXR is likely not involved in T₃ responsiveness (Chandra et al, 2008; Lefebvre et al, 2010).

Positive TRE

The genes which are stimulated by thyroid hormones contain a positive thyroid response element (pTRE). Mostly pTRE are located upstream from the transcription start site but they can also be present in the intron or 3' flanking region. A typical pTRE consists of a core consensus hexanucleotide half site (A/G) GGT(C/G)A. The half site binding motif can be further arranged as palindromes (TRE PAL), direct repeats (DR) and inverted palindrome (TRE IP) separated by six of nucleotides. The most common motif is a direct repeat separated by 4 nucleotides (DR4) (Bassett et al, 2003). The common TREs are shown in **Table 1-2**.

Table 1-1. Examples of genes regulated by T₃

Induced genes	Inhibited genes
Pyruvate dehydrogenase kinase 4	Thyroid stimulating gene α
Phosphoenolpyruvate carboxykinase	Thyroid stimulating gene β
Carnitine palmitoyltransferase 1A	Prohormone convertase
Malic enzyme	Necdin
Growth hormone	Sterol element binding protein
Mylein basic protein	Superoxide mutase
Fatty acid synthetase	Type II deiodinase
Myosin heavy chain	Epidermal growth factor receptor

Table 1-2. Consensus positive thyroid response elements

Type of TRE	Nucleotide sequence
Consensus half site	(A/G)GGT(C/G)A
Direct repeat 4(DR-4)	AGGTCANNNNAGGTCA
Inverted palindrome	TGACCTNNNNNNAGGTCA
Palindrome	AGGTCATGACCT

Mechanism of Gene Activation by T₃

Thyroid hormone induces the expression of a number of genes involved in metabolism. The molecular mechanism of gene up regulation by T₃ is better understood than gene repression. For positively regulated genes, TRs bind primarily as heterodimers with RXR. The unliganded TR in a positive TRE is associated with corepressors like SMRT, NCoR1 and HDACs leading to basal gene repression. Upon T₃ binding, the helix 12 of TR undergoes conformational changes and the corepressors dissociate from the TR. The repressor complex is replaced by coactivators, such as the steroid receptor SRC-1 and the CREB-binding protein (CBP) and p300. These coactivators CBP/p300 possess intrinsic histone acetylase activity, which neutralizes the positive charge of lysine residues of the histones, thereby loosening the wrapping of negatively charged DNA against the histones. This leads to formation of open chromatin structure allowing transcription factor and basal transcriptional machinery to access the gene promoter causing gene activation (Grimaldi et al, 2013; Harvey & Williams, 2002). A model of gene activation by T₃ is shown in **Figure 1-5**.

Mechanism of Gene Inhibition by T₃

Although one activity of liganded TR is to stimulate transcription, an important regulatory role of T₃ involves gene repression (Feng et al, 2000; Flores-Morales et al, 2002). Examples of genes inhibited by T₃ include TSH α , TSH β , neclin, prolactin, and others. TSH α and TSH β are crucial targets for negative feedback by T₃ in pituitary. T₃ suppresses many genes in liver (Weitzel et al, 2001). Unlike transcriptional stimulation, the mechanisms by which T₃ represses gene expression are poorly understood. In contrast to positively regulated genes, many of the negatively regulated genes are stimulated by unliganded TR and repressed by liganded TR. Different theories have been proposed to explain the T₃ mediated gene inhibition (Santos et al, 2011; Weitzel, 2008). Outlined below are some of the proposed possible models by which liganded TR inhibits gene transcription.

Direct DNA-Binding Model

TR binds directly to negative thyroid response elements (nTRE). This model hypothesizes that the negatively regulated genes contain a negative thyroid response element known as a nTRE. Binding of TR to this nTRE is responsible for gene repression by T₃.

Repression through negative TREs is considered to be one of the major mechanisms of gene repression by TR (Duran-Sandoval et al). Some of the reported nTREs are summarized in **Table 1-3**. The nucleotides critical for T₃ responsiveness are underlined. According to this mechanism, TR via its DNA binding domain binds directly to nTRE and causes transcriptional repression in a T₃ dependent manner. Negative TREs, have been identified in the promoters of genes like TSH α and TSH β . In contrast to

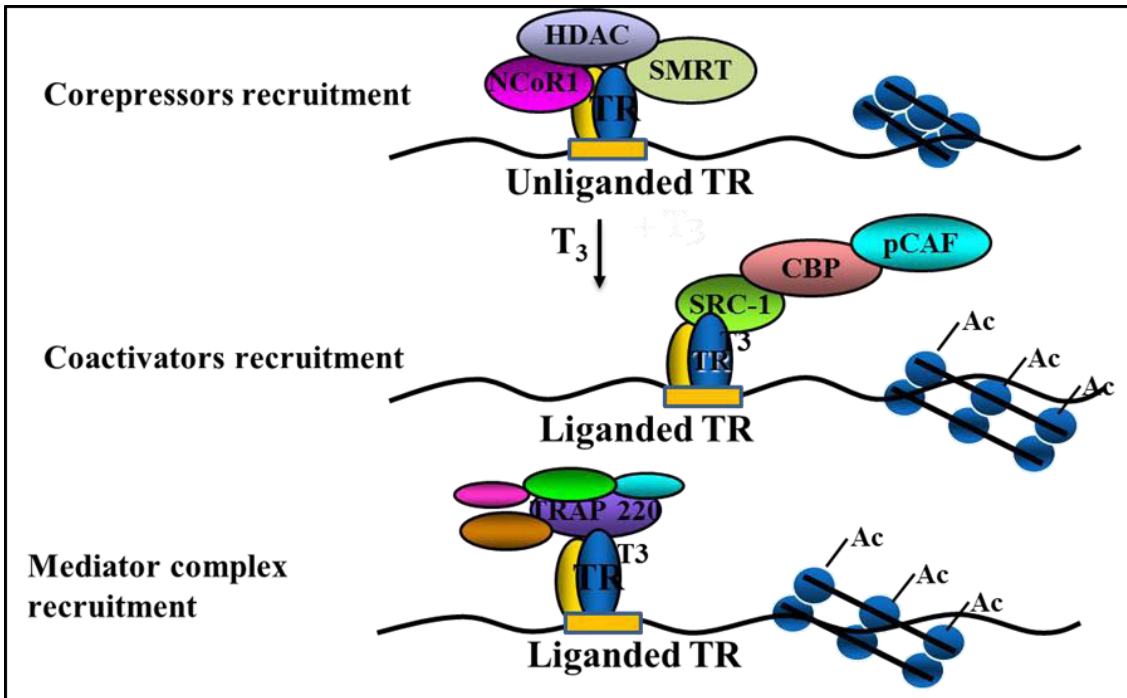


Figure 1-5. Classical model of gene activation by thyroid hormone

Mechanism of classical gene regulation by TR involves transcriptional repression via recruitment of corepressors by unliganded TR. Liganded TR mediated activation involves recruitment of coactivator complexes with HAT activity.

Table 1-3. Examples of negative thyroid response elements

Genes	Negative thyroid response element
Thyroid stimulating gene β	CGCCAGTGCAAAGTAA (Z box)
E2F	TCCGGACAAAGCCTGCGC (Z box)
Necdin	TGTCGGAACAAAGTAAGG (Z box)
Superoxide dismutase	GCGAGGCGATTGGTTTGGGGCA
CD44	CCTCTCTTTGGGTGTGTT
Epidermal growth factor	GCCCGAGTCCCCGCCTCGCC
Preprothyrotropin releasing hormone	CCCGCTGACCTCACA
Sterol regulatory element binding protein	GCCTGACAGGTGAAATCGGC
Prohormone convertase 1	TCCACTCA GCCTGGAGACCGA
Prohormone convertase 2	AAGAGAGGCCATGTGTGTCG
β amyloid precursor protein	GGGCAGAGCAAGGACG
Sodium potassium ATPase α 3	CTGATTGGCCAAGAGCCCCGCCTC

positive TREs, the sequence requirements of negative TREs (nTRE) are poorly defined. Generally, the nTREs have been identified close to the transcription start site and are called Z boxes (CAAAG). nTREs loosely resembling to TRE-like half sites have been reported. However, the binding of thyroid receptor to these nTREs is very weak compared to DR4. Since the nTRE and pTRE differ considerably from each other, questions were raised as to whether the TR binds directly to these elements. The concept of a nTRE was strengthened by the studies of Sibuwasa et al where they found that a TR β defective in DNA binding was not able to suppress the expression of TSH gene both in vitro and in vivo. These studies suggested that TR does binds directly to nTRE. However the question of how the nTRE leads to gene repression rather than activation has not been addressed. Below are a few mechanisms by which the nTRE could inhibit the gene expression.

Interference Model

In this case, the nTRE functions via an interference mechanism (**Figure 1-6**). For example, the nTRE in TSH β gene lies near the transcription start site and thereby interferes with formation of transcription initiation complex. Another possibility is that the nTRE might overlap with the binding sites of other transcription factors. Binding of T₃ to the liganded TR prevents the binding of the transcription factor to its promoter element leading to gene suppression. Examples include the β amyloid precursor protein in which the nTRE overlaps with Sp1 binding site. Binding of TR β precludes binding of Sp1 thereby inhibiting Sp1 mediated induction. Likewise in epidermal growth factor receptor gene promoter repression results from inference with Sp1 (Xu et al, 1993). Similarly for CTCF-binding factors, nTRE and CRE overlap and mutation of CRE eliminates the T₃ effect.

Role Reversal Model

According to this mechanism the function of the coregulator proteins is reversed (**Figure 1-7**). In a nTRE, coactivators promote repression while the corepressors lead to gene activation. This mechanism has been proposed for a few negatively regulated genes. It was reported that SMRT activated the TSH α via the nTRE (Berghagen et al, 2002). Similarly for TRH gene, corepressors were found to cause the basal activation rather than inhibition. Likewise, a TR mutant, defective in corepressor binding surface, was unable to activate a nTRE in SOD gene promoter (Santos et al, 2006). Similarly, in SRC-1 knockout mice, deletion of SRC-1 affected the T₃ mediated induction of positively regulated genes, but surprisingly the T₃ inhibition of TSH β gene was also attenuated. This suggests that for some negatively regulated genes coactivators might acts as corepressors. Both promoter and cell environment are responsible for this role reversal.

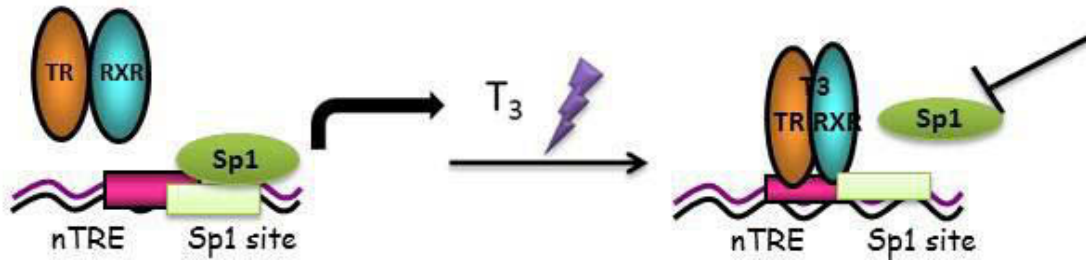


Figure 1-6. Interference model of gene repression

Nuclear receptor interferes with the activation of other transcription factors. For example TR prevents the binding of Sp1 to the β amyloid precursor gene.

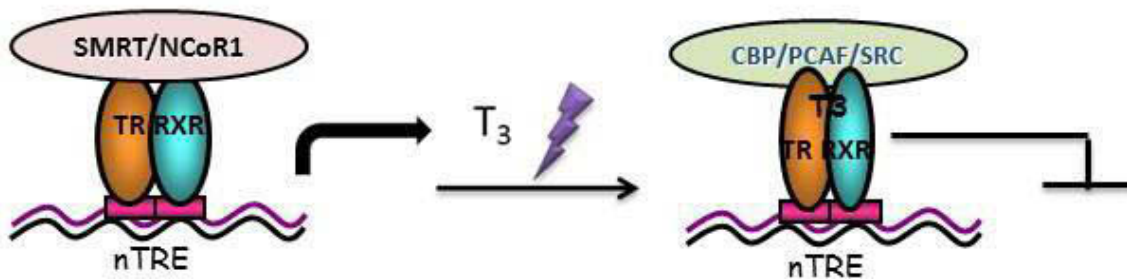


Figure 1-7. Coregulator role reversal model

The functions of regulatory proteins are reversed. Corepressor SMRT activates the TSH α via the nTRE rather than inhibiting it.

Inverse Recruitment Model

This model proposes that in some nTRE there is inverse recruitment of coregulator proteins. Unlike the pTRE, the unliganded TR on the nTRE might be associated with coactivator leading to gene activation while liganded TR recruits corepressors causing gene inhibition.

This hypothesis is supported by a recent study (You et al, 2010) where mice were made defective in the interaction of NCoR1 with HDAC3 by creating a mutation in the deacetylase domain of NCoR1. In these mice, positively regulated T₃ responsive genes which are inhibited by unliganded TR were activated, while negatively T₃ regulated genes like TSH α and deiodinase 2 were modestly induced by T₃. A model of inverse recruitment is shown in **Figure 1-8**. This suggests that on negatively regulated genes like TSH α and deiodinase 2 liganded TR might be associated with NCoR1. The exact mechanism responsible for the switching of the coregulator proteins is not known. One possible explanation for this inverse recruitment is DNA sequence (TRE) acts as allosteric modulator for TR. Binding of TR can cause conformational changes in the TR receptor such that liganded TR has affinity for corepressors rather than coactivators.

Recruitment of Ligand-Dependent Corepressors

Recruitment of coactivator by liganded nuclear receptors cannot fully account for the actions of the nuclear receptors. This model proposes that ligand dependent corepressors might be involved in negative gene regulation by nuclear receptors or some coregulators might function as coactivator or corepressors in a gene dependent manner (**Figure 1-9**). Examples of agonist dependent corepressors include receptor interacting protein 140 (RIP140). RIP140 was initially identified as coactivator recruited to liganded NRs including estrogen receptor alpha (ER α), TR α/β , androgen receptor (AR), vitamin D receptor (VDR), PPAR α and Liver x receptor α (LXR α) (Augereau et al, 2006). Later it was found that RIP140 acts as a corepressor by competing for binding of coactivator to liganded nuclear receptors. Furthermore, RIP140 recruits HDAC1 and 3 in case of RAR/RXR heterodimer. Ligand dependent corepressor (LCoR) is another agonist bound corepressors. It was first found to bind with the ER α receptor and later studies showed that it interacted with other nuclear receptors. LCoR interacts with HDAC3 and HDAC6. Other examples of agonist bound corepressors include preferentially expressed antigen in melanoma (PRAME), repressor of estrogen activity (REA) etc.

Transrepression

This mechanism involves the protein- protein interaction of nuclear receptors with other transcription factors. In this case, nuclear receptors do not bind the gene promoter directly, but instead interfere with the function of transcription factors through protein-protein interaction (**Figure 1-10**) (Pascual & Glass, 2006). In addition to binding to T₃ response element, the DBD of TR can interact with other transcription factors. Nuclear

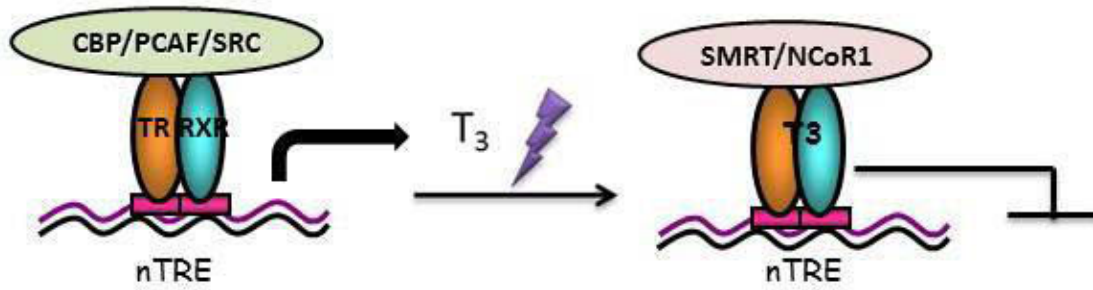


Figure 1-8. Inverse recruitment of corepressors to ligand bound thyroid receptor

In this mechanism corepressors are associated with ligand bound TR. NCoR1 has been found to be associated with ligand bound TR in nTRE of TSH α gene.

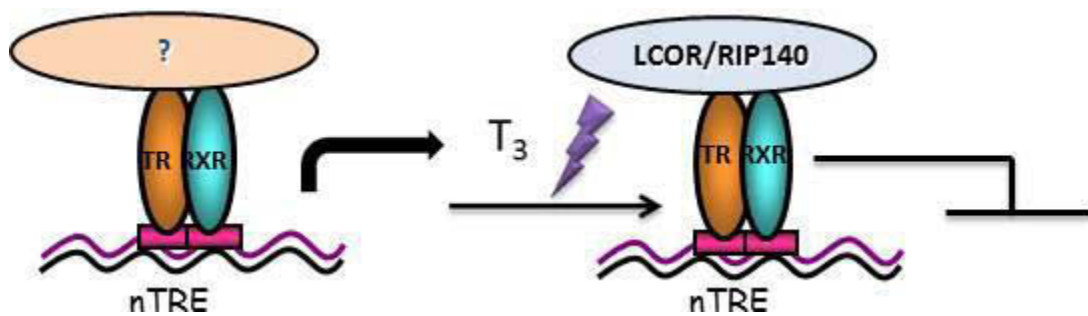


Figure 1-9. Repression by recruitment of ligand dependent corepressors

Ligand dependent co repressors (LCoR1) are recruited to TR in response to T₃ binding leading to gene silencing.

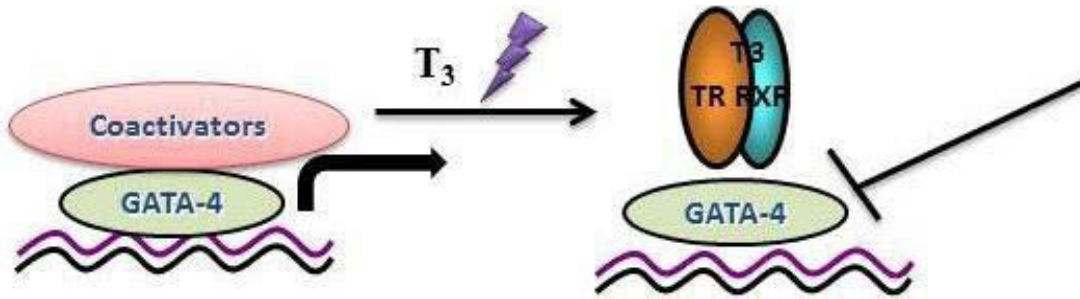


Figure 1-10. Transcriptional repression by transrepression mechanism

Ligand bound TR interferes with the binding of GATA-4 and prevents GATA-2 mediated transcription. Similarly, ligand bound GR interacts and prevents AP-1 mediated gene activation.

receptors have been shown to inhibit the inflammatory genes via direct interaction of NRs with NF-KB and AP-1. For example the DBD of GR interacts with NF-KB and AP-1 (Caldenhoven et al, 1995). Similarly PPAR α interferes with the DNA binding of AP-1 and NF-KB in IL-6 mediated induction in vascular smooth muscle cells (Delerive et al, 1999).

Regarding transrepression by TR, with some of the genes repressed by T₃, the DBD for TR is not required. T₃ inhibits hepatic angiotensin like-3 (ANGPTL3) gene expression via interactions of TR β with HNF4 bound to the ANGPTL3 proximal promoter (Fugier et al, 2006). Similarly another study on TSH β suggests that TR via its DBD interacts with transcription factor GATA-2, and liganded TR prevents the binding of GATA to its response element. This model is very well established with respect to glucocorticoid receptors (GR). Glucocorticoids are known to inhibit the expression of various inflammatory genes by tethering of GR to the NF-KB and AP-1 proteins.

Squelching or Sequestering Model

According to this model, the soluble non DNA bound TR in the nucleus sequesters the cofactors from the DNA bound TR-cofactor complexes. In the presence of T₃, soluble TR competes for coactivator thereby limiting the amount of coactivator to DNA bound TR leading to gene repression. The idea of this hypothesis is supported by a study by Gill et al where they found that overexpression of GAL4 inhibited the expression of genes not containing the GAL4 binding sites. It was proposed that transcriptional activator GAL4 was squelching the transcriptional machinery (TATA binding proteins and RNA polymerase II). This model had been proposed to the negatively regulated genes which do not require the DBD of TR for their inhibition (Gill & Ptashne, 1988).

Thyroid Hormone Actions

T₃ modulates lipid metabolism, plasma lipids and cardiovascular function. The physiological actions of T₃ are mediated through TR α and TR β (Forrest & Vennstrom, 2000; Wu & Koenig, 2000; Zabrocka & Klimek, 2004). The important functions of thyroid hormone are summarized below:

Effect of T₃ in Liver

Liver plays a critical role in metabolism and about 5% of genes in liver are regulated by T₃. Lipid and glucose metabolism are the two of the major physiological processes modulated by T₃. T₃ regulates every aspect of carbohydrate metabolism in the liver. It induces gluconeogenesis by increasing the expression of phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6-P) genes. T₃ reduces lipogenesis by inhibiting the expression of lipogenic gene sterol regulatory element binding protein

(SREBP-1c). T₃ regulates cholesterol metabolism. It induces the expression of the LDL-receptor (LDL-R), increasing the hepatic uptake of LDL from blood. Expression of CYP7A1, the enzyme involved bile acid synthesis from cholesterol, is induced by T₃. The net result of elevated T₃ is increased cholesterol clearance (Pramfalk et al, 2011; Weiss et al, 1998; Yen, 2001).

Effect of T₃ in Heart

Thyroid hormone increases heart rate, cardiac contractility and cardiac output. The actions of T₃ in heart are mainly mediated by the TR α isoform. T₃ induces expression of the β 1-adrenergic receptor via TR α and thereby elevates β -adrenergic responsiveness. Additionally, T₃ increases the expression of genes critical for cardiac function. By inducing the expression of myosin heavy chain and sarcoplasmic reticulum Ca²⁺-ATPase, T₃ enhances cardiac output and decreases relaxation time (Danzi & Klein, 2002; Silva & Bianco, 2008).

Effect of T₃ in Adipose Tissue

Thyroid hormone regulates the function of both white adipose tissue (WAT) and brown adipose tissue (BAT). In WAT, it induces differentiation from pre-adipocytes to adipocytes. T₃ promotes both lipogenesis and lipolysis. It induces lipolysis by increasing sensitivity of adipose tissue to adrenergic stimulation and increasing the activity of hormone sensitive lipase. In BAT, T₃ promotes thermogenesis by enhancing the response of norepinephrine signaling pathway and inducing the expression of uncoupling protein 1 gene (Silva, 1995; Silva, 2011).

Effect of T₃ in Brain

T₃ is essential for normal brain development. Hypothyroidism in neonatal period leads to mental retardation and neurological defects. Hypothyroidism in neonatal rats leads to decreased axonal growth and dendritic arborization in cerebellum, cerebral cortex and hippocampus (Pilhatsch et al, 2010; Wallis et al, 2010).

TR β Agonists

Therapeutic potential of thyroid hormone as a lipid-lowering and anti-obesity agent remains largely hampered by dose-limiting cardiac effects, muscle wasting and osteoporosis mediated by the TR α isoform. Considerable effort has been made to develop TR β selective modulators due to their preferential effect on liver metabolism (Baxter & Webb, 2009). Examples of some of the known TR β agonists are given below:

GC-1

GC-1 named as sobetirome, is a selective thyromimetic with a 10-fold preferential affinity for TR β 1 over TR α 1. It accumulates preferentially in liver as compared to other tissues. In rodents and primates, it lowered cholesterol levels and reduced hyperlipidemia. GC-1 increased hepatic fatty acid oxidation in mitochondria and peroxisomes but to a lesser extent than T₃. Importantly heart rate and cardiac performance were not altered (Baxter et al, 2004; Johansson et al, 2005; Perra et al, 2008).

