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Regulation of Pyruvate Dehydrogenase Kinase 4 by Thyroid Hormone / Role of Peroxisome Proliferator Activated Receptor Gamma Coactivator-1 alpha and Ccaat Enhancer Binding Protein

Abstract

Pyruvate dehydrogenase kinase 4 (PDK4) regulates pyruvate oxidation through the phosphorylation and inhibition of the pyruvate dehydrogenase complex (PDC). The PDC catalyzes the conversion of pyruvate to acetyl-CoA and it is an important control point in glucose and pyruvate metabolism. Previous studies had reported that PDK4 gene expression is induced by thyroid hormone (T3). These studies did not investigate the mechanisms by which T3 regulated PDK4 gene expression. I have examined the role of the thyroid hormone receptor (TR), transcriptional coactivators especially the peroxisome proliferator activated receptor gamma coactivator-1 (PGC-1 α) and other transcription factors that act as accessory factors in T3 actions.

Thyroid hormone receptors (TR α or TR β) are part of the nuclear receptor family and have been implicated in the induction of many genes by T3. The nuclear receptors constitute a broad class of transcription factors that have the ability to bind to DNA and regulate the expression of the genes in a ligand dependent manner. To identify a binding site for the TR β in the promoter of the PDK4 gene, I transfected serial deletions of this promoter ligated to the luciferase reporter gene into the human hepatoma (HepG2) cells. The TR β binding site was characterized by gel shift mobility assays, site directed mutagenesis and *in vivo* and *in vitro* luciferase constructs transfection techniques. In addition, I have used the chromatin immunoprecipitation (ChIP) technique to confirm the binding of the TR β to the PDK4 gene promoter.

Transcription coregulators bind to nuclear receptors to facilitate or inhibit the transcription of the target genes. I have explored the role of several coregulators including the transcriptional coactivator peroxisome proliferator activated receptor gamma coactivator-1 (PGC-1 α). Previous studies demonstrated that PGC-1 α could induce the PDK4 gene. Here, I found that T3 increases PGC-1 α abundance and association with the PDK4 gene. In addition, adenoviral shRNA-mediated knock-down of PGC-1 α reduced the T3 induction of PDK4 and several other genes. These data suggest that PGC-1 α participates in the T3 induction of multiple genes in the liver. In addition, I investigated the role of several transcription factors including estrogen related receptor (ERR α), CCAAT/enhancer binding protein beta (C/EBP β) and the forkhead transcription factor (FOXO1) in the activation of the PDK4 gene by T3. ERR α and FoxO1 recruit PGC-1 α to the proximal PDK4 promoter and are involved in the synergistic stimulation of the PDK4 gene by T3 and PGC-1 α . C/EBP β was identified as an important accessory factor in the T3 induction of the PDK4 gene. Knock-down of C/EBP β with adenoviral shRNA decreased both the basal expression and T3 stimulation of PDK4. Overall, my results showed that T3 induces PDK4 gene expression through a TR β binding site in the promoter of the PDK4 gene and that the two transcription coregulators PGC-1 α and C/EBP β enhance this induction.

Finally, I have tested the ability of T3 to induce a group of transcriptional regulators and metabolic genes. After 24 hours, 27 genes were found to be induced by T3 including several coactivators. These results suggest that T3 may induce a network of transcriptional regulators to further amplify the actions of T3.

Thus, my studies have characterized the mechanism of the activation of an important metabolic gene, PDK4 gene, by T3 and identified the transcription factors that are involved in this mechanism. Also, my studies have provided a broad insight of the hepatic transcription factors network that work coordinately to facilitate the action of the T3 in the liver. This work may open a door to further investigate therapeutic targets in this network to modulate metabolic disease processes that are affected by the thyroid hormone.

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**REGULATION OF PYRUVATE DEHYDROGENASE KINASE 4 BY THYROID
HORMONE: ROLE OF PEROXISOME PROLIFERATOR ACTIVATED
RECEPTOR GAMMA COACTIVATOR-1 ALPHA AND CCAAT ENHANCER
BINDING PROTEIN**

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
Ramy Naguib Attia
May 2009

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DEDICATION

This dissertation is dedicated to my precious parents
Mr. & Mrs. Raafat Naguib Attia

And to my lovely wife
Dr. Riham Ishak

And to my sweet son
Mr. Ryan Ramy Naguib

And to my beloved brother
Dr. Raed Raafat Naguib

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ABSTRACT

Pyruvate dehydrogenase kinase 4 (PDK4) regulates pyruvate oxidation through the phosphorylation and inhibition of the pyruvate dehydrogenase complex (PDC). The PDC catalyzes the conversion of pyruvate to acetyl-CoA and it is an important control point in glucose and pyruvate metabolism. Previous studies had reported that PDK4 gene expression is induced by thyroid hormone (T3). These studies did not investigate the mechanisms by which T3 regulated PDK4 gene expression. I have examined the role of the thyroid hormone receptor (TR), transcriptional coactivators especially the peroxisome proliferator activated receptor gamma coactivator-1 (PGC-1 α) and other transcription factors that act as accessory factors in T3 actions.

Thyroid hormone receptors (TR α or TR β) are part of the nuclear receptor family and have been implicated in the induction of many genes by T3. The nuclear receptors constitute a broad class of transcription factors that have the ability to bind to DNA and regulate the expression of the genes in a ligand dependent manner. To identify a binding site for the TR β in the promoter of the PDK4 gene, I transfected serial deletions of this promoter ligated to the luciferase reporter gene into the human hepatoma (HepG2) cells. The TR β binding site was characterized by gel shift mobility assays, site directed mutagenesis and *in vivo* and *in vitro* luciferase constructs transfection techniques. In addition, I have used the chromatin immunoprecipitation (ChIP) technique to confirm the binding of the TR β to the PDK4 gene promoter.

Transcription coregulators bind to nuclear receptors to facilitate or inhibit the transcription of the target genes. I have explored the role of several coregulators including the transcriptional coactivator peroxisome proliferator activated receptor gamma coactivator-1 (PGC-1 α). Previous studies demonstrated that PGC-1 α could induce the PDK4 gene. Here, I found that T3 increases PGC-1 α abundance and association with the PDK4 gene. In addition, adenoviral shRNA-mediated knock-down of PGC-1 α reduced the T3 induction of PDK4 and several other genes. These data suggest that PGC-1 α participates in the T3 induction of multiple genes in the liver. In addition, I investigated the role of several transcription factors including estrogen related receptor (ERR α), CCAAT/enhancer binding protein beta (C/EBP β) and the forkhead transcription factor (FOXO1) in the activation of the PDK4 gene by T3. ERR α and FoxO1 recruit PGC-1 α to the proximal PDK4 promoter and are involved in the synergistic stimulation of the PDK4 gene by T3 and PGC-1 α . C/EBP β was identified as an important accessory factor in the T3 induction of the PDK4 gene. Knock-down of C/EBP β with adenoviral shRNA decreased both the basal expression and T3 stimulation of PDK4. Overall, my results showed that T3 induces PDK4 gene expression through a TR β binding site in the promoter of the PDK4 gene and that the two transcription coregulators PGC-1 α and C/EBP β enhance this induction.

Finally, I have tested the ability of T3 to induce a group of transcriptional regulators and metabolic genes. After 24 hours, 27 genes were found to be induced by T3

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Thus, my studies have characterized the mechanism of the activation of an important metabolic gene, PDK4 gene, by T3 and identified the transcription factors that are involved in this mechanism. Also, my studies have provided a broad insight of the hepatic transcription factors network that work coordinately to facilitate the action of the T3 in the liver. This work may open a door to further investigate therapeutic targets in this network to modulate metabolic disease processes that are affected by the thyroid hormone.

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LIST OF ABBREVIATIONS

CBP	cAMP response element-binding protein (CREB) binding protein
C/EBP α	CCAAT enhancer binding protein alpha
C/EBP β	CCAAT enhancer binding protein beta
ChIP	Chromatin Immunoprecipitation
CPT-1	carnitine palmitoyltransferase-1
CREB	cAMP response element-binding protein
DBD	DNA binding domain
DR4	direct repeats 4
ERR α	estrogen-related receptor alpha
ERR γ	estrogen-related receptor gamma
Fabp	fatty acid binding protein
FAS	fatty acid synthetase
FOXO	forkhead transcription factor
G6pase	glucose-6-phosphatase
GR	glucocorticoid receptor
HAT	histone acetyltransferase
HDAC	histone deacetylase
HNF-4	hepatic nuclear factor 4
LBD	ligand binding protein
MCAD	medium chain acyl-CoA dehydrogenase
MEF2C	myocyte enhancer factor 2C
NAD ⁺	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide hydride
NcoR	nuclear corepressor
NRF-1	nuclear respiratory factor 1

p/CAF.....	p300/CBP-associated factor
PDC.....	pyruvate dehydrogenase complex
PDK.....	pyruvate dehydrogenase kinase
PDP	pyruvate dehydrogenase phosphatase
PEPCK	phosphoenolpyruvate carboxykinase
PGC-1 α	peroxisome proliferator-activated receptor gamma coactivator 1 alpha
PGC-1 β	peroxisome proliferator-activated receptor gamma coactivator 1 beta
PPAR α	peroxisomal proliferator activated receptor alpha
PPAR δ	peroxisomal proliferator activated receptor delta
PPAR γ	peroxisomal proliferator activated receptor gamma
PRC	PGC-1 related coactivator
rPDK4	rat pyruvate dehydrogenase kinase 4
RXR	retinoid X receptor
shRNA.....	short hairpin RNA
SMRT.....	silencing mediator for RXR and TR
SRC-1.....	steroid receptor coactivator 1
T3	triiodothyronine
T4	tetraiodothyronine
TR	thyroid receptor
TRE.....	thyroid response element
TSH.....	thyroid stimulating hormone
UCP-1	uncoupling protein 1
UCP-2	uncoupling protein 2
UCP-3	uncoupling protein 3

CHAPTER 1: INTRODUCTION

This chapter provides an overview of the topics examined in this dissertation including thyroid hormone biology, the functions of the transcription coregulators and finally the role and the regulation of pyruvate dehydrogenase kinase 4.

1.1 Thyroid hormone

1.1.1 General biology

Thyroid hormone plays an important role on various aspects of metabolism, development and differentiation of cells (Yen, 2001). The thyroid gland secretes the thyroid hormones, T4 and the more biologically active form T3. Most of the T4 undergoes peripheral 5'-deiodination into the T3 form (Braverman et al., 1970; Kohrle, 2000). T3 increases the metabolic rate by stimulating almost every tissue in the body to produce mitochondrial proteins and by increasing the oxygen utilization in cells (Oppenheimer et al., 1987). Early studies showed that T3 treatment of hypothyroid rats triggers RNA synthesis in liver and alters the transcription of target genes (Miksicek & Towle, 1982; Tata et al., 1963; Tata & Widnell, 1966). T3 mediates its effect on gene expression through binding to the thyroid hormone receptors (TRs) (Munoz et al., 1988; Schueler et al., 1990). Thyroid hormone receptors belong to the superfamily of nuclear hormone receptors, which are a class of ligand activated proteins that, when bound to specific sequences of DNA serve as transcriptional regulators within the cell nucleus. This family of nuclear receptors controls the development and differentiation of skin, bone, behavioral centers in the brain as well as the continual regulation of reproductive tissues and metabolism (Alaynick, 2008; Chung & Cooney, 2003; Francis et al., 2003). Currently about 48 nuclear hormone receptors have been discovered in human and they have been categorized according to their sequence homology into 6 subfamilies (Laudet, 1997; Zhang et al., 2004c).

The specific sequence that TRs bind to in the DNA sequence are known as T3 response elements (TREs). TRs can bind to these sequences with or without ligand to produce its positive or negative regulation on the target genes (Rastinejad et al., 1995; Samuels et al., 1988). A putative consensus hexamer half-site sequence of [(G/A)GGT(C/G)A] was characterized for the TREs, based on mutational and sequence comparison studies. There is considerable variation in the primary nucleotide sequence as well as the number, spacing, and orientation of the half-sites (Cheng, 2000; Williams & Brent, 1995). The half-sites for the TREs can be arranged as palindromes (TRE_{pal}), direct repeats (DRs), and inverted palindromes (IPs) and the optimal spacing between these half-site arrangements are zero, four, and six nucleotides, respectively. The most common TRE contains a direct repeat separated by 4 nucleotides (DR4) (Glass, 1994; Williams & Brent, 1995).

1.1.2 Thyroid hormone receptors

There are two major TR isoforms encoded on separate genes, designated as TR α and TR β . The primary transcript for each gene can be alternatively spliced, generating four thyroid hormone-binding receptors, namely TR α_1 , TR α_2 , TR β_1 , TR β_2 and TR β_3 (Chassande, 2003; Williams, 2000). All the TR isoforms bind thyroid hormone and mediate gene regulation except for TR α_2 which encodes an altered ligand binding domain of unknown function (Munroe & Lazar, 1991). The TR isoforms in human, rat and mouse are highly homologous with respect to their amino acid sequences (Lazar, 1993). The TRs have several conserved domains including an A/B domain in the amino terminus and a DNA binding domains containing two zinc fingers. Importantly, the ligand binding domain (LBD) contains the activation function-2 (AF-2) peptide that interacts with coactivators. TR α_1 , TR α_2 , TR β_1 and TR β_3 are expressed in most of the rat, mouse, and human tissues although their tissue specific abundance differs greatly (Bradley et al., 1992; Hodin et al., 1990). However, the TR β_2 is restricted to pituitary and particular brain regions of the rodent (Bradley et al., 1992; Hodin et al., 1990; Lechan et al., 1994; Wood et al., 1991) and has not yet been detected in the human. Although both TR α_1 and TR β_1 are expressed in most tissues, TR α_1 is highly expressed in skeletal muscle and brown adipose tissue whereas the TR β_1 is the most abundant form of TR in liver, kidney and brain (Lazar, 1993). In heart, TR α_1 is most predominant TR isoform and is responsible for mediating thyroid hormone action in myocardiocytes, while in liver TR β_1 mediates the action of the thyroid hormone (Dillmann & Gloss, 2002).

TRs can bind to the TREs as a monomer, homodimer or heterodimer with the retinoid X receptor (RXR). This heterodimerization with RXR enables the TRs to bind to asymmetric TREs and direct repeats separated by four nucleotides. In the great majority of genes, TR binds as a heterodimer with RXR (Glass, 1994; Ribeiro et al., 1994).

Thyroid hormone plays an important role in the regulation of genes involved in metabolism and development. The development of transgenic and knockout mouse models for the different TRs isoforms have helped us in understanding the molecular mechanisms of T3 action in normal and disease states. For example, the role of the thyroid hormone in pituitary gland had been identified by knockout studies done on the TR β_2 isoform, and it showed that TR β_2 may be playing the predominant role in regulating the thyroid stimulating hormone (TSH) (Abel et al., 1999). In heart knockout studies for the TR α_1 and TR β had showed that T3 maintains baseline heart rate through the TR α_1 , whereas T3 stimulates heart rate through TR β (Johansson et al., 1999). In addition, the role of T3 in lipid synthesis and degradation had been examined in mice expressing the mutant TR β . These mice had elevated serum cholesterol levels compared with control animals in both hypothyroid and hyperthyroid states (Hayashi et al., 1996). Moreover, using a mouse model of resistance to thyroid hormone where a point mutation in the ligand-binding domain of TR β abolished its ability to bind to T3, Koshi Hashimoto reported cross talk between the liver X receptor α (LXR α) and the TR β to regulate the cholesterol uptake and metabolism in liver. (Hashimoto et al., 2006). On the other hand, TR $\beta^{-/-}$ mice showed lowered stimulation of the cholesterol 7- α hydroxylase gene (CYP7A) mRNA level by T₃ but not in TR $\alpha_1^{-/-}$ mice. These results indicate that TR β not

the TR α is the mediator of T₃ action on CYP7A gene, the rate limiting enzyme in the process of the degradation of the cholesterol into bile acids, and consequently it is one of the major regulators of cholesterol metabolism *in vivo*. However, T₃-deficient TR β ^{-/-} mice showed an augmented CYP7A response after challenge with dietary cholesterol, and these animals did not develop hypercholesterolemia to the extent as did wild type controls. The latter results lend strong support to the concept that TRs may exert regulatory effects *in vivo* independent of T₃ (Gullberg et al., 2000).

1.1.3 Thyroid hormone effects on glucose and fatty acids metabolism

Lipid and glucose metabolism are among the many physiological processes that are regulated by thyroid hormone. In fact, there is a strong link between thyroid hormone disorders and number of widespread metabolic diseases including diabetes (Crunkhorn & Patti, 2008; Johnson, 2006; Wu, 2007), obesity (Kozłowska & Rosolowska-Huszcz, 2004; Naslund, 2000; Ribeiro, 2008) and cardiovascular disease (Gomberg-Maitland & Frishman, 1998; Klein & Ojamaa, 2001). Along with heart and muscle, the liver is one of the main organs affected by T₃ (Weitzel, 2003).

