Regulation of Apolipoprotein C-III Gene Expression by Nuclear Receptors Hepatocyte Nuclear Factor 4 Alpha and Chicken Ovalbumin Upstream Promoter Transcription Factor II, but not Retinoids in Hepatic Cells

Meredith Lee Howell
mhowell14@utk.edu

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Guoxun Chen, Major Professor

We have read this thesis and recommend its acceptance:

Jay Whelan, Ling Zhao

Accepted for the Council:

Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)
Regulation of Apolipoprotein C-III Gene Expression by Nuclear Receptors Hepatocyte Nuclear Factor 4 Alpha and Chicken Ovalbumin Upstream Promoter Transcription Factor II, but not Retinoids in Hepatic Cells

A Thesis Presented for the Master of Science Degree
The University of Tennessee, Knoxville

Meredith Lee Howell
August 2013
DEDICATION

To my mother, Nancy Hanks Howell, whose unconditional love and support has been the driving force for me to see this thesis to fruition

To my brother, Evan James Howell, who I consider my very best friend in life and a source of great joy and humor throughout my graduate school experience

And to my grandparents, DeWitt Sadler Hanks and Christine Prickett Hanks, whose loving memory is a source of inspiration and blessing in my life
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ABSTRACT
Retinoic acid (RA) treatment induces hyperlipidemia in humans and animals. RA regulates the expression levels of various genes through the induction, repression, or coactivation of nuclear receptors, mediating its effects. RA-induced hyperlipidemia has been attributed to the induction of apolipoprotein CIII (gene, Apoc3), which inhibits lipoprotein lipase activity (LPL). We have shown that vitamin A (VA) status and retinoid treatment regulates hepatic lipogenic gene expression, suggesting that the induction of lipogenic genes may also contribute to hyperlipidemia. To test the hypothesis that retinoids may not affect Apoc3 expression, we analyzed the expression levels of Apoc3 mRNA in response to retinoid treatments or adenovirus-mediated over-expression of nuclear receptors mediating RA responses in primary rat hepatocytes and HL1C rat hepatoma cells using real-time PCR. We report that retinoids did not induce Apoc3 mRNA expression in these cells. The over-expression of hepatocyte nuclear factor 4 alpha (HNF4α [alpha]) or chicken ovalbumin upstream promoter transcription factor 2 (COUP-TFII) significantly induced or inhibited the Apoc3 mRNA level, respectively. Therefore, it is concluded that retinoid treatment could not directly induce Apoc3 mRNA levels in rat hepatocytes. Instead, the hepatic expression of Apoc3 mRNA may be controlled by the expression levels of HNF α and COUP-TFII. Furthermore, the mRNA level of Apoc3 in isolated and cultured hepatocytes of Zucker Lean (ZL) and Zucker Fatty (ZF) was not significantly changed. However, the Rarb and Srebp-1c mRNA levels, two RA-responsive genes, are significantly higher in the liver tissue and isolated hepatocytes from ZF rats than
that from ZL rats. Therefore, we conclude that RA-induced hyperlipidemia may not be attributed to the direct induction of Apoc3 mRNA level in hepatocytes by RA, but at least in part to the RA-mediated induction of lipogenic genes such as Srebp1-c.
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CHAPTER I

INTRODUCTION
The World Health Organization (WHO) estimates that 1 billion individuals worldwide are diagnosed as overweight or obese based on mean body mass index. The current prevalence of overweight and obesity in the United States is 68%, relative to approximately 47% in the 1970s (1). Often times, this increase in body weight is associated with an increase in blood triglycerides, defined as hyperlipidemia. This condition acts as a severe risk factor for co-morbidities associated with obesity such as coronary artery disease (CAD) and diabetes mellitus (DM). The fundamental causes of this obesity epidemic can be summarized by the increased availability of calorically dense foods and a simultaneous decrease in physical activity. However, the pathogenesis of obesity and associated co-morbidities is multi-factorial and the environmental changes that have contributed to the increased prevalence of obesity must be addressed. Despite the obvious link between nutrition and metabolic diseases, the roles of individual micronutrients in the development of obesity and diabetes have not been actively investigated.