MB07811

MB07811 is a TR β selective liver prodrug. In the liver, it is metabolized into an active metabolite MB07344. In a study by Cable et al, MB07811 reduced hepatic steatosis and hepatic triglycerides in both rats and mice. It induced CPT1a and PGC-1 α while inhibiting SREBP-1c expression (Cable et al, 2009; Erion et al, 2007).

KB-141

KB-141 is another TR β agonist which is more selective for stimulating metabolic rate and is 30-fold more selective for cholesterol lowering than for positive chronotropic effects. In primates, it caused significant cholesterol reduction and weight loss and (Grover et al, 2005). In ob/ob mice, KB141 was found to reduce serum triglycerides and non-esterified free fatty acid (Amorim et al, 2009).

GC-24

GC-24 has demonstrated ability to reduce body fat accumulation and to prevent liver steatosis in rats with diet-induced obesity and increasing energy expenditure. However, GC-24 reduced only marginally total cholesterol levels and did not have any effect on free fatty acids or IL-6 levels (Amorim et al, 2009).

KB-2115

KB-2115 is the only TR β selective compound that has been used in human trials (Tancevski et al, 2011). In 2 week clinical trials, KB2115 lowered the LDL cholesterol by 40%. In another clinical trial, the patients were already on statin therapy. Following 100 μ g or 200 μ g dose of KB2115 for 12 weeks there was a 30% decrease in the LDL cholesterol and a significant reduction in triglycerides. There was no change in heart rate and muscle strength (Ladenson et al, 2010).

Phospholipase A2

Phospholipases A2 are a family of esterase enzymes which hydrolyze the second carbon of membrane phospholipids to release non-esterified free fatty acid and lysophospholipids. These products generated could either serve as secondary messengers or be metabolized to generate bioactive molecules. For example, the free fatty acid (arachidonic acid) is a precursor for inflammatory mediators such as prostaglandins. Similarly the lysophospholipids (lysophosphatidylcholine or lysophosphatidic acid) may be converted to lipid mediators like platelet activating factor. In mammals, more than 30 enzymes having phospholipase activity have been identified (Murakami & Lambeau, 2013). The reaction catalyzed by of phospholipase A2 is shown in **Figure 1-11**.

Classification of Phospholipases

Based on their Ca^{2+} ion requirement and cellular localization, PLA2 enzymes have been classified into four groups: cytosolic phospholipase A2, secretory phospholipase A2, calcium independent phospholipase A2 and lipoprotein associated phospholipase A2 (Balsinde et al, 2002; Dennis et al, 2011; Jaross et al, 2002; Kudo & Murakami, 2002).

Cytosolic phospholipase A2 (cPLA2s). cPLA2 belong to the category of intracellular PLA2. These are located in the cytosol and migrate to nuclear membranes in response to inflammatory stimuli. They usually have a molecular mass greater than 60 kDa and require Ca^{2+} ion for their enzymatic activity. This family has a conserved N-terminal C2 domain for calcium dependent association with cellular membrane. The cPLA2s have a preference for the phospholipids containing arachidonic acid. This category includes 6 isoforms which includes cPLA2 IV A (cPLA2 α), cPLA2 IVB (cPLA2 β), cPLA2 IVC (cPLA2 γ) cPLA2 IVD (cPLA2 ζ), cPLA2 IVE and cPLA2 IVF (Burke & Dennis, 2009; Lucas & Dennis, 2004).

Secretory phospholipases (sPLA2s). sPLA2 are stored in cytoplasmic granules and are secreted into the extracellular environment in response to stimuli. These are low molecular weight phospholipases with a size of 14-18 kDa. These enzymes require mM concentrations of calcium for their enzymatic activity. They contain a highly conserved Ca^{2+} binding loop (CXCGXGG) and a catalytic (DXCCXXHD) domain. This group is cationic in nature and have 6-8 disulphide bonds which impart high degree of stability to these enzymes. Currently 11 sPLA2s have been identified in humans. This class includes PLA2gIB, PLA2gIIA, PLA2g IIC, PLA2gIID, PLA2gIIE, PLA2gIIF, PLA2gIII, PLA2gV, and PLA2gX (Dennis et al, 2011).

Calcium-independent phospholipase A2 (iPLA2s). iPLA2 also called patatin like phospholipases, are high molecular weight cytosolic phospholipases which do not

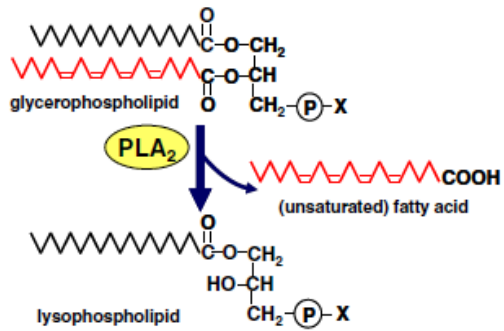


Figure 1-11. Enzymatic reaction catalyzed by PLA₂

Phospholipases hydrolyze the membrane phospholipids to release free fatty acid and lysophospholipids. Based on the cellular localization, phospholipases have been divided into intracellular cytosolic PLA₂, calcium independent PLA₂ and extracellular secreted PLA₂.

require Ca^{2+} ion for their enzymatic activity. Group VI iPLA2 is an 85-88kDa enzyme. This class includes phospholipase group PLA2gVIA, PLA2gVIB, PLA2gVIC, PLA2GVID, PLA2GVIE, and PLA2GVIF. These are also known as lipase type enzymes since many of them act on neutral lipids like triglycerides rather than acting on phospholipases (Kudo & Murakami, 2002).

Lipoprotein-associated phospholipase A2 (Lp-PLA2). It is a 45kDa sPLA2 secreted by the macrophages, T lymphocytes and mast cells. This enzyme has platelet-activating factor acetyl hydrolase activity along with phospholipase activity. Lp-PLA2 is usually associated with oxidized plasma lipoproteins (Murakami, 2004).

General Functions of Secretory Phospholipases

Secretory phospholipases have a wide variety of functions. sPLA2s can act on the cellular membranes in an autocrine and paracrine manner. Besides generating arachidonic acid, they also generate other saturated, mono and polyunsaturated fatty acids. Various non-cellular phospholipids such as microbial membrane, dietary phospholipids, lipoproteins and pulmonary surfactants are their other targets. sPLA2 stimulates inflammation independent of its catalytic activity. sPLA2 binds to the M-type PLA2R receptors present on the surface of mast cells, neutrophils, macrophages and induce the production of cytokines by these cells. However, the binding of sPLA2 to PLA2R1 internalizes sPLA2 into phagolysosomes leading to its degradation (Mallat et al, 2007). The physiological actions of sPLA2s are shown in **Figure 1-12**.

sPLA2g2a

PLA2g2a is a secretory PLA2 which is also known as non-pancreatic or inflammatory PLA2. It was first purified from the platelet and the synovial fluids of patients suffering from arthritis (Pruzanski & Vadas, 1988).

Enzymatic Properties

PLA2g2a is highly cationic in nature due to the presence of histidine, arginine and lysine residues. It has high affinity for anionic phospholipids such as phosphatidylserine and phosphatidyl ethanolamine and phosphatidylglycerol as compared to neutral phosphatidylcholine (Dennis et al, 2011).

Expression

High levels of this enzyme have been found in plasma and other biological fluids of patients suffering from various inflammatory diseases. Many cell types including

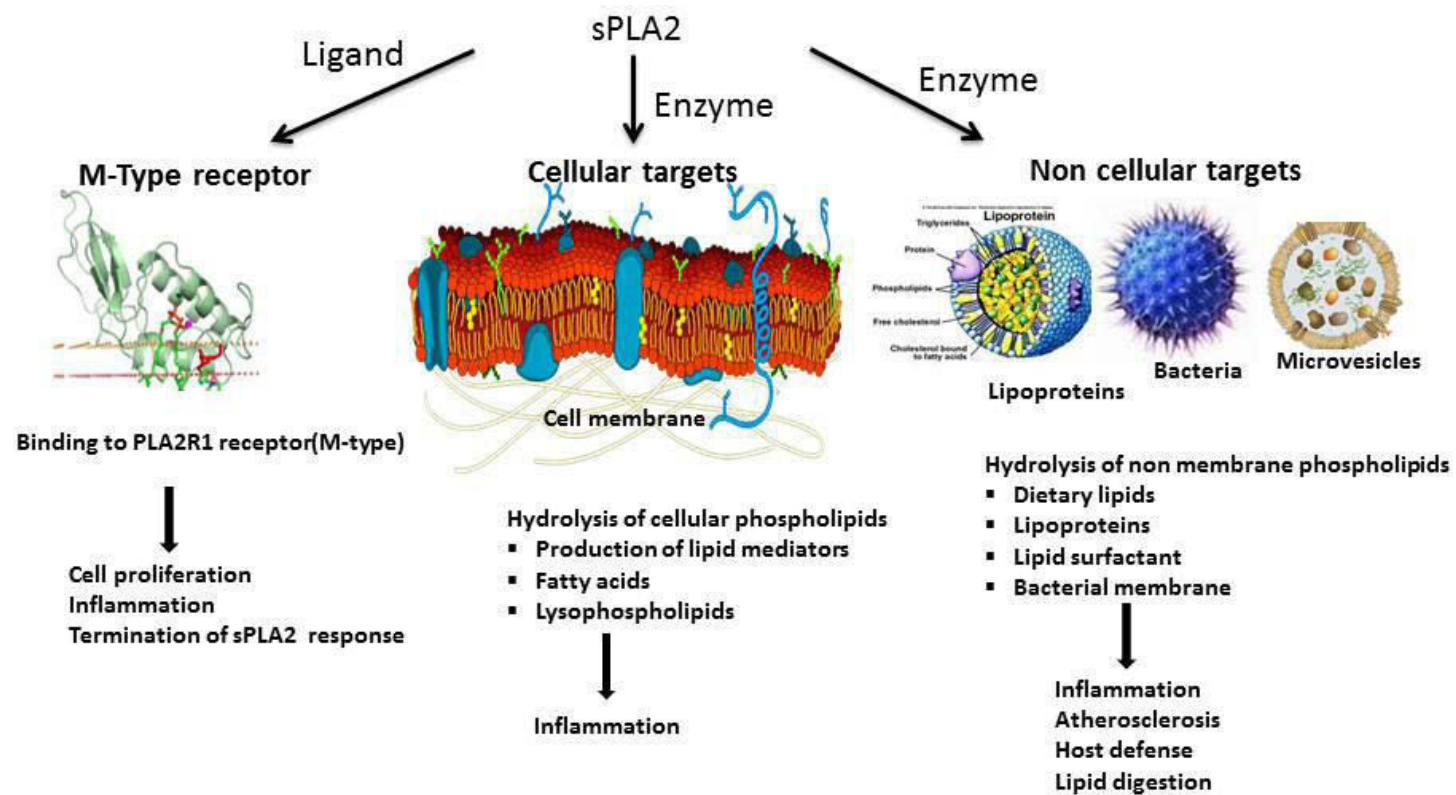


Figure 1-12. General functions of secretory phospholipases

Based on their enzymatic properties, sPLA2 can hydrolyze membrane phospholipids, and various other non-cellular targets like dietary phospholipids, microbial membrane and micro vesicles. By binding to M-type receptor via their non-enzymatic properties they could promote cell proliferation, and inflammation.

hepatocytes, vascular smooth muscle cells and endothelial cells secrete PLA2g2a. Liver is one of the major contributors of sPLA2 in human plasma along with vascular smooth muscle cells. Liver biopsies of patients with acute pancreatitis revealed increased PLA2g2a expression in liver and elevated serum PLA2g2a levels.

Regulation of PLA2g2a

Expression of PLA2g2a is induced by various stimuli including interleukin 1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor α (TNF α), lipopolysaccharides (LPS) and cyclic AMP (Massaad et al, 2000). IL-1 β induces the transcription of PLA2g2a through NF-KB and peroxisome-proliferator activated receptors while cAMP acts via CCAAT enhancer binding protein (CEBP β) to stimulate PLA2g2a (Antonio et al, 2002; Couturier et al, 2000; Fan et al, 1997).

Functions of PLA2g2a

Role of PLA2g2a in inflammation. The fact that PLA2g2a is highly abundant in the biological fluids of patients suffering from inflammatory diseases like arthritis, sepsis and myocardial infarctions led to the hypothesis that PLA2g2a promotes inflammation. In a study (Boillard et al, 2010), PLA2g2a knockout BALB/c mice had attenuated joint inflammation as compared to wild type BALB/c mice.

Role of PLA2g2a in atherosclerosis. PLA2g2a has proatherogenic properties and elevated levels of PLA2g2a are a biomarker for cardiovascular diseases (Kugiyama et al, 1999). The first evidence for the role of PLA2g2a in atherosclerosis came from a study with PLA2g2a Tg mice. The C57BL/6 mice do not express PLA2g2a due to frame shift mutation in exon 3. The human PLA2g2a was introduced into C57BL/6 mice. These PLA2g2a Tg mice when fed on a high cholesterol atherogenic diet showed increased atherosclerotic lesions and reduced plasma HDL (Ivandic et al, 1999). Transplantation of bone marrow from PLA2g2a Tg mice into LDL receptor deficient mice resulted in a significant increase in atherosclerotic lesions. Overexpression of PLA2g2a in mouse macrophages accelerated the development of arterial wall lesions and the movement of cholesterol from LDL to foam cells (Ghesquiere et al, 2005; Webb et al, 2003). Varespladib an inhibitor of sPLA2 had been reported to prevent the development of atherosclerosis in guinea pigs (Leite et al, 2009). In a study a high-fat diet model, Varespladib (A-002) reduced aortic atherosclerosis by 50% and reduced plasma total cholesterol levels. Oral dosing of A-002 for 16 weeks significantly reduced aortic atherosclerosis in the ApoE $^{-/-}$ mice model. The reduction was associated with a significant decrease in total cholesterol (Fraser et al, 2009).

Role of PLA2g2a in metabolic syndrome. Transgenic mice were generated that express the human PLA2g2a gene regulated by the human promoter. When these mice were placed on a high cholesterol diet, there was elevated accumulation of cholesterol in the liver suggesting that PLA2g2a impacts hepatic cholesterol uptake (Eckey et al, 2004). Another study provided evidence of possible role of PLA2g2a in diet induced obesity and metabolic dysfunction. In high fat diet induced obesity rat models the levels of PLA2g2a were elevated 20 fold in white adipose tissue. Inhibition of PLA2g2a by the selective PLA2g2a inhibitor KH064 protected rats against diet induced metabolic dysfunction and adiposity. They proposed that PLA2g2a inhibition improved adipose tissue function via stimulating lipolysis there by decreasing fat stores in adipose tissue. Genes involved in lipid metabolism and energy expenditure like peroxisome- proliferator activator receptor, adiponectin, hormone sensitive lipase were induced by KH064.

Role of PLA2g2a in bacterial infection. PLA2g2a can hydrolyze the bacterial membrane phospholipids like phosphatidyl ethanolamine and phosphatidylglycerol thereby providing antibacterial activity. Tg mice overexpressing PLA2g2a showed decreased mortality against Gram negative bacterial infection (Laine et al, 1999; Laine et al, 2000).

sPLA2g1b

sPLA2g1b often named as pancreatic phospholipase A2 is mainly expressed in pancreatic acinar cells. It is found within zymogen granules of the acinar cells. It will promote lysophospholipid absorption. PLA2g1b KO mice when fed on high fat/carbohydrate diet showed resistance to the effects of diet-induced obesity, diabetes and hyperlipidemia. PLA2g1b gene lies in a locus for obesity susceptibility in humans (Wilson et al, 2006). PLA2g1b KO mice showed increased glucose tolerance and improved insulin sensitivity as compared to wild type mice (Hollie & Hui, 2011; Labonte et al, 2010). The PLA2g1b inhibitor methyl indoxam was able to reduce diet induced obesity, hypercholesterolemia and hyperglycemia in the rats fed on high fat high carbohydrate diet.

sPLA2g3

Unlike other sPLA2s, PLA2g3 is large protein of 55 kDa. It is highly expressed in epididymal epithelium and dorsal root ganglionic neurons. PLA2g3 KO mice show reduced sperm motility and reduced ability to fertilize intact eggs suggesting the role of PLA2g3 in sperm maturation (Sato et al, 2010). In addition, PLA2g3 can hydrolyze HDL and LDL. PLA2g3-modified LDL promoted the formation of lipid droplet-rich foam cells from macrophages. Furthermore, atherosclerosis was exacerbated in PLA2g3 Tg × apoE KO mice fed a high cholesterol diet (Sato et al, 2008). PLA2g3Tg mice also showed chronic inflammation and dermatitis.

sPLA2g5

PLA2g5 is highly expressed in macrophages, bronchial epithelium, and myocardium. In the lung, PLA2g5 expression is elevated in mice models of asthma or acute respiratory distress syndrome (ARDS) mice models (Munoz et al, 2007; Munoz et al, 2009). PLA2g5 transgenic mice showed reduced lung surfactant phospholipids like diapaimitoyl phosphatidylcholine and phosphatidylglycerol leading to respiratory failure and neonatal death (Ohtsuki et al, 2006). PLA2g5 is expressed in human atherosclerotic lesions and has been proposed to play an important role in promoting atherosclerosis (Bostrom et al, 2007). It was reported that PLA2g5 hydrolyzed LDL were more susceptible to form macrophage foam cell formation.

sPLA2g10

PLA2g10 is expressed in neutrophils, intima of human atherosclerotic lesion and in alveolar macrophages and airway epithelial cells. Similar to PLA2g1b, PLA2g10 is produced as a zymogen (Cupillard et al, 1997). It will modify LDL and promote foam cell formation and atherosclerosis. PLA2g10 knockout mice have reduced allergen induced asthma (Henderson et al, 2007).

CHAPTER 2. RESEARCH OBJECTIVE

Hypothesis-1

T₃ status modulates sPLA2 expression by inhibiting the expression of PLA2g2a and other sPLA2 isoforms in liver.

Hypothesis-2

The second hypothesis was that T₃ inhibits the expression of PLA2g2a via a negative thyroid response element and that nuclear corepressors are recruited to nTRE in a T₃ dependent manner.

Aim 1: To Characterize the Regulation of PLA2g2a by Thyroid Hormone in Liver

Clinical studies have correlated hypothyroidism with low grade inflammation, elevated risk of hepatic steatosis and atherosclerosis. (Ichiki, 2010; Kvetny et al, 2004). PLA2g2a is involved in inflammation, atherosclerosis and hyperlipidemia. The linkage of hypothyroidism and inflammation led us to investigate the modulation of PLA2g2a expression by T₃. Since the liver is one of the major contributors to the total pool of PLA2g2a in plasma, I propose to characterize the regulation of PLA2g2a and other sPLA2s by thyroid hormone in liver.

Specific aim 1-1: To investigate the effect of thyroid hormone status on the regulation of PLA2g2a and other sPLA2 in liver in vitro and in vivo. I will study the effect of T₃ on PLA2g2a expression in rat hepatocytes and human hepatoma cells. I will investigate whether the thyroid status regulates the endogenous sPLA2 gene expression *in vivo* in rats. Rats will be made hypothyroid by providing an iodine free diet supplemented with propylthiouracil (PTU) for five weeks. T₃ will be administered to make them hyperthyroid. The effect of thyroid status will be then measured on the abundance of PLA2g2a and other sPLA2 isoforms.

Specific aim 1-2: To study the effect of thyroid hormone on cytokines mediated induction of PLA2g2a gene. Since the PLA2g2a is an inflammatory enzyme, expression of PLA2g2a is induced by various cytokines like interleukin 1(IL-1), interleukin-6 (IL-6), tumor necrosis factor α (TNF α), and lipopolysaccharides (LPS). I will investigate whether T₃ can block the cytokine mediated induction of PLA2g2a. Rat hepatocytes and HepG2 cells will be treated with cytokines TNF α and IL-6, respectively. The effect of T₃ will be then studied on the cytokine mediated induction of PLA2g2a.

Aim 2: To Identify the Mechanism of Negative Gene Regulation of PLA2g2a by T₃

The mechanisms by which T₃ inhibits hepatic gene expression are not understood. I will address this question by using PLA2g2a as my model gene. I hypothesize that T₃ reduces PLA2g2a expression via a nTRE and that the liganded TRβ recruits corepressors to the PLA2g2a gene to inhibit the expression of PLA2g2a gene.

Specific aim 2-1: To identify the negative thyroid response element in PLA2g2a promoter. A consensus motif for the nTRE has not been defined. The nTREs do not resemble a classical positive TRE (pTRE) consisting of an AGGTCA motif separated by four nucleotides (DR4). To identify the T₃ responsive element, I will clone the rat PLA2g2a promoter and fuse it to the luciferase (luc) reporter. To localize the nTRE response element I will create 5' serial deletions of the PLA2g2a promoter. The binding of TRβ will be tested. Site directed mutagenesis of PLA2g2a-luc will be conducted to identify T₃ inhibitory elements functionally. I will ligate the nTRE in front of TK-luciferase to determine if PLA2g2a promoter context is critical for the inhibition by T₃. This experiment will determine if the sequence of the nTRE is sufficient to impart inhibition by T₃ or if other factors bound to the PLA2g2a promoter are needed for the repression of gene expression.

Specific aim 2-2: Identification of the coregulatory proteins involved in PLA2g2a repression by T₃. I hypothesize that on a nTRE TRβ recruits corepressors to inhibit PLA2g2a transcription. Several approaches will be used to investigate whether corepressors are involved in the T₃ mediated repression or not. I will knockdown NCoR1 and SMRT using siRNA. I will test whether the T₃ inhibition of PLA2g2a is lost by corepressor knockdown. CHIP assays will be conducted to assess the T₃ dependent recruitment of NCoR1 and SMRT to the PLA2g2a gene. I will make TRβ mutants with altered amino acids in helices 1 and 3 which are known to be defective in corepressors binding. These mutations will diminish the interaction of TRβ with NCoR1 and SMRT (Collingwood et al, 1998; Marimuthu et al, 2002a). I will identify the other genes which require the nuclear corepressors for T₃ mediated inhibition of gene expression.

CHAPTER 3. REGULATION OF SECRETORY PHOSPHOLIPASES BY THYROID HORMONE*

Introduction

Several studies have correlated hypothyroidism with elevated risk of atherosclerosis, hepatic steatosis and components of the metabolic syndrome including hyperlipidemia and obesity (Baxter & Webb, 2009). Chronic low-grade inflammation is associated with obesity and hypothyroidism (Garces et al, 2010; Kvetny et al, 2004). The Secretory phospholipase PLA2g2a enhance the progression of several chronic inflammatory diseases including arthritis and atherosclerosis (Murakami et al, 2011). PLA2g2a promotes conversion of LDL to the more atherogenic oxidized LDL (Lambeau & Gelb, 2008). Most studies on PLA2g2a expression have been conducted in vascular cells and macrophages with respect to atherosclerosis and arthritis (Murakami et al, 2010). However, the liver is one of the major contributors to the total pool of extracellular sPLA2 (Exeter et al, 2012; Grass et al, 1996) and hepatocytes secrete PLA2g2a in response to cytokines (Adamson et al, 1994; Crowl et al, 1991; Nevalainen et al, 1996). Since PLA2g2a expression is elevated in various inflammatory states and hepatocytes actively secrete PLA2g2a, we investigated the regulation of PLA2g2a in liver. The potential linkage of hypothyroidism with inflammation led us to examine the modulation of PLA2g2a expression by T₃.

In this chapter, I characterized the regulation of PLA2g2a by T₃ in liver. I found that T₃ decreases both the mRNA and protein abundance of PLA2g2a by 60% in primary rat hepatocytes as well as in human hepatoma cell lines (HepG2). I found that the thyroid status regulates endogenous PLA2g2a gene expression in rats and BALB/c mice. Hyperthyroid rats had significantly lower levels of PLA2g2a mRNA and proteins as compared to hypothyroid rats Likewise, other sPLA2 isoforms including PLA2g1b, PLA2g3 and PLA2g5 were inhibited in hyperthyroid rats. Similarly BALB/c hyperthyroid mice had significantly lower levels of PLA2g2a as compared to hypothyroid mice. Moreover, T₃ also reversed the cytokines mediated induction of PLA2g2a by 50%. Both in primary rat hepatocytes and HepG2 cells TNF and IL-6 mediated induction of PLA2g2a was inhibited by T₃. I discovered that PLA2g2a is induced by high fat diet both in primary rat hepatocytes and *in vivo* in BALB/c mice.