In liver, T₃ increases the abundance of a number of genes potentially involved in hepatic triglyceride production, including spot 14 and fatty acid transporter protein as well as a range of genes involved in hepatic lipogenesis and low density lipoprotein receptor expression (Feng et al., 2000; Ness & Zhao, 1994). Paradoxically, T₃ simultaneously induces genes involved in lipolysis including lipoprotein lipase (Pykalisto et al., 1976; Wahrenberg et al., 1994).

Regarding glucose metabolism, T₃ stimulates almost all aspects of carbohydrate metabolism, including enhancement of insulin-dependent entry of glucose into cells and increased gluconeogenesis through elevating the transcription of key gluconeogenic enzymes, such as glucose-6-phosphatase (G6P) and phosphoenolpyruvate carboxykinase (PEPCK) (Fowden et al., 2001; Park et al., 1997). In addition, T₃ promotes glycogenolysis to generate free glucose (Johnson, 2006).

Previous studies had detected at least two different gene expression pattern mediated by T₃. Whereas limited number of genes were rapidly induced by T₃ within 6 hours only of *in vivo* T₃ treatment, the majority of T₃ regulated genes needed 24-48 hours to be induced by the T₃ treatment (Weitzel et al., 2003a; Weitzel et al., 2001). This observation led to the assumption that T₃ can mediate gene expression by several mechanisms. One direct mechanism for the T₃ mediated gene regulation is through binding of T₃ to its TRE site on targeted gene promoter. The other mechanism is through the induction of an intermediate factor such as, the peroxisome proliferator activated receptor gamma coactivator-1 or the nuclear respiratory factor or CCAAT/enhancer binding protein beta that can in turn promote the induction of the targeted gene (Park et al., 1997; Weitzel et al., 2001; Zhang et al., 2004b).

Although detailed investigations have been performed to identify the mechanism by which T3 regulated different metabolic genes including phosphoenolpyruvate carboxykinase (PEPCK), carnitine palmitoyltransferase 1a (CPT-1a), apolipoprotein B (ApoB) and uncoupling protein 3 (UCP3) (de Lange et al., 2007; Jackson-Hayes et al., 2003; Mukhopadhyay et al., 2003; Park et al., 1999; Zhang et al., 2004b), the mechanism of the induction of the pyruvate dehydrogenase kinase 4 (PDK4) gene by T3 had not been identified (Sugden et al., 1996). Understanding the mechanism of activation of PDK4 by T3 is an important step to fully understand the metabolic effect of the T3 on fatty acid and glucose metabolism as PDK4 is a key regulator in the pyruvate metabolism, which in turn affects both glucose and fatty acid metabolism (Jeoung & Harris, 2008; Sugden & Holness, 2002).

1.1.4 Mechanism of the regulation of genes by thyroid hormone

The thyroid gland produces tetraiodothyronine (T4), triiodothyronine (T3), and 3,3',5'-triiodothyronine (rT3), however, the T4 is quantitatively the major secretory product (Moreno et al., 2008). There are three types of deiodinases, and they are responsible for the peripheral 5'-deiodination of T4 and T3 (Bianco et al., 2002; Kuiper et al., 2005). The first type is deiodinase I (D1) which is found in peripheral tissues such as liver and kidney and is responsible for the conversion of the majority of T4 to T3 in the blood circulation, while deiodinase II (D2) is predominant in brain, pituitary, and brown adipose tissue and primarily converts T4 to T3 for intracellular use. The third type is deiodinase III (D3), which is responsible for the generation of reverse T3 (rT3), which is the inactive form of the hormone. D3 is found primarily in placenta, brain, and skin (Yen, 2001). D1 and D3 are also involved in the further degradation of T3 or T4 to distinct diiodothyronines: 3, 5-diiodothyronine (T2); 3, 3'-diiodothyronine (3, 3'-T2); and 3', 5'-diiodothyronine (3', 5'-T2). And finally these, in turn, can be transformed into monoiodothyronine (T1) and subsequently into thyronine (T0) (Moreno et al., 2008).

T3 binds to the thyroid hormone receptor (TR) which in turn binds to the thyroid response element (TRE) to modulate the transcription of target genes. However, the unliganded TRs can still bind to the TRE in the promoter of the targeted gene and can repress basal transcription of positively T3 regulated genes (Baniahmad et al., 1992; Brent et al., 1989). This basal repression is due to the direct interaction of unliganded TRs with corepressor molecules that typically possess the histone deacetylase activity and can interfere with the assembly of a functional preinitiation complex at the promoter of the target gene (Baniahmad et al., 1993; Tong et al., 1995). Nuclear receptor corepressor (NCoR) and the silencing mediator for retinoic acid receptor and thyroid receptor (SMRT) are corepressors that interact with unliganded TR (Horlein et al., 1995; Lee et al., 1995). The consensus LXXI/HIXXXI/L sequences within the NCoR and SMRT interact with the amino acid residues on helices 3, 5, and 6, which are part of the ligand pocket and hinge region of the TRs (Nagy et al., 1999; Perissi et al., 1999). These corepressors interact with other repressors as Sin 3 and histone deacetylase 1 (HDAC1), which in turn promotes histone deacetylation and results in basal transcription repression (Heinzel et al., 1997; Nagy et al., 1997).

When the ligand T3 is bound to the TR, it triggers significant conformational changes (Bourguet et al., 2000), including the repositioning of the helix 12 containing the core of AF-2 in the TR LBD (Glass & Rosenfeld, 2000), that results in release of the corepressor complex and recruitment of a coactivator complex to enhance transcription of the target gene (Onate et al., 1995). Most of the characterized coactivators interact with the LBDs of the TRs, through helical LXXLL motifs present within these coactivators (Heery et al., 1997). Several coactivators for the thyroid hormone transcription induction have been identified including the CREB binding protein (CBP) and the p300 protein complex, steroid receptor coactivator-1 (SRC-1) and PGC-1 α (Chakravarti et al., 1996; Kamei et al., 1996; Rosenfeld et al., 2006; Weiss et al., 1999; Wu et al., 2002; Zhang et al., 2004b). These coactivators such as the CREB/p300 complex in turn interact and recruit the PCAF (p300/CBP-associated factor) to TRE site of the target gene. The PCAF has a histone acetyltransferase activity (HAT) which promotes histone acetylation and transcription activation (Ogryzko et al., 1998).

Therefore, the TR can shift between the unliganded and liganded form with T3 causing the association or dissociation of coactivators or corepressors of the transcription machinery for the target genes (**Figure 1.1**) (Yen, 2001; Yen et al., 2006). As I mentioned before T3 can increase cardiac function via the TR α while elevating hepatic metabolism via TR β . Thus, developing therapeutic agents that can promote the induction of metabolic genes by activating specific isoform of the TR can be beneficial for treating metabolic diseases as hypercholesterolemia or obesity without provoking T3 cardiac side effects. Preliminary studies in animals with TR β selective agonists have led to a lowering of the triglyceride and cholesterol levels in animal models with less cardiac side effects (Baxter et al., 2004; Grover et al., 2004; Perra et al., 2008; Trost et al., 2000). All these facts show that understanding of the mechanism by which T3 regulate metabolic genes can lead to the identification of more therapeutic targets within its network of regulated genes for curing of related metabolic diseases.

1.2 Transcription factors and coactivators

1.2.1 General biology

Transcription factors and their coregulators coordinately regulate many biological programs at the transcriptional level (Handschin & Spiegelman, 2006; Schrem et al., 2002). Transcription factors control gene expression by binding to specific sequences frequently in the promoter upstream of the DNA of target genes and thereby control the transcription of their genetic information from DNA to RNA (Karin, 1990; Latchman, 1997). Transcription factors are composed of two essential functional regions: a DNA-binding domain and a transactivation domain. The DNA-binding domain is composed of amino acids that recognize specific DNA bases, while the activator domain of transcription factors contain the amino acids responsible for interacting with the components of the transcriptional apparatus (RNA polymerase) and with other regulatory proteins (transcription coactivators) (Agalioti et al., 2000; Senger et al., 2000).

Figure 1.1: Schematic model for the action of T3 on the regulation of gene transcription in liver cells.

Abbreviations: CBP, cAMP-response element-binding protein; HDAC, histone deacetylase; P/CAF, p300/CBP-associated factor; RXR, retinoid X receptor; TRβ, thyroid hormone receptor beta; TRE, thyroid hormone-response element.

Transcription coregulators are those proteins that either activate or repress the transcription of specific genes by interacting and regulating the transcription factors (Glass & Rosenfeld, 2000). Transcription coactivators are the coregulators that activate gene transcription, while those that repress it are known as corepressors. Transcriptional coactivators or corepressors regulate the transcription by binding to other DNA-bound factors without directly contacting DNA themselves (McKenna et al., 1999; Xu, 2005). Two different mechanisms have been identified for transcription coregulators to regulate the transcription of genes. The first mechanism for transcription coregulators is to modify chromatin structure through covalent modification of histones by acetylation, phosphorylation or methylation (Glass & Rosenfeld, 2000; Nowak & Corces, 2004; Stewart et al., 2005). The second mechanism is to modify the conformation of chromatin by an ATP-dependent process through altering chromatin structure by changing the location or conformation of the nucleosome. These structural changes do not involve covalent modification, and can either activate or repress the transcription (Aoyagi et al., 2005; Kingston & Narlikar, 1999).

Based on the structure of its DNA-binding domain, transcription factors are typically categorized into four groups including: basic domains, zinc-coordinating domains, helix-turn-helix domains and beta-scaffold domains with minor groove contacts (Stegmaier et al., 2004). The thyroid hormone receptor (TR) belongs to a zinc finger type of transcription factors, while the CCAAT/enhancer binding protein beta (C/EBP β) belongs to the transcription factors with basic domains (leucine zipper type). On the other hand, one common feature of nuclear receptor coactivators is that they contain one or more LXXLL binding motifs. Different examples of transcription coactivators are listed here, androgen receptor associated protein, cAMP response element-binding protein-binding protein (CBP), coactivator-associated arginine methyltransferase 1 (CARM1), steroid receptor coactivator (SRC), p300/CBP associating factor (p/CAF), and proliferator activated receptor gamma coactivator 1 (PGC-1) (Cai et al., 2005; El Messaoudi et al., 2006; Xu et al., 1999).

In this dissertation I am focusing on the effect of two transcription factors the TR and the C/EBP β on the activation of the PDK4 gene and the role of one transcription coactivator, the PGC-1 α . I have discussed the thyroid receptor biology and function in the first part of this chapter and I will give an overview on the PGC-1 α and the C/EBP β in the following part of the introduction.

1.2.2 PGC-1 α as a transcription coactivator

Mitochondrial biogenesis, hepatic gluconeogenesis, and β -oxidation of fatty acids are among many metabolic processes that are regulated by the transcription coactivator proliferator activated receptor gamma coactivator-1 alpha (PGC-1 α) (Puigserver & Spiegelman, 2003). PGC-1 α belongs to a family of transcriptional coactivators that includes PGC-1 β and PGC-1 related coactivator (PRC) (Finck & Kelly, 2006). PGC-1 α and PGC-1 β are expressed in brown adipose tissue (BAT), heart, skeletal muscle, brain, kidney and liver (Finck & Kelly, 2006), but there is less information about the expression

patterns of PRC (Andersson & Scarpulla, 2001). Physiological conditions that demand increased mitochondrial energy production such as exercising, fasting and cold exposure highly induce the expression of PGC-1 α in skeletal muscle, in the heart, liver and brown adipose tissue (Baar et al., 2002; Lehman et al., 2000; Puigserver et al., 1998; Rhee et al., 2003; Terada et al., 2002). Interestingly, fasting but not cold exposure induces PGC-1 β , indicating that there are unique regulatory mechanisms of regulation for each coactivator and each activator may be involved in different metabolic pathways (Lin et al., 2002; Lin et al., 2003).

PGC-1 has been implicated in the activation of many transcription factors including a number of nuclear receptors such as the peroxisome proliferator-activated receptors (PPAR) both the α and the β/δ isoforms (Vega et al., 2000; Wang et al., 2003), retinoid receptors (Puigserver et al., 1998), glucocorticoid receptor (Knutti et al., 2000), estrogen receptor (Knutti et al., 2000; Puigserver et al., 1998), farnesyl X receptor (FXR) (Zhang et al., 2004a), pregnane X receptor (PXR) (Bhalla et al., 2004), hepatic nuclear factor-4 (HNF-4) (Rhee et al., 2003), liver X receptor (LXR) (Lin et al., 2005b), and the estrogen-related receptors (ERRs) (Huss et al., 2002; Schreiber et al., 2003).

PGC-1 α was originally discovered as a cold-inducible transcription coactivator of adaptive thermogenesis in brown adipose tissue (BAT) (Puigserver et al., 1998). PGC-1 α promotes mitochondrial biogenesis both in BAT and skeletal muscles through regulating the nuclear respiratory factors (NRF-1 and NRF-2) (Leone et al., 2005; Wu et al., 1999). Recent evidence also implicates PGC-1 α in the regulation of glucose metabolism in muscles as it activates expression of glucose transporter 4 (GLUT4) in cultured skeletal muscle cells by coactivating myocyte enhancer factor-2 (MEF-2c) (Michael et al., 2001). Moreover, PGC-1 α regulated fatty acid oxidation and fuel selection in muscle by activating the expression of the gene encoding pyruvate dehydrogenase kinase 4 via its NR partner, ERR α (Wende et al., 2005). Finally in liver and heart, PGC-1 α promotes different metabolic processes. First, PGC-1 α activate expression of PPAR α target genes involved in hepatic and cardiac fatty acid oxidation (Huss & Kelly, 2004; Leone et al., 2005; Vega et al., 2000). Second, PGC-1 α coactivates HNF-4 α and FOXO1 to drive expression of genes involved in gluconeogenesis in liver (Puigserver et al., 2003). Third, PGC-1 α activates mitochondrial biogenesis in cultured cardiac myocytes (Lehman et al., 2000).

PGC-1 α knockout mice further emphasized its important role for appropriate adaptation to the metabolic and physiologic stressors. Leone et al showed that PGC-1 α ^{-/-} mice were unable to maintain core body temperature following exposure to cold, consistent with an altered thermogenic response (Leone et al., 2005). Also, they develop hepatic steatosis after a short period of starvation due to a combination of reduced mitochondrial respiratory capacity and an increased expression of lipogenic genes (Leone et al., 2005). On the other hand, the Spiegelman group reported that PGC-1 α ^{-/-} mice are paradoxically lean and resistant to diet-induced obesity and that they did not develop hepatic steatosis. They showed diminished expression of genes involved in mitochondrial fatty acid oxidation, the TCA cycle, and oxidative phosphorylation (Lin et al., 2004). The mechanistic basis for the dissimilarities between the 2 lines of PGC-1 α ^{-/-} mice is unclear (Finck & Kelly, 2006). However, both of them reported that PGC-1 α ^{-/-} mice are cold

intolerant and they have a marked vacuolar lesions in the central nervous system together with compromised the capacity of their hearts to perform basal and strenuous aerobic work. Moreover, M. Patti had supported the central role of the PGC-1 system in human body metabolism as she reported that decreased PGC-1 α and PGC-1 β expression in human may be responsible for decreased expression of NRF-dependent genes, contributing to the metabolic disturbances characteristic of insulin resistance in type 2 diabetes mellitus patients (Patti et al., 2003).

The expression and activity of PGC-1 α are linked to a variety of upstream cellular signaling pathways. Glucocorticoids and cAMP increased PGC-1 α gene transcription in liver (Bernal-Mizrachi et al., 2003; Puigserver et al., 1998). While in muscles, the AMP-activated protein kinase (AMPK), calcineurin A and calcium/calmodulin-dependent protein kinase (CaMK) and p38 mitogen-activated protein kinase (p38 MAPK) have been shown to activate PGC-1 α (Fan et al., 2004; Schaeffer et al., 2004; Zong et al., 2002). The upstream signaling events and downstream gene regulatory actions for the PGC-1 α coactivator in different body tissues are illustrated in **Figure 1.2** (Finck & Kelly, 2006; Lin et al., 2005a).