Vitamin A (VA, retinol) and molecules with similar physiological functions (retinoids) comprise a group of critical and physiologically active molecules for vision, normal embryonic development, immune function, and cellular differentiation (2). Accordingly, it has invited therapeutic potentials in dermatology and cancer treatments, which have enveloped much of retinoid research historically. Even among the first clinical studies (3)(4)(5) on patients treated with isotretinoin (13 cis-RA), alterations in lipid metabolism were noted and evoked questions on the VA’s roles in lipid metabolism. More recently, retinoids have been proposed to play roles in energy homeostasis such as adaptive thermogenesis and adipogenesis (6). This comes as our
understanding of ligand-receptor interactions for VA metabolites is cultivated which is consistent with clinical observations using VA derivatives therapeutically. With the development of structural analogs and antagonists for the activation of nuclear receptors mediating retinoid effects, the physiological consequences of VA deficiency and toxicity have shifted our understanding of VA’s role in the development of metabolic disease.

Obesity and comorbidities are the physiological consequences of a disruption in the regulation of body energy storage, which is associated with profound changes in hepatic glucose and lipid metabolism. These changes are often attributed to the expression levels of hepatic genes involved in glucose and lipid metabolism. VA status and retinoids affect hepatic glucose and fatty acid metabolism directly through the regulation of the expression of genes involved or indirectly via controlling insulin secretion and action (7)(5). One such change that can contribute to elevated plasma triglyceride levels is the induction of Apoc3, which historically has been the culprit of RA-induced hyperlipidemia. In our studies, we have observed the significant changes in Srebp1-c, a transcription factor that activates fatty acid synthesis, in response to VA status. In the current model of RA-induced hyperlipidemia, little consideration has been given to the impact this could have on the development of hyperlipidemia in patients undergoing VA therapy. We hypothesize that Srebp1-c acts as the major contributor to RA-induced hyperlipidemia, based on our original observations (9).

It is imperative to understand the molecular mechanisms that VA employs to regulate hepatic and glucose metabolism, the impact of VA status on the development of obesity and diabetes, as well as the physiological effects of retinoids on hormonal
action leading to insulin resistance. This understanding may provide a novel therapeutic
technique for controlling abnormal hepatic glucose and lipid metabolism, and thus
alleviating diabetic and obese phenotypes.
CHAPTER II

LITERATURE REVIEW
2.1. Overview of VA

2.1.1 Vitamin A Structure and Function

VA is a fat-soluble vitamin that exists in multiple isomeric forms and promotes the maintenance and formation of various body tissues. The retinoids designation collectively refers to a family of molecules structurally similar containing a 20 carbon structure with a methyl substituted cyclohexenyl ring (beta-ionone ring) and a tetraene side chain containing diverse molecular arrangements that control their functions (10). The parent molecule, retinol (ROL), contains a hydroxyl group at carbon-15 and has been identified as the least potent form of VA, however, can serve as a precursor to more active forms. The oxidized product of retinol, retinal (RAL) contains an aldehyde group while the biologically active form of VA; retinoic acid (RA) contains a carboxylic acid group. VA can also be esterified for storage with the addition of a fatty acyl group at carbon-15 to generate retinyl ester (RE) (10). Carotenoids, the provitamin form of VA, usually consist of 40 carbon atoms, conjugated double bonds, and may contain one or two cyclic structures at the end of their chain. This lends to their ability to be metabolized to form one or two ROL molecules after processing depending on the enzymatic system used (10).

2.1.2 Dietary Sources of VA

Because retinoids are considered essential micronutrients, meaning they cannot be synthesized de novo, the recommended daily intake and sources are important to discuss. VA is structurally specific to the type of food products containing it, namely animal or plant products. There are a variety of naturally occurring foods rich in VA sources. These foods are typical of the Western diet, suggesting that the majority of North Americans do meet the dietary recommendations for this micronutrient, potentially
in excess. The animal-derived products contain preformed VA in the form of ROL or RE and plant products deliver the provitamin form called carotenoids, which can serve as a precursor for ROL (10). Dietary provitamin A carotenoids such as β-carotene, α-carotene, and β-cryptoxanthin can be identified in the pigments of certain yellow, red, orange, or purple fruits and vegetables and additionally in red palm oil (10). The animal products rich in VA content include liver, milk, cheddar cheese, and egg substitutes. To prevent the consequences of a deficiency or toxicity of VA, the RDA for men (900 µg ROL activity equivalents (RAE)) and women (700 µg RAE) have been established (10). If these recommendations are not met, the consequences can cause systemic disruption of various developmental and metabolic processes.