In conclusion, found that T₃ inhibits the expression of PLA2g2a and other sPLA2 isoforms in primary rat hepatocytes, human HepG2 cell lines as well as *in vivo* in both rats and mice livers. My data suggest that the thyroid status may modulate aspects of the inflammatory response.

* Adapted with permission. Sharma P, Thakran S, Deng X, Elam MB, Park EA. (2013). Nuclear Corepressors Mediate the Repression of Phospholipase A2 Group IIa Gene Transcription by Thyroid Hormone J. Biol Chem, 2013 June 7; 288(23):16321-33.

Material and Methods

Primary Rat Hepatocyte Cell Culture and Treatment

Rat hepatocytes were prepared by collagenase perfusion as described previously (Zhang et al, 2004). Hepatocytes (3×10^6 in 60mm dishes) were maintained for 12 h in RPMI 1640 media and 10% fetal bovine serum. The cells were then washed two times with 1XPBS. RPMI 1640 media without serum was then added to the cells. The cells were treated with 100 nM T_3 for 24 h. $TNF\alpha$ was added to the cells at a concentration of 25 ng/mL.

RNA Extraction

Growth media was aspirated from the plates and 1mL of RNA stat 60 was added to each 60 mm plates. After 5 minutes of incubation at room temperature, cells were scrapped and transferred to 1.5ml eppendorf tubes. 0.2 mL of chloroform was added to the homogenate and the tube were shaken vigorously for 15 sec. Tubes were then stored at RT for 10 min, followed by centrifugation at 12000g for 20 min at 4 °C .The aqueous layer was transferred to fresh tubes and the RNA was precipitated by using 0.5 mL of isopropanol and centrifuging at 12000 g for 20 min. The supernatant was removed and the pellet was washed with 75% ethanol by centrifuging at 7500 rcf for 5 minutes. Pellets were air dried and dissolved in nuclease free water. RNA was purified with the Qiagen RNeasy Mini Kit (74104) and quantified using a NanoDrop machine.

Animals and Treatments

Adult male Sprague Dawley rats and BALB/c mice were housed under controlled conditions (22 °C, constant humidity, 12 h/12 h dark/light cycle) in the animal care facility of the University of Tennessee Health Science Center. Hypothyroidism was induced in both by feeding an iodine-free diet containing 0.15% propylthiouracil (Teklad 95125) for 5 weeks. The rats/mice were given intraperitoneal injection of T_3 (0.33 mg/kg and 0.11 mg/kg of body weight respectively) (Cook et al, 2001) after 24 hrs another bolus of same dose of T_3 was given. Rats / mice were sacrificed after 24 hrs and livers were isolated for RNA and protein. For high fat diet experiments BALB/c mice were divided into three groups 1) chow fed (Teklad Diet 06101), 2) high fat diet (HFD) (TD 95217), 3) hypothyroid (PTU) (TD 120714). The energy content of the HFD was 45% fat and 40% carbohydrate while the chow diet has 65% carbohydrate and 17% fat. C57BL/6 hPLA2gIIA+ mouse contained the human PLA2g2a gene under the regulation of its own promoter. These mice were provided to us by Dr. Eric Boilard in Quebec Canada. To induce hypothyroidism, PTU was added at 0.05% in the iodine free diet for 6 weeks. Each group of mice has seven female mice. C57BL/6 hPLA2gIIA+ were made hyperthyroid by giving them two intraperitoneal injection of T_3 (0.11 mg/kg of body weight) for two days. After 24 hrs of second injection mice were sacrificed blood and

liver were collected.

ELISA

HepG2 cells were plated in 24 well plates at a density of 0.2×10^6 cells/well in DMEM media supplemented with 5% fetal bovine serum (FBS) and 5% bovine serum (BS) and 1% penicillin and streptomycin. The following day serum free DMEM was added to the cells and cells were treated with desired treatments. After 24 hours cell culture media was collected in eppendorf tubes and centrifuged at 1200 rpm to ensure that the cell supernatant was free of cell debris. ELISA was performed for PLA2g2a (human Type IIA) using EIA Kit from Cayman Chemicals (585000).

Western Blot

Western blot analysis was performed on whole cell extracts from rat hepatocytes and rat liver (Attia et al, 2011). Cells/ rat liver were harvested in RIPA buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA pH 8.0, 1% Triton, 1 mM benzamidine, 0.5 mM PMSF and protease inhibitor cocktail) by sonicating at medium setting for 30 seconds. The lysate were kept on ice for 30 minutes. Cell debris was removed by centrifugation at 12,000 rpm for 20 min at 4°C. The supernatant was then transferred to fresh tubes. An equal amount of protein was loaded on a 3-8% Tris-acetate acrylamide gel and transferred to a 0.22- μ m nitrocellulose membrane (Bio-Rad, Hercules, CA). The membranes were immunoblotted with primary antibodies PLA2g2a (Abcam) and Actin (A3853, Sigma) in Tris buffered saline with Tween 20 containing 5% nonfat dry milk powder. Membranes were incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody. Immunoreactive proteins were detected using Super signal West femto chemiluminescent substrate (Thermo Scientific).

Statistical Analysis

The Student's one/two tailed t test or ANOVA was used to analyze the data. The error bar indicates S.E.M. Significance is calculated relative control vs. T₃ (* = *p* value < 0.05; ** = *p* value <0.01; *** = *p* value <0.001).

Real Time PCR

2.5 μ g of RNA was reverse transcribed to cDNA using Superscript III (Invitrogen). The resulting cDNA was diluted 1:5 in nuclease free water for real time PCR reactions. The parameters for real time PCR were as follows: 95°C for 5 min, 40 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 10 s. The target genes were normalized with the 18S gene. Quantification of the PCR products was carried out using $\Delta\Delta$ Ct method. List of the forward and reverse is provided in **Table 3-1**.

Table 3-1. List of primers for RT-PCR

Genes	Primers
Rat PLA2g2a F	CATGGCCTTTGGCTCAATTCAGGT
Rat PLA2g2a R	ACAGTCATGAGTCACACAGCACCA
Rat PLA2g1b (qiagen)	QT00179529
Rat PLA2g3 F	ACAGCCTTGAACCTTCTGGTCCACT
Rat PLA2g3 R	GCTTTGAGCAGGTTGAAGCGTTG
Rat PLA2g5 F	AACTGTGTGGTCTTGAACCTCCGT
Rat PLA2g5 R	ACACACTCTCATGCAGCCTACCAT
Rat 18 S F	CGGCTACCACATCCAAGGAA
Rat 18 S R	TTTTTCGTCACTACCTCCCCG
Rat FASN F	TGGAGAAGCCCAGGAACAACATCAT
Rat FASN R	ACCGAGTAATGCCGTTTCAGTTCCT
Rat SREBP-1c F	CATGGATTGCACATTTGAAGAC
Rat SREBP-1c R	GCAGGAGAAGAGAAGCTCTCAG
Rat PDK4 F	GGATTACTGACCGCCTCTTTAGTT
Rat PDK4 R	GCATTCCGTGAATTGTCCATC
Rat CPT-1a F	CGGTTCAAGAATGGCATCATC
Rat CPT-1a R	TCACACCCACCACCACGAT
Hu PLA2g2a F	CATGGCCTTTGGCTCAATTCAGGT
Hu PLA2g2a R	AGGCTGGAAATCTGCTGGATGTCT
Hu PLA2g1b (qiagen)	QT00000637
Hu CPT1-a F	TGGCGTCTGAGAAGCATCAGCATA
Hu CPT1-a R	ACACCACGTAAAGGCAGAAGAGGT
Hu PEPCK F	ATGTCAACTGGTTCCGGAAGGACA
Hu PEPCK R	TTTCAGGTTTCAGGGCATCCTCCTT
Hu FASN F	AGGTTTGATGCCTCCTTCTTCGGA
Hu FASN R	TGGCTTCATAGGTGACTTCCAGCA
Hu PDK4 F	ACAGAGCCTGATGGATTTGGTGGA
Mice PLA2g2a F	AGCCTCGATCATGGCCTTT
Mice PLA2g2a R	GCCGAATCATTTCGCCAAA
Mice PEPCK	QT00153013
Mice CPT-1A	QT00106820
Mice PDK4	QT00157248

Results

Thyroid Hormone Inhibits PLA2g2a Expression in Primary Rat Hepatocytes

My first experiment characterized the regulation of PLA2g2a gene expression by T₃. Primary rat hepatocytes were treated with 100 nM T₃ for 24 h. The next day cells were harvested and the mRNA and protein abundance of PLA2g2a was measured. In hepatocytes, there was a 48% decrease in the PLA2g2a mRNA levels (**Figure 3-1A**). The PLA2g2a protein abundance was reduced nearly 70% by T₃ (**Figure 3-1B**). To examine whether T₃ had similar effects in human hepatoma cells, HepG2 cells were treated with T₃. Both the levels of mRNA as well as secreted PLA2g2a protein in cells media as measured by ELISA were decreased 50% (**Figure 3-1C** and **3-1D**). These data suggests that T₃ inhibits the expression of PLA2g2a in human and primary hepatocytes.

T₃ Has No Effect on the mRNA Stability of PLA2g2a

To rule out the possibility of nongenomic effects of T₃, I conducted a time course study for PLA2g2a mRNA abundance. No rapid T₃ effect was observed and a significant reduction in PLA2g2a mRNA levels was seen after 12 h (**Figure 3-2A**). These results suggested that the T₃ inhibits PLA2g2a via genomic actions. T₃ could inhibit PLA2g2a gene expression by decreasing its mRNA stability. To test this possibility I had treated primary rat hepatocytes with T₃, actinomycin and actinomycin D along with T₃. I found that the mRNA half-life of PLA2g2a is nearly 12 hours as shown in **Figure 3-2B**. The rate of mRNA decay was identical for actinomycin D and actinomycin D with T₃ treated cells suggesting that T₃ does not impact the mRNA stability (**Figure 3-2B**).

Thyroid Hormone Decreases the Cytokine Mediated Induction of PLA2g2a

Since PLA2g2a is an inflammatory enzyme and is induced by various cytokines, I investigated whether T₃ could block the cytokine mediated induction of PLA2g2a. I treated rat hepatocytes and HepG2 cells with TNF α and IL-6, respectively. Addition of T₃ decreased the TNF α induced expression of PLA2g2a mRNA from 7 to 2.5-fold (**Figure 3-3A**). Similarly, T₃ treatment reduced the IL-6 mediated induction of PLA2g2a mRNA from 10 to 5-fold (**Figure 3-3B**) in HepG2 cells. The levels of secreted PLA2g2a protein as measured by ELISA were decreased following T₃ treatment (**Figure 3-3C**).

Effect of Thyroid Hormone Status on sPLA2 Expression in Rats

Next, I investigated whether the thyroid status regulated the endogenous sPLA2 gene expression *in vivo*. Rats were made hypothyroid by providing an iodine free diet supplemented with propylthiouracil (PTU) for five weeks. T₃ was administered twice (0.33 mg/kg body wt.) at 24 hrs intervals. T₃ administration decreased the hepatic

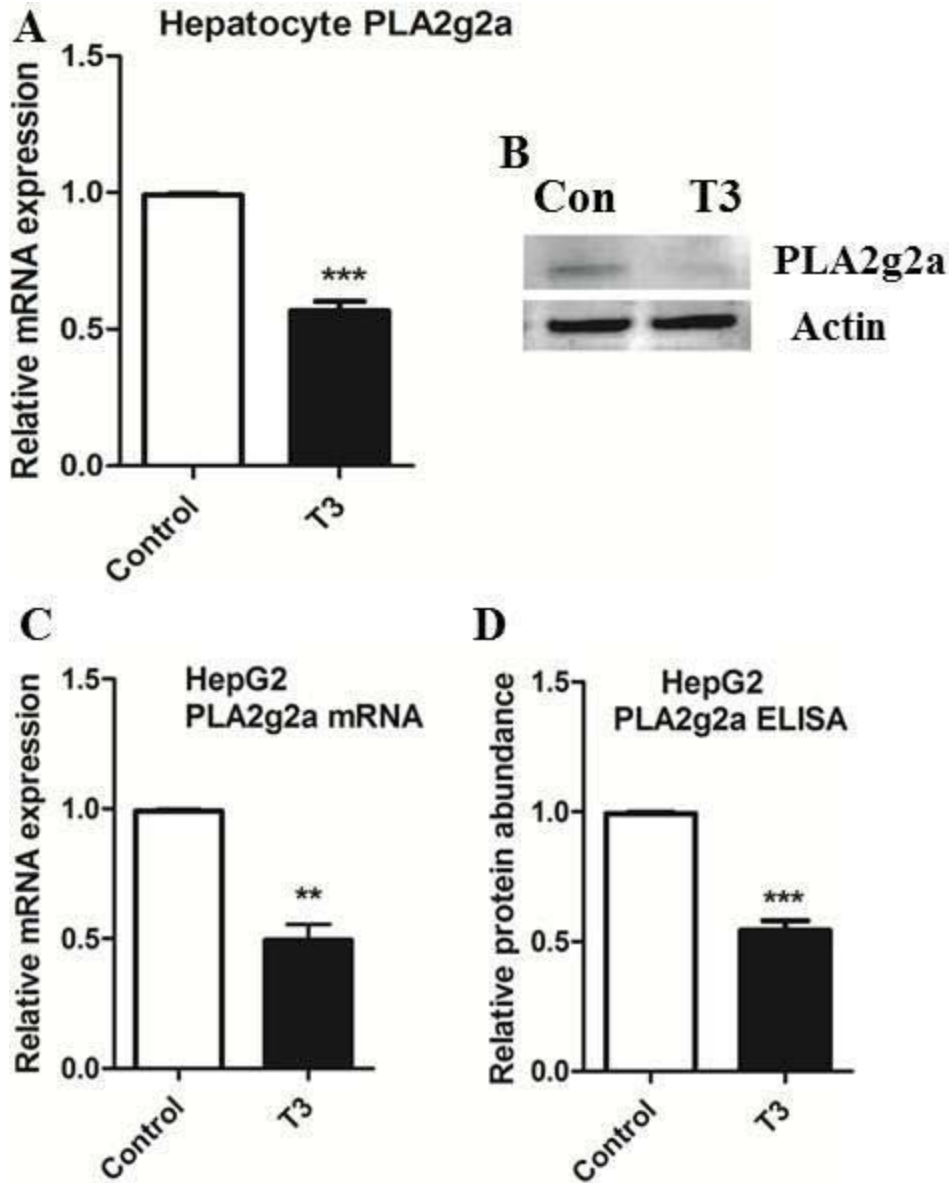


Figure 3-1. Thyroid hormone inhibits the expression of PLA2g2a

A) Rat hepatocytes were treated with 100 nM thyroid hormone (T₃) for 24 hrs. PLA2g2a mRNA levels were measured by real time PCR. B) PLA2g2a protein levels were measured by western analysis in rat hepatocytes. C) HepG2 cells were treated with 100 nM thyroid hormone (T₃) for 24 hrs. PLA2g2a mRNA levels were measured by real time PCR. D) PLA2g2a protein levels in HepG2 cells were measured by ELISA. Data are expressed as the relative RNA or protein expression. All experiments were repeated 4 to 6 times. The data are expressed as the mean of the fold induction by T₃ ± S.E.M of mRNA abundance relative to untreated cells (** = *p* value <0.01; *** = *p* value <0.001).

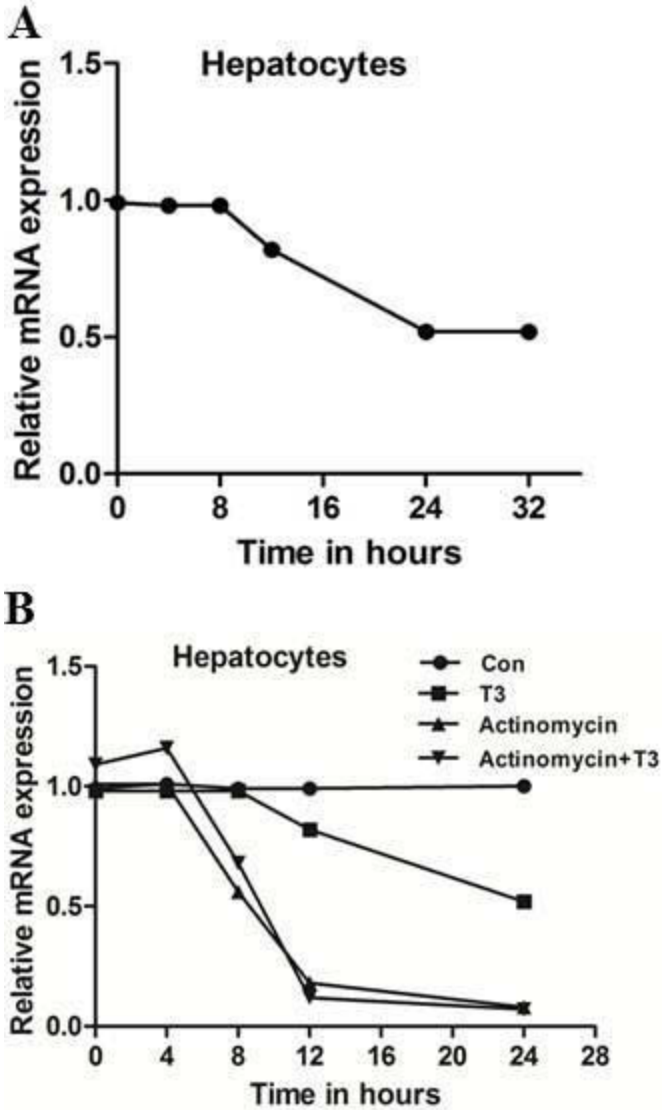


Figure 3-2. T₃ has no effect on the mRNA stability of PLA2g2a

A) Rat hepatocytes were treated with T₃ for various times and PLA2g2a mRNA abundance was measured. B) Hepatocytes were exposed to T₃ or 5 μg/ mL actinomycin D or both. PLA2g2a mRNA levels in HepG2 cells were assessed the indicated time points. Data are expressed as the relative RNA. All experiments were repeated 3-4 times. The data are expressed as percent inhibition.

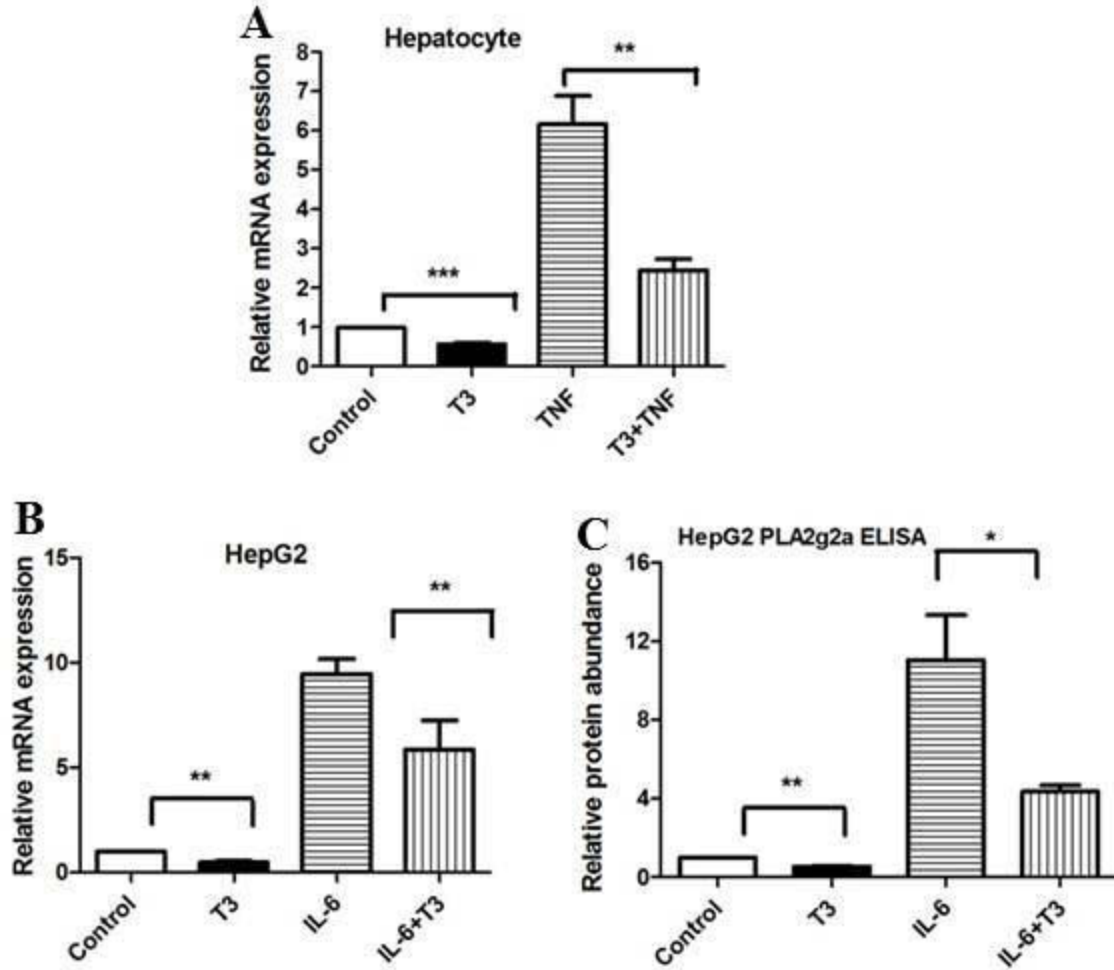


Figure 3-3. T₃ inhibits the cytokine mediated induction of PLA2g2a

A) Rat hepatocytes were treated with 25 ng/mL TNF α or 100 nM T₃ or both for 24 hrs. PLA2g2a mRNA abundance was assessed. B) HepG2 cells were treated with 10 ng/mL IL-6 or 100 nM T₃ for 24 h. RNA abundance was determined. C) Media was collected from HepG2 cells treated with IL-6 or T₃ and the PLA2g2a levels were determined by ELISA. All experiments were repeated 4 to 6 times. The data are expressed as the mean of the fold induction \pm S.E.M of mRNA abundance relative to untreated control cells (* = p value < 0.05; ** = p value < 0.01; *** = p value < 0.001).

expression of PLA2g2a mRNA. The isoform of sPLA2 were similarly inhibited by T₃ suggesting that T₃ modulates additional secretory PLA2 genes (**Figure 3-4A** and **3-4B**). Expression of the lipogenic gene SREBP-1c gene was decreased by T₃ administration (**Figure 3-4C**). Expression of other positively regulated genes was examined. Carnitine palmitoyltransferase (CPT1a), phosphoenolpyruvate carboxykinase (PEPCK) and pyruvate dehydrogenase kinase (PDK4) were all induced by T₃ (**Figure 3-4D-F**). The abundance of the PLA2g2a protein was decreased by T₃ administration (**Figure 3-4G**).

T₃ Inhibits PLA2g2a in BALB/c Mice

Since C57BL/6 have a “natural” knockout of PLA2g2a while BALB/c mice express PLA2g2a. I tested whether the thyroid status similarly regulated PLA2g2a expression in BALB/c mice. Mice were made hypothyroid by providing an iodine free diet supplemented with 0.15% PTU for five weeks. T₃ was administered twice at lower dose (0.11 mg/Kg body wt.) at 24 hrs intervals for two consecutive days. Liver were then harvested. RNA abundance of PLA2g2a (**Figure 3-5A**) and other positive control genes were measured (**Figure 3-5B-D**). In hyperthyroid BALB/c mice PLA2g2a levels were significantly decreased while the positive control genes like PDK4 and PEPCK were induced.