The most important aspect regarding the PGC-1 coactivator system related to this dissertation is the ability of PGC-1 α to coactivate the thyroid receptor and to induce the PDK4 gene (Puigserver et al., 1998; Wende et al., 2005). In addition, previous studies had reported that T3 highly induced the hepatic PGC-1 α mRNA expression (Weitzel et al., 2001). Moreover, previous studies from our lab had reported that overexpression of the PGC-1 α enhanced the induction of carnitine palmitoyltransferase 1a (CPT-1a) by thyroid hormone. All this information had linked the T3 effect on metabolic processes with the PGC-1 α , but the role of the PGC-1 α in the induction of PDK4 by T3 had not been investigated. Therefore, this dissertation aimed to answer this question, is the PGC-1 α is important for the hepatic action of T3 on PDK4 gene and other metabolic genes?

1.2.3 C/EBP β as a transcription factor

The CCAAT/enhancer binding protein beta (C/EBP β) is a heat-stable DNA-binding protein found in rat liver nuclei. It belongs to a distinct subfamily of transcription factors called C/EBPs with several members (C/EBP α , C/EBP β , C/EBP γ , C/EBP δ , C/EBP ϵ , and C/EBP ζ). They are encoded by separate genes located on different chromosomes (Landschulz et al., 1988; Williams et al., 1991). The C/EBPs are characterized by their ability to regulate different metabolic genes through binding selectively to the CCAAT motif in the promoters of these genes (Schrem et al., 2004). This group of proteins shares the highly conserved two homologous domains, the C-terminal basic DNA binding domain and the leucine zipper dimerization domain. In addition, they contain two other less homologous domains, the N-terminal activation domain and the attenuation domain (Arizmendi et al., 1999).

The expression patterns for the C/EBP isoforms are quite different as the C/EBP α is expressed in liver, adipose, intestine, lung, adrenal gland, placenta, ovary and peripheral blood mononuclear cells (PBMCs), while both C/EBP β and C/EBP δ are

Figure 1.2: The upstream signaling events and downstream gene regulatory actions for the PGC-1 α coactivator in different body tissues.

The schematic indicates the upstream signaling events and downstream gene regulatory cascade for the actions of the PGC-1 α coactivator in different tissues. Interaction of PGC-1 α with its cognate transcription factor targets is shown linked to specific organ systems, together the target genes regulated and the altered metabolic process due to this regulation.

expressed in liver, intestine, lung and adipose tissue. C/EBP β is also detectable in kidney, heart, and spleen by Northern analysis in the mouse (Cao et al., 1991). On the other hand, C/EBP ϵ is expressed only in myeloid and lymphoid lineages. Finally, C/EBP γ and C/EBP ζ are expressed ubiquitously (Lekstrom-Himes & Xanthopoulos, 1998).

It is noteworthy that C/EBPs can bind to the DNA as homodimers or as heterodimers with other transcription factors (Schrem et al., 2004). Previous studies had demonstrated the ability of the C/EBP family to form heterodimers with members of the cAMP-responsive-element-binding protein/ activating transcription factor (CREB/ATF) family of transcription factors especially ATF-2 and C/ATF (Fawcett et al., 1999; Shuman et al., 1997; Vallejo et al., 1993). C/EBP-ATF heterodimers bind to selective DNA sequences that are uniquely different than that for the C/EBP and ATF homodimers, as the C/EBP-ATF heterodimers bind to a so-called C/EBP-ATF composite site in the promoter region of regulated genes. C/ATF-C/EBP β heterodimers bind to a subclass of asymmetric cAMP response elements exemplified by those in the phosphoenolpyruvate carboxykinase and proenkephalin genes (Vallejo et al., 1993).

C/EBP β expression is highly induced by lipopolysaccharide (LPS), IL-6, IL-1, dexamethasone, and glucagon, suggesting a role in the mediation of the inflammatory response (Alam et al., 1992; An et al., 1996; Matsuno et al., 1996). In adipose tissue, C/EBP β is involved in the transcription of fat-specific genes (Christy et al., 1989; Yeh et al., 1995) and participates in the differentiation of pre-adipocytes (Bezy et al., 2007; Hamm et al., 2001). In addition, C/EBP β regulates carbohydrate and lipid metabolism through binding and activating a variety of genes encoding key metabolic enzymes including, but not limited to, phosphoenolpyruvate carboxykinase (PEPCK) and tyrosine aminotransferase (Garlatti et al., 1993; Park et al., 1990; Park et al., 1999; Wang et al., 1996), fatty acid synthesis enzyme acetyl-CoA carboxylase (Tae et al., 1994) and the albumin gene (Friedman et al., 1989).

Several hormones including insulin, glucocorticoids and thyroid hormone regulate the transcription of the C/EBP β and through this regulation they further control the expression of gluconeogenic genes (Duong et al., 2002; Ghosh et al., 2001; Menendez-Hurtado et al., 1997; Park et al., 1999). However, the most relevant point regarding this dissertation is the effect of thyroid hormone on the expression of C/EBP β that had been reported by Menendez-Hurtado (Menendez-Hurtado et al., 1997). He found that during development, congenital hypothyroidism caused a significant decrease in both C/EBP α and C/EBP β gene expression at early stages of postnatal development in liver (Menendez-Hurtado et al., 1997). This effect was tissue-specific since thyroid hormone had no effect on both C/EBPs mRNA levels in brown fat. Upon injection of those hypothyroid animals with thyroid hormone, there was a slow recovery of hepatic C/EBP α and C/EBP β mRNA levels. Also, hypothyroid neonates had shown diminished C/EBP α and C/EBP β protein levels, and the kinetics of induction of these proteins by thyroid hormone was faster than the one observed for the corresponding transcripts suggesting a translational or post-translational regulation of these genes as the major component of

thyroid hormone action on these proteins (Menendez-Hurtado et al., 1997). Also, studies from our lab had linked C/EBP β with the induction of PEPCK by thyroid hormone (Park et al., 1999). These information led us to investigate the role of C/EBP β in induction of another important metabolic gene, the PDK4, by thyroid hormone.

1.3 Pyruvate dehydrogenase kinase 4 (PDK4)

1.3.1 General biology

The pyruvate dehydrogenase complex (PDC) catalyzes the irreversible oxidative decarboxylation of pyruvate into acetyl-CoA. This reaction links the utilization of glycogen, glucose, and lactate with the citric acid cycle as well as fatty acid oxidation and synthesis, since acetyl-CoA can be used for fatty acid and cholesterol synthesis in the liver and fatty acid synthesis in adipose tissue. Prolonged fasting or high fat diets inactivate the PDC in order to minimize loss of pyruvate carbon to conserve it for maintaining the blood glucose levels needed through gluconeogenesis. Another important reason for inactivating the PDC is to promote the usage of fatty acids as their primary energy source in as many tissues as possible. This in turn conserves body protein because amino acids are not oxidized for energy and less amino acid carbon has to be used for glucose synthesis (Randle, 1986; Sugden et al., 2001b).

All these observations indicate that regulating PDC is an important step in fuel selection for energy production in animals during different nutritional and hormonal states. Thus, the regulation of PDC is critical in glucose and fatty acids metabolism. PDC is regulated by the reversible phosphorylation reaction. Pyruvate dehydrogenase kinase (PDK) inactivates PDC by phosphorylation and pyruvate dehydrogenase phosphatase (PDP) activates PDC by dephosphorylation. **Figure 1.3** illustrates the role of the PDK and PDP genes in the overall regulation process of the PDC activity (Sugden & Holness, 2003).

1.3.2 The role of the PDK in the regulation of the PDC

The mammalian pyruvate dehydrogenase complex is composed of three components: Pyruvate dehydrogenase (E1), the dihydrolipoamide acetyltransferase subunits (E2) and the dihydrolipoamide dehydrogenase (E3). E2 forms the core of the complex, while E1 is the enzyme responsible for decarboxylation of pyruvate. E1 binds noncovalently to an E1-binding domain on E2 as heterotetrameric $\alpha_2\beta_2$ structure. E3 is the enzyme responsible for oxidation of the reduced lipoyl groups of E2 and the subsequent production of NADH, and it binds to the complex by the E3-binding protein (E3BP), as twelve E3-binding proteins anchor 12 E3 homodimers to each pyruvate dehydrogenase complex. Thus, each pyruvate dehydrogenase complex is composed of 216 subunits (60 E2, 60 E1 α , 60 E1 β , 12 E3BP, and 24 E3) that sum up to about 9.5 MD (Harris et al., 2002).

Figure 1.3: The role of the PDK and PDP in the regulation process of the PDC and the related metabolic processes.

PDC catalyzes the irreversible oxidative decarboxylation of pyruvate into acetyl-CoA. The PDC is regulated by the reversible phosphorylation reaction. Pyruvate dehydrogenase kinase (PDK) inactivates PDC by phosphorylation and pyruvate dehydrogenase phosphatase (PDP) activates PDC by dephosphorylation. The regulation of the PDC links with the glucose and fatty acid metabolism. NADH, nicotinamide adenine dinucleotide hydride; FFA, free fatty acids; PDP, pyruvate dehydrogenase; PEPC, phosphoenolpyruvate carboxykinase.

Although PDC activity is directly inhibited by the acetyl-CoA and NADH, the two byproducts of fatty acid and pyruvate oxidation, covalent modification of the PDC at the serine residues appears to be more important for regulating the PDC activity (Harris et al., 2002). Two regulatory enzymes are involved in this covalent modification, the PDK which inactivates the PDC through phosphorylation and the PDP which activate the PDC through dephosphorylation. Four PDK isoenzymes (PDK1, PDK2, PDK3 and PDK4) and two PDP isoenzymes (PDP1 and PDP2) are expressed in mammals (Bowker-Kinley et al., 1998; Huang et al., 1998). The phosphorylation and the dephosphorylation occur at three serine residues number 203, 264 and 271 on the α -subunit of the E1 element of the PDC (Harris et al., 2002; Sugden & Holness, 2003).

1.3.3 PDK gene expression

PDK consists of two subunits, the catalytically active α -subunit and the regulatory β -subunit. Four isoenzymes of PDK have been identified in mammalian tissues (PDK1, PDK2, PDK3 and PDK4) (Bowker-Kinley et al., 1998; Kwon & Harris, 2004; Rowles et al., 1996). The primary structure of PDK was determined for the first time by isolating a cDNA encoding a 48-kDa form of PDK, later termed PDK from a rat heart cDNA library (Popov et al., 1993). Based on analysis of the deduced amino acid sequences and crystal structures studies, it had been suggested that PDK does not belong to the eukaryotic serine/threonine protein kinases but rather it resembles the prokaryotic histidine protein kinases. Therefore, it had been assigned to the ATPase/kinase superfamily (composed of bacterial histidine protein kinases, DNA gyrases, and molecular chaperone Hsp90) (Bowker-Kinley et al., 1998; Popov et al., 1993; Steussy et al., 2001).

There is 66-74% homology in the primary structures between the four PDK isoenzymes: PDK3 and PDK4 are the most distinct and PDK1 and PDK2 the most conserved (Popov et al., 1994). Each PDK isoform is extremely similar in sequence between species, and PDK1 and PDK2 are >95% identical between rat and human (Sugden & Holness, 2003). The PDK2, PDK3, and PDK4 share the same molecular weight which corresponds to a 45-kDa, while only PDK1 has molecular weight of 48 KDa (Sugden & Holness, 2003).

The mammalian PDK isoenzymes exhibit tissue-specific expression patterns (Bowker-Kinley et al., 1998). PDK1 expression has been detected in heart (Sugden et al., 2000b; Wu et al., 1998), the pancreatic islet (Sugden et al., 2001a) and skeletal muscle (Peters et al., 2001). PDK2 is expressed ubiquitously with particularly high expression in heart, liver, and kidney (Bowker-Kinley et al., 1998), while PDK3 is expressed in testis, kidney and brain (Huang et al., 1998). Finally, PDK4 is highly expressed in several tissues, including heart (Sugden et al., 2000b; Wu et al., 1998), skeletal muscle (Holness et al., 2002; Holness et al., 2000; Peters et al., 2001), liver (Holness et al., 2003; Sugden et al., 2002), kidney (Bowker-Kinley et al., 1998; Sugden et al., 2001b) and the pancreatic islet (Sugden et al., 2001a).

PDK isoenzymes show different kinetic parameters for phosphorylation site specificity in the regulation of PDC activity. As I mentioned before, the phosphorylation of α -subunit of the E1 element of the PDC, occurs at three specific serine residues, serine-264 (designated phosphorylation site 1), serine-271 (phosphorylation site 2) and serine-203 (phosphorylation site 3) (Harris et al., 2002; Holness & Sugden, 2003; Sugden & Holness, 2003). PDC can be inactivated by phosphorylation of each site alone, although phosphorylation of site 1 is most rapid and site 3 phosphorylation least rapid. All four PDKs exhibit higher activity towards site 1 of free E1 compared with sites 2 and 3. PDK2 exhibits the highest activity and PDK3 the lowest activity towards site 1. Hence, PDK2 activity may account for much of the short-term inhibition of PDC observed on the transition from the fed to the fasted state through phosphorylation of site 1. PDK4 exhibits a much higher activity towards site 2 compared with PDK1, PDK2 and PDK3 (Korotchkina & Patel, 2001). All four PDKs can phosphorylate sites 1 and 2, but site 3 can be phosphorylated only by PDK1, this was demonstrated using recombinant mutant proteins of the E1 element of the PDC with a single functional phosphorylation site (Kolobova et al., 2001; Korotchkina & Patel, 2001).

1.3.4 The role of the PDK4 gene in metabolic processes

PDK4 is expressed in heart, skeletal muscle, liver, kidney and in the pancreatic islets. PDK4 is involved in the regulation of different metabolic processes within these tissues. In heart for example, Harris et al reported marked increase in the abundance of the PDK4 mRNA and protein in the hearts of rats that had been starved or rendered diabetic with streptozotocin (Wu et al., 1998). Re-feeding of starved rats and insulin treatment of diabetic rats very effectively reversed the increase in PDK4 protein, mRNA levels and restored PDK enzyme activity to levels of chow-fed control rats. On the other hand, little or no change in the amounts of PDK1 and PDK2 protein and the abundance of their messages occurred in response to starvation and diabetes, indicating that control of the amount of PDK4 is important in long-term regulation of the activity of the pyruvate dehydrogenase complex in rat heart (Wu et al., 1998). Also, Mary Sugden reported that hyperthyroidism and high fat feeding induced the expression of the PDK4 in hearts of rats (Sugden et al., 2000b), while in liver high-fat feeding increased hepatic PDK2, but not PDK4, protein expression whereas hyperthyroidism increased both hepatic PDK2 and PDK4 protein expression (Holness et al., 2003). However, Huang et al reported that free fatty acids are effective inducers of PDK4 expression in 7800C1 hepatoma cells (Huang et al., 2002). Finally, high fat diets also induced the mRNA levels of the PDK4 gene in skeletal muscle (Sparks et al., 2006). In addition, starvation induced the PDK4 gene expression on both the RNA and protein levels not only in heart but in different mammalian tissues including liver and kidney (Wu et al., 2000). All these facts suggest that PDK4 may be the most important means of regulation of PDC activity during starvation and in diet-induced obesity.

Therefore, PDK4 is involved in the regulation of glucose synthesis and fatty acid oxidation as PDK4 upregulation during starvation may participate in directing available pyruvate toward oxaloacetate formation in liver and kidney or to the lactate output rather

than oxidation in case of skeletal muscle, with subsequent use for glucose synthesis for entry into the gluconeogenic pathway and glucose synthesis (Sparks et al., 2006; Sugden & Holness, 2002, 2003; Sugden et al., 2000a). Indeed, blood concentrations of glucose, lactate, pyruvate, and alanine are low in PDK4-knockout (PDK4^{-/-}) mice in the starved state (Jeoung et al., 2006), consistent with an important role for PDK4 in maintaining glucose levels during starvation. In addition, a recent PDK4^{-/-} mice model showed lower fasting blood glucose levels, a slightly improved glucose tolerance, and slightly greater insulin sensitivity compared with wild-type mice (Jeoung & Harris, 2008). In the same time, these PDK4^{-/-} mice have elevated levels of nonesterified fatty acids, ketone bodies, and branched-chain amino acids, consistent with slower rates of fatty acid oxidation.

Several transcription factors and coactivators have been implicated in the regulation of the PDK4 gene expression and its downstream metabolic processes. Activation of PPAR α *in vivo* enhances hepatic and renal PDK4 protein expression in the fed state promoting fatty acid oxidation (Huang et al., 2002; Sugden et al., 2001c). Furthermore, the upregulation of hepatic and renal PDK4 protein expression normally evoked in response to prolonged starvation is attenuated in PPAR α -deficient mice (Sugden et al., 2001b; Sugden et al., 2002). In contrast, skeletal muscle PDK4 gene expression is suppressed by treatment of insulin-resistant rats with PPAR agonists, promoting lipid entrapment in adipose tissue (Way et al., 2001). Interestingly, the transcriptional coactivator PGC-1 α that is in the core of this dissertation coactivates PDK4 gene expression via the orphan nuclear receptor ERR α , exerting reciprocal inhibitory influences on glucose catabolism while increasing alternate mitochondrial oxidative pathways in skeletal muscle (Wende et al., 2005). In addition, recent studies have also shown that the forkhead transcription factor FOXO1 and the glucocorticoid receptor (GR), two additional potential PGC-1 α partners, directly regulate PDK4 expression through consensus binding sites in the PDK4 gene promoter (Furuyama et al., 2003; Kwon et al., 2004).