### 2.1.3 Uptake of Dietary Retinoids

VA’s, in the form of preformed retinoids or provitamin form, initial uptake begins in the lumen of the intestine (6). It is apparent that for optimal retinoid absorption, fat must be consumed to act as a facilitator for incorporation of retinoids into chylomicrons for transport to various tissues (7). To incorporate the retinoid into the chylomicron within the enterocyte, ROL can be taken up directly into the lumen for entry into the enterocyte while VA in the form of RE must be hydrolyzed first to enter the enterocyte. It can be acted upon by a brush-border enzyme hydrolase or a preliminary hydrolase. The preliminary hydrolysis is achieved by a retinyl ester hydrolase (REH), named pancreatic triglyceride lipase (PTL), an observation made in both rat and human studies (7). PTL is also responsible for the hydrolysis of cholesterol esters. Another REH proposed to act in synergy with PTL is pancreatic-lipase related protein 2 (PLPR2) working to optimize
retinoid absorption by catalyzing the hydrolysis of RE. Following hydrolysis, ROL can now be taken up by the enterocyte (7).

Dietary β-carotene must also be enzymatically cleaved in the enterocyte to subsequently be converted back into ROL and then RE. This RE will be incorporated into the nascent chylomicron for transport (6). This provitamin form will enter the enterocyte with the help of scavenger receptor class B, type 1 or SR-B1. SR-B1 also facilitates the transport of cholesterol into the enterocyte (6). The central cleavage of β-carotene by β-carotene monoxygenase type I (Bcmo1) will result in the creation of one to two RAL molecules (7). This aldehyde can then be reduced to ROL by retinal reductases, which currently are not well-characterized (6).

Once in the enterocyte, ROL will be bound to cellular retinol-binding protein II (CRBP-II) and then esterified back to RE which is catalyzed by two different enzymes, lecithin: retinol acyltransferase (LRAT) and intestinal acyl-CoA: retinol acyltransferase, (ARAT) (6, 7). However, approximately 90% of the catalytic activity is attributed to LRAT (7). After esterification, RE will be packed with dietary fat and cholesterol into nascent chylomicrons for entrance into the lymphatic circulation system. This process is illustrated in Figure 2.1 below.
Figure 2.1 Schematic of retinoid uptake within the intestine. (11) Abbreviations:
2.1.4 Retinoid Transport in Circulation

Because VA in its alcohol form, ROL, has the ability to disrupt membrane function, it must be transported through the blood bound to transporters (12). Extracellular transport of these retinoids can be in the form RE, incorporated into chylomicrons in the lymphatic system as previously described, and/or attached to retinol-binding proteins in the bloodstream (RBP) (9). Moreover, ROL can be carried through the serum bound to transthyretin, a carrier of thyroxine (T4) and ROL (13), which is synthesized by hepatocytes to aid in the transport of VA. Also, the liver is the primary site of VA metabolism, which allows the body to meet tissue needs for VA through the release into circulation.

2.1.5 Body Stores

The liver serves as the principle site of VA storage. In fact, in times of VA adequacy, over 90% of stored VA is in the liver as the RE form. It is important to note that whole-body VA status can affect the efficiency of hepatic VA storage. Using radioisotopic methods, it was found that dietary VA retention can be as high as 50% in times of VA homeostasis; this efficient retention can be compromised in individuals of lower VA status (10).