Effect of Hypothyroidism and High Diet on the Expression of PLA2g2a

Since PLA2g2a was induced in the adipose tissue of rats which were on a high fat diet (Iyer et al, 2012), I examined the ability of long chain fatty acids (LCFA) to directly increase the expression of PLA2g2a. Addition of oleic acid (18:1) to hepatocytes elevated PLA2g2a mRNA 3 fold, and this stimulation was inhibited by T₃ (**Figure 3-6A**).

The effect of high fat diet was evaluated in BALB/c mice. These mice were divided into three experimental groups: 1) chow fed (Teklad Diet 06101), 2) hypothyroid (PTU) (TD 120714) 3) and high fat diet (HFD) (TD 95217). Hypothyroidism increases the expression of PLA2g2a around 1.8 fold while PLA2g2a was induced in liver nearly 3.8 fold in response to a high fat diet (**Figure 3-6B**).

T₃ Inhibits the Expression of PLA2g2a in C57BL/6 hPLA2gIIA+ Mice

In this experiment (**Figure 3-7A-I**), I tested the effect of hypo and hyperthyroidism in C57BL/6 hPLA2gIIA+ mice. These C57BL/6 hPLA2gIIA+ mice express human PLA2g2a. This experiment was important for demonstrating the feasibility of the future mouse experiments where we propose to identify the role of PLA2g2a in liver. As shown in **Figure 3-7A** hyperthyroid rats had significantly lower levels of PLA2g2a. Similarly other sPLA2 isoforms (**Figure 3-7B-D**) excluding PLA2g1b were inhibited in hyperthyroid rats. Negative control gene SREBP-1c (**Figure 3-6E**) was also inhibited while positive control genes were stimulated (**Figure 3-7F-H**).

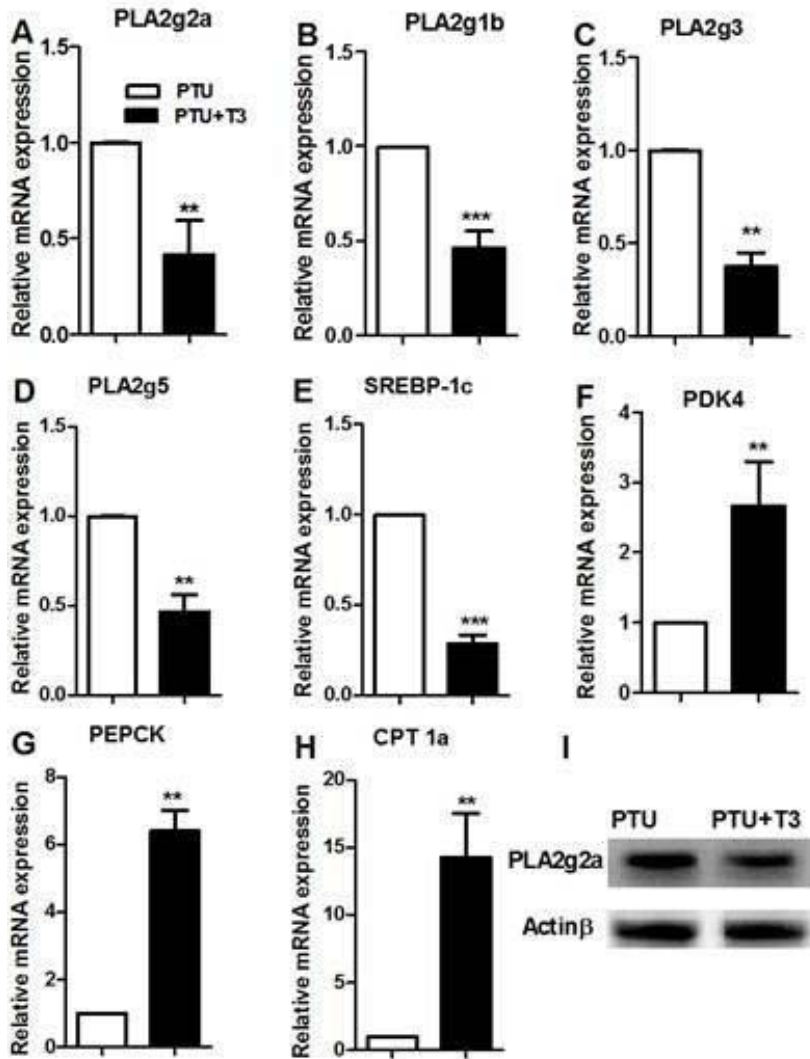


Figure 3-4. T₃ inhibits the expression of PLA2g2a *in vivo* in rats

A) Rats were made hypothyroid with an iodine free diet and the addition of PTU. After 5 weeks, the animals were sacrificed and mRNA was harvested from the liver. The mRNA levels were measured for A) PLA2g2a, B) PLA2g1b, C) PLA2g3, D) PLA2g5, E) SREBP-1c, F) PDK4, G) PEPCK, H) CPT1a. I) PLA2g2a protein abundance was measured by western blotting. Values are the average of RNA from 4 rats. The data are expressed as the mean of the fold change by PTU+T₃ ± S.E.M of mRNA abundance relative to PTU treated rats (** = *p* value <0.01; *** = *p* value <0.001).

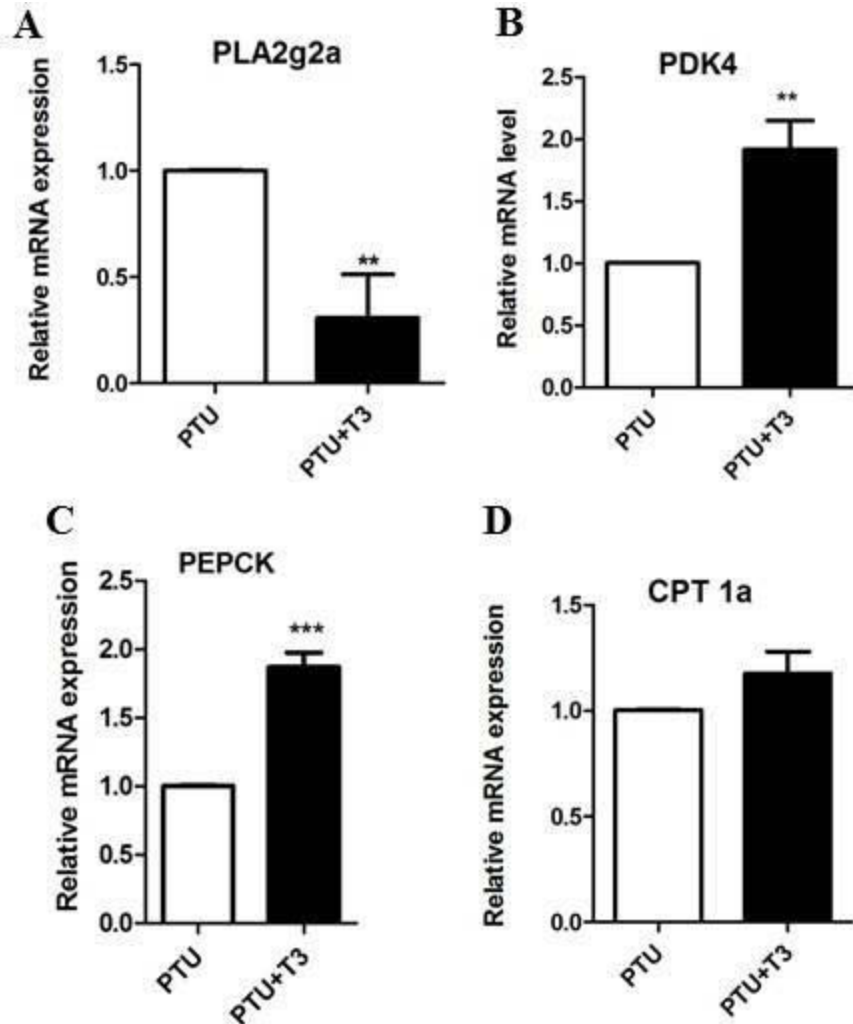


Figure 3-5. T₃ regulates PLA2g2a in BALB/c mice

A) BALB/c mice were made hypothyroid as mentioned previously. Hyperthyroidism was induced by injecting them with a lower dose of T₃ (0.11mg/kg body wt.) for two days. Mice were sacrificed and liver were harvested for RNA. The mRNA levels were measured for A) PLA2g2a, B) PDK4, C) PEPCK D) CPT1a. Values are the average of RNA from 5 mice. The data are expressed as the mean of the fold induction by PTU+T₃ ± S.E.M of mRNA abundance relative to PTU treated mice (** = *p* value <0.01; *** = *p* value <0.001).

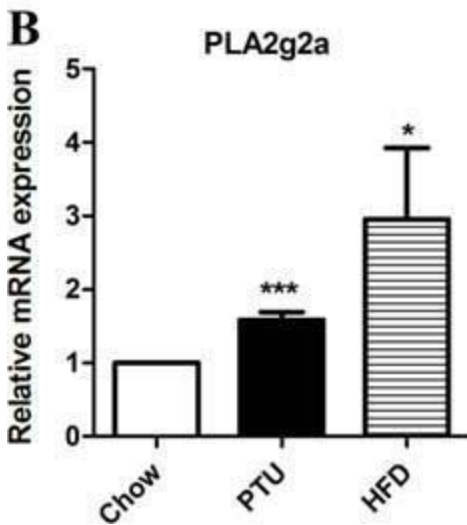
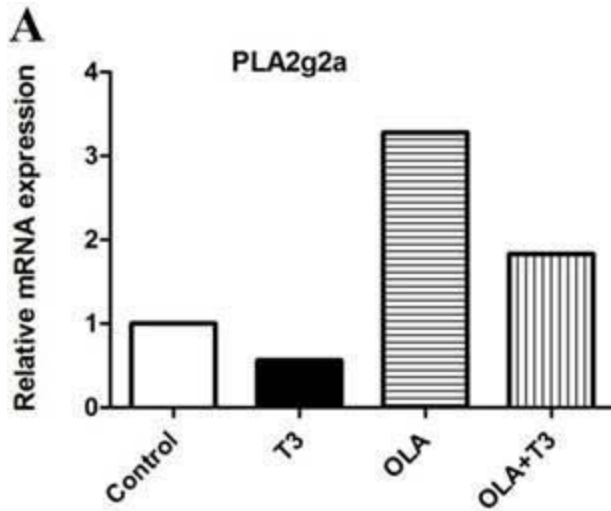


Figure 3-6. PLA2g2a is induced by high fat diet and hypothyroidism

A) Primary rat hepatocytes were treated with 100 nM T₃ or oleic acid (18:1) or both. Cells were harvested and PLA2g2a was measured. B) BALB/c mice were kept on chow diet, iodine deficient PTU diet and high fat diet for 12 weeks. Mice were sacrificed after 12 weeks and liver was harvested for mRNA analysis. The levels of PLA2g2a were measured by RT-PCR.

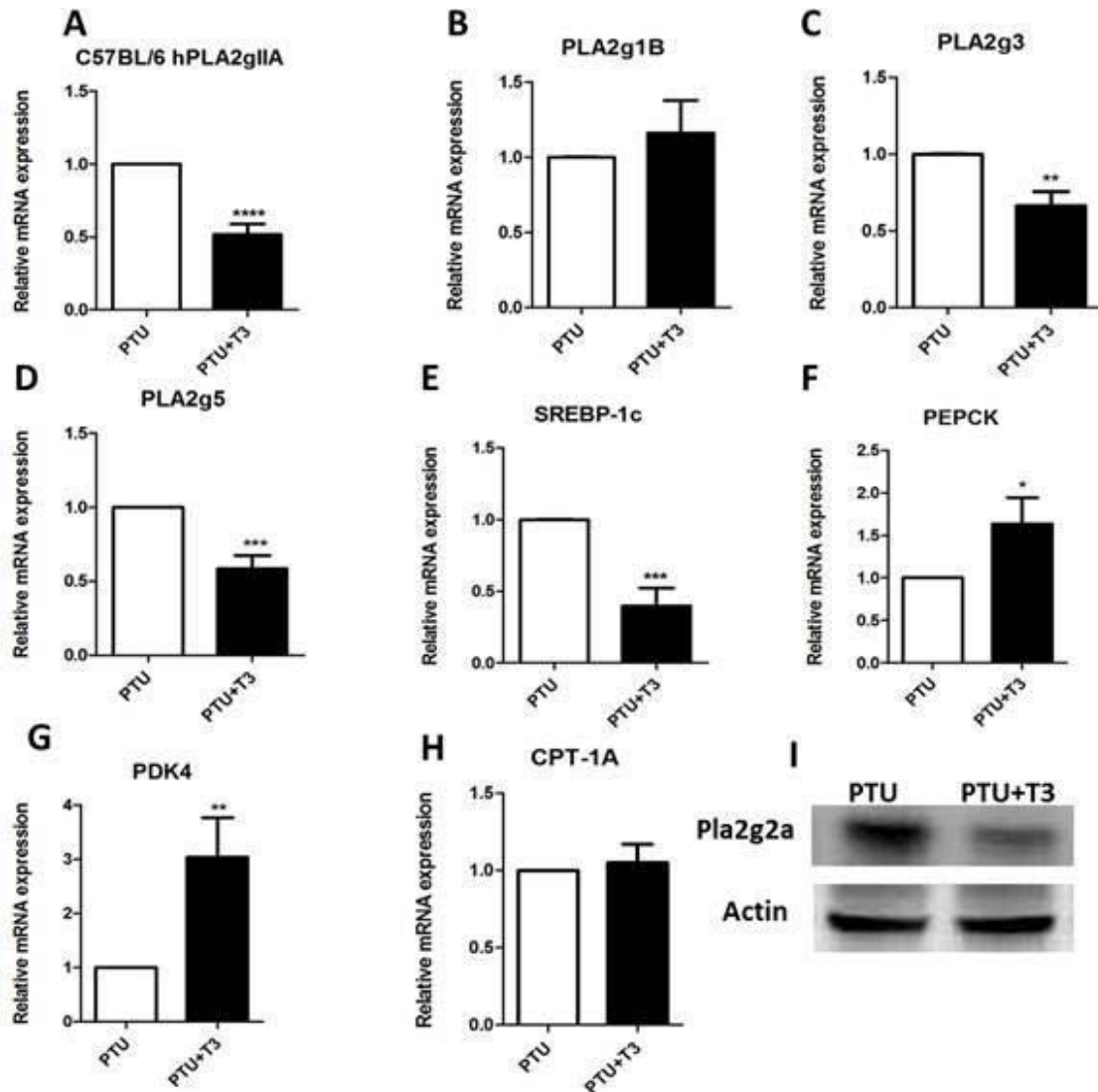


Figure 3-7. T₃ inhibits sPLA2s in transgenic C57BL/6 hPLA2gIIA⁺ mice models

C57BL/6 hPLA2gIIA⁺ mice were kept on iodine free and PTU supplemented diet for 5 weeks. After 5 weeks, the mice were sacrificed and mRNA was harvested from the liver. The mRNA levels were measured for A) PLA2g2a, B) PLA2g1b, C) PLA2g3, D) PLA2g5, E) SREBP-1c; F) PEPCK, G) PDK4, and H) CPT-1a, I) PLA2g2a protein abundance was measured by western blotting. Values are the average of RNA from 7 mice. The data are expressed as the mean of the fold induction by PTU+T₃ ± S.E.M of mRNA abundance relative to PTU treated mice. (* = *p* value < 0.05; ** = *p* value < 0.01; *** = *p* value < 0.001).

T₃. The abundance of the PLA2g2a protein was decreased by T₃ administration (**Figure 3-7I**). The serum profile of hypo and hyperthyroid C57BL/6 hPLA2gIIA+ mice is shown in **Table 3-2**. Serum levels of free unbound T₃ (FT3), cholesterol (CHL), triglycerides (TGS), high density lipoproteins (HDL) and low density lipoproteins (LDL) were measured.

Table 3-2. Serum profiles of C57BL/6 hPLA2gIIA+ mice

Mice	CHL (mg/dl)	TGs (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	FT3 (pg/ml)
PTU Mice 1	145	48	85	52	1.1
PTU Mice 2	188	43	104	75	<1
PTU Mice 3	152	60	89	51	1.8
PTU Mice 4	131	49	85	36	3.3
PTU Mice 5	174	59	98	64	1.5
PTU Mice 6	166	59	99	55	1.3
PTU Mice 7	157	69	96	47	1.1
PTU+T3 Mice 1	41	36	30	4	14.3
PTU+T3 Mice 2	47	55	38	0	18.3
PTU+T3 Mice 3	53	52	44	0	24
PTU+T3 Mice 4	37	55	27	0	16.4
PTU+T3 Mice 5	23	22	17	1.5	20
PTU+T3 Mice 6	53	38	40	5	17
PTU+T3 Mice 7	30	42	23	0	20.1

CHAPTER 4. NUCLEAR COREPRESSORS MEDIATED THE INHIBITION OF PLA2G2A GENE TRANSCRIPTION BY THYROID HORMONE*

Introduction

The genomic mechanisms by which T₃ induces gene expression include the ligand mediated activation of TR β , the dissociation of corepressors and subsequent recruitment of coactivators (Cheng et al, 2010b). Although, many genes are repressed by T₃ the mechanisms by which T₃ represses gene expression are not as well understood. Several hypotheses have been proposed to explain the TR mediated gene repression (Santos et al, 2011; Weitzel, 2008). However despite considerable effort, no consensus model has been proposed. In this chapter I had investigated the mechanism of PLA2g2a repression by thyroid hormone.

Material and Methods

Cloning of Rat PLA2g2a Promoter

Genomic DNA was isolated from a rat tail using Qiagen genomic DNA isolation kit (51304). The rat PLA2g2a promoter region was obtained from the PCR amplification of the genomic DNA. The forward primers contain Sac I restriction sites while and the reverse primers contain a Bgl II restriction sites. The PCR products were cut with appropriate restriction enzymes and were cloned into PGL4 expression vector. The primers used to amplify the PLA2g2a promoter are listed in **Table 4-1**.

Transient Transfections of Luciferase Vectors

HepG2 cells were seeded 4 hours prior to transfections and were maintained in DMEM media supplemented with 5% FBS, 5% BS and 1% penicillin and streptomycin. The cells were than transfected by calcium phosphate method. Transfections included 2 μ g of PLA2g2a luciferase reporters, 1 μ g of SV40-TR β and 0.1 μ g of TK-renilla. On the next day, cells were washed twice with phosphate-buffered saline (PBS) and the media was changed to serum free DMEM media and 100 nM of T₃ added. Cells were harvested after 24 h of treatment in passive lysis buffer. Luciferase assays were conducted from the cell lysate using the Promega Dual Luciferase kit (Cat No. E1960). Protein content in each lysate was determined by BCA method. Luciferase values were normalized for protein content and renilla luciferase activity to account for cell density and transfection efficiency, respectively.

* Adapted with permission. Sharma P, Thakran S, Deng X, Elam MB, Park EA. (2013). Nuclear Corepressors Mediate the Repression of Phospholipase A2 Group IIa Gene Transcription by Thyroid Hormone *J. Biol Chem*, 2013 June 7; 288(23):16321-33.

Table 4-1. List of primers used for cloning PLA2g2a promoter

Nucleotides	Primer sequence 5' to 3'
-448 F.P	AGTGAGCTCATGAATGACTGACACGTGAATTAAG
-378 F.P	AGTGAGCTCACACTCATTCCCTCCCCTGT
-297 F.P	AGTGAGCTCTGGGCTTTTGGAAAGTTCTC
-203 F.P	AGTGAGCTCACCCATGGTATGAGGGCTTT
-175 F.P	AGTGAGCTCTCAAGGCTGTTCTGCCAGCTGTT
-119 F.P	AGTGAGCTCGTATGCCCGTCTGTGAATCC
-49 F.P	AGTGAGCTCCCCTGCAGAGGGAAGAGCTATTA
-24 F.P	AGTGAGCTCGAGCATTGGGAGTACATGCCCA
+58 R.P	AGTAGATCTTGGGCATGTACCTCTTGAT

Real Time PCR

RNA was isolated from primary rat hepatocytes using RNA-Stat-60 (Tel-Test). Isolated RNA was then purified with the Qiagen RNeasy Mini Kit (74104). The concentration of each sample was then measured by using the Nanodrop machine. For RT-PCR, 2.5 µg of cDNA was prepared using Superscript III (Invitrogen) from the RNA samples. The resulting cDNA was diluted 1:6 in nuclease free water for real time PCR reactions. The parameters for real time PCR were as follows: 95°C for 5 min, 40 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 10 s. Primers were used at a final concentration of 0.1 µM. The target genes were normalized with the 18S gene. Quantification of the PCR products was carried out using $\Delta\Delta C_t$ method. The forward and reverse primers used for real time PCR are provided in **Table 4-2**.

ELISA

HepG2 cells were grown in 24 well plates at a density of 0.2×10^6 cells/well. The growth media consists of 5% FBS and 5% BS and 1% antibiotics penicillin and streptomycin. Next day cells were washed twice with 1XPBS and were kept on serum free DMEM as media. Cells were then given the desired treatments. After 18 hours of treatment, cell culture media was collected in eppendorf tubes and centrifuged at 1200 rpm to remove any cell debris. The ELISA was performed on the supernatant cell culture media for PLA2g2a (human Type IIA) using EIA Kit from Cayman chemicals (585000).

Electrophoretic Mobility Shift Assay

To conduct electrophoretic mobility shift assays, double-stranded oligonucleotides were labeled with klenow enzyme and [α - 32 P] dCTP. Oligonucleotides were designed to contain sequences representing the nTRE. Recombinant histidine tagged TR β (His-TR β) and RXR α were prepared in the BL21 *E. coli* strain as described previously (Jansen et al, 2000). The protein-DNA binding mixtures contained labeled probe (60,000 cpm) in 80 mM KCl, 25 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol and polydeoxyinosine-deoxycytidine (dI-dC). In the supershift assays antibodies were added prior to the addition of nuclear proteins. TR β supershift antibody was purchased from santa cruz. The binding reactions were incubated at room temperature for 20 min and then resolved on 5% non-denaturing acrylamide gels (80:1 acrylamide/bisacrylamide) in Tris-glycine running buffer (22 mM Tris and 190 mM glycine) (Jansen et al, 2000). The electrophoresis was then carried out 160 volts for 70 minutes.

Site-Directed Mutagenesis of the PLA2g2a Promoter

The Quick-change-XL site directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) was used to alter nucleotides in nTRE in the -448/+58 PLA2g2a-luciferase.

Table 4-2. List of RT-PCR primers

Nucleotides	Primer sequence 5' to 3'
Rat PLA2g2a F	CATGGCCTTTGGCTCAATTCAGGT
Rat PLA2g2a R	ACAGTCATGAGTCACACAGCACCA
Rat PLA2g1b qiagen.	QT00179529
Rat 18 S F	CGGCTACCACATCCAAGGAA
Rat 18 S R	TTTTCGTCACTACCTCCCCG
Rat FASN F	TGGAGAAGCCCAGGAACAACATCAT
Rat FASN R	ACCGAGTAATGCCGTTTCAGTTCCT
Rat SREBP-1c F	CATGGATTGCACATTTGAAGAC
Rat SREBP-1c R	GCAGGAGAAGAGAAGCTCTCAG
Rat PDK4 F	GGATTACTGACCGCCTCTTTAGTT
Rat PDK4 R	GCATTCCGTGAATTGTCCATC
Rat CPT-1a F	CGGTTCAAGAATGGCATCATC
Rat CPT-1a R	TCACACCCACCACCACGAT
PLA2g2a RT-PCR F	CATGGCCTTTGGCTCAATTCAGGT
PLA2g2a RT-PCR R	AGGCTGGAAATCTGCTGGATGTCT
Hu PLA2g1b qiagen	QT00000637
Hu CPT1-a F	TGGCGTCTGAGAAGCATCAGCATA
Hu CPT1-a R	ACACCACGTAAAGGCAGAAGAGGT
Hu PEPCK F	ATGTCAACTGGTTCCGGAAGGACA
HU PEPCK R	TTTCAGGTTTCAGGGCATCCTCCTT
Hu FASN F	AGGTTTGATGCCTCCTTCTTCGGA
Hu FASN R	TGGCTTCATAGGTGACTTCCAGCA
Hu PDK4 F	ACAGAGCCTGATGGATTTGGTGGGA
Hu PDK4 R	TGACTGGGTCAACTGTACAGGCAT

The forward (FP) and reverse primers (RP) used to introduce these mutations are shown in **Table 4-3**.

siRNA Knockdown Experiment

Small interfering RNA (siRNA) against human SMRT and NCoR1 and RNA interference-negative control were purchased from Dharmacon. HepG2 cells were grown in 60mm plates and were transfected with the siRNA against SMRT, NCoR1 or nonspecific RNA (200 pmol) using Lipofectamine 2000 (Invitrogen). Knockdown of SMRT and NCoR1 was confirmed by real-time PCR and western blot. After 16 hours of transfection, cells were treated with 250 nM T₃ in serum free medium for 24 h. Forty-eight hours after transfection, cells were harvested for RNA and proteins.