CHAPTER 2: RESEARCH OBJECTIVES AND SPECIFIC AIMS

2.1 Research objectives

2.1.1 Characterize the regulation of PDK4 gene by thyroid hormone

Thyroid hormone (T3) is a key regulator of several processes including development, differentiation and metabolic balance. Among its cellular metabolic effects is the stimulation of fatty acid oxidation which is accompanied by a reduction in glucose utilization. This particular effect can be explained, in part, by the ability of T3 to induce the hepatic pyruvate dehydrogenase kinase (PDK4) gene expression. PDK4 suppresses glucose oxidation by its inhibitory effect on the pyruvate dehydrogenase complex (PDC) leading to an increase in fatty acid oxidation. T3 mediates its effect on gene expression of other genes through binding to a thyroid hormone receptor which is a nuclear receptor bound to the thyroid hormone response elements (TRE), frequently found in the promoters of these target genes. Upon T3 binding to the thyroid hormone receptor, the receptor undergoes a conformational change and recruits transcriptional coregulators which facilitate the regulation of transcription by T3. Peroxisome proliferator-activated receptor gamma coactivator-1 (PGC-1) and the CCAAT/enhancer binding protein (C/EBP) are among these transcription coregulators. Previous studies in our lab have demonstrated that PGC-1 α and C/EBP β are coactivators for the T3 induction of CPT-1a and PEPCK, respectively. In addition, the forkhead transcription factor (FOXO1) and the estrogen related receptor α (ERR α) are involved in the transcription regulation of PDK4 and other metabolic genes.

The induction of PDK4 gene by T3 was already documented; however, the mechanism of this activation has not been investigated. Both, the TRE site in the promoter of the PDK4 gene, and the transcription coregulators or the accessory factors that are involved in this activation, have not been identified. Therefore, the first hypothesis is that T3 activates PDK4 gene expression directly through a TRE site or indirectly through the recruitment of transcription factors and accessory proteins or through both mechanisms. To address this hypothesis, I characterized the PDK4 gene promoter to locate a TRE site, and I also tested whether PGC-1 α and C/EBP β participate in the T3 induction of the PDK4 gene. Also, the role of accessory factors as the ERR α and the FOXO1 in this induction was examined.

2.1.2 Study the network of the transcription factors involved in the early response of hepatic metabolic genes to thyroid hormone

Thyroid hormone regulates the expression of key metabolic genes including phosphoenolpyruvate carboxykinase (PEPCK), carnitine palmitoyltransferase 1a (CPT-1a), apolipoprotein B (ApoB) and uncoupling protein 3 (UCP3). However, the detailed regulation of these genes is not fully understood. Moreover, previous studies

demonstrated that T3 elevates the abundance of many transcription factors and coactivators associated with mitochondrial biogenesis and lipid metabolism, such as PGC-1 α , ERR α and lipins. Therefore, the second hypothesis is that T3 stimulates metabolism in part by increasing a network of transcription factors that induce metabolic genes which may not contain a TRE site within their promoters. To address this hypothesis, I have examined the effect of treating primary hepatocytes with T3 for 24 hours on 90 transcription factors and metabolic genes using superarray® real time PCR technology.

2.2 Specific aims

The following are the specific aims for this dissertation:

- **SPECIFIC AIM 1:** To characterize regulation of PDK4 gene by thyroid hormone.
- **SPECIFIC AIM 2:** To determine if T3 elevates expression of transcription factors involved in the regulation of metabolic genes.

CHAPTER 3: RESULTS AND EXPERIMENTAL PROCEDURES

3.1 Results

3.1.1 Identifying thyroid response elements (TREs) sites within the rPDK4 gene promoter

Transcription of the PDK4 gene in the liver is induced by T3 in hyperthyroid rats (Sugden et al., 1996). However, the position of the TRE within the rPDK4 gene had not been determined. Therefore, the initial experiments were designed to localize and confirm the region in the rPDK4 promoter required for inducing rPDK4 transcription by T3. To localize a TRE in the PDK4 gene, I cotransfected HepG2 cells with rPDK4-luciferase reporter constructs and an expression vector for TR β . First, I tested serial deletions of the PDK4 promoter driving the luciferase reporter. Deletion of the 283 nucleotides between -1256 to -973 of the rPDK4 promoter abolished T3 responsiveness from 5.1 ± 0.4 fold induction of the -1256/+78 rPDK4 luciferase vector to 0.8 ± 0.1 with the -973/+78 rPDK4 luciferase vector (**Figure 3.1A**). The results are expressed as the relative induction by T3 $\pm$ S.E. by comparing the T3 induction of vectors in the T3 treated cells to the non-treated ones. These data suggested that there was a TRE in this region of the PDK4 promoter. I identified two potential TREs sites within this region of the rPDK4 promoter. These TREs resemble the well established putative consensus hexamer half-site sequence [(G/A)GGT(C/G)A] arranged as a direct repeat separated by four nucleotides (DR4) which is the optimal binding site for TR/RXR. The putative TREs are shown in **Figure 3.1B** together with a model of the rat PDK4 promoter identifying the binding sites for ERR α , FOXO1 and PGC-1 α .

I used site-directed mutagenesis of the PDK4 promoter to determine if either of these potential TREs was important for the T3 induction. I disrupted the TRE1 by changing the (GGT) in the second hexamer half site sequence into (AAC), and for the TRE2 I changed the (GG) in its second hexamer half site into (AA). Primers used to establish these mutations are listed in the experimental procedures section. To determine the effect of these mutations on the T3 responsiveness, I transfected HepG2 cells with the TRE1 mutant -1256/+78, TRE2 mutant -1256/+78 or the wild type -1256/+78 and tested for T3 responsiveness by luciferase assays. TRE1 mutation caused a very significant reduction in the T3 responsiveness (1.5 ± 0.2 fold, 60% less than the wild type 3.8 ± 0.4 fold, $p = 0.0019$). On the other hand, the TRE2 mutation did not alter the T3 responsiveness (4.4 ± 1.4 , $p = 0.299$) (**Figure 3.1C**).

To further confirm the role of TRE1 in the T3 induction of the rPDK4 gene promoter, we performed an *in vivo* transfection into the livers of hyperthyroid rats (**Figure 3.1D**). This experiment was conducted in collaboration with Dr. Gene Ness lab in University of South Florida, and was performed by his graduate student Lindsey Boone. The -1256/+78 rPDK4 luciferase vector wild type and the TRE1 mutant were transfected by electroporation into different spots of the same rat liver.

Figure 3.1: Localization and identification of the T3 responsive regions within the rPDK4 promoter that enhance T3 responsiveness.

A. HepG2 cells were transiently transfected with 2 μ g of different rPDK4 luciferase reporters, 1 μ g of RSV-TR β , and 0.1 μ g of TK-renilla. For hormone treatment, cells were incubated either in serum-free DMEM or DMEM containing 100 nM T3 for 24 h. All transfections were performed in duplicate and repeated three to six times. Luciferase and renilla assays were performed in the same tube. Luciferase activity was corrected for both protein content and renilla activity which served as a transfection control. Results are expressed as the relative induction by T3 $\pm$ S.E. by comparing the T3 induction of vectors in the treated cells to the non-treated ones. The significance is calculated in relative to the shortest construct of the rPDK4-luciferase (-578/+78) (** = p value 0.001 to 0.01, *** = p value <0.001). B. A model of the rat PDK4 promoter is shown that contains the sites for the binding of the ERR α , FOXO1 and PGC-1 α , also the sequence of the region between -1256 to -973 for the rPDK4 promoter with underlined potential TREs. C. HepG2 cells were transiently transfected with 2 μ g of various -1256/+78 rPDK4 constructs, 1 μ g of RSV-TR β , and 0.1 μ g of TK-renilla. For hormone treatment, cells were incubated either in serum-free DMEM or DMEM containing 100 nM T3 for 24 h. All transfections were performed in duplicate and repeated three to six times. Luciferase assays were performed as described above. Results are expressed as the relative induction by T3 $\pm$ S.E. by comparing the T3 induction of vectors in the treated cells to the non-treated ones. The significance is calculated in relative to the wild type rPDK4 -1256/+78-luciferase (** = p value 0.001 to 0.01). D. Hypothyroid rats were transfected *in vivo* by electroporation of liver as outlined in the experimental procedures section. The transfection included the -1256/+78 wild type rPDK4-luciferase or the TRE1 mut rPDK4-luciferase plasmids. TK-renilla was included as a control. The hypothyroid rats were exposed to T3 three days before electroporation, with an additional half dose injection prior to the day of electroporation. The values are the average of four hypothyroid untreated rats and four T3 treated rats.

A.

B.

-1256 ggagcagagtttcacgagagatagaaattagacatcagtcattgtgactaggttagctctcttaggggaa -1187
-1186 aaggcaaagtaagagtgcacctagggtcaccatgactttcatcacaccacatcctccttacatgcgagg -1117
TRE1 site
-1116 gctaaggggcaccttgggtaacaaactgcctttgctgccttggtccattccattccatttgaaccgagtcgt -1044
TRE2 site
-1043 tatcattgtcccttatcaacagtccatttcgagtgaacagaagagcgagagaggttaggatgagagtttt -973

C.

D.

Figure 3.1: (continued).

Four hypothyroid rats were treated with T3 for 3 days before electroporation; with an additional half dose injection the day prior to electroporation. Another hypothyroid four rats were left untreated. In rats with T3 treatment, the expression of the wild type -1256/+78 rPDK4 luciferase promoter increased significantly by 3.1 ± 0.6 fold ($p = 0.01$) in comparison to the mutant one, while in non-treated rats the induction of the wild type form was not significantly different than that of the mutant form being only 1.4 ± 0.2 fold (**Figure 3.1D**).

Finally, gel shift mobility assays were conducted with the wild type and the mutants oligomers for the TRE1 site to test the ability of the TR β to bind this element *in vitro*. The results indicated that the TR β can bind as a heterodimer with RXR to the wild type of the TRE1 site. However, TR β did not bind the mutated TRE (**Figure 3.2**). The sequence of the oligomers used in this gel shift assays are indicated at the bottom of the panel.

These data prove that we had discovered a TRE site localized within the -1112 to -1096 region of the rPDK4 gene promoter, and that disruption of this site by site directed mutagenesis reduced the ability of the T3 to induce the rPDK4 gene.

3.1.2 Association of the TR β , PGC-1 α and C/EBP β with the PDK4 gene promoter *in vivo*

Previous studies from our laboratory have implicated the involvement of both peroxisome proliferator activated receptor gamma coactivator-1 alpha (PGC-1 α) and CCAAT/enhancer binding protein beta (C/EBP β) in the activation of different metabolic genes including the CPT-1 α and PEPCK (Park et al., 1999; Song et al., 2004; Zhang et al., 2004b). To determine whether TR β , PGC-1 α and C/EBP β were associated with the PDK4 gene *in vivo*, we conducted ChIP assays (**Figure 3.3**). Rat primary hepatocytes were exposed to 100nM T3 for 24 h or left in serum-free media. We cross-linked the hepatocytes with 1% formaldehyde prior to conducting immunoprecipitations with the TR β or PGC-1 α or C/EBP β antibodies. As a control, we used the immunoprecipitation with rabbit pre-immune serum. We created PCR primers sets for the proximal, the TRE region, and an upstream promoter region of the PDK4 gene, and they are listed in the experimental procedures section. The upstream region served as a negative control. The data from the ChIP assays indicated that prior to T3 treatment both TR β and C/EBP β are associated with the proximal and the promoter region of the PDK4 gene around the TRE site. However, they are not associated with the upstream region of the PDK4 gene and the addition of T3 to hepatocytes did not increase the association of TR β and C/EBP β with the proximal or the TRE region. On the other hand, the ChIP data show that prior to T3 treatment PGC-1 α can associate to the proximal region of the PDK4 gene promoter, and to a little extent to the TRE region of the promoter. Following treatment of hepatocytes with T3, the association of PGC-1 α with the proximal region of the PDK4 gene increased by 1.85 ± 0.2 fold but T3 did not increase its association with the TRE region of the promoter (**Figure 3.3**). The TR β , C/EBP β and PGC-1 α were associated to different extents with both the proximal and the TRE regions of the PDK4

TRβ	-	+	-	+
RXR	-	+	-	+

TRE1 Wild type

TRE1 Mutant

TRE1 WT top oligo
TRE1 Mut top oligo

GGGCTAAGGGCACCTTGGGTAACAAA
GGGCTAAGGGCACCTTGA**ACA**ACAAA

Figure 3.2: TR β binding site in the rPDK4 promoter.

A gel shift mobility assay was conducted as described in the experimental procedures section using the oligomers for the potential TRE1 site and its mutated version. The sequences for these oligomers are given below the gel shift with the mutated nucleotides shown in bold, and the sequence for the wild type TRE is underlined.

Figure 3.3: TR β , C/EBP β and PGC-1 α are associated with the rPDK4 gene promoter *in vivo*.

A. Chromatin immunoprecipitation assays (ChIP) were conducted on primary rat hepatocytes. Hepatocytes were treated with 100nM thyroid hormone (T3) for 24 hours and then cross linked with 1% formaldehyde as described in materials and methods. Antibodies to A. TR β , B. PGC-1 α and C. C/EBP β , or immunoglobulin G (IgG) were used for immunoprecipitations. The amplified PCR products using primers for the proximal, The PDK4-TRE and upstream region of the rPDK4 gene were resolved on an agarose gel. D. The association of TR β , C/EBP β and PGC-1 α with PDK4-TRE and proximal were quantified using quantity one software. These data are the average $\pm$ SE of four independent ChIP assays (** = p value 0.001 to 0.01).

A.

B.

C.

D.

Figure 3.3: (continued).

gene promoter but none of them were detected at the upstream control region of the PDK4 promoter. The data indicate that T3 through TR β , C/EBP β and PGC-1 α can regulate the PDK4 transcription. This regulation may involve a looping of the proximal region of the promoter with its distal region that contains the TRE site.

3.1.3 Thyroid hormone treatment increases the mRNA of both the PDK4 and PGC-1 α genes in primary rat hepatocytes.

I examined the effect of thyroid hormone (T3) on the abundance of both PGC-1 α and PDK4 mRNA in primary rat hepatocytes. Primary rat hepatocytes were treated with T3 for 24 h and then RNA was isolated. The mRNA abundance was measured by real time PCR. Both the PGC-1 α and the PDK4 mRNA abundance were significantly increased 2.6 ± 0.7 fold and 2.2 ± 0.3 fold after T3 treatment in rat hepatocytes, respectively (**Figure 3.4A**). These data indicate that T3 can increase PGC-1 α mRNA abundance in the liver. However, previous studies from our laboratory showed that PGC-1 α mRNA abundance did not change in heart after T3 treatment suggesting that T3 induces PGC-1 α in a tissue-specific manner (Zhang et al., 2004b). Next, I tested whether PGC-1 α and PDK4 protein abundance were increased in response to T3 administration in rat hepatocytes. Using Western analyses to assess PGC-1 α levels, we found that the PGC-1 α and the PDK4 protein abundance were increased 2.2 ± 0.2 fold and 2 ± 0.2 fold in response to T3 treatment, respectively (**Figure 3.4B**). These experiments provide further confirmation that the PGC-1 α is a T3-responsive gene and supports our hypothesis that PGC-1 α is a coactivator in T3 action for the PDK4 gene.

3.1.4 PGC-1 α enhances the thyroid hormone induction of the PDK4 gene and the role of the forkhead transcription factor (FOXO1) and the estrogen related receptor α (ERR α) in this enhancement.