2.1.6 Hepatic Retinoid Metabolism: VA Storage and Conversion to Active Metabolites

The liver acts as the primary site of VA storage and postprandial uptake after uptake and processing by the small intestine (14)(11). ROL and RE can be delivered to the liver via two routes, either associated with lipoproteins via the lymphatic to blood circulation system or bound to a RBP in the bloodstream (11). Entrance of bound ROL
is achieved by receptor-mediated endocytosis mediated by retinoic acid (RA) gene 6 (STRA6) (15). In contrast, the RE-rich lipoprotein (chylomicron remnants) will be associated with early endosomes and undergo hydrolysis via intracellular hydrolases in order to enter the hepatocyte (15). It has been documented that the hydrolysis is facilitated by a number of enzymes classified as retinyl ester hydrolases, carboxylesterases, and lipases (11).

Once hydrolysis is achieved, the ROL has diverse fates (16). It can be transported to a specialized cell in the liver known as hepatic stellate cells (HSC) where it will be re-esterified for storage as RE in the lipid droplets (17). This seems to be a mechanism to prevent VA deficiency by increased storage if VA mobilization is needed in times of deficiency (11). Once in the HSC, the presence of the enzyme lecithin: retinol acyl:CoA transferase (LRAT) will catalyze the esterification of ROL for storage as RE in the lipid droplets of the HSC. The other constituents stored in these droplets include triglycerides, cholesterol, cholesteryl ester, phospholipids, and free fatty acids (18).

In contrast to storage, the ROL available in the liver can be oxidized to RAL and consequently the active form of VA, RA. It is generally accepted that this conversion to the active form and later effects on gene expression will take place in target cells, meaning the liver is not the only site of VA storage but also the site of RA synthesis. However, since RA-responsive nuclear receptors have been identified and expressed in the liver, the hepatic conversion of ROL to its biologically active form is important to discuss. The conversion of ROL to RA is a two-step reaction with the rate-limiting step being the oxidation of ROL to RAL. The second step is the irreversible oxidation of RAL to the active metabolite RA (2). The enzymes involved in the oxidative steps towards
production of RA seem to be tissue-specific and product specific. For example, current literature divides the enzymes into three categories: alcohol dehydrogenases, aldehyde dehydrogenases, and cytochrome P450s (16). Those enzymes facilitating the reversible oxidation/reduction reaction of ROL to RAL are termed dehydrogenases and exhibit properties as an alcohol dehydrogenase (ADH) or a short-chain dehydrogenase reductase (SDR) (19). For the irreversible oxidation of RAL to RA, enzymes are classified in the aldehyde dehydrogenase family (ALDH). This complex network of enzymes consists of 17 different isoforms as a whole, all proposed to exhibit essential properties to retinoid homeostasis confirmed through knock-out and transgenic rodent models (19). This process is illustrated in Figure 2.2 (16).
Figure 2.2 Hepatic Retinoid Metabolism. The oxidation of retinol into retinaldehyde and retinoic acid and diverse fates of retinol in hepatic processing (16).
Last, another important family of enzymes to consider in retinoid homeostasis is the CYP26 enzyme group. *Cyp26a1* (cytochrome P450 hydroxylase 26A) is responsible for modification of RA molecules to facilitate RA disposal. It serves as the primary CYP26 enzyme expressed in the liver, contributing the largest amount of activity in the clearance of RA from humans (14)(20). This gene irreversibly oxidizes RA into more polar metabolites for excretion. Because *Cyp26a1* contains a RA response element (RARE) in its promoter region, it is deemed likely that the transcription of the mRNA of this enzyme will dynamically reflect the levels of RA in the cell and in turn promote oxidation of RA into more polar metabolites (2).

### 2.1.7 Activation of Retinoic Acid Receptor (RAR) and Retinoid X Receptor (RXR)

The biologically active metabolite of VA is RA, which exists in multiple isomeric forms. RA isomers activate two families of nuclear receptors: RA receptors (RARα, β, and γ; activated by all-trans and 9-cis RA) and retinoid X receptors (RXRα, β, and γ; activated by 9-cis RA). RAR/RXR hetero- and RXR/RXR homo-dimers bind to RAREs in the promoters of RA responsive genes and regulate their expression upon activation (21). Physiological consequences of this activation varies depending on receptor moiety and succeeding recruitment of cofactor complexes (22). It has been indicated RXR can act as a universal dimerization partner for other families of nuclear receptors including peroxisome proliferator activated receptors (PPARs), liver X receptor (LXR), farsenoid X receptor (FXR), pregnane X receptor (PXR), thyroid hormone receptor (TR), and vitamin D receptor (VDR) (23). This suggests a complex transcriptional network that allows the bioactive form of VA, RA, to exert its biological activity.
2.2 Vitamin A in Health and Disease