Western Blot Analysis

Western blot analysis was performed on whole cell extracts from HepG2 cells and rat hepatocytes (Attia et al, 2011). Cells were harvested in RIPA buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA pH 8.0, 1% Triton, 1 mM benzamidine, 0.5 mM PMSF and protease inhibitor mixture from Sigma). The cells were kept on ice for 30 minutes. Cell debris was removed by centrifugation at 12,000 rpm for 20 min at 4°C. Protein was quantified by the BCA method. An equal amount of protein was loaded on a 3-8% Tris-acetate acrylamide gel and transferred to a 0.45- μ m nitrocellulose membrane (Bio-Rad). The membranes were immunoblotted with primary antibodies NCoR1 (5948, Cell Signaling), SMRT (06-891, Millipore) and Actin (A3853, Sigma) in Tris buffered saline with Tween 20 containing 5% nonfat dry milk powder. Membranes were incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody. Immunoreactive proteins were detected using Supersignal West Femto Chemiluminescent Substrate (Thermo Scientific).

Immobilized Template Assay

To characterize the binding of TR β to the PLA2g2a promoter region immobilized template assays were carried out. PLA2g2a promoter fragments corresponding to regions -119/+58 and the +1108 to +1256 control region were PCR amplified from genomic DNA using the 5' biotinylated forward primer and the reverse primers. The PCR products were purified with a gel extraction kit (Qiagen, M-280). Streptavidin coated Dynabeads (Invitrogen) were resuspended in equilibration buffer (5 mM Tris-HCl (pH 7.5), 1mM EDTA, 1M NaCl) and then conjugated with 70 ng biotinylated template for 30 min at room temperature with constant agitation. The immobilized templates were concentrated with a magnetic particle concentrator, washed once with 5 mM Tris-HCl, pH 7.5, 1mM EDTA, 1M NaCl, 0.05% Tween 20 and subsequently with binding buffer (20 mM HEPES (pH 7.6), 4 mM MgCl₂, 80 mM KCl, 0.08 mM EDTA, 8 mM DTT, 10% glycerol, and 0.05% Tween 20). The beads were concentrated by a magnetic particle

Table 4-3. List site directed mutagenesis primers

Mutants	Primer sequence 5' to 3'
-102mut F	CCGTCTGTGAATCCATGCGCAGGCCACACCCACCT CC
-102mut R	GTGGGTGTGGCCAAATAACATCTTCACAGACGGGC ATAC
-97mut F	CCGTCTGTGAATCCATGCGCAGGCCACACCCACCT CC
-97mut R	GGAGGTGGGTGTGGCCTGCGCATGGATTCACAGAC GG
-92mut F	CGTCTGTGAATCCATTATTTATAGCACCCACCTCC CCATCCCTG
-92mut R	CAGGGATGGGGAGGTGGGTGCTATAAAATAATGG ATTCACAGACG
-87mut F	GTGAATCCATTATTTGGCCAAGATAACCTCCCCAT CCCTGTGGC
-87mut R	GCCACAGGGATGGGGAGGTTATCTTGGCCAAATAA TGGATTCAC
-82mut F	CATTATTTGGCCACACCCTATGTCCCATCCCTGTGG CTCTC
-82mut R	GAGAGCCACAGGGATGGGACATAGGGTGTGGCCA AATAATG

concentrator and resuspended in 25 μ l of binding buffer along with TR β , RXR α , poly deoxyinosine-deoxycytidine (dI-dC) and 100 nM T₃ for 20 min at room temperature. The Dynabeads were washed four times with binding buffer, resuspended in 3 X SDS-loading buffers, resolved by 4-12% bis-tris gel and analyzed via immunoblotting. The primers used for immobilized template assay are listed in **Table 4-4**.

Chromatin Immunoprecipitation Assays

ChIP assays were conducted according to manufacturer's protocol for the Millipore Magna ChIP kit (17-610) with minor modifications. Rat hepatocytes were grown in RPMI 1640 media containing 5% fetal bovine serum and 5% calf serum for 24 hrs. Cells were treated with 100 nM T₃ overnight in serum-free media. Next day cells were cross linked with 1% formaldehyde for 10 min at room temperature and sonicated as previously described (Attia et al, 2011) to give DNA fragments between 500-800bp. The supernatant chromatin was precleared and immunoprecipitated with the control antibody IgG (sc-2027, Santa Cruz), anti-TR β (MA1-216, Thermo Scientific), anti-NCoR1 (5948, Cell Signaling) or anti-SMRT (17-10057, Millipore) overnight at 4°C along with magnetic protein G beads. The beads were washed and the DNA eluted. Eluted DNA was purified using the PCR purification kit (Qiagen 28104). DNA was subjected to 32 cycles of PCR using 3-5 μ L of DNA. PCR products were analyzed on 2% Nusieve 3:1 agarose (Lonza, Walkersville, MD) and visualized with Multimage Light Cabinet with Alpha Imager EP software. The primers used to amplify portions of the PLA2g2a promoter are provided in **Table 4-5**.

Results

T₃ Inhibits PLA2g2a at Promoter Level

To determine if T₃ directly regulates PLA2g2a gene expression, the rat PLA2g2a promoter was cloned and fused to the luciferase (luc) reporter. The -448/+58 PLA2g2a-luc vector was transfected into HepG2 cells along with TR β using the transient transfection method. HepG2 cells were then treated with T₃ for 24 hrs. T₃ decreased the activity of -448/+58 PLA2g2a-luc nearly by 70%. This result suggests that PLA2g2a is regulated by T₃ at promoter level and T₃ inhibits PLA2g2a expression by inhibiting its transcription. Various conflicting reports have been published on the requirement of DNA binding domain (DBD) for gene repression (Shibusawa et al, 2003). To evaluate the role of the TR β DBD, I made a TR β mutant with only the ligand binding domain (TR β -LBD) and lacking DBD. TR β -LBD was transfected with -448/+58 PLA2g2a-luc into the HepG2 cells. Unlike full length TR β , unliganded TR β -LBD failed to exhibit the T₃ mediated repression (**Figure 4-1**). These data suggest that the intact DNA binding domain is critical for T₃ effect and TR β might bind directly to PLA2g2a gene promoter.

Table 4-4. List of primers used for immobilized template assay

Nucleotides	Primer sequence 5' to 3'
-119PLA2g2a F	GTATGCCCGTCTGTGAATCC
+58 PLA2g2a R	TGGGCATGTACCTCTTGGAT
+1158 PLA2g2a F	GCTATGGCTTCTACGGTTGC
1256 PLA2g2a R	CGGTGGGACACTGAGGTAGT

Table 4-5. List of primers used for ChIP assay

Nucleotides	Primer sequence 5' to 3'
-180 rat PLA2g2aF	GCCCGTCTGTGAATCCATTATTTGGC
+61 rat PLA2g2a R	CAAATGCATCCAAAGGGCAGGAGT
+1639 rat PLA2g2a F	CACACACATGCATGCTGGGAACTT
+1900 rat PLA2g2a R	GCTTAGGCTGCCTTTGAGTTCTCT

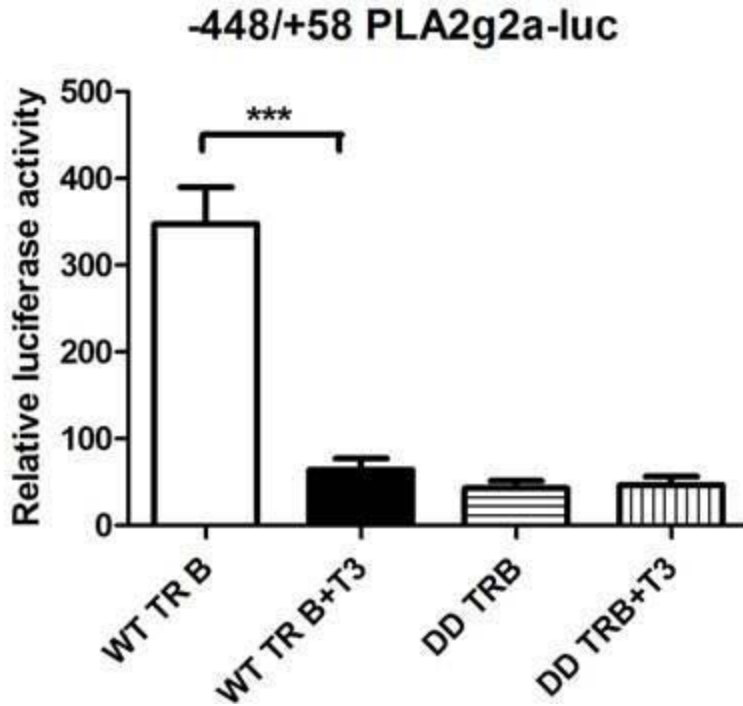


Figure 4-1. T₃ inhibits PLA2g2a at promoter level

HepG2 cells were transfected with 2 µg of -448/+58 PLA2g2a luciferase, 1 µg of SV40-TRβ or TRβ-LBD and 0.1 µg of TK-renilla and treated with or without T₃ for 24 hours. The data are expressed as the relative luciferase activity. Luciferase activity was corrected for both protein content and Renilla activity. All experiments were repeated 3 to 4 times. The error bar indicates S.E.M. Significance is calculated relative wild TRβ treated with T₃ (***) = *p* value <0.001).

Localization of a T₃ Responsive Element in the PLA2g2a Gene Promoter

The next experiments were designed to identify the thyroid response element in the PLA2g2a promoter. A series of deletions of the PLA2g2a gene were made between -448 and -24 region. The serial deletion removed approximately 20 to 80 bp fragments from the 5' end of the promoter in the -448/+58 PLA2g2a-luc vector. The diagrammatic representation of 5' serial deletions of PLA2g2a is shown in **Figure 4-2A**. These PLA2g2a-luc constructs were then transfected into HepG2 cells and luciferase assays were conducted. The luciferase activity of serial deletion was compared to the -448/+58 construct. The results are expressed in terms of percent inhibition. Deletion of the promoter region between -102/-82 resulted in loss of repression by T₃, suggesting that a negative thyroid response element (nTRE) was located in the -102/-82 region proximal promoter region (**Figure 4-2B**).

TRβ Binds Directly to the Proximal Promoter Region of PLA2g2a

Since the TRβ mutant lacking the DBD was not able to inhibit PLA2g2a-luc the DNA binding domain of TRβ was required to represses PLA2g2a. We next tested whether TRβ binds directly to the PLA2g2a promoter using immobilized template assays. I generated a biotinylated -119/+58 fragment of the PLA2g2a promoter and a control +1108/+1256 region. The biotinylated DNA templates were incubated with the recombinant TRβ and RXRα with and without T₃. The biotinylated templates along with the bound proteins were precipitated by streptavidin beads. The beads were washed and the bound proteins were eluted and analyzed by western analysis. TRβ bound as a heterodimer with RXRα to the -119/+58 region, and T₃ had no effect on the binding. In contrast, no binding was observed in control +1108/+1256 region (**Figure 4-3**).

Characterization of the nTRE in the PLA2g2a Promoter

Next, I examined whether TRβ binds directly to the nTRE (-102/-82) of the PLA2g2a gene. Electrophoretic mobility shift experiments were performed using purified recombinant TRβ or RXRα proteins. Binding studies were carried out with a ³²P labeled PLA2g2a nTRE oligomer. The DNA/protein complexes were resolved on a non-denaturing acrylamide gel. The results showed that neither TRβ nor RXRα alone could bind to the -102/-82 region (**Figure 4-4A lanes 2 and 3**). TRβ along with RXRα bound as a heterodimer to -102/-82 region while T₃ had no effect on binding (**Figure 4-4A lanes 4 and 5**). To confirm the specificity of the protein binding, antibodies to TRβ and IgG were used. The TRβ antibody disrupted the complex showing that TRβ was present in the complex (**Figure 4-4A lane 6**), whereas a nonspecific antibody had no effect (**Figure 4-4A lane 7**). To check the specificity of the sequence, competition assays were conducted using a 10-fold excess of double-stranded unlabeled -102/-82 oligomer, an idealized TRE (DR4) or a nonspecific unlabeled oligomer. The unlabeled -102/-82 oligomer competed with the TRβ binding (**Figure 4-4B lane 3**) and the DR4 completely disrupted binding while nonspecific oligomers had no effect (**Figure 4-4B lanes 4 and 5**).

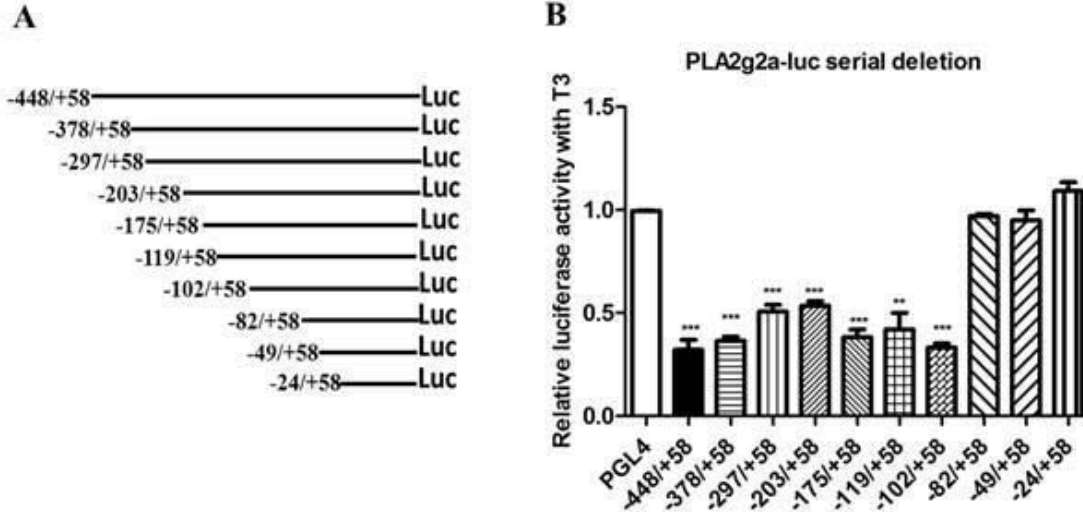


Figure 4-2. Localization of thyroid response element

A) A model of the serial deletions of the rat PLA2g2a promoter ligated in front of the luciferase reporter gene (PLA2g2a-luc) is shown. B) HepG2 cells were transfected with various 5' serial deletions of PLA2g2a-luc and an expression vector for TR β . Cells were treated with T₃ for 24 hours. The data are expressed as relative inhibition with T₃. All transfections were repeated 4 to 6 times. The significance is calculated relative to the empty vector PGL4. The error bar indicates S.E.M (* = p value < 0.05; ** = p value < 0.01; *** = p value < 0.001).

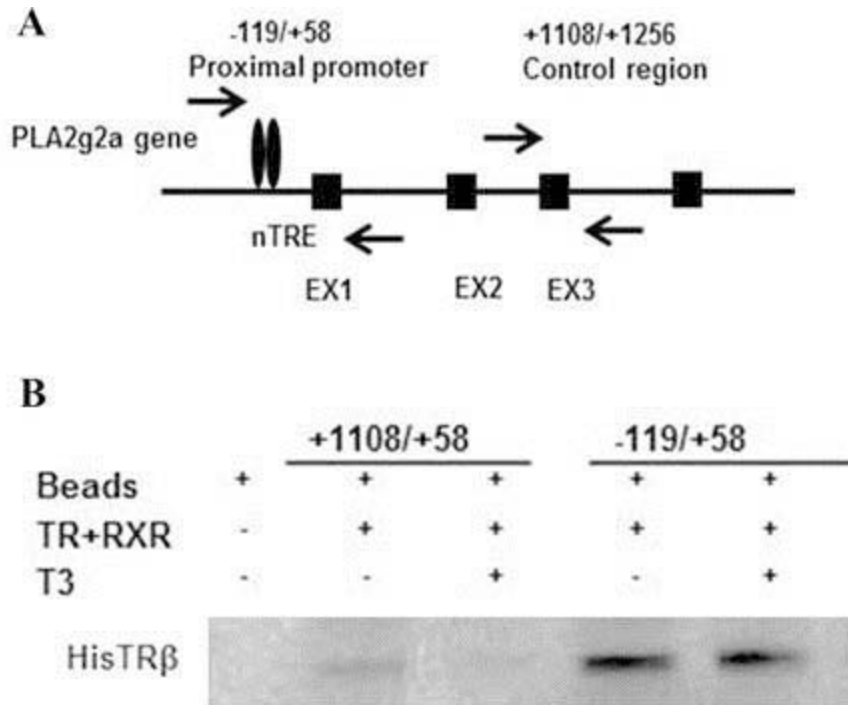


Figure 4-3. TR β binds directly to the proximal promoter region of PLA2g2a

A) Schematic representation of PLA2g2a core promoter primers and control primers used to generate biotinylated DNA. B) The immobilized template assay was conducted with the biotinylated DNA corresponding to region -119/+58 and +1158/+1256 control region. The biotinylated DNA was incubated with His-TR β and His-RXR α with and without T₃. The protein DNA complexes were resolved on Bis-Tris 4-12% gel and probed for TR β by western blotting.

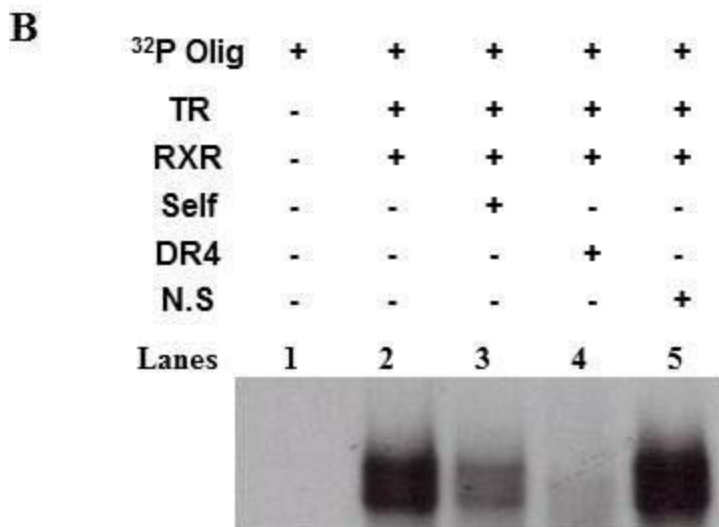
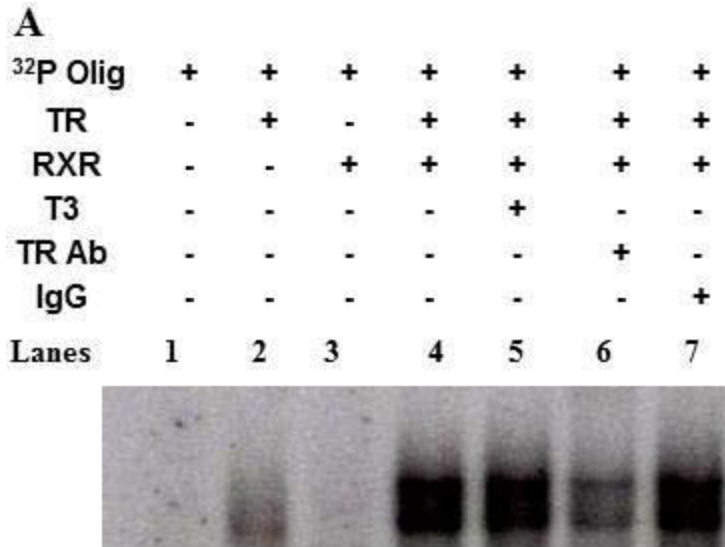


Figure 4-4. Characterization of negative thyroid response element

A) Double-stranded oligonucleotides were constructed that encompassed the -102/-82 nucleotides in the PLA2g2a gene. The ³²P-radiolabeled double-stranded oligomer representing the -102/-82 element was incubated with purified recombinant TR β and RXR α . Antibodies to TR β and IgG as well as 100 nM of T₃ were added. B) To assess the specificity of the TR-RXR α binding, a 10-fold excess of the competitor oligomers was added. Competition assays were conducted using double-stranded unlabeled -102/-82 direct repeat of AGGTCA separated by 4 nucleotides (DR4) and nonspecific oligomer (NS).

The DR4 oligomer competed more strongly than the self -102/-82 oligomer suggesting that the TR β /RXR α heterodimer had more affinity for DR4 as compared to nTRE.

nTRE by Itself Conferred T₃ Responsiveness

To determine if the nTRE conferred T₃ responsiveness to a neutral promoter, I ligated two copies of the nTRE (-102/-82) element in front of SV40-luciferase. This -102/-82 PLA2g2a- SV40 luc along with TR β and TK-renilla was transfected into HepG2 cells. The nTRE PLA2g2a- SV40 luc reporter was efficiently repressed in the presence of T₃. As shown in **Figure 4-5**, T₃ does not have any effect on empty SV40-luciferase, while on -102/-82 SV40-Luc was inhibited nearly 60%. These data showed that the nTRE could repress transcription in the absence of other proteins associated with the PLA2g2a promoter.

TRE-Like Half Site GGCCA is Critical for Regulation of PLA2g2a by T₃

For identification of the exact nucleotides critical for TR β /RXR α binding, I made mutations in the nTRE. Five different mutants having five base pair mutations in the -102/-82 region were designed and named as -102 mut, -97 mut, -92 mut, -87 mut and -82 mut. Competition analyses were conducted using these mutants. The different mutant sequences are shown in **Figure 4-6A**. The DNA protein complex was competed by a 10-fold excess unlabeled wild type -102/-82 regions (**Figure 4-6B lane 3**). Mutant -97, -92 and -82 were unable to compete (**Figure 4-6B lanes 5, 6 and 8**) while mutants -102 and -87 competed (**Figure 4-6B lanes 4 and 7**). These data indicate that the nucleotides within the -97/-93, -92/-88 and -82/-79 nucleotides are involved in TR β /RXR α binding. Each of the mutations was introduced into the -448/+58 PLA2g2a-luc and transfected into HepG2 cells. The -92/-88 mutation eliminated the ability of unliganded TR β to induce PLA2g2a and blocked the inhibition by T₃ (**Figure 4-7**). These data demonstrate that there is an nTRE in the promoter of the PLA2g2a gene and the single TRE-like half site GGCCA is critical for regulation of PLA2g2a by T₃.

I also tested whether the nTRE was required for the T₃ inhibition of the TNF α induction of PLA2g2a. The TNF α stimulation of PLA2g2a-luc was reduced by T₃, but expression of the PLA2g2a-luc vector with the disrupted nTRE (mut-92) was not blocked by T₃ indicating that T₃ inhibits cytokine action through this element (**Figure 4-8**).