In the next experiments I examined the ability of PGC-1 α to enhance the T3 induction of the PDK4 gene. I conducted co-transfections of pSV-PGC-1 α with the wild type -1256/+78 rPDK4-luc or the forkhead transcription factor (FOXO1) mutant at position -347 or the estrogen related receptor α (ERR α) mutants at positions -338 and -370 of the -1256/+78 rPDK4-luc. Transfections were conducted in HepG2 hepatoma cells. Primers used to establish the mutations in the -1256/+78 rPDK4-luc are listed in the experimental procedures section. Addition of 100nM of T3 for 24 hours stimulated the wild type version of -1256/+78 rPDK4-luc vector by 8.4 ± 2 fold. In these experiments, overexpression of PGC-1 α induced it by 4.3 ± 0.7 fold. When T3 was added in the presence of PGC-1 α , I obtained a synergistic effect with 23.2 ± 4 fold induction. Also, when treating the -338 ERR α mutant, -370 ERR α mutant and -347 FOXO1 mutant versions of the rPDK4 -1256/+78 with T3 all of them showed luciferase induction by similar levels 7 ± 1.2 fold, 6.1 ± 1.3 fold and 7.1 ± 0.7 fold, respectively. However, the overexpression of PGC-1 α did not induce either of the ERR α mutants or induced the -347 FOXO1 mutant by 3.1 ± 0.6 fold only, which is still lower than the wild type -1256/+78 induction.

Figure 3.4: Thyroid hormone increases PDK4 and PGC-1 α mRNA and protein abundance in primary hepatocytes.

A. Primary rat hepatocytes were plated on collagen-coated plates for 16 h. T3 was added at a concentration of 100 nM to the hepatocytes for 24 h. RNA was harvested, and the abundance of PDK4 and PGC-1 α mRNA was determined by real time PCR. The data are presented as the fold induction of mRNA abundance by T3 (average $\pm$ SE) from four independent hepatocytes preparations, and the control samples were assigned a relative value of 1. B. The expression of the PDK4 and PGC-1 α proteins was monitored by Western blot analysis using specific antibodies (1: 1000, Santa Cruz). The data are presented as the fold induction of protein abundance by T3 (average $\pm$ SE) from three independent hepatocytes preparations, and the control samples were assigned a relative value of 1 (* = p value 0.01 to 0.05, ** = p value 0.001 to 0.01).

A.

B.

But astonishing, when T3 was added in the presence of PGC-1 α , I still obtained a significant synergistic effect for all the mutants versions of the -1256/+78 as follows: 15.5 ± 3.5 fold for the -338 ERR α mutant, 11.6 ± 2 fold for the -370 ERR α mutant and 20.1 ± 4.4 fold for the -347 FOXO1 mutant (**Figure 3.5**). These data indicate that PGC-1 α stimulates PDK4 gene primarily through the -338 to -370 region of the rPDK4 promoter but, more importantly, that PGC-1 α can act as a coactivator for the T3 induction of the PDK4 gene either through binding to its elements in the -338 to -370 region or may be through interacting with the thyroid receptor itself. For this reason we still observed a significant synergistic effect with T3 addition even though I mutated both ERR α sites in the promoter and PGC-1 α alone by itself could not induce the PDK4 promoter. Supporting this concept are the ChIP data showing association of PGC-1 α with the rPDK4 TRE site **Figure 3.3D**. Moreover, previous studies had detected an interaction between the PGC-1 α and both the TR β and RXR *in vitro* and *in vivo* (Delerive et al., 2002; Puigserver et al., 1998; Wu et al., 2002). To further confirm this possibility I used adenoviral delivery of shRNA specific for silencing the PGC-1 α in primary hepatocytes to see the effect of reduction in the PGC-1 α gene expression on the hepatic T3 induction of PDK4 gene as.

3.1.5 Knocking down of the PGC-1 α in rat primary hepatocytes affects the ability of thyroid hormone to induce different metabolic genes.

To test the role of the PGC-1 α in the T3 induction of hepatic metabolic gene expression, I infected rat primary hepatocytes with adenovirus encoding the shRNA to silence the PGC-1 α (Ad-siPGC-1 α) for 16 hours prior to treatment of cells with T3 for additional 24 hours. Adenovirus encoding control shRNA that does not silence any rat genes was used as a control (Ad-NC). Ad-siPGC-1 α significantly knocked down the expression of PGC-1 α by 70% compared to the Ad-NC in hepatocytes non-treated with T3 (0.3 ± 0.04 fold relative to Ad-NC infected hepatocytes non treated with T3). In addition, the induction of PGC-1 α by T3 treatment was reduced in hepatocytes infected with Ad-siPGC-1 α (0.6 ± 0.04 fold relative to Ad-NC infected hepatocytes untreated with T3) in comparison to that infected with Ad-NC (6.2 ± 3 relative to Ad-NC infected hepatocytes untreated with T3) (**Figure 3.6A**). The knock down of the PGC-1 α in primary hepatocytes was accompanied by inability of T3 to induced the expression of PDK4, CPT-1 α , PEPCK and C/EBP β , while T3 had increased all these genes in hepatocytes infected with Ad-NC by 3.3 ± 0.9 fold, 3.9 ± 1 fold, 3.12 ± 0.52 fold, 1.7 ± 0.35 fold, respectively (**Figure 3.6B-E**). This impairment of the T3 induction for these metabolic genes after PGC-1 α silencing indicates that PGC-1 α is an important coactivator in the T3 induction process of these genes in primary hepatocytes.

I have shown in this first part of the results chapter of this dissertation the ability of thyroid hormone to induce the PDK4 gene through a TRE and that PGC-1 α enhanced this induction. When PGC-1 α was completely silenced, thyroid hormone lost its ability to activate the PDK4 gene indicating that PGC-1 α is an important coactivator in this process.

Figure 3.5: PGC-1 α enhances the T3 induction of rPDK4 gene.

HepG2 hepatoma cells were transiently transfected with 2 μ g of different rPDK4-Luc constructs, 1 μ g of pSV-PGC-1 α or pSV, 1.0 μ g of RSV-TR β , and 0.1 μ g of TK-*Renilla*. T3 was added at a concentration of 100 nM for 24 h. All transfections were performed in duplicate and repeated three to six times. Luciferase and renilla assays were performed in the same tube. Luciferase activity was corrected for both protein content and renilla activity which served as a transfection control. Results are expressed as fold induction by PGC-1 α or T3 compared to the untreated cells in each data set (* = p value 0.01 to 0.05, ** = p value 0.001 to 0.01, *** = p value <0.001).

Figure 3.6: The effect of knocking down the PGC-1 α on the induction gene expression of the PDK4, CPT-1a, PEPCK and C/EBP β by T3.

Rat primary hepatocytes were infected with adenoviral vectors expressing shRNA for PGC-1 α (Ad-siPGC-1 α), or expressing control-shRNA (Ad-NC). After 24 h, hepatocytes were treated with T3 at a concentration of 100nM for another 24 hours. RNA was isolated from the cells, and the mRNA abundance of the indicated genes was measured by real time PCR. 18S rRNA was used as the control. The infections were repeated three times on independent plates of cells. A. fold induction of the PGC-1 α gene expression in hepatocytes in comparison to hepatocytes infected with Ad-NC and non-treated with T3. B. PDK4, C. CPT-1a, D. PEPCK and E. C/EBP β mRNAs abundance were also measured in the same time with measuring the PGC-1 α gene expression. The data are expressed as the mean of the fold induction $\pm$ S.E. of mRNA abundance relative to cells non-treated with T3 (* = p value 0.01 to 0.05, ** = p value 0.001 to 0.01).

A.

B.

C.

D.

Figure 3.6: (continued).

E.

Figure 3.6: (continued).

To further investigate the mechanism of PDK4 induction by thyroid hormone, I tested the role of another coregulator, C/EBP β , that was associated with the PDK4 gene promoter by ChIP technique in the induction of PDK4 by thyroid hormone (refer to **Figure 3.3C**).

3.1.6 Overexpression of C/EBP β induces the rPDK4 gene luciferase promoter.

Previous studies from our laboratory had shown that C/EBP β is implicated in the T3 induction of the PEPCK gene (Park et al., 1999). Therefore, in the following part of this dissertation I investigated the role of the C/EBP β as a coregulator in the induction of the PDK4 gene by T3. In the first experiment, I tested the ability of the C/EBP β to induce the PDK4 gene promoter in the absence of T3. I conducted co-transfections of different serial deletions constructs of the rPDK4 promoter driving a luciferase reporter and MSV-C/EBP β in HepG2 hepatoma cells and measured the level of induction for these luciferase constructs. Luciferase assays showed that deletion of the 210 nucleotides between -788 to -578 of the rPDK4 promoter significantly reduced C/EBP β responsiveness from 7.6 ± 0.7 fold to 3.3 ± 0.5 fold ($p = 0.001$), and also the deletion of the 255 nucleotides between -325 to -70 of the rPDK4 promoter significantly reduced C/EBP β responsiveness from 4.4 ± 0.8 fold to 1.00 ± 0.3 fold ($p = 0.005$) (**Figure 3.7**). These data indicate that C/EBP β can induce PDK4 gene expression and that at least two sites in the PDK4 gene promoter between nucleotides -788 to -578 and -325 to -70 are essential for its action. These data are consistent with the ChIP data (**Figure 3.3C**) where C/EBP β was associated with both the proximal and the TRE site of rPDK4 gene promoter.

3.1.7 Thyroid hormone treatment increases the C/EBP β protein abundance in primary hepatocytes.

The next question investigated whether C/EBP β was regulated by T3. I treated primary rat hepatocytes with 100nM T3 for 24 h and extracted proteins. Using Western analyses to assess C/EBP β protein level, I found that the C/EBP β protein abundance was significantly increased by 2.1 ± 0.4 fold in response to T3 treatment (**Figure 3.8**). This experiment demonstrated that C/EBP β abundance is modulated by T3 and raised the possibility that it may be a coregulator in T3 action for the PDK4 gene.

3.1.8 C/EBP β enhances the thyroid hormone induction of rPDK4 gene.

In the next experiment, I examined the ability of the C/EBP β to enhance the T3 induction of the PDK4 gene. I conducted co-transfections of the wild type -1256/+78 rPDK4-luc and MSV-C/EBP β in HepG2 hepatoma cells with and without T3 addition. Addition of 100nM of T3 for 24 hours stimulated the wild type version of -1256/+78 rPDK4-luc vector by 5.3 ± 1.1 fold. In these experiments, overexpression of C/EBP β induced the PDK4-luc vector 4.7 ± 1.3 fold.

Figure 3.7: Overexpression of C/EBPβ induces the rPDK4 promoter.

HepG2 cells were transiently transfected with 2 µg of different rPDK4 constructs, 1 µg of MSV-C/EBPβ, and 0.1 µg of TK-renilla. All transfections were performed in duplicate and repeated three at least three times. Luciferase and renilla assays were performed in the same tube. Luciferase activity was corrected for both protein content and renilla activity which served as a transfection control. Results are expressed as the relative induction by T3 ± S.E by comparing the T3 induction of vectors in the treated cells to the non-treated ones. The significance is calculated in relative to the shortest version of the rPDK4-luciferase (-70/+78) (** = p value 0.001 to 0.01, *** = p value <0.001).

Figure 3.8: Thyroid hormone increases the C/EBPβ protein abundance in primary hepatocytes.

The expression of the C/EBPβ proteins was monitored by Western blot analysis using specific antibodies (1: 1000, Santa Cruz). The data are presented as the fold induction of protein abundance by T3 (average ± SE) from three independent hepatocytes preparations, and the control samples were assigned a relative value of 1 (* = p value 0.01 to 0.05).

When T3 was added in the presence of C/EBP β , I obtained a significant synergistic effect with a 16.9 ± 5 fold induction (**Figure 3.9**). These data indicate that C/EBP β can activate the PDK4 gene promoter and can act synergistically with the T3 to amplify its effect on the PDK4 gene promoter.

To further demonstrate and confirm the role of the C/EBP β protein in the induction of PDK4 transcription, I tested a set of specific dominant negative vectors. Hep4-C/EBP is a dominant negative C/EBP vector, which has the C/EBP leucine zipper attached to an acidic amphipathic helix containing 4 heptads repeats (Krylov et al., 1995). The amphipathic helix interacts with the basic region of C/EBP proteins to form a strong non-DNA binding heterodimer and inhibits all C/EBP proteins including both alpha and beta isoforms (Krylov et al., 1995). For controls, I used the hep4-CREB which is a dominant negative CREB protein with a CREB leucine zipper and an amphipathic helix (Ahn et al., 1998) and the hep4-Fos which is a dominant negative Jun vector with the Fos leucine zipper and an amphipathic helix (Olive et al., 1997). To examine the effect of these dominant negative proteins on the basal induction of the PDK4 gene, the -1256/+78 PDK4-luc vector was cotransfected with three dominant negative vectors into HepG2 cells and after 48 hours from the transfection, proteins were extracted from the cells and tested for the luciferase level induction. The dominant negative C/EBP reduced the basal levels of the PDK4 gene significantly by 32% ($p= 0.02$), while the dominant negative FOS did not have any effect on the basal level of the PDK4 gene and the dominant negative CREB even significantly doubled its basal level ($p= 0.0007$) (**Figure 3.10A**). The reason for the induction of the PDK4 gene basal level by hep4-CREB is not known.

To confirm the role of the C/EBP proteins in the induction of the PDK4 gene by T3, I cotransfected the -1256/+78 PDK4-luc vector with three dominant negative vectors into HepG2 cells and treated them with 100nM T3. Proteins were extracted from the cells and tested for the luciferase activity. Cotransfection with hep4-C/EBP inhibited the 4.5 ± 1.3 fold induction of T3. Both the dominant negative CREB and FOS did not affect at all the induction by T3 (**Figure 3.10B**).

These data indicate that C/EBP β induces the expression of the PDK4 gene and enhances the T3 induction of this gene. Inhibition of the C/EBP isoforms by dominant negative vectors abolishes the ability of T3 to induce PDK4 gene. However, the dominant negative vectors cannot differentiate between the two isoforms of the C/EBP and therefore, to pinpoint which isoform this involved in the T3 induction of the PDK4 gene, I used adenoviral shRNA delivery to specifically inhibit the C/EBP β isoform in the primary rat hepatocytes.

3.1.9 Knocking down C/EBP β reduces the ability of thyroid hormone to induce PDK4 gene expression in primary rat hepatocytes.

To test the role of the C/EBP β in the T3 induction of PDK4 gene expression, I infected rat primary hepatocytes with adenovirus encoding the shRNA to silence specifically the C/EBP β (Ad-siC/EBP β).

Figure 3.9: C/EBP β enhances the T3 induction of the rPDK4 gene.

HepG2 hepatoma cells were transiently transfected with 2 μ g of -1256/+78 rPDK4-Luc constructs, 1 μ g of MSV-C/EBP β or MSV, 1.0 μ g of RSV-TR β , and 0.1 μ g of TK-Renilla. T3 was added at a concentration of 100 nM for 24 h. All transfections were performed in duplicate and repeated three times. Luciferase assays were performed as described in **Figure 3.8**. Results are expressed as fold induction by PGC-1 α or T3 *versus* the untreated cells in each data set (* = p value 0.01 to 0.05, ** = p value 0.001 to 0.01).

Figure 3.10: The effect of inhibiting the C/EBP, FOS and CREB on the induction of rPDK4 by T3.

A. The effect of inhibiting the C/EBP, FOS and CREB on the basal expression of the -1256/+78 rPDK4 luc. HepG2 hepatoma cells were transiently transfected with 2 µg of -1256/+78 rPDK4-Luc constructs, 1 µg of PSV-40, hep4-C/EBP, hep4-FOS or hep4-CREB, 1.0 µg of RSV-TRβ, and 0.1 µg of TK-Renilla. Forty-eight hours after the transfection, cells were collected. All transfections were performed in duplicate and repeated three times. Luciferase and renilla assays were performed in the same tube. Luciferase activity was corrected for both protein content and renilla activity which served as a transfection control. Results are expressed as fold induction by PGC-1α or T3 *versus* the untreated cells in each data set (* = p value 0.01 to 0.05, *** = p value <0.001). B. The effect of inhibiting the C/EBP, FOS and CREB on the induction of the -1256/+78 rPDK4 luc by T3. HepG2 hepatoma cells were transiently transfected with 2 µg of -1256/+78 rPDK4-Luc constructs, 1 µg of PSV-40, hep4-C/EBP, hep4-FOS or hep4-CREB, 1.0 µg of RSV-TRβ, and 0.1 µg of TK-Renilla. T3 was added at a concentration of 100 nM for 24 h. All transfections were performed in duplicate and repeated three times. Luciferase assays were performed as described above. Results are expressed as fold induction by PGC-1α or T3 *versus* the untreated cells in each data set (* = p value 0.01 to 0.05, ** = p value 0.001 to 0.01).

A.

B.