2.2.1 Consequences of VA Deficiency

VA deficiency is caused by a lack of VA availability and stores in the human body. The WHO states approximately 13.8 million children suffer to varying degrees of VA deficiency evidenced by visual losses (24). Signs and symptoms of vitamin A deficiency (VAD) include but are not limited to: xerophthalmia, anorexia, compromised growth, increased vulnerability to infections, augmented development of hair follicles, and keratinization of epithelial cells with associated failure to properly differentiate. VAD can be caused by compromising nutrition factors including iron deficiency and chronic alcohol consumption. Because ROL and ethanol are metabolized in similar pathways, there is the potential for competition for enzymatic breakdown. Not surprisingly, individuals with chronic alcohol consumption may be subject to VAD due to malabsorption of essential micronutrients and depletion of hepatic VA stores. VAD can be corrected through oral supplementation, food fortification, and dietary diversity. In order to preserve public health in developing countries, government has sought to fortify staple foods of different regions with VA to prevent undesirable consequences of VAD.

2.2.2 Toxicity: Hypervitaminosis A

Although VA is an essential micronutrient, a tolerable upper limit (UL) has been established in order to prevent adverse physiological effects resulting from excessive VA intake. Hypervitaminosis A can be caused by acute or chronic exposure to high doses of VA. The severity of the system’s response is dose-dependent and varies with molecular arrangements of retinoid structures (25). Acute hypervitaminosis A can occur as a result of a single-dose of VA administration and is typically presented with nausea,
vomiting, double vision, headache, dizziness, and desquamation of skin (26). However, chronic excessive intake (i.e., VA oral supplementation) is manifested by anorexia, alopecia, bone and muscle pain, and liver damage—most often associated with VA supplementation (26). Excess VA can also be teratogenic, increasing the risk of malformations in the fetus, evident in several animal models (27). After the introduction of retinoids for treatment of skin disorders in humans, a rise in spontaneous abortions and birth defects was observed in women ingesting 13-cis-RA (isotretinoin, Accutane®) and etretinate (Tegison, Tigason). As a result, these drugs became contraindicated during pregnancy (27). Additionally, toxic effects on the liver may include hyperplasia of the stellate cells, hypertrophy, fibrogenesis, sclerosis of veins, portal hypertension, and congestion of perisinusoid cells, which can accelerate the development of cirrhosis (26).

2.2.3 Role of VA in Cellular Differentiation

VA, specifically the biologically active RA, is necessary for normal cell differentiation for specific cell types. For labile cells, VA will induce cell differentiation and inhibit the cell-division cycle. Epithelium depends on VA for maintenance of homeostatic function and structure (28). Consequently, VA isomers have been widely utilized topically and systemically for skin disorders. RA functions to activate gene transcription for the synthesis of keratin proteins (12), a key component of skin and hair. Moreover, it has been reported that hormones and their signaling cascades can regulate mesenchymal stem cells differentiation characteristics for osteoblasts and adipocytes. Recently, RA has been shown to inhibit adipocyte differentiation and stimulate osteoblastogenesis, affecting the balance of this process (29).
2.3 Roles of VA in Energy Metabolism

2.3.1 VA Affects Hepatic Glucose Metabolism

For glucose utilization in hepatocytes, it is first phosphorylated into glucose-6-phosphate by hexokinase D, glucokinase (GK). Insulin induces the hepatic expression levels of GK gene (Gck) in the liver. It has been shown that all-trans ROL, RAL, and RA are able to synergize with insulin to induce Gck expression via the activation of RAR/RXR in primary rat hepatocytes (30). Furthermore, the Gck expression level is reduced in the VAD rats in comparison to the VA sufficient (VAS) controls. RA treatment rapidly recovered this reduction (30).

The liver generates glucose via gluconeogenesis in response to nutritional