Coactivators Participate in Unliganded TR β Mediated Induction of PLA2g2a

Unlike positively regulated genes, some studies on negatively regulated genes have suggested that the unliganded TR β instead of being inhibitory is stimulatory (Furumoto et al, 2005; Liu et al, 2011). To order to determine if unliganded TR β could stimulate the PLA2g2a gene, I transfected SV40-TR β with PLA2g2a-luc. In the absence of ligand I observed that, TR β stimulated the PLA2g2a promoter nearly 2.5 fold as

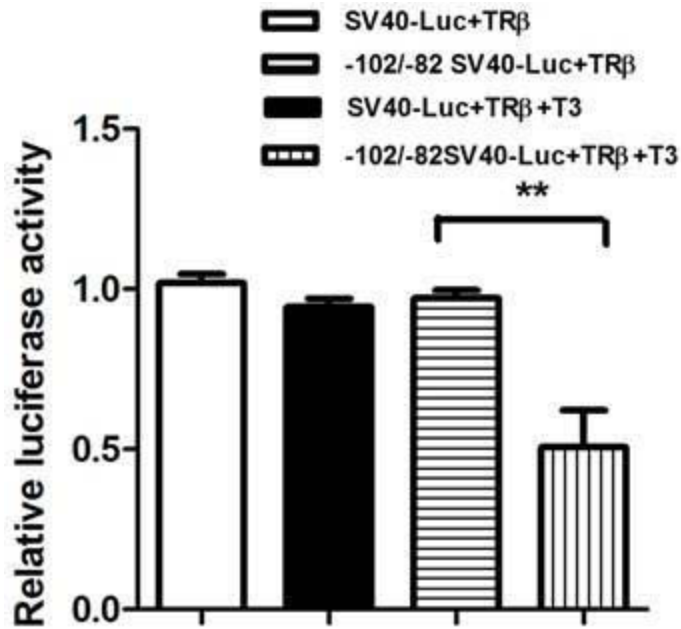


Figure 4-5. nTRE (-102/-82 region) by itself conferred T₃ responsiveness

The -102/-82 element was cloned into a luciferase reporter plasmid in front of the SV40 promoter (-102/-82 SV40-luc). This reporter was cotransfected with TR β into HepG2 cells in the presence or absence of T₃. Cells were treated with T₃ for 24 hrs. The transfections were repeated four times. Luciferase activity was corrected for both protein content and renilla activity. The error bar indicates S.E.M (** = *p* value < 0.01).

A

-102 WT-	TCCAT	TATTT	GGCCA	CACCC	ACCTC
-102 Mut-	GATGT	TATTT	GGCCA	CACCC	ACCTC
-97 Mut-	TCCAT	GCGCA	GGCCA	CACCC	ACCTC
-92 Mut-	TCCAT	TATTT	TATAG	CACCC	ACCTC
-87 Mut-	TCCAT	TATTT	GGCCA	AGATA	ACCTC
-82 Mut-	TCCAT	TATTT	GGCCA	CACCC	TATGT

B

³² P Oligo	+	+	+	+	+	+	+	+
TR+RXR	-	+	+	+	+	+	+	+
Self(cold)	-	-	+	-	-	-	-	-
-102 mut	-	-	-	+	-	-	-	-
-97 mut	-	-	-	-	+	-	-	-
-92 mut	-	-	-	-	-	+	-	-
-87 mut	-	-	-	-	-	-	+	-
-82 mut	-	-	-	-	-	-	-	+



Lanes 1 2 3 4 5 6 7 8

Figure 4-6. Characterization of the thyroid response element

Mutations in the -102/-82 element were made to identify the nucleotides necessary for TR β binding. A) The sequence of the various nucleotide substitutions is shown. B) Competition gel shift assays were conducted with a 10-fold excess of unlabeled -102/-82 mutants and purified TR β and RXR α proteins.

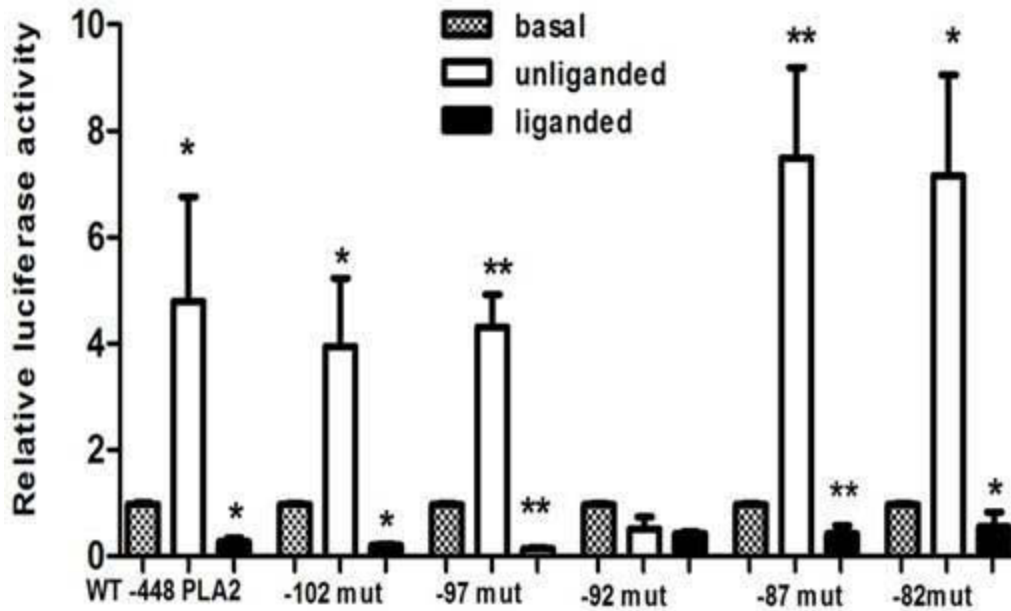


Figure 4-7. Identification of nucleotides critical for T₃ responsiveness

A) Different mutants of the PLA2g2a promoter corresponding to the gel shift oligomer sequences were introduced by site directed mutagenesis. HepG2 cells were transiently transfected with 2 µg of different PLA2g2a luciferase mutants, 1 µg of SV40-TRβ and 0.1 µg of TK-renilla. Cells were treated with or without T₃ for 24 hours. All transfections were repeated 4 times. Luciferase activity was corrected for both protein content and renilla activity. The error bar indicates S.E.M (* = *p* value < 0.05; ** = *p* value < 0.01).

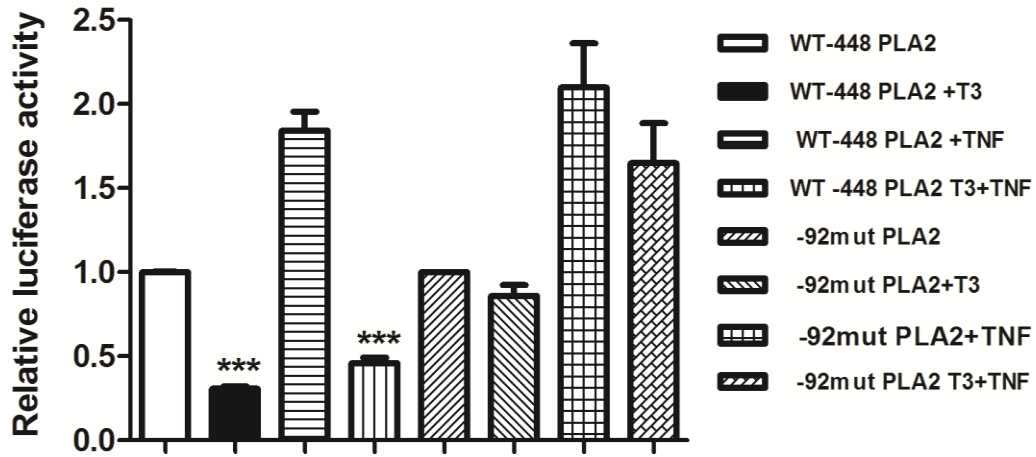


Figure 4-8. nTRE was required for the T₃ inhibition of the TNF α induction of PLA2g2a

HepG2 cells were transfected with PLA2g2a-luc and treated with 25 ng/ml TNF α or 100 nM T₃. All transfections were repeated 4 times. Luciferase activity was corrected for both protein content and renilla activity. The error bar indicates S.E.M (***) = *p* value <0.001).

compared to the basal levels (**Figure 4-9A**).

I next investigated the effect of a constitutively active TR β using the full length TR β fused to viral protein activation domain (TR β -VP16). A DR4 containing reporter was strongly induced by TR β -VP16 (**Figure 4-9B**). PLA2g2a expression was markedly reduced by TR β -VP16 and reduction was unchanged by T₃ (**Figure 4-9C**). These results support the concept that the activated TR β represses PLA2g2a. I hypothesized two possible mechanisms that might be involved in PLA2g2a repression. The first possibility was a role reversal mechanism where the function of coregulators is reversed so that the coactivator causes gene repression while corepressors do the opposite. Another possibility was the inverse recruitment of coregulators i.e. the coactivators is associated with unliganded TR β while T₃ binding leads to corepressor recruitment. To understand the role of coactivators in PLA2g2a regulation, TR β was transfected with coactivator CBP with and without T₃. CBP increased the TR β mediated induction of PLA2g2a and had no effect on T₃ dependent repression (**Figure 4-9D**). This suggested the possibility that the inverse recruitment mechanism might account for T₃ mediated regulation of PLA2g2a.

TR β Mutant Defective in Corepressors Binding Leads to Loss of T₃ Effect

I introduced previously characterized mutations (Collingwood et al, 1998; Marimuthu et al, 2002b) in the hinge region of TR β (mut214) to reduce the interactions of TR β with corepressors. Two other mutations in the AF-2 domain of TR β , which were defective in coactivator binding, were made (mut454 and mut457). T₃ mediated repression of PLA2g2a-luc was relieved 50% by the TR β mut214. In contrast this mutation had no effect on the T₃ induction of the CPT-luc. Similarly, mutations in the AF-2 domain (TR β mut454) decrease the positive actions of T₃ on CPT-luc and had no effect on T₃ mediated repression of -448/+58 PLA2g2a-luc. Surprisingly, the TR β mut457 did not repress PLA2g2a (**Figure 4-10A** and **4-10B**). The corepressors and coactivators share some interactive surfaces within TR β so that a single mutation could decrease association of both classes of coregulators (Watson et al, 2012). Overall, these data suggested that corepressors were involved in the inhibition of PLA2g2a by T₃.

T₃ Inhibition of PLA2g2a Requires Corepressors

To assess the role of corepressors in T₃ mediated inhibition of PLA2g2a, I knocked down NCoR1 and SMRT in HepG2 cells by siRNA. Knockdown of both corepressors was confirmed at the mRNA and protein levels (**Figure 4-11A-D**). Cells were treated with T₃. Knockdown of NCoR1 and SMRT reduced the ability of T₃ to inhibit PLA2g2a (**Figure 4-12A** and **B**). In contrast, T₃ mediated induction of positively regulated gene PEPCK was not affected by corepressor knockdown (**Figure 4-12C**). This experiment suggests that corepressors participate in T₃ mediated inhibition of PLA2g2a.

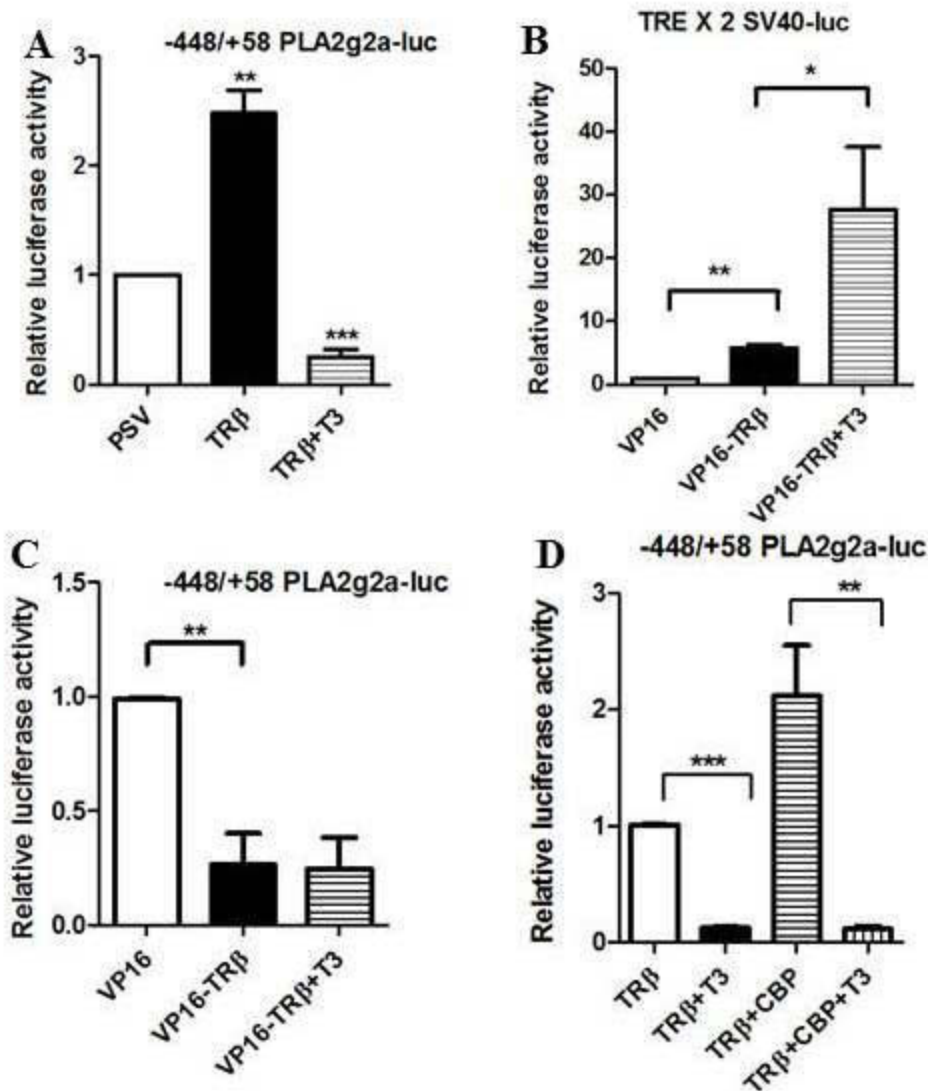


Figure 4-9. Activated TRβ receptor represses PLA2g2a and coactivators participate in the induction of PLA2g2a by unliganded TRβ

HepG2 cells were transiently transfected with 2 μg of PLA2g2a luciferase reporters, 1 μg of null vector pSV-sport or SV40-TRβ and 0.1 μg of TK-renilla. T₃ was added for 24 hours. B) HepG2 cells were transiently transfected with 2 μg of TRE X 2 SV40-luc luciferase reporters, 1 μg of VP16-TRβ and 0.1 μg of TK-renilla. T₃ was added for 24 hours. C) PLA2g2a-luc was tested with VP16-TRβ as above. D) HepG2 cells were transfected with PLA2g2a-luc, SV40-TRβ or CBP. T₃ was added for 24 hrs. The error bars indicates S.E.M (* = *p* value < 0.05; ** = *p* value < 0.01 *** = *p* value < 0.001).

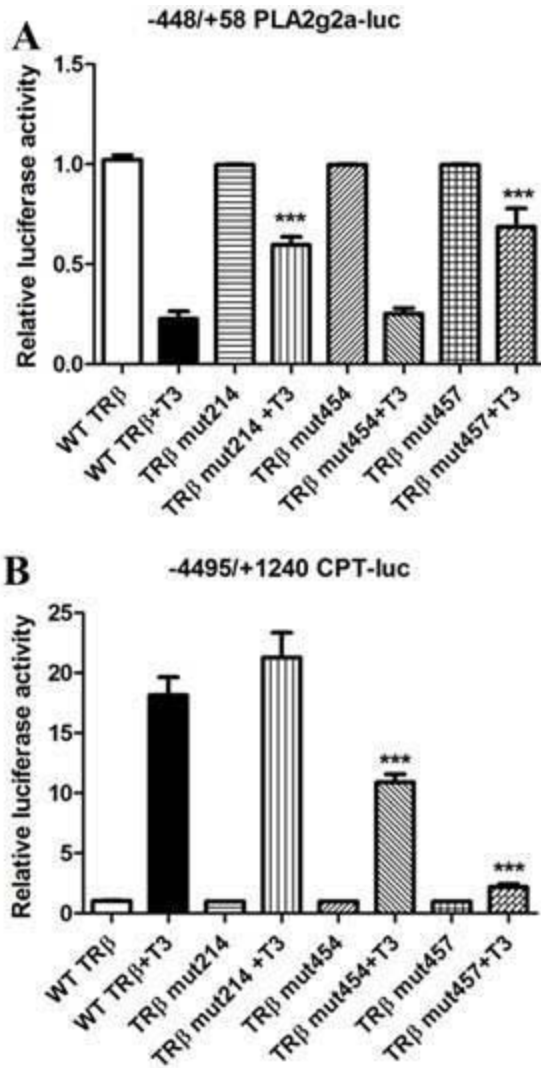


Figure 4-10. Corepressors participate in the T_3 mediated inhibition of PLA2g2a

A) PLA2g2a was transfected with three different TR β vectors carrying single amino acid substitutions. B) -4495/+1240 CPT1a-luc was transfected with the same TR β expression vectors. The data are expressed as the relative luciferase activity. Luciferase activity was corrected for both protein content and renilla activity. All experiments are repeated 3 to 4 times. The error bar indicates S.E.M. Significance is calculated relative to wild TR β treated with T_3 (***) = p value < 0.001).

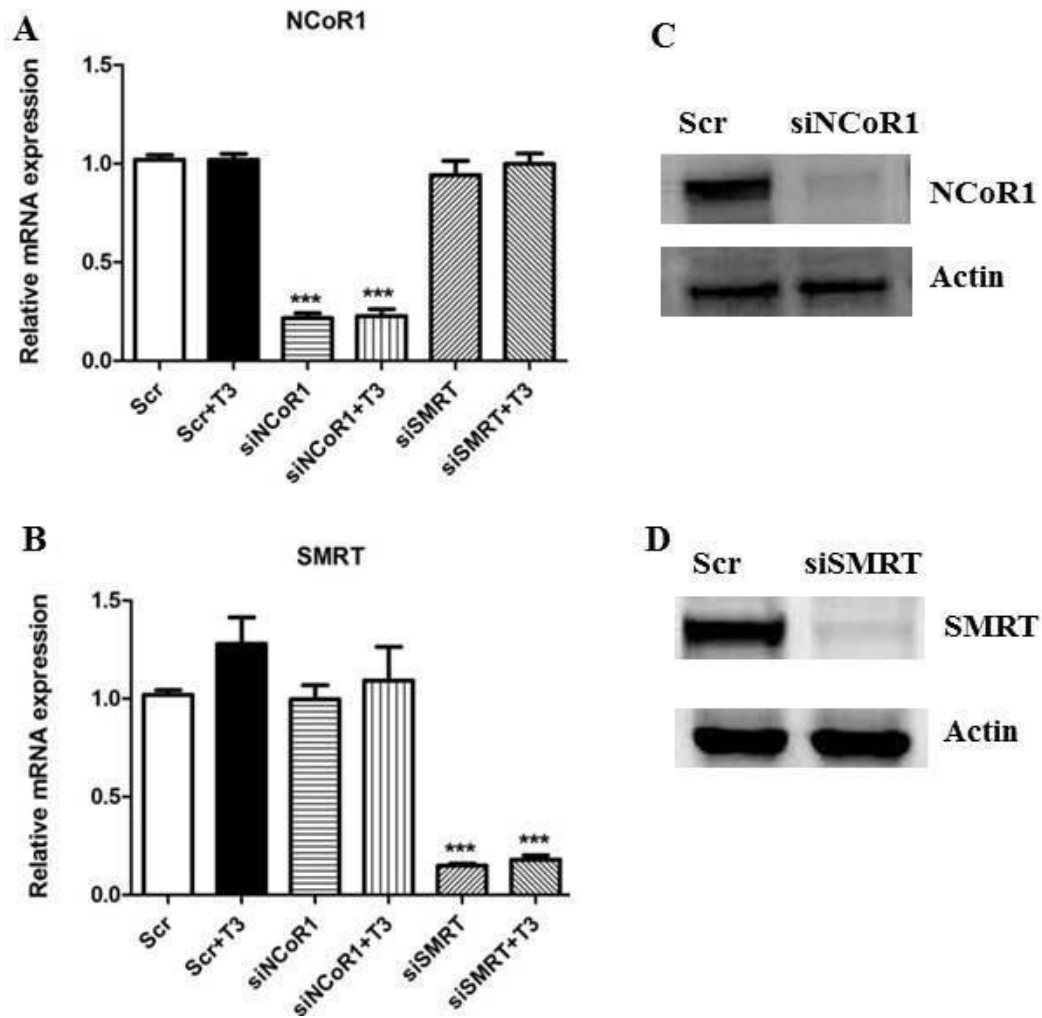


Figure 4-11. Knockdown of corepressors by siRNA

HepG2 cells were transfected with siNCoR1 and siSMRT or scrambled siRNA overnight. A) The knockdown of NCoR1 mRNA was assessed by real time PCR. B) The knockdown of SMRT RNA abundance was measured (***) = p value <0.001). The protein abundance of C) NCoR1 and D) SMRT following knockdown is shown by western blot analysis.

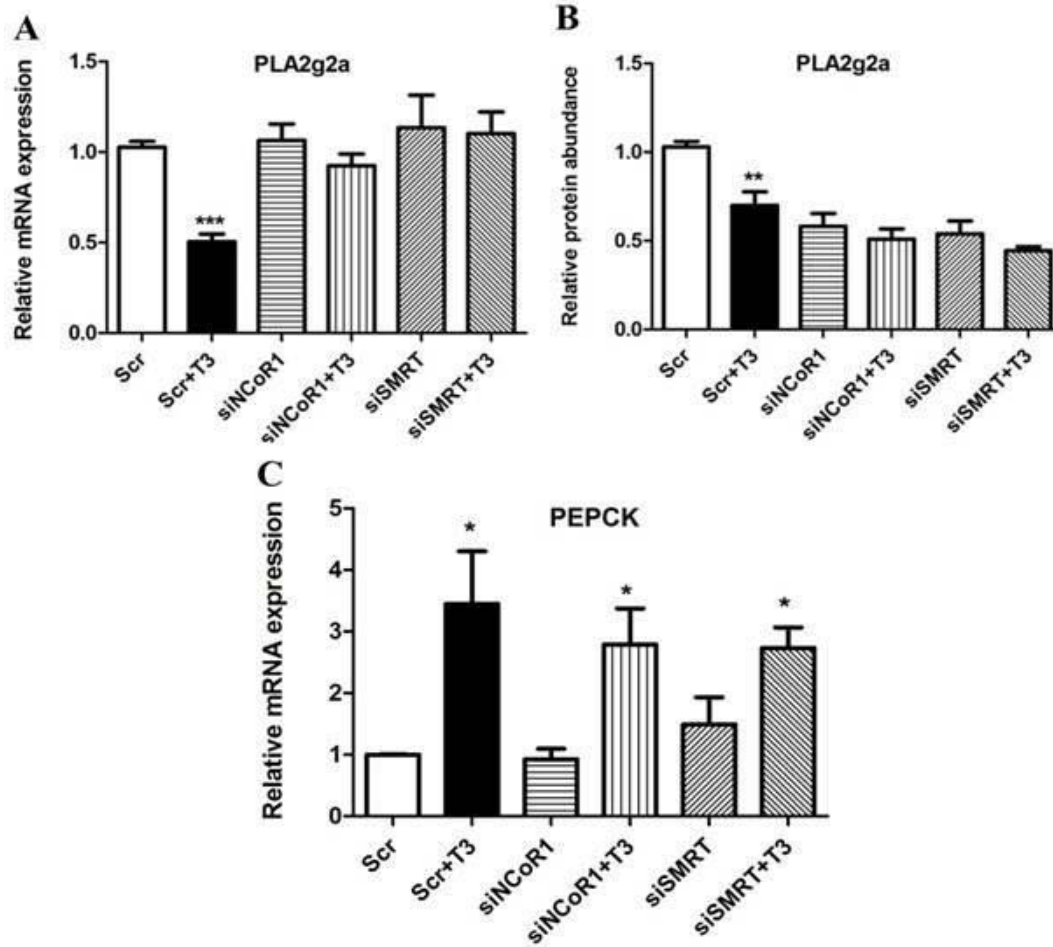


Figure 4-12. Corepressors are involved in T₃ mediated repression of PLA2g2a

HepG2 cells were transfected with siNCoR1 and siSMRT or scrambled siRNA overnight. The following day cells were treated with 100 nM T₃ for 24 hrs. A) The mRNA abundance of PLA2g2a was measured. B) The protein abundance of PLA2g2a was measured by ELISA. C) The mRNA levels of the PEPCK gene were measured. The data are expressed as the relative expression \pm S.E. of mRNA abundance of T₃ and untreated HepG2 cells (* = *p* value 0.01 to 0.05; ** = *p* value <0.01; *** = *p* value <0.001).