Hepatocytes were transduced with the adenoviruses for 16 hours prior to treatment of cells with T3 for another 24 hours. Adenovirus encoding control shRNA that does not silence any rat genes was used as a control (Ad-control). I obtained these adenoviruses from Dr. Jacob E. Friedman from University of Colorado at Denver (Schroeder-Gloeckler et al., 2007). Ad-siC/EBP β significantly knocked down the expression of C/EBP β by 65% compared to the Ad-NC in hepatocytes untreated with T3 (0.4 ± 0.02 fold relative to Ad-control infected hepatocytes untreated with T3). In addition, the induction of C/EBP β by T3 was reduced in hepatocytes infected with Ad-siC/EBP β (0.4 ± 0.04 fold relative to Ad-control infected hepatocytes untreated with T3) in comparison to that infected with Ad-control (1.8 ± 0.3 relative to Ad-control infected hepatocytes untreated with T3) (**Figure 3.11A**). The knock down of the C/EBP β in primary hepatocytes was accompanied by a significant reduction in the T3 induction of PDK4, as T3 induced PDK4 only by 1.55 ± 0.24 fold while T3 had increased PDK4 in hepatocytes infected with Ad-control by 2.3 ± 0.2 fold (**Figure 3.11B**). This reduction of the T3 induction for the PDK4 gene expression after C/EBP β silencing indicates that C/EBP β is an important coregulator in the T3 induction process of the PDK4 gene expression in primary hepatocytes.

From the previous experiments, I have confirmed the induction of PDK4 gene by T3 treatment. I also showed that both the PGC-1 α and C/EBP β are important coregulators for this induction. However, previous studies had shown that thyroid hormone induces a number of metabolic genes. This implies a large network of genes and transcription factors that are involved in the T3 cellular and genomic actions (Weitzel et al., 2001; Yen et al., 2006). Therefore, I designed the next experiment to identify the early response network of transcription factors and metabolic genes that are regulated following 24 hours of T3 treatment involved in the thyroid hormone metabolic action.

3.1.10 Thyroid hormone treatment induces a network of transcription factors and different metabolic genes in primary rat hepatocytes.

Thyroid hormone regulates the expression of key metabolic genes including phosphoenolpyruvate carboxykinase (PEPCK), carnitine palmitoyltransferase 1a (CPT-1a), apolipoprotein B (ApoB) and uncoupling protein 3 (UCP3) (Boss et al., 2000; Jackson-Hayes et al., 2003; Mukhopadhyay et al., 2003; Park et al., 1997; Yen et al., 2006). Also, previous studies demonstrated that T3 elevates the abundance of many transcription factors and coactivators associated with mitochondrial biogenesis and lipid metabolism, such as PGC-1 α and ERR α (Xie et al., 1999; Zhang et al., 2004b). These facts indicate that T3 may stimulate metabolism in part by increasing a network of transcription factors that induce different metabolic genes which may or may not contain a TRE site within their promoters. To address this possibility, I treated primary rat hepatocytes with 100nM T3 for 24 hours, and then RNA was isolated from these hepatocytes and from non-treated ones. RNA was assessed on a superarray, containing 90 real time PCR primers for genes of transcription factors and metabolic genes, according to the manufacturer procedures using the Roche Light cycler 480 machine. A complete list of these genes is provided in the experimental procedures section.

Figure 3.11: The effect of knocking down the C/EBP β on the induction gene expression of the PDK4 gene by T3.

Rat primary hepatocytes were infected with adenoviral vectors expressing shRNA for C/EBP β (Ad-siC/EBP β) or expressing control-shRNA (Ad-control). After 24 h, hepatocytes were treated with T3 at a concentration 100nM for another 24 hours. RNA was isolated from the cells, and the A. PDK4 and B. C/EBP β mRNA abundance was measured by real time PCR. 18 S rRNA was used as the control. The infections were repeated four to seven times on independent plates of cells. The data are expressed as the mean of the fold induction $\pm$ S.E. of mRNA abundance relative to cells non-treated with T3.

A.

B.

The results from four independent hepatocytes preparations showed up-regulation of 27 genes by more than 1.5 fold relative to non-treated cells (**Figure 3.12**). Thyroid treatment significantly induced genes involved in glucose homeostasis and gluconeogenesis such as, glucose-6-phosphatase (G6pase) by 1.7 ± 0.2 fold, phosphoenolpyruvate carboxykinase (PEPCK) by 3.7 ± 1.1 fold, solute carrier family 2 (facilitated glucose transporter), member 4 (Slc2a4) by 3.2 ± 0.6 fold, cytochrome P450 2E1 (Cyp2e1) by 2.2 ± 0.7 fold (Lieber, 1999). On the other hand, T3 treatment induced genes involved in fatty acid oxidation and metabolism such as, 3-ketoacyl-Coenzyme A thiolase alpha subunit (Hadha) by 2.5 ± 0.05 fold, fatty acid binding protein 3/small heterodimer partner (Fabp3/SHP) by 2.5 ± 0.8 fold, solute carrier family 25 carnitine/acylcarnitine translocase member 20 (Slc25a20) by 2 ± 0.5 fold, fatty acid binding protein 1 (Fabp1) by 2.7 ± 1 fold, carnitine palmitoyltransferase 1a (CPT-1a) by 2 ± 0.6 fold, and acetyl-Coenzyme A carboxylase beta (Acacb) by 2.6 ± 0.8 fold. Interestingly T3 significantly induced genes involved in fatty acid and lipid synthesis including, solute carrier family 27 fatty acid transporter member 5 (Slc27a5) by 1.9 ± 0.3 fold, fatty acid synthase (FAS) by 2.3 ± 0.3 fold.

Also, T3 treatment induced number of transcription factors such as, GA binding protein transcription factor, beta subunit 1 (Gabpb1) by 1.9 ± 0.4 fold, peroxisome proliferative activated receptor, delta (Ppar δ) by 3.1 ± 1 fold, estrogen related receptor gamma (EsRR γ) by 2.5 ± 0.9 , CCAAT/enhancer binding protein beta (C/EBP β) by 2 ± 0.7 , sterol regulatory element-binding transcription factor 1 (Srebf1) by 3.5 ± 0.6 (Zhang et al., 2003), Sirtuin 1 (Sirt-1) which deacetylates proteins that contribute to cellular regulation (Frye, 1999), and the homeobox protein NK-2 homolog (Nkx2.5) that binds and activates the promoter of thyroid specific genes such as thyroglobulin, thyroperoxidase, and thyrotropin receptor (Civitareale et al., 1989; Dentice et al., 2006; Francis-Lang et al., 1992), it was increased by 2.8 ± 0.7 fold. In addition, T3 treatment showed induction of number of transcription coactivators such as, peroxisome proliferative activated receptor gamma coactivator-1 alpha (PGC-1 α) by 2.3 ± 0.9 fold, peroxisome proliferative activated receptor gamma coactivator-1 beta (PGC-1 β) by 3 ± 1.1 fold, also T3 induced both lipin gene isoforms, 1 and 2, by 2.1 ± 0.7 and 3.6 ± 1.6 fold, respectively.

Finally T3 treatment induced number of miscellaneous proteins involved in different metabolic processes including, deiodinase, iodothyronine, type II (Dio2) which is responsible for the activation of thyroid hormone by converting the prohormone thyroxine (T4) by outer ring deiodination (ORD) to bioactive triiodothyronine (T3) (Yen, 2001), it was increased by 3.7 ± 1 fold, pyruvate dehydrogenase kinase isoform 4 (PDK4) by 3 ± 0.7 fold and both isoforms of the uncoupling proteins, 2 and 3 (UCP2 and UCP3), involved in thermogenesis, insulin secretion and fatty acid transportation anions (Fisler & Warden, 2006), T3 induced UCP2 and UCP3 by 3.2 ± 1.5 fold and 3.5 ± 1.6 , respectively.

Figure 3.12: Effect of T3 on transcription factor and metabolic gene abundance in primary rat hepatocytes.

Primary rat hepatocytes were plated on collagen-coated plates for 16 h. T3 was added at a concentration of 100 nM to the hepatocytes for 24 h. RNA was harvested using RNA-STAT 60 and treated with Turbo DNase I to remove contaminating genomic DNA and further purified using the Qiagen RNeasy kit. The extracted total RNA was converted into cDNA using superarray RT² First Strand Kit. The abundance of the transcription factors and metabolic genes mRNA was determined by superarray® real time PCR (RT² Profiler™ PCR Array System, SABiosciences cat # CAPR-0529F). This superarray contains primers for 90 transcription factors/metabolic genes and 6 controls. The cDNA was assessed on the superarray according to the manufacturer protocol with a Roche light cycler 480. The data are presented as the fold induction of mRNA abundance by T3 (average $\pm$ SE) from four independent hepatocytes preparations and the control samples were assigned a relative value of 1. Only the genes that were induced by T3 treatment by more than 1.5 fold relative to non-treated cells are represented in this figure (* = p value 0.01 to 0.05, ** = p value 0.001 to 0.01, *** = p value <0.001).

Fold induction of mRNA by T3 hormone compared to non-treated cells

3.2 Experimental procedures

3.2.1 Transient transfection of luciferase vectors

PDK4-luciferase constructs (PDK4-luc) were transiently transfected into HepG2 cells by the calcium phosphate method as previously described (Zhang et al., 2004b). Transfections included 2 µg of PDK4-luciferase along with SV40-thyroid hormone receptor β (SV40-TR β), PSV-PGC-1 α and TK-Renilla. For experiments with C/EBP β ; MSV-C/EBP β was included in the transfection. Dominant negative vectors for C/EBP (hep4-CEBP), CREB (hep4-CREB) and FOS (hep4-FOS) were also transfected. Cells were transfected in Dulbecco's modified Eagle's medium containing 5% calf serum, 5% fetal calf serum and incubated overnight at 37 °C. Following two washes with phosphate-buffered saline, the medium was replaced by Dulbecco's modified Eagle's medium containing no serum. Cells were treated with 100 nM T3 for 24 h. After T3 treatment, cells were lysed in Passive Lysis Buffer (Promega). Both luciferase and *Renilla* activity were measured using Promega dual luciferase reporter kit (cat# E1980). Protein content in each lysate was determined by Pierce BCA Protein assay kit (cat# 23225). Luciferase activity was corrected for both protein content and *Renilla* activity to account for cell density and transfection efficiency, respectively.

3.2.2 Site directed mutagenesis of the rPDK4 promoter

Stratagene's Quick-change-XL site directed mutagenesis kit was used to carry out mutation in the potential TREs in the -1256/+78 rPDK4-luciferase vector. TRE1 was disrupted by changing the (GGT) in its second hexamer half site sequence into (AAC), while the TRE2 was disrupted by changing only two nucleotides (GG) in its second hexamer half site into (AA). In addition, site directed mutagenesis technique was used to carry out mutations in the ERR α and FOXO1 binding sites in the -1256/+78 rPDK4-luciferase vector. Mutations of the ERR α binding site are named Mut338 and Mut370, while the mutation of the FOXO1 binding site is named Mut347. Primers used to establish all these mutations are listed in **Table 3.1**, mutated nucleotides are in bold.

3.2.3 *In vivo* luciferase transfection

In vivo transfection of the wild type and mutated TRE1 -1256/+78 was done by electroporation in hypothyroid (by hypophysectomy) rats livers. Four hypothyroid rats were used without thyroid treatment and another 4 rats were treated with T3 three days prior to electroporation, with an additional half-dose injection the day before electroporation. This experiment was done in Dr. Gene Ness lab in University of South Florida. Electroporation was performed as previously described (Lagor et al., 2007). Approximately 16 µg of plasmid in 40 µl of sterile saline was subcapsular injected using a 30-gauge, 3/8-in length needle and it was visible below the capsular surface as a blanched out area after injection. A circular six-node electrode was placed on the liver

Table 3.1: Sequences of the primers used for the site-directed mutagenesis of the rPDK4 promoter.

TRE region	Primer sequence
MutTRE1 Forward	5'-GAGGGCTAAGGGCACCTTGA A CAACAAACTGCCTTTGCTGC-3'
MutTRE1 Reverse	5'-CTCCCGATTCCCGTGGA ACTTT GTTGTTTGACGGAAACGACG-3'
MutTRE2 Forward	5'-GAGTGAACAGAAGAGCGAGAGAA A TTAGGATGAGAGTTTTATGAC-3'
MutTRE2 Reverse	5'-CTCACTTGTCTTCTCGCTCTCT TTA ATCCTACTCTCAAATACTG-3'
Mut338 Forward	5'-AGATGGCTCCTGAGTTGTAAACAA AA ACAAGTCTGGGCGGG-3'
Mut338 Reverse	5'-CCCGCCCAGACTTG TTTT TGTTTACAACCTCAGGAGCCATCT-3'
Mut347 forward	5'-TGACATTGAGATGGCTCCTGAGTT TG CAACAAGGACAAGTCTGGGC-3'
Mut347 Reverse	5'-GCCCAGACTTGTCTTGTT G CAAACTCAGGAGCCATCTCAATGTCA-3'
Mut370 forward	5'-CAGTTTCTGGCGAGGAATGCGTG TTCTG TGAGATGGCTCCTGAGTTGTAA-3'
Mut370 Reverse	5'-TTACAACCTCAGGAGCCATCTC CGA ACACGCATTCTCGCCAGAACTG-3'

area where the plasmid was visible, so that each rat liver was electroporated with both the wild type and the mutated version of the -1256/+78 plasmid, in different spots of the liver. Electrodes were sunk to a controlled depth of 2 mm using a rubber spacer. 6 x 150-msec pulses at 100 V/cm, with a 150-msec rest between pulses were used to perform the transfection for optimal gene delivery to the liver with minimal tissue damage. To control for transfection efficiency, a plasmid-encoding renilla luciferase was cotransfected. After electroporation, the liver was placed back in the abdomen, and the wound was closed with surgical staples. At the time of surgery, animals were given a single subcutaneous injection of ketoprofen (5 mg/kg) for analgesia.

The results are expressed as the mean of luciferase activity of the wild type -1256/+78 rPDK4-luciferase to that of the TRE1 mutated -1256/+78-luciferase for the hypothyroid rats or T3-treated rats.

3.2.4 Electrophoretic mobility shift

Electrophoretic mobility shift assays were conducted by labeling double-stranded oligonucleotides with Klenow enzyme and [α -³²P]dCTP. Oligonucleotides were designed that contain sequences for the TRE1. The mutated oligomers for each site (See **Figure 3.2** for oligomer sequences) are identical to the mutations introduced into the PDK4-luc by site directed mutagenesis. His-tagged TR β and RXR proteins were used to determine the ability of binding the TR β -RXR complex to bind to either the wild type or the mutated oligos (Bahouth et al., 1997; Park et al., 1997). The protein-DNA binding mixtures contained labeled probe (30,000 cpm) and the purified protein in 80 mM KCl, 25 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol and poly deoxyinosine-deoxycytidine; which is a nonspecific competitor. 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). The electrophoresis was carried out at 180 volts for 80 min at 4 °C.

3.2.5 Chromatin immunoprecipitation (ChIP) assay

The ChIP assays were performed as follows. Rat primary hepatocytes were prepared extracted from rat liver tissues by collagenase perfusion as previously described (Deng et al., 2002). Rat primary hepatocytes (3×10^6 in 60-mm dishes) were maintained for 12 h in priming media of RPMI 1640 and 10% fetal bovine serum. Following two washes with phosphate-buffered saline, the medium was replaced by RPMI 1640 medium containing no serum. Then cells were treated with 100 nM T3 for 24 h. Cross-linking was performed with 1% formaldehyde for 15 min at room temperature and stopped by adding 125 mM glycine to each plate for 5 min. Cross-linked hepatocytes were washed twice with ice-cold phosphate-buffered saline. Cells were scraped from the plate and collected by centrifuging for 5 min at 2,000 rpm. ChIP assays were conducted with slight modifications following the protocol given by the Millipore Magna

ChIP kit (#17-610). The cell pellets were resuspended in 200 μ l of cell lysis buffer, containing protease inhibitor mixtures (P8340, Sigma). Samples were incubated on ice for 15 min and vortexed once, followed by homogenization using a Dounce homogenizer. The homogenate was pelleted, resuspended in nuclear lysis buffer, and sonicated to 400-600bp 9 x 30 seconds in ice water, followed by centrifugation at 14,000 rpm for 10 min. Chromatin preparations were diluted with dilution buffer and protease inhibitor cocktail and designated as input samples (no antibody), used for immunoprecipitation with the desired antibody, or with the control antibody rabbit IgG (Santa Cruz # sc-2027). Antibodies used were anti-TR β (sc-67122, Santa Cruz Biotechnology), anti-PGC-1 α (sc-13067, Santa Cruz Biotechnology.), or anti-C/EBP β (sc-150, Santa Cruz Biotechnology). Samples were left rotating overnight at 4° with the antibody and the A magnetic beads. The magnetic beads were pelleted and washed with a low-salt immune complex wash buffer, high-salt immune complex wash buffer, lithium-chloride immune complex wash buffer, and TE buffer. DNA was eluted using the ChIP elution buffer and treated with proteinase K for 4 hours at 65°C followed by 10 minutes at 95 °C. DNA was purified using the Qiaquick PCR purification kit (Qiagen, #28104).