Corepressors are Recruited to the PLA2g2a Gene by T₃

Since corepressors were involved in T₃ dependent repression of PLA2g2a, we next asked whether corepressors are associated with the PLA2g2a gene. The relative position of ChIP primers is shown in **Figure 4-13A**. I conducted ChIP assays with antibodies to TR β , NCoR1 and SMRT. For these experiments, I used rat hepatocytes since I had identified an nTRE in the PLA2g2a gene. My data indicated that TR β is associated with the PLA2g2a promoter (**Figure 4-13B**) and that addition of T₃ increased the association of corepressors NCoR1 and SMRT with the PLA2g2a gene promoter (**Figure 4-13C and 13D**). These data support my hypothesis that T₃ suppresses PLA2g2a gene expression in part by the recruitment of corepressors.

Corepressors are Required for the T₃ Inhibition of Other Genes

Next, I tested whether NCoR1 or SMRT were needed for the T₃ repression of other genes by using siRNA mediated knockdown of these corepressors in HepG2 cells. We evaluated the expression of the following 12 known T₃ responsive genes by real time PCR (Lin et al, 2013; Sadana et al, 2011): A kinase anchor protein 4 (AKAP-4), serpin peptidase inhibitor member 2 (SERPINE), solute carrier family 2 member 1 (SLC2A1), family with sequence similarity 46, member A (FAM46A), solute carrier family 26, member 3 (SLC26A3), sorbin and SH3 domain containing 1 (SORBS1), CD24 molecule (CD24), heparan sulfate 3-O-sulfotransferase 3A1 (HS3T3A1), KIAA1199 protein (KIAA1199), solute carrier family 1 member 4 (SLCA4), secretagogin EF-hand calcium binding protein (SCGN), serine/threonine kinase 17b (STK-17B). Of these T₃ repressed genes, five genes including AKAP-4, SERPINE, SLCA4, STK-17B and HST3A1 had decreased T₃ responsiveness with corepressor knockdown while others were still inhibited by T₃ as shown in **Figure 4-14**. These data suggest that for some genes corepressors are involved in liganded TR β mediated inhibition of gene expression while different mechanisms might be involved in repression of other genes by T₃.

Figure 4-13. Corepressors are recruited to the PLA2g2a gene by T₃

Hepatocytes were treated with T₃ for 24 hrs. Cells were cross-linked and the DNA sheared for chromatin immunoprecipitation assays. A) A model of the PLA2g2a promoter and the location of the primers are shown. B) The TRβ antibody (TRβ-Ab) was used for immunoprecipitation. PCR products for the proximal promoter and the third intron are shown. C) ChIP experiments were conducted with an antibody to NCoR1 D) ChIP experiments were carried out with a SMRT antibody.

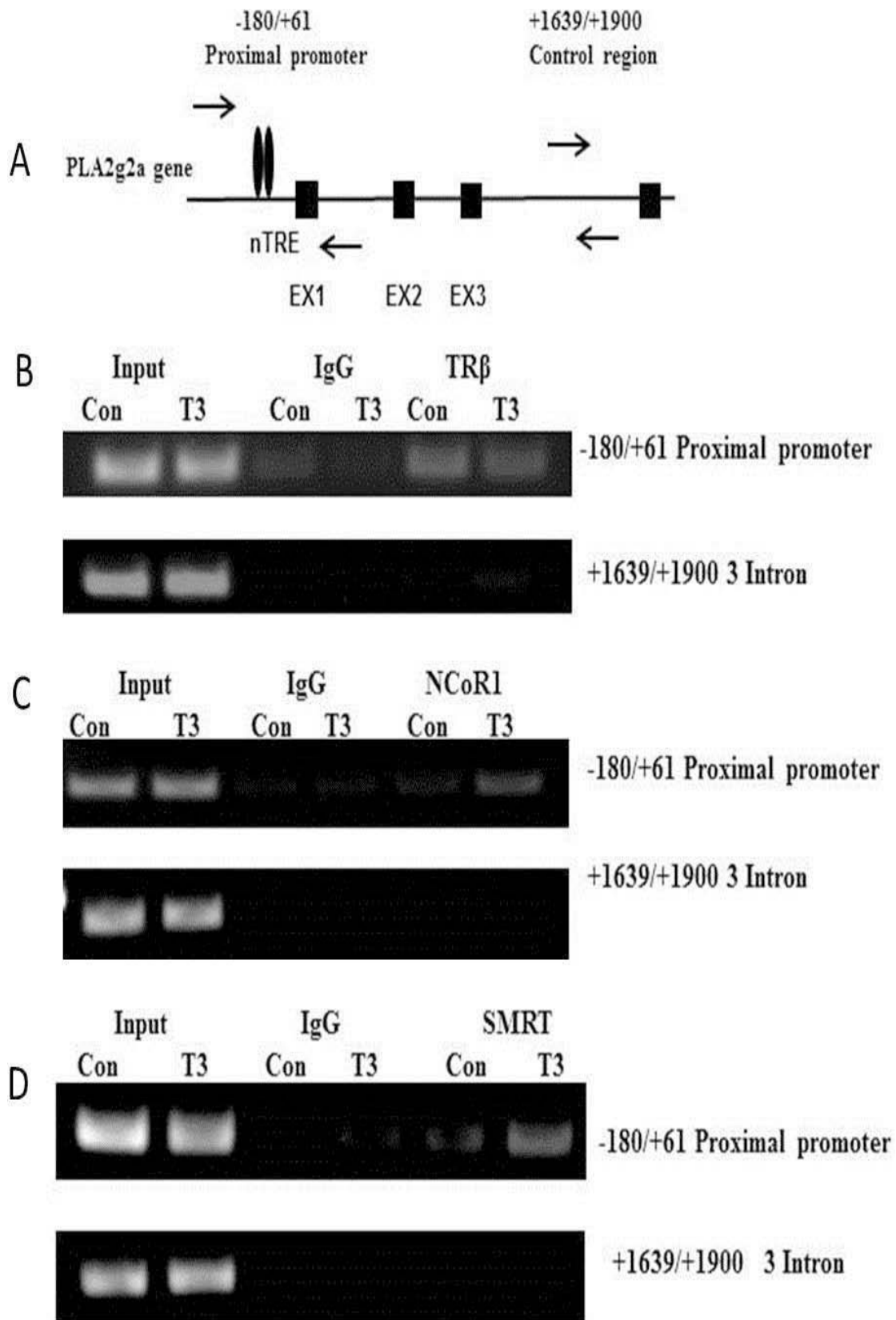
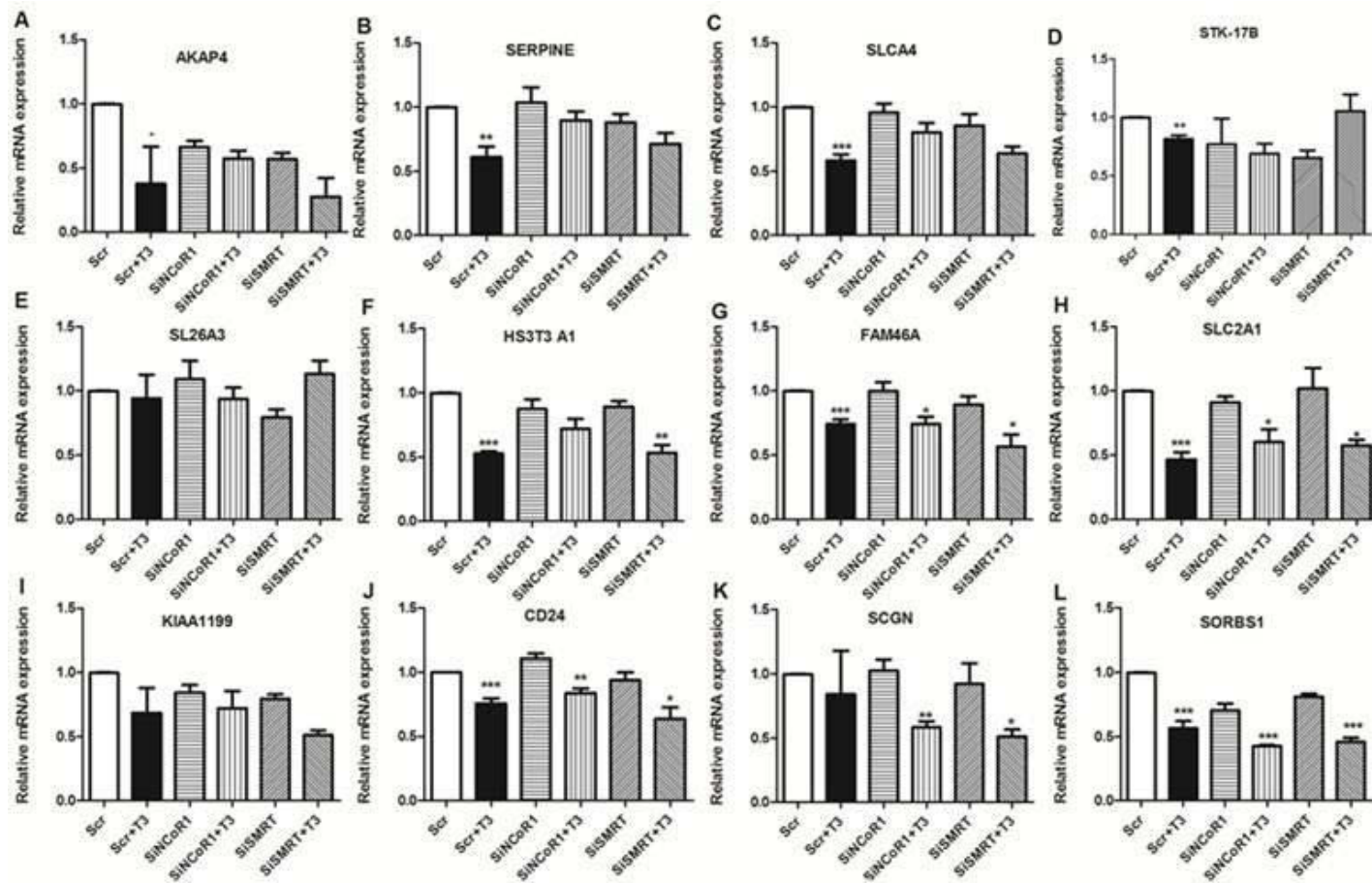


Figure 4-14. Corepressors participate in the T₃ inhibition of several hepatic genes

HepG2 cells were treated with siNCoR1 or siSMRT. Cells were treated with 100 nM T₃ for 24 hrs. The mRNA abundance was determined by real time PCR: A) AKAP-4, B) SERPINE, C) S SLCA4, D) STK17-B, E) SLC26A3, F) HS3T3A1, G) FAM46A, H) SLC2A1, I) KIAA1199, J) CD24, K) SCGN and L) SORBS1. The data are expressed as the relative expression \pm S.E. of mRNA abundance of T₃ and untreated HepG2 cells (* = *p* value 0.01 to 0.05; ** = *p* value <0.01; *** = *p* value <0.001).



CHAPTER 5. DISCUSSION

Chronic low-grade inflammation is associated with obesity and hypothyroidism (Garces et al, 2010; Kvetny et al, 2004). Secretory phospholipases are involved in various inflammatory diseases including arthritis and atherosclerosis (Murakami et al, 2011). The regulation and role of PLA2g2a has been studied in vascular smooth muscle cells and macrophages in relation to atherosclerosis. (Murakami et al, 2010). Since PLA2g2a expression is elevated in various inflammatory states and hepatocytes actively secrete PLA2g2a, I investigated the regulation of PLA2g2a in liver.

Modulation of PLA2g2a Gene Expression by Thyroid Hormone in Liver

In Chapter 3, I examined the regulation of PLA2g2a in liver by T₃. There have been a very limited number of reports linking T₃ with the expression of sPLA2. One group found that PLA2g2a expression in astrocytes was reduced by T₃ (Thomas et al, 2000). They reported that in brain astrocyte cells (DITNC1) T₃ decreased sPLA2 activity. At a 10nM concentration of T₃, there was an approximate 50% decrease in sPLA2 activity. In addition to T₃, they tested a pharmacologically T₃ related compound CGS23425, which similarly inhibited the sPLA2 activity. Another study reported decreased expression of PLA2g2a in hypophysectomized rats treated with T₃ (Boone et al, 2011). Microarray analysis of the RNA isolated from liver of hypophysectomized rats treated with T₃ showed a profound decreased in the PLA2g2a levels followed by T₃ treatment.

In my studies, I characterized the regulation of PLA2g2a and other sPLA2 isoforms by T₃ in liver. I found that T₃ decreased both the mRNA and protein abundance of PLA2g2a by 60% in primary rat hepatocytes. T₃ similarly inhibited the expression of PLA2g2a in human hepatoma cell lines (HepG2). I found that the thyroid status regulated endogenous PLA2g2a gene expression. Hyperthyroid rats had significantly lower levels of PLA2g2a as compared to hypothyroid at rats the both mRNA and proteins level. I observed that other sPLA2 isoforms including PLA2g1b, PLA2g3 and PLA2g5 were also inhibited in hyperthyroid rats. Similarly in BALB/c mice, hyperthyroidism negatively regulated the expression of PLA2g2a as compared to hypothyroid mice. Furthermore, T₃ reversed the cytokine mediated induction of PLA2g2a by 50%. In both primary rat hepatocytes and HepG2 cells the TNF and IL-6 mediated induction of PLA2g2a was inhibited by T₃.

Recent studies using PLA2g2a inhibitors have suggested that inhibition of PLA2g2a confers resistance to diet-induced obesity (Iyer et al, 2012). Inhibition of PLA2g2a resulted in beneficial changes in hepatic metabolic gene expression including the induction of peroxisome proliferator activated receptor coactivator (PGC-1 α) and the inhibition of SREBP-1c. However, most PLA2g2a inhibitors have activity against all sPLA2 isoforms so the precise contribution of PLA2g2a cannot be defined. Knockout of the PLA2g1b isoform ameliorated the effects of high fat diets in part through the

decreased intestinal absorption of lipids (Huggins et al, 2002; Hui et al, 2009). Overall, these studies suggest that the secretory PLA2 isoforms contribute to the pathology of diet-induced obesity.

Nonalcoholic fatty liver disease (NAFLD) is a common form of liver disease. It is characterized by accumulation of triglycerides and hepatic inflammation. Several TR β selective agonists including GC-1 and KB2115 have been shown to lower cholesterol and stimulate hepatic metabolism. I believe that T₃ and TR β agonists by the virtue of their ability to inhibit sPLA2 and lipogenic genes like sterol receptor element binding protein (SREBP-1c) as well as by inducing genes involved in fatty acid oxidation like carnitine palmitoyltransferase 1a (CPT1a) possess potential as treatments for hepatic steatosis. I postulate that TR β ligands could reverse hepatic steatosis and will improve the metabolic profile first by decreasing sPLA2 expression and the associated inflammatory response and secondly by reducing lipotoxicity via the stimulation of hepatic fatty acid oxidation and inhibition of lipogenesis.

Nuclear Corepressors Mediated the Inhibition of PLA2g2a Gene Transcription by Thyroid Hormone

In Chapter 4, I explored the mechanism of inhibition of the PLA2g2a gene by thyroid hormone. The genomic mechanisms by which T₃ induces gene expression include the ligand mediated activation of TR β , the dissociation of corepressors and subsequent recruitment of coactivators (Cheng et al, 2010b). Interestingly, T₃ inhibits many genes in liver. Different mechanisms have been proposed to explain the TR mediated gene repression (Santos et al, 2011; Weitzel, 2008). However despite considerable effort, no consensus model has been proposed.

The transrepression hypothesis suggests that the nuclear receptor does not bind the gene promoter directly, but instead interferes with the function of transcription factors. For example, T₃ inhibits hepatic ANGPTL3 gene expression via interactions of TR β with HNF4 bound to the ANGPTL3 proximal promoter (Fugier et al, 2006). The glucocorticoid receptor suppresses expression of numerous inflammatory genes via interactions with NF-KB and fos/jun (Rhen & Cidlowski, 2005). However, T₃ does not inhibit PLA2g2a through either the NF-KB or C/EBP β binding sites suggesting that transrepression is not the mechanism by which T₃ represses PLA2g2a. In addition, the DBD of TR β is required for transcriptional repression.

An alternate hypothesis suggests that TR β binds to nTREs in gene promoters repressed by T₃ (Kim et al, 2005; Lin et al, 2000; Santos et al, 2006). The mouse SREBP-1c gene is inhibited via a nTRE in the promoter that binds TR β /RXR α (Hashimoto et al, 2006). The SREBP-1c nTRE contains a single AGGTCA-like motif but to date a consensus motif for the nTRE does not exist. Z elements, which are DNA sequences often close to transcription start sites, have been described as nTREs. A core sequence (CAAAG) has been delineated (Nygard et al, 2006; Nygard et al, 2003; Sasaki et al, 1999) for few negatively regulated genes but this sequence is not found within the

PLA2g2a nTRE. Negative TREs do not resemble a classical positive TRE consisting of an AGGTCA motif separated by four nucleotides (DR4). The β amyloid precursor protein exhibits a variation of the nTRE in that the nTRE overlaps with Sp1 binding site. Binding of TR β precludes binding of Sp1 thereby inhibiting Sp1 mediated induction (Villa et al, 2004). To investigate the PLA2g2a repression, we cloned the rat PLA2g2a promoter and identified a nTRE. I provided several lines of evidence that this nTRE requires TR β binding. First, TR β and RXR α bind as heterodimers as shown by gel shift assays and immobilized template assays. The TR β mutant lacking DNA binding domain had no effect on PLA2g2a transcription suggesting that the DNA binding domain is critical for the T₃ inhibition. The -102/-82 (nTRE) region was sufficient for the T₃ mediated suppression of SV40-luc. Other evidence supporting association of TR β with this region of the promoter included CHIP assays and mutagenesis studies.

A role reversal model has been proposed for a few negatively regulated genes in which a corepressor such as SMRT functions as an activator or reciprocally a coactivator represses (Santos et al, 2011). It was reported that SMRT activated the TSH α via the nTRE. (Berghagen et al, 2002). A TR, which had a defective corepressor binding surface, was unable to activate a nTRE (Ortiga-Carvalho et al, 2005). In my studies, transfection of coactivators CBP had no effect on T₃ repression of PLA2g2a, instead it increased the unliganded TR β mediated induction of PLA2g2a. Also, disruption of the corepressor binding site of TR β decreased the T₃ inhibition of PLA2g2a suggesting that role reversal was not the mechanism by which T₃ acted.

For genes that are induced by T₃, the unliganded TR β represses gene expression via the recruitment of corepressors (Zhang & Lazar, 2000). The inverse recruitment hypothesis proposes that the liganded TR recruits corepressors rather than coactivators. This hypothesis is supported by a recent study of mice harboring a mutation in the deacetylase domain of NCoR1 which was defective in interaction with HDAC3. In these mice, positive T₃ responsive genes which are normally inhibited by unliganded TR were activated, while negatively T₃ regulated genes like TSH α and deiodinase 2 were modestly induced by T₃ (You et al, 2010). This suggests that on some negatively regulated genes liganded TR might be associated with NCoR1.

In my studies, I found that the unliganded TR β stimulated PLA2g2a transcription leading me to speculate that coactivators were involved in the unliganded TR β mediated induction of PLA2g2a, while corepressors were associated with the liganded TR β on PLA2g2a promoter. To validate my hypothesis, I knocked down NCoR1 and SMRT by siRNA in T₃ treated the cells. Knockdown of these corepressors alleviated the T₃ inhibition of PLA2g2a while there was no effect on the T₃ stimulation of the positively regulated PEPCK gene. To extend my findings to other T₃ mediated negatively regulated genes I conducted additional experiments to determine whether other genes need NCoR1 or SMRT for transcriptional repression by T₃. I analyzed the expression of 14 genes that are known to be inhibited by T₃. I found that for five of these genes the T₃ mediated suppression of gene expression was reduced by NCoR1 or SMRT knockdown. I found that the effect of T₃ was lost in the AKAP, STK-17B, SERPINE and SLCA4 genes suggesting that T₃ recruits corepressors to inhibit other genes. These data suggest that for

some additional genes repressed by T₃ require corepressors like NCoR1 or SMRT. However, not all the T₃ suppressed genes utilize NCoR1 or SMRT suggesting either redundancy of the corepressors functions or that additional mechanism are involved.

Using CHIP assays, I discovered that NCoR1 and SMRT are recruited to PLA2g2a gene promoter in a T₃ dependent manner. The possible model of inhibition of PLA2g2a by T₃ is shown in **Figure 5-1**. I believe that corepressors are recruited to the PLA2g2a gene in a ligand dependent manner while coactivators may be associated with the unliganded receptor. The basis for this inverse recruitment of corepressors by the liganded TR β is not understood. One possible explanation is that the DNA sequence acts as an allosteric modulator for TR β and could govern its conformation and hence determine its association with coregulators (Putcha & Fernandez, 2009). It has been shown that the sequence of the binding site for the GR determines its transcriptional activity (Meijsing et al, 2009). In conclusion, our results demonstrate that T₃ regulates PLA2g2a gene expression *in vitro* and *in vivo* in rat liver. The nTRE in the PLA2g2a gene is sufficient for T₃ mediated inhibition. Our data indicate that T₃ represses PLA2g2a gene by an inverse recruitment mechanism in which the liganded TR β has corepressors associated with it.

In conclusion, my study has enhanced understanding of the molecular mechanisms of T₃ action and provides a cellular mechanism by which T₃ inhibits PLA2g2a expression. I have identified a novel inverse recruitment mechanism in which unliganded TR β is stimulatory while liganded TR β recruits corepressors to inhibit PLA2g2a expression. In addition to PLA2g2a, I discovered other novel genes which require corepressors for T₃ mediated inhibition.

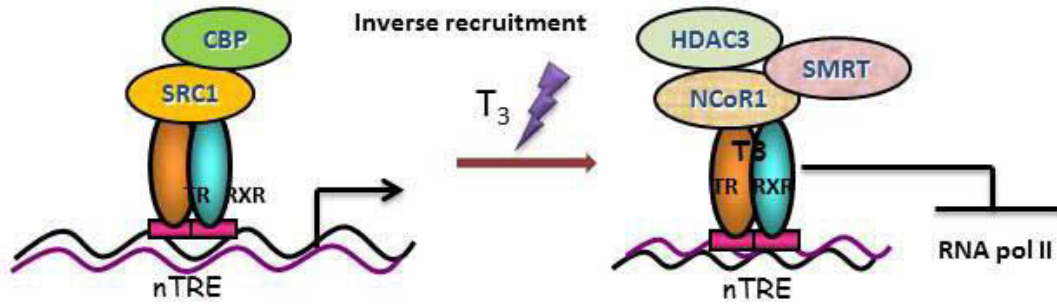


Figure 5-1. Possible model for T_3 mediated repression of PLA2g2a

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APPENDIX. SUPPLEMENTAL DATA

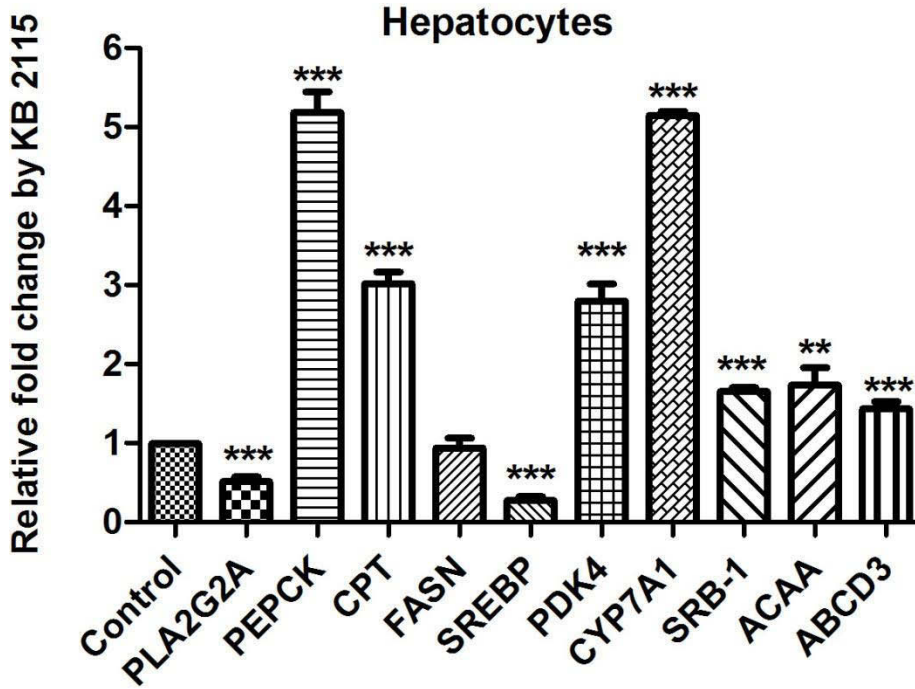


Figure A-1. Hepatic gene regulation by TR β agonist KB2115

Rat hepatocytes were treated with 1 μ M KB2115 for 24 hrs. RNA was harvested and assessed for PLA2g2a mRNA abundance by real time PCR. Experiments were repeated 3 times. (** = p value 0.001 to 0.01, *** = p value <0.001).