A total of 3–6 μ l of purified sample was used in 35 cycles of PCR. Primers for regions of the target genes are listed in **Table 3.2**. The PCR products were analyzed on 2% Nusieve 3:1 agarose (Cambrex, 50094) and visualized with MultiImage Light Cabinet with Alpha Imager EP software. The relative intensity of the bands was determined using AlphaView version 1.2.0.1 (Alpha Innotech Corporation).

3.2.6 Real-time PCR

For real time PCR, cDNA was prepared using RNA isolated from primary rat hepatocytes. The RNA was isolated with RNA-Stat-60 (Tel-test) (Zhang et al., 2006). The RNA was then treated with DNase I (2 units) at 37 °C for 1 h followed by addition of DNase Inactivation Reagent (Ambion). The concentration of each sample containing DNA free RNA was measured using Nano drop machine (Thermo scientific). Equal amounts of DNA-free RNA were used for first-strand cDNA synthesis. Two μ g of DNA-free RNA extracted from different cells was converted to cDNA using Superscript reverse transcriptase III and random hexamers (Invitrogen). The parameters for real time PCR were as follows: 95 °C for 10 min, 40 cycles of 95 °C 30 s and 60 °C 1 min. The final concentration of primers in each well in the PCR plates was 0.1 μ M. . Four μ l of 1:5 of each cDNA was used as a template to assess target genes and the normalize control 18S gene. The following forward (FP) and reverse primers (RP) were used for real time PCR to quantitate mRNA abundance: PDK4 forward primer, ggattactgaccgcctcttagt; PDK4 reverse primer, gcattccgtgaattgtccatc; PGC-1 α forward primer, atgaatgcagcggctcttagc; PGC-1 α reverse primer, aacaatggcagggtttgttc; CPT-1a forward primer, cgggtcaagaatggcatcatc; CPT-1a reverse primer, tcacaccaccaccacgat; 18S forward primer, cggctaccacatccaaggaa; 18S reverse primer, ttttcgtcactacctcccg. While the primers for both the C/EBP β and the PEPCK were obtained from Qiagen (cat # QT00366478 and QT01619975, respectively).

Table 3.2: Sequences of the primers used in the ChIP assay to demonstrate interactions of TR β , PGC-1 α and C/EBP β with the PDK4 gene.

Regions of PDK4 gene amplified	Forward primer (FP)	Reverse primer (RP)
rPDK4, -591/-338 (proximal)	TAAGGCTATTTAGGCAGTTT	CCAGACTTGTCTTGTTTAC
rPDK4, -1535/-1228 (TRE site)	AGTGTCTCCACCAGATTGT	CTAAGAGAGCTAACCTAGT
rPDK4, -6634/-6377 (upstream)	TATGAGAAGTGCTGCAATAA	CCAGACTTGTCTTGTTTAC

The results are relative quantification relates the PCR signal of the target transcript in treated cells to that of the untreated control cells using the delta delta CT method as described previously (Livak & Schmittgen, 2001).

3.2.7 Western blot

Western analysis was performed on whole cell extracts from non-treated primary rat hepatocytes or treated with 100nM of T3 for 24 hours. Cells were lysed in RIPA buffer (20 mM Tris-Cl, pH 7.4, 150 mM NaCl, 0.5% deoxycholate, 5 mM EDTA pH 8.0, 0.1% SDS, and diluted protease inhibitor mixture). The cells were vortexed for 1 minute. The cell membranes was removed by centrifugation for 25 min at 4 °C. An equal amount of protein was loaded onto a 12% SDS-PAGE gel and transferred to a 0.45-μm pure nitrocellulose membrane (Bio-Rad). Blots were immunoblotted with primary antibodies (anti-PGC-1α, anti-C/EBPβ and anti-PDK4) in phosphate-buffered saline containing 5% nonfat dry milk powder and were incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody. Immunoreactive proteins were identified using Super Signal West Femto Chemiluminescence Substrate (Pierce). The ChemiDoc™ XRS gel documentation system (Bio-Rad) was used to quantify the immunoreactive proteins. Actin was used as the loading control for each lane.

3.2.8 Adenoviral infection

Purified adenoviruses encoding shRNA specific for the PGC-1α (Ad-siPGC-1α) and control adenoviruses encoding non-template shRNA (Ad-NC) were amplified by Viraquest incorporation. The purified adenoviruses encoding shRNA designed for C/EBPβ targeting its 3'-untranslated region (5'-CCGGGCCCTGAGTAATCAC-3') were obtained from Dr. Jacob E. Friedman from University of Colorado, together with its control adenoviruses encoding non-template shRNA (Ad-control). Primary rat hepatocytes were plated at a density of 3×10^6 in a 60-mm dish in RPMI 1640 media (3 plates for each condition). Five hours after plating the cells, media was aspirated and 2 ml of purified adenoviruses was added to the cells in the presence of 8ug/ml polybrene. The media was changed 24 hours post transduction and the cells were treated with 100nM T3 for another 24 hours in serum free RPMI 1640. The hepatocytes were harvested and RNA was isolated using RNA Stat 60 and converted to cDNA after DNase treatment and real-time PCR was performed according to the instructions stated under the real-time PCR section (see section 3.2.6).

3.2.9 Superarray® real time PCR

To investigate the role of T3 in the expression of different transcription factors, superarray® real time PCR (SABioscience) was performed. The superarray is a custom designed real time plate, containing primers for 90 transcription factors and 6 controls, complete list of the genes is provided in **Table 3.3**. RNA was extracted from non-treated

rat primary hepatocytes and hepatocytes treated with T3 100nM for 24 hours as mentioned in section **3.2.6 Real-time PCR**. The extracted RNA was converted to cDNA using first strand synthesis kit from SABioscience, the produced cDNA was then assessed on the superarray according to the manufacturer procedures using the Roche Light cycler 480 machine. The parameters for real time PCR were as follows: 95 °C for 10 min, 40 cycles of 95 °C 15 s and 60 °C 1 min.

Table 3.3: Complete list of the genes primers on the superarray real time plate.

Gene name	Ref seq	Gene symbol
1. Acyl-CoA synthetase long-chain family member 1	NM_012820	Acs1 1
2. Carnitine palmitoyltransferase 1a, liver	NM_031559	Cpt1a
3. Carnitine palmitoyltransferase 1b, muscle	NM_013200	Cpt1b
4. Carnitine palmitoyltransferase 1c	XM_001075677	Cpt1c
5. Carnitine acetyltransferase	NM_001004085	Crat
6. Solute carrier family 25 (mitochondrial carnitine/acylcarnitine translocase), member 20	NM_053965	Slc25a20
7. Carnitine palmitoyltransferase 2	NM_012930	Cpt2
8. Acyl-Coenzyme A dehydrogenase, very long chain	NM_012891	Acadv1
9. Acetyl-coenzyme A dehydrogenase, medium chain	NM_016986	Acadm
10. Cytochrome P450, family 2, subfamily e, polypeptide 1	NM_031543	Cyp2e1
11. Malonyl-CoA decarboxylase	NM_053477	Mlycd
12. Acetyl-Coenzyme A carboxylase beta	NM_053922	Acacb
13. Hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme, alpha subunit	NM_130826	Hadha
14. Cd36 antigen	NM_031561	Cd36
15. Solute carrier family 27 (fatty acid transporter), member 1	NM_053580.2	Slc27a1
16. Solute carrier family 27 (fatty acid transporter), member 2	NM_031736.1	Slc27a2
17. Solute carrier family 27 (fatty acid transporter), member 4	XM_231115	Slc27a4
18. Solute carrier family 27 (fatty acid transporter), member 5	NM_024143	Slc27a5
19. Solute carrier family 2 (facilitated glucose transporter), member 2	NM_012879	Slc2a2
20. Solute carrier family 2 (facilitated glucose transporter), member 4	NM_012751	Slc2a4
21. Hexokinase 2	NM_012735	Hk2
22. Phosphoenolpyruvate carboxykinase 1	NM_198780	Pck1
23. Glucose-6-phosphatase, catalytic	NM_013098	G6pc

Table 3.3: (continued).

Gene name	Ref seq	Gene symbol
24. Pyruvate dehydrogenase kinase, isoenzyme 2	NM_030872	Pdk2
25. Pyruvate dehydrogenase kinase, isoenzyme 4	NM_053551	Pdk4
26. Acetyl-coenzyme A acetyltransferase 1	NM_017075	Acat1
27. 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2	NM_173094	Hmgcs2
28. 3-hydroxy-3-methylglutaryl-Coenzyme A lyase	NM_024386	Hmgcl
29. 3-hydroxybutyrate dehydrogenase, type 1	NM_053995	Bdh1
30. 3-hydroxybutyrate dehydrogenase, type 2 (predicted)	XM_215705	Bdh2
31. Stearoyl-Coenzyme A desaturase 1	NM_139192	Scd1
32. Fatty acid desaturase 2	NM_031344	Fads2
33. Acyl-Coenzyme A oxidase 1, palmitoyl	NM_017340	Acox1
34. Acyl-Coenzyme A oxidase 3, pristanoyl	NM_053339	Acox3
35. Hydroxysteroid (17-beta) dehydrogenase 4	NM_024392	Hsd17b4
36. Acetyl-Coenzyme A acyltransferase 1	NM_012489	Acaa1
37. Alpha-methylacyl-CoA racemase	NM_012816	Amacr
38. 2-4-dienoyl-Coenzyme A reductase 2, peroxisomal	NM_171996	Decr2
39. Acyl-CoA thioesterase 8	NM_130756	Acot8
40. Phytanoyl-CoA hydroxylase	NM_053674	Phyh
41. ATP-binding cassette, sub-family D (ALD), member 1 (predicted)	XM_343840	Abcd1
42. Carnitine O-octanoyltransferase	NM_031987	Crot
43. ATP-binding cassette, sub-family D (ALD), member 2	NM_033352	Abcd2
44. ATP-binding cassette, sub-family D (ALD), member 3	NM_012804	Abcd3
45. Nuclear receptor subfamily 0, group B, member 2	NM_057133	Nr0b2
46. Apolipoprotein A-IV	NM_012737	Apoa4
47. Peroxisomal biogenesis factor 11A	NM_053487	Pex11a
48. Acyl-CoA thioesterase 12	NM_130747	Acot12
49. Fatty acid synthase	NM_017332	Fasn
50. Acetyl-coenzyme A carboxylase alpha	NM_022193	Acaca

Table 3.3: (continued).

Gene name	Ref seq	Gene symbol
51. Protein phosphatase 2C, magnesium dependent, catalytic subunit	NM_019372	Ppm2c
52. Pyruvate dehydrogenase phosphatase isoenzyme 2	NM_145091	Pdp2
53. Fatty acid binding protein 1, liver	NM_012556	Fabp1
54. Fatty acid binding protein 3	NM_024162	Fabp3/SHP
55. Insulin receptor substrate 2	XM_573948	Irs2
56. Thyroid hormone receptor alpha	NM_001017960	Thra
57. Thyroid hormone receptor beta	NM_012672	Thrb
58. Peroxisome proliferator activated receptor alpha	NM_013196	Ppara
59. Peroxisome proliferator activated receptor delta	NM_013141	Ppard
60. Peroxisome proliferator activated receptor gamma	NM_013124	Pparg
61. Estrogen related receptor, alpha	NM_001008511	Esrra
62. Estrogen-related receptor gamma	NM_203336	Esrrg
63. CCAAT/enhancer binding protein (C/EBP), alpha	NM_012524	Cebpa
64. CCAAT/enhancer binding protein (C/EBP), beta	NM_024125	Cebpb
65. CCAAT/enhancer binding protein (C/EBP), beta	NM_024125	Cebpd
66. Sirtuin 1 ((silent mating type information regulation 2, homolog) 1	XM_228146	Sirt1
67. Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha	NM_031347	Ppargc1a
68. Peroxisome proliferative activated receptor, gamma, coactivator 1 beta	NM_176075	Ppargc1b
69. Forkhead box O1A	XM_342244	Foxo1a
70. Forkhead box O3a (predicted)	XM_215421	Foxo3a
71. Forkhead box A2	NM_012743	HNF3b/FoxA2
72. Lipin 1	NM_001012111	Lpin1
73. Lipin 2 (predicted)	XM_237521	Lpin2
74. Sterol regulatory element binding factor 1	XM_213329	Srebf1
75. Nuclear respiratory factor 1 (predicted)	XM_231566	Nrf1
76. GA repeat binding protein, beta 1	XM_344606	Gabpb1
77. Similar to myocyte enhancer factor 2C	NM_001014035	LOC309957

Table 3.3: (continued).

Gene name	Ref seq	Gene symbol
78. Nuclear receptor subfamily 2, group F, member 2	NM_080778	Nr2f2
79. Uncoupling protein 2 (mitochondrial, proton carrier)	NM_019354	Ucp2
80. Uncoupling protein 3 (mitochondrial, proton carrier)	NM_013167	Ucp3
81. Diacylglycerol O-acyltransferase 1	NM_053437	Dgat1
82. Diacylglycerol O-acyltransferase homolog 2 (mouse)	NM_001012345	Dgat2
83. Superoxide dismutase 1	NM_017050	Sod1
84. Superoxide dismutase 2, mitochondrial	NM_017051	Sod2
85. Cytochrome c oxidase subunit IV isoform 1	NM_017202	Cox4i1
86. Citrate synthase	NM_130755	Cs
87. Deiodinase, iodothyronine, type II	NM_031720	Dio2
88. NK2 transcription factor related, locus 5 (Drosophila)	NM_053651	Nkx2-5
89. Actin, beta	NM_031144	ActB
90. Ribosomal protein L13A	NM_173340	RPL13A
91. Glyceraldehyde-3-phosphate dehydrogenase	NM_017008	GAPDH
92. Genomic DNA Contamination control		
93. Reverse Transcriptase control		
94. Positive PCR control		
95. Positive PCR control		

CHAPTER 4: DISCUSSION

Both thyroid hormone (T3) and the transcriptional coregulators (transcriptional factors & coactivators) play an important role in regulating the transcription of genes involved in the glucose and fatty acid metabolism. In this study, I focused on the role of the thyroid hormone receptor beta (TR β), proliferator-activated receptor gamma coactivator-1 alpha (PGC-1 α) and the CCAAT/enhancer binding protein beta (C/EBP β) in the regulation of pyruvate dehydrogenase kinase 4 (PDK4) by thyroid hormone.

Pyruvate dehydrogenase kinases decrease the pyruvate dehydrogenase complex (PDC) activity by phosphorylation, thereby inhibiting the conversion of pyruvate to acetyl-CoA. Changes in PDC activity link glucose metabolism to fatty acid metabolism. PDKs facilitate gluconeogenesis by inhibiting PDC and preserving the three carbon compounds (pyruvate, lactate and alanine) as substrates for gluconeogenesis. At the same time, PDC inhibition (via PDKs) allows the oxidation of fatty acids to be the primary source of energy. Elevated gluconeogenesis and disturbance in fatty acid metabolism are observed in metabolic diseases as diabetes, obesity and hyperthyroidism (Basu et al., 2005; Baxter et al., 2004; Chevalier et al., 2006; Crunkhorn & Patti, 2008; Kabir et al., 2005; Wensaas et al., 2009). My studies that focused on the mechanism by which thyroid hormone regulates the transcription of the PDK4 gene and the role of the transcriptional coregulators in this regulation, have shed light on the role of thyroid hormone in both the glucose and fatty acid metabolism. In addition, I have provided a broad picture of the network of transcription factors and coactivators involved in the hepatic regulation of T3 responsive metabolic genes.

4.1 Regulation of the PDK4 gene expression

4.1.1 The role of the thyroid hormone in the PDK4 gene expression

T3 is a prominent regulator of the PDK4 gene expression and activity in heart, liver and skeletal muscles (Holness et al., 2003; Sugden et al., 2000a; Sugden et al., 2000b). The transient transfection experiments done in this study showed that indeed T3 can induce PDK4 gene expression *in vitro* in hepatoma HepG2 cells and *in vivo* in hyperthyroid rats. This stimulation was mediated through a TRE site found in the PDK4 gene promoter designated as (agggcacccttgggtaa) which resembles the direct repeat separated by 4 nucleotides (DR4) type of TRE (Williams & Brent, 1995). We were the first to identify a TRE site within the rPDK4 promoter indicating that T3 can activate the PDK4 gene expression directly and not through an intermediate factor. However, the identification of a TRE does not rule out a role of intermediate factors in the activation of the T3 induction. Therefore, I studied the role PGC-1 α and C/EBP β as two potential intermediate factors in this induction.

4.1.2 The role of the PGC-1 α in the induction of the PDK4 by thyroid hormone

Previous studies showed that there are links between the T3 metabolic actions and PGC-1 α . First, PGC-1 α coactivated the thyroid receptors (Puigserver et al., 1998; Wu et al., 2002). PGC-1 α can also influence thyroid hormone action by altering RNA processing of TR α transcripts, thus modulating the ratio of TR α_1 and TR α_2 isoforms which is accompanied by a decrease in 5'-deiodinase expression (Thijssen-Timmer et al., 2006). Second, Weitzel et al reported that the hepatic PGC-1 α mRNA expression is induced by T3 treatment (Weitzel et al., 2001). Third, the enhancement of the induction of carnitine palmitoyltransferase 1a (CPT-1a) by thyroid hormone in the presence of PGC-1 α was reported in previous studies (Zhang et al., 2004b). Finally, a recent review by Crunkhorn et al emphasized the potential linkage between PGC-1 coactivators and thyroid hormone action on the mitochondrial oxidation pathway (Crunkhorn & Patti, 2008).

In agreement with the proposed role of the PGC-1 α in the T3 action, my experiments showed an association between the PGC-1 α and both the proximal and the TRE site in the PDK4 promoter *in vivo* indicated by ChIP technique. Previous studies had reported that PGC-1 α stimulated PDK4 (Wende et al., 2005). This association was increased significantly by thyroid hormone. Moreover, T3 treatment induced the mRNA and the protein level of both PDK4 and PGC-1 α . In addition, overexpression of PGC-1 α showed significant synergistic effect on the T3 induction of PDK4 gene expression. However, this synergistic effect was not abolished by mutating either the ERR α or the FOXO1 binding sites, indicating that PGC-1 α is involved in the PDK4 regulation not only via the ERR α action but it can also exert its action through interacting with the TRs (Puigserver et al., 1998; Wu et al., 2002).

Adenoviral-mediated silencing of the PGC-1 α was utilized to investigate the role of the PGC-1 α in the induction of PDK4 and other metabolic genes by T3. This experiment showed that silencing PGC-1 α abolished the ability of T3 to induce PDK4, CPT-1a, PEPCK and C/EBP β gene expression indicating that PGC-1 α is an important coactivator for the T3 induction of these genes in primary hepatocytes. This experiment contrasts with a recent study conducted by Wetzel's group, as they reported that silencing PGC-1 α had no effect on the induction of gene expression by T3 in rat pituitary (GC) cells (Wulf et al., 2007). In contrast with our studies, they measured the T3 effect on two other genes, glycerol-3-phosphate dehydrogenase (GPDH) and adenine nucleotide translocator 2 (ANT2). More importantly, they used GC pituitary cells, which are subclone of the rat GH₃ pituitary tumor cell line and possess a different gene expression pattern than that of primary hepatocytes. The proliferation of GC cells are highly induced by T3 treatment (Barrera-Hernandez et al., 1999; Kitagawa et al., 1987; Laverriere et al., 1986; Miller et al., 2001; Nass et al., 2000). On the other hand, T3 has no effect on the proliferation of hepatocytes *in vitro* (Kariv et al., 2003). All these data suggest that the T3 regulation in pituitary GC cells is likely different than that for primary hepatocytes. It is also noteworthy that PGC-1 α is underexpressed in immortalized tumor cell lines as it was reported in human hepatoma cells (Martinez-Jimenez et al., 2006). In addition, The role of PGC-1 α in the T3 mediated gene expression in heart is still controversial. Two studies

had indicated that PGC-1 is potentially involved in mediating myocardial-specific remodeling of mitochondria in response to thyroid hormone treatment (Goldenthal et al., 2004; McClure et al., 2005). However, two other studies had ruled out a role for the PGC-1 α in the cardiac mitochondrial adaptations by T3 (Athea et al., 2007; Irrcher et al., 2003), although, Irrcher et al stated that PGC-1 α is still involved in the mitochondrial adaptation by T3 treatment in liver and skeletal muscles indicating that PGC-1 α involvement in the T3 mediated gene expression is potentially tissue specific (Irrcher et al., 2003).

On the other hand, number of previous studies support the results from the adenoviral-mediated silencing of PGC-1 α experiment. One of these studies was conducted by the Spiegelman's group as they reported that C/EBP β was abnormally activated in the fed state in the PGC-1 α null mice (Lin et al., 2004). Normally, fasting not feeding induced C/EBP β in wild type mice (Lin et al., 2004). C/EBP δ was also induced in the fed liver lacking PGC-1 α , while C/EBP α is expressed normally with respect to PGC-1 α genotypes indicating that silencing PGC-1 α alters expression of specific C/EBP isoforms. The aberrant induction of C/EBP β and C/EBP δ , however, is absent when PGC-1 α -deficient hepatocytes are grown in cell culture, suggesting that systemic signals are likely responsible for their increased expression (Lin et al., 2004). Although, this study had linked the PGC-1 α to the C/EBP β gene induction by another physiological process which is feeding or fasting status and not T3 action, however, it supports our results that PGC-1 α is an important regulator of C/EBP β gene expression. Also, as supportive evidence Crunkhorn and Patti had stated in their review, that their unpublished data indicate that siRNA targeting PGC-1 α or PGC-1 β decreased the mRNA of TR α_1 and mitochondrial genes in skeletal muscle (Crunkhorn & Patti, 2008).

Based on the transient transfection, ChIP, and adenovirus mediating knockdown experiments done in this dissertation, we can model the mechanism by which PGC-1 α coactivate the induction of PDK4 gene expression by T3 (**Figure 4.1**). In this model, T3 increases the abundance of the PGC-1 α so that more PGC-1 α will be available to stimulate the PDK4 gene. PGC-1 α can induce the PDK4 gene expression through the interaction with the ERR α at the proximal part of the PDK4 promoter (Wende et al., 2005; Zhang et al., 2006). Also, PGC-1 α can synergistically coactivate the T3 induction of the PDK4 gene by interacting with the TR and RXR at the TRE site of the PDK4 promoter, even after disrupting the ERR α binding sites.

4.1.3 The role of the C/EBP β in the induction of the PDK4 by thyroid hormone

Although, our lab and others had reported that C/EBP α is involved in the T3 induction of number of genes (Jackson-Hayes et al., 2003; Jurado et al., 2002; Park et al., 1997; Yin et al., 2005), to our knowledge only two studies had linked the C/EBP β to T3 action. The first study was done by Menendez-Hurtado, in which he reported that both C/EBP α and C/EBP β gene expression decreased in liver of congenital hypothyroid rats during development (Menendez-Hurtado et al., 1997). Upon injection of those hypothyroid animals with thyroid hormone, there was an increase of hepatic C/EBP α and

Figure 4.1: PGC-1 α is a coactivator in the T3 induction of the PDK4 gene expression.

T3 activates the PDK4 gene expression directly through a TRE site and indirectly by stimulating the expression of the PGC-1 α gene expression. Also, T3 increases the association between PGC-1 α and the PDK4 promoter.

C/EBP β mRNA and protein levels (Menendez-Hurtado et al., 1997). The second study was carried out by our lab, and we reported that C/EBP β overexpression enhanced the T3 induction of PEPCK (Park et al., 1999).

In this study we are the first to report that overexpression of the C/EBP β in transient transfections induced the PDK4 promoter. In addition, C/EBP β enhanced the T3 induction of PDK4 gene expression. Also, T3 treatment induced the C/EBP β gene expression in primary hepatocytes. Moreover, adenoviral-mediated silencing of C/EBP β experiments demonstrated that C/EBP β is required for full induction of PDK4 mRNA by T3, as knocking down the C/EBP β reduced the ability of T3 to induce PDK4 gene expression. The Ad-siC/EBP β did not reduce significantly the basal levels of PDK4 mRNA levels. These results are similar to studies done by the Friedman's group, where they reported that C/EBP β is not essential for maintenance of the basal PEPCK mRNA levels in C/EBP β knockout mice (Arizmendi et al., 1999). However, C/EBP β deletion limited the full induction of PEPCK and glucose 6-phosphatase genes in streptozotocin induced diabetic mice (Arizmendi et al., 1999). Although this study had linked C/EBP β as essential factor for metabolic and gene regulatory responses to diabetes, it did not connect C/EBP β to T3 action. However, C/EBP β does modulate gluconeogenesis, a process regulated by both PDK4 gene and T3 (Fowden et al., 2001; Park et al., 1997; Sparks et al., 2006; Sugden & Holness, 2002). It is also noteworthy that C/EBP β participates in the regulation of the PEPCK gene in response to different stimulus like cAMP, glucocorticoids and insulin (Croniger et al., 2001; Duong et al., 2002; Yamada et al., 1999).

4.2 Transcription factors and metabolic genes involved in the thyroid hormone network in primary hepatocytes

Previous studies had showed that T3 regulates different aspects of both glucose and fatty acid metabolism. T3 stimulates gluconeogenesis, cellular glucose uptake and glycogenolysis (Crunkhorn & Patti, 2008; Fowden et al., 2001; Johnson, 2006; Park et al., 1997). In addition, T3 stimulates triglyceride production, lipogenesis, lipolysis and fatty acid oxidation (Feng et al., 2000; Moreno et al., 2008; Ness & Zhao, 1994; Pykalisto et al., 1976; Ribeiro, 2008; Wahrenberg et al., 1994; Zhang et al., 2004b).

T3 exerts all these metabolic effects through inducing a network of transcription coregulators and metabolic genes (Weitzel et al., 2003a; Weitzel et al., 2003b; Weitzel et al., 2001). In this dissertation I used superarray® real time PCR arrays to identify selected metabolic genes and transcription coregulators that are regulated by 24 hours of T3 treatment in primary hepatocytes. Although, a similar experiment had been performed by the Weitzel's group, this experiment was done using a broad microarray technique unlike my experimental approach which focused on transcription coregulators and metabolic genes involved in glucose and fatty acid metabolism (Weitzel et al., 2003a). Also, microarray sensitivity is different than real-time PCR sensitivity (Kubista et al., 2006). Finally Weitzel's experiment was done using RNA extracted from T3-treated

hypothyroid rats and outcomes from these rats can be different than that done using RNA extracted from cultured T3-treated primary hepatocytes from normal rats.

Indeed out of the 27 genes upregulated by T3 after 24 hours of treatment that I have reported in this study, only 4 genes, the two transcription coactivators PGC-1 α and PGC-1 β , Cytochrome P450 which is involved in both fatty acid oxidation and gluconeogenesis (Lieber, 1999), and fatty acid binding protein 1 (Fabp1) involved in fatty acid metabolism (Kabir et al., 2005), are in common with that reported by Weitzel (Weitzel et al., 2003a). However, many of the genes detected in my study had been reported by previous studies to be stimulated by T3. In this study we reported the upregulation of some transcription factors like sterol regulatory element binding transcription factor (Srebf1), C/EBP β , peroxisomal proliferator activated receptor delta (PPAR δ). These factors had been reported to be induced by T3 treatment by other studies (de Lange et al., 2007; Menendez-Hurtado et al., 1997; Zhang et al., 2003). On the other hand, to our knowledge we are the first to report the T3 induction of transcription factors lipin gene isoforms 1 and 2, ERR γ , GA binding protein transcription factor, beta subunit 1 (Gabpb1), Sirtuin 1 (Sirt-1) and transcription coactivator homeobox protein NK-2 (NKx2.5). It is important to mention in this aspect that lipin 1 was reported to be an amplifier of the PGC-1 α /PPAR α -mediated control of hepatic lipid metabolism and it exerts its action by direct interacting with both PGC-1 α and PPAR α (Finck et al., 2006). Also, Sirt-1 had been involved in the activation of PGC-1 α by deacetylation (Gerhart-Hines et al., 2007; Markus & Morris, 2008). Thus, upregulation of lipin 1 and Sirt-1 by T3 may be another way to regulate the activity of PGC-1 α .

Also, in this study we reported the T3 induction of genes involved in gluconeogenesis, PEPCK and G6pase and solute carrier family 2 (facilitated glucose transporter), member 4 (Slc2a4). All of them had been previously reported to be T3 responsive genes (Fowden et al., 2001; Park et al., 1997; Torrance et al., 1997). For genes involved in fatty acid oxidation and synthesis, we are the first to report the T3 induction of 3-ketoacyl-Coenzyme A thiolase alpha subunit (Hadha), fatty acid binding protein 3/small heterodimer partner (Fabp3/SHP), solute carrier family 25 carnitine/acylcarnitine translocase member 20 (Slc25a20), solute carrier family 27 fatty acid transporter member 5 (Slc27a5) and acetyl-Coenzyme A carboxylase beta (Acacb). In addition, we reported the upregulation of carnitine palmitoyltransferase 1a (CPT-1a) and fatty acid synthase (FAS) by T3 treatment, which had been previously documented (Xiong et al., 1998; Zhang et al., 2004b).

Finally, using this superarray technique we reported the T3 induction of miscellaneous genes. First, deiodinase type II (Dio2) which is responsible for generation of T3 from T4 predominantly in brain, pituitary, and brown adipose tissue in mammals (Yen, 2001) and we detected its hepatic upregulation in response to T3 treatment. It was recently reported that Dio2 mRNA was detectable by real time PCR in livers of cows and in the livers of liver X receptors null mice (Capuco et al., 2008; Kalaany et al., 2005). Second, we confirmed PDK4 (gene of interest of this dissertation) upregulation by T3 treatment. At last, uncoupling proteins 2 and 3 (UCP2 and UCP3) was also found to be T3 responsive genes. UCP3 was previously reported to be induced by T3 treatment (de

Lange et al., 2007), however to our knowledge we are the first to report upregulationn of UCP2 by T3 treatment.

Thus, this part of the dissertation has pinpointed the network of transcription coregulators and metabolic genes involved in T3 hepatic action. Further studies using knockout mice or mice injected using virus mediating silencing of these genes can indicate its importance in the T3 mediated hepatic gene expression.

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APPENDIX: SUPPLEMENTAL DATA FOR CHAPTER 4

PGC-1 α is involved in the activation of different metabolic genes by thyroid hormone. To further investigate the role of the PGC-1 α in the T3 activation of other metabolic genes, we examined whether we could observe increased association of PGC-1 α to PDK4, CPT-1a and PEPCK in hypothyroid and hyperthyroid rats. Rats were made hypothyroid by the administration of propylthiouracil (PTU) and then they were treated with 100nM of T3 for 24 hours. Pieces of the liver were minced in 1% formaldehyde and then used in ChIP assays. Again we detected PGC-1 α in the proximal region of the PDK4 gene promoter, and this association was increased by 5.9 ± 0.5 fold after T3 treatment. However, we did not observe any association of PGC-1 α with the TRE region of the PDK4 gene promoter. In addition, we observed increased association of the PGC-1 α both the first intron and the TRE region of the CPT-1a gene promoter after T3 treatment by 6.4 ± 0.8 fold and 2.2 ± 0.5 fold, respectively. Also, increased association of the PGC-1 α with proximal region of the PEPCK gene promoter by 3.6 ± 0.8 fold was detected after T3 treatment (**Figure A.1A and A.1B**).

T3 not only increased the association of the PGC-1 α with the above genes promoters, but it also increased the abundance of the PGC-1 α protein itself in these hyperthyroid rats by 2.8 ± 0.3 fold (**Figure A.1C**). The increased abundance and association of the PGC-1 α with different metabolic genes after T3 treatment suggests that T3 activation can work through the PGC-1 α in the activation PDK4 and other metabolic genes.

Figure A.1: PGC-1 α association with T3 responsive genes is elevated in hyperthyroid rats.

A. ChIP assays were conducted in liver samples from hypothyroid rats that were untreated or exposed to T3 for 24 hours. The ChIP assays were conducted as outlined in the materials and methods. Various portions of the PDK4, CPT-1a and PEPCK genes were analyzed for the binding of PGC-1 α . Representative PCR reactions are shown. B. The binding of PGC-1 α to the various genes was quantified using quantity one software. The results are the summary of four independent hypothyroid and T3 rats. C. The abundance of PGC-1 α was quantified using western blot analysis. The PGC-1 α abundance was assessed using protein extracts from the same rats that were used for the ChIP assays.

A.

B.

C.

Figure A.1: (continued).

VITA

Ramy Raafat Naguib Attia was born in Cairo, Egypt, in the year 1978. He received his bachelor degree in Pharmacy from Cairo University in 2001. He worked in EVA Pharmaceutical Incorporation in Egypt for the research and development department till May 2004. He joined the Ph.D. integrated program of biomedical science at University of Tennessee Health Science Center (UTHSC) in August 2004. During his doctoral study, Ramy Naguib Attia received two travel awards from ASBMB to attend the FASEB meeting in 2008 and 2009.