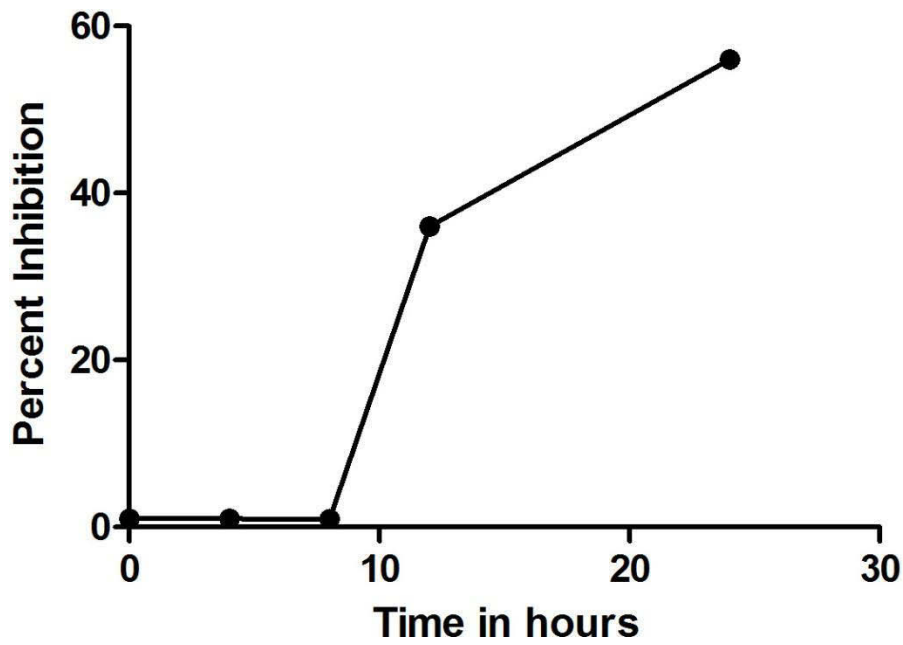


Figure A-2. Time course for PLA2g2a regulation by KB2115

Rat hepatocytes were treated with KB2115 for various times and PLA2g2a mRNA abundance was measured. Experiment was repeated 3-4 times. The data is expressed as the percent inhibition.

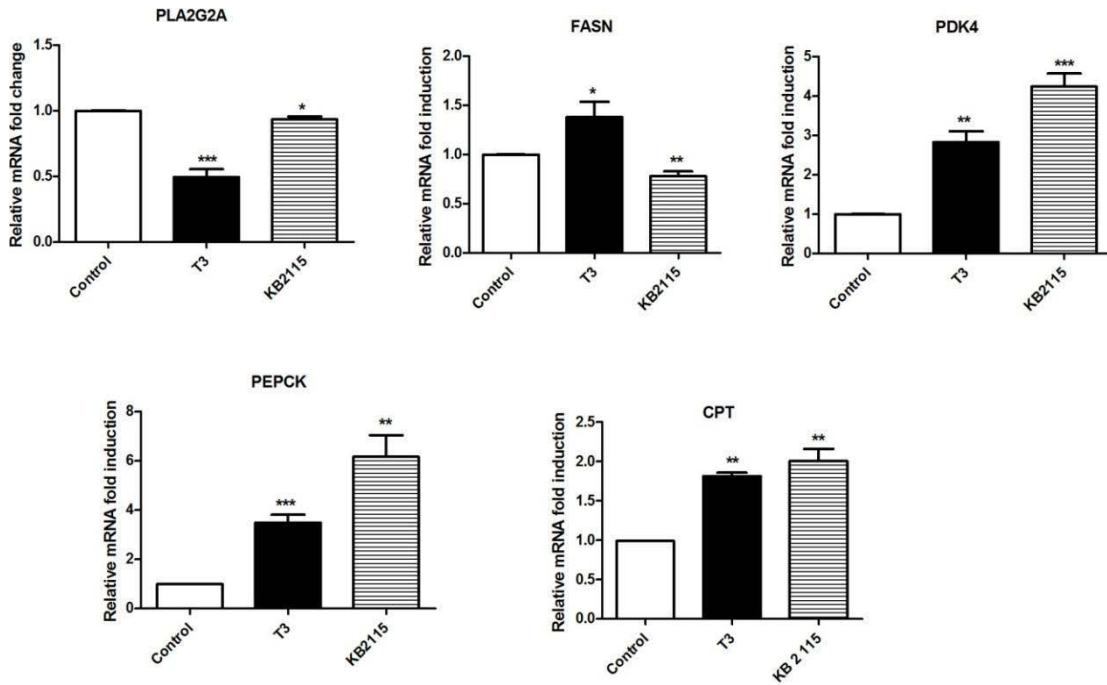


Figure A-3. Comparison of KB2115 and T₃ mediated regulation of hepatic genes

HepG2 cells were treated with 100 nM of T₃ or 1 μM of KB2115 for 24 hrs. RNA was harvested and assessed for various T₃ responsive genes by real time PCR. Experiments were repeated 4 times. (* = *p* value < 0.05; ** = *p* value < 0.01; *** = *p* value < 0.001).

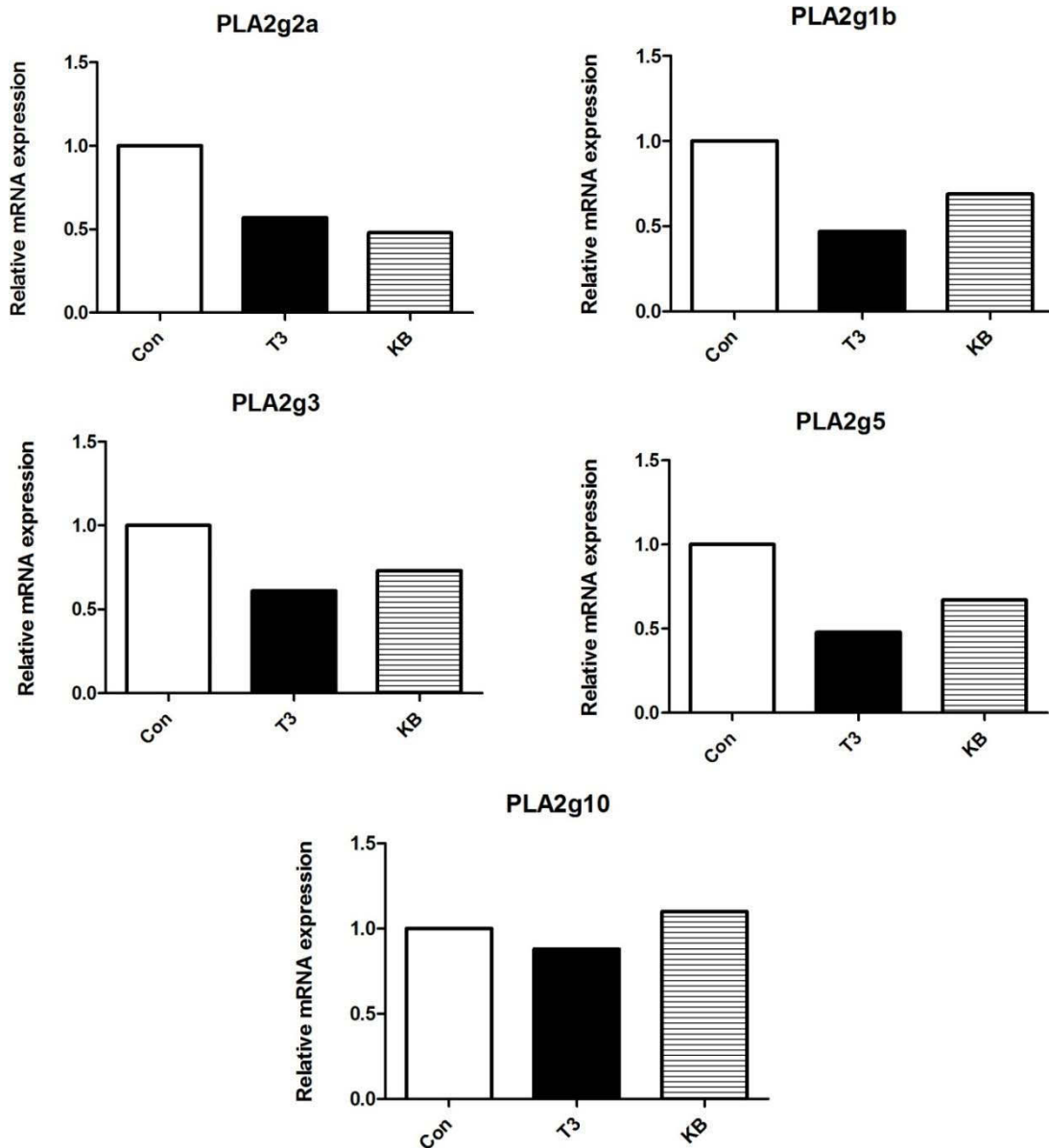


Figure A-4. Comparison of KB2115 and T₃ mediated regulation of the regulation sPLA2 isoforms in hepatocytes

HepG2 cells were treated with 100 nM of T₃ or 1 μM of KB2115 for 24 hrs. RNA was harvested and assessed for mRNA abundance of sPLA2s was measured by real time PCR.

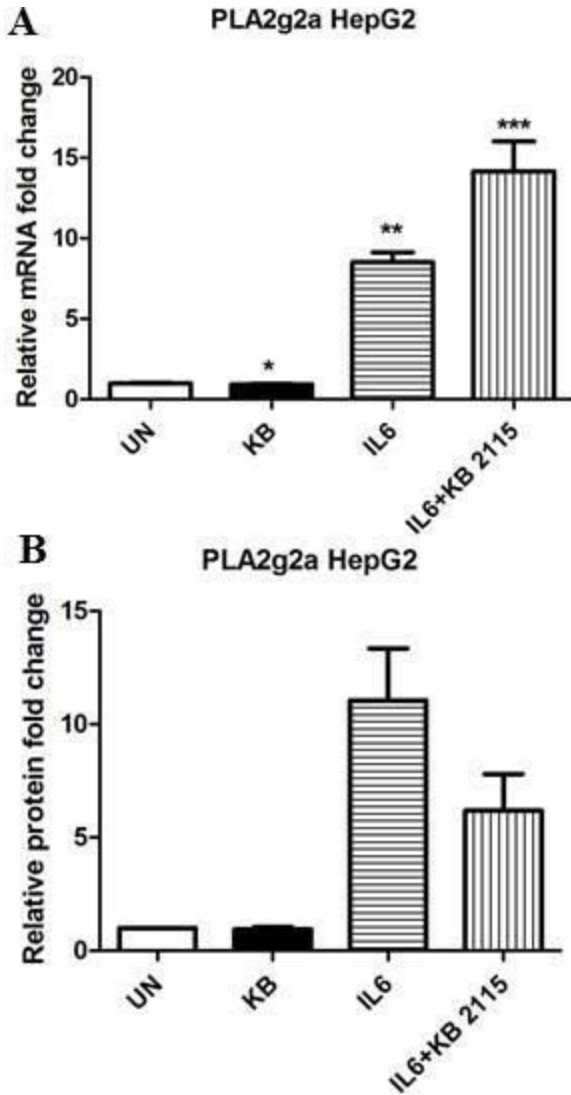


Figure A-5. Effect of KB2115 on cytokines mediated induction of PLA2g2a

HepG2 cells were treated with 10 ng/mL IL-6 or 100 nM T₃ or both for 24 hrs. PLA2g2a mRNA abundance was assessed. B) Media was collected from HepG2 cells treated with IL-6 or T₃ and the PLA2g2a levels were determined by ELISA. All experiments were repeated 3 times. The data are expressed as the mean of the fold induction \pm S.E.M of mRNA abundance relative to untreated control cells (* = *p* value < 0.05; ** = *p* value < 0.01; *** = *p* value < 0.001).

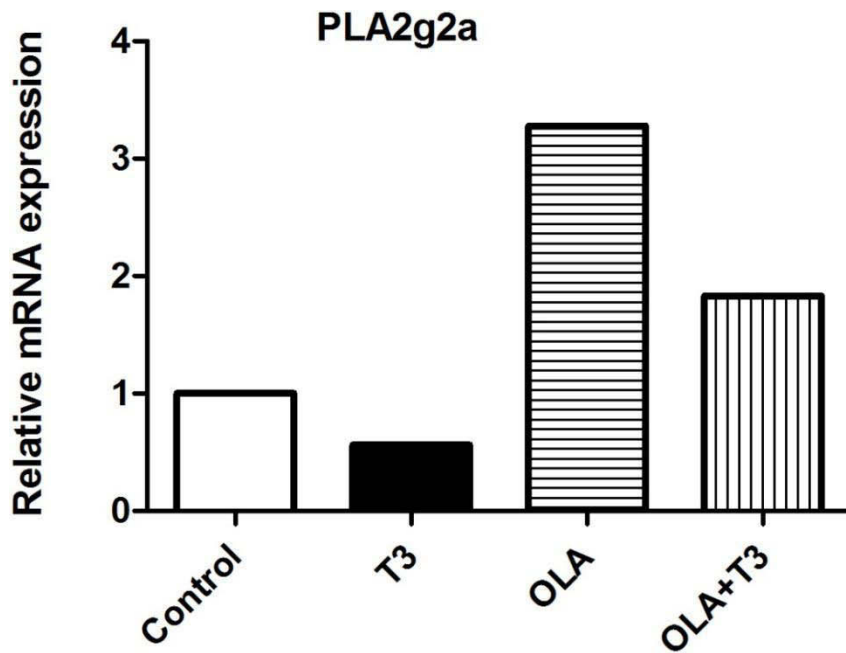


Figure A-6. T₃ and KB2115 decreases fatty acid mediated induction of PLA2g2a

Rat hepatocytes were treated with 100 nM T₃, 1 μ M KB2115 or 100 nM of oleic acid for 24 hrs. PLA2g2a mRNA levels were measured by real time PCR.

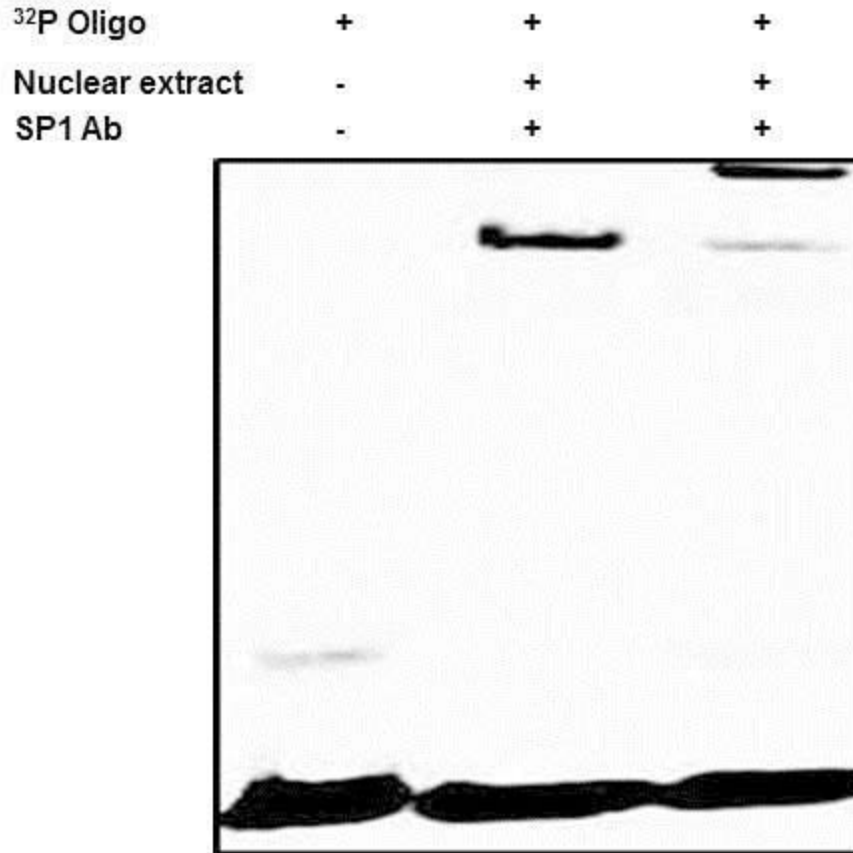


Figure A-7. Identification of Sp1 binding to -102/-72 proximal promoter region PLA2g2a

A biotinylated oligomer representing the sequence between -102/-72 was incubated with rat liver nuclear extract. Antibody to Sp1 was added. The resulting complexes were resolved on a 4-6% non-denaturing polyacrylamide gel.

³² P DR-4	+	+	+	+
TR	-	+	-	+
RXR	-	-	+	+

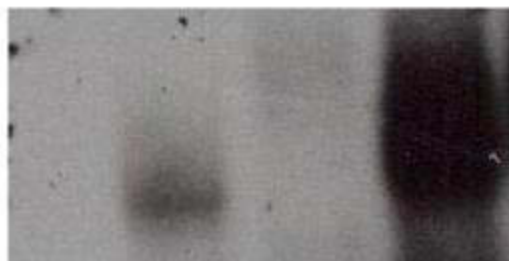


Figure A-8. Binding of purified recombinant TR β and RXR α . with DR-4

The double -stranded oligonucleotides corresponding to DR-4 were constructed and labeled with the ³²P. The-radiolabeled DR-4 was incubated with purified recombinant TR β and RXR α .

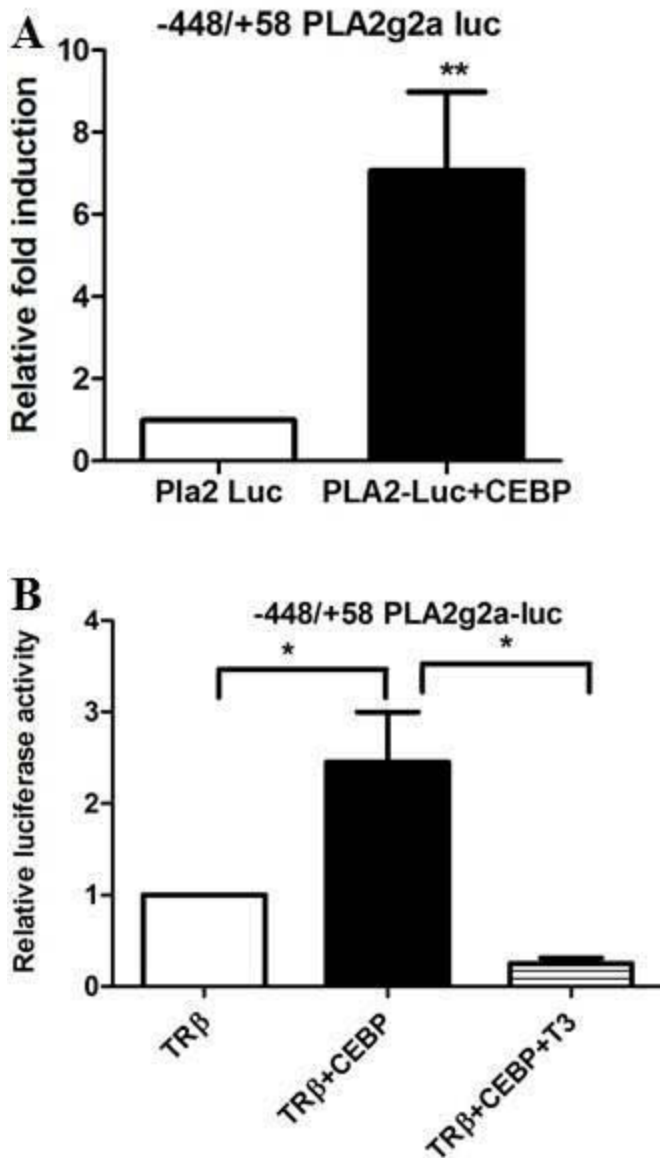


Figure A-9. CEBPβ induces the expression of PLA2g2a luciferase

HepG2 cells were transiently transfected with 2 μg of different PLA2g2a luciferase mutants, 1 μg of SV40-TRβ, 1 μg of MSV CEBPβ and 0.1 μg of TK-renilla. Cells were treated with or without T₃ for 24 hours. All transfections were repeated 4 times. Luciferase activity was corrected for both protein content and renilla activity. The error bar indicates S.E.M (* = *p* value < 0.05; ** = *p* value < 0.01).

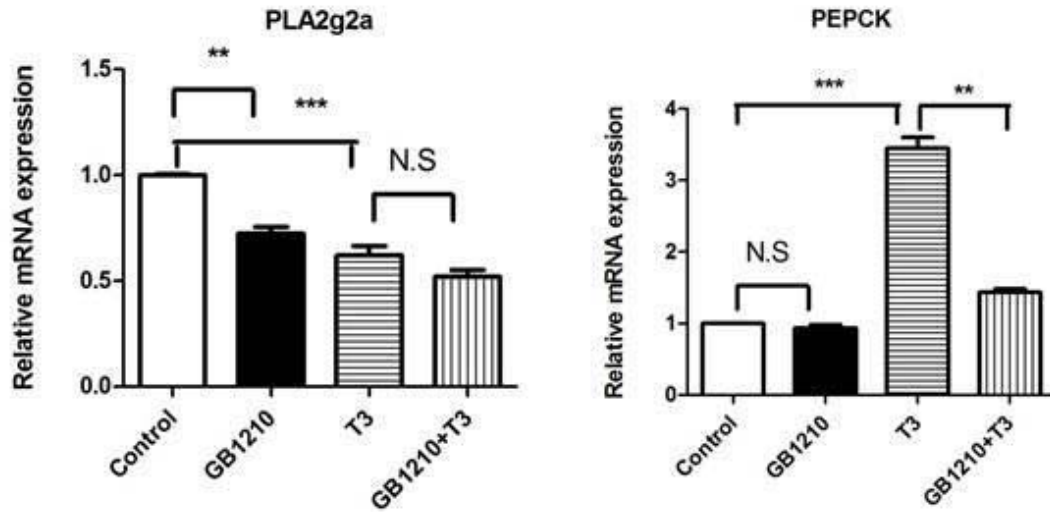


Figure A-10. Inhibitor of steroid receptor coactivator-TR β interactions represses PLA2g2a gene expression

HepG2 cells were exposed to T₃ (100 nM) or GB1210, the SRC recruitment inhibitor, (1 μ M) for 24 h. The mRNA abundance of A) PLA2g2a, B) PEPCK was measured by RT-PCR. All experiments are repeated 3 times (* = p value < 0.05, ** = p value 0.001 to 0.01, *** = p value <0.001).

VITA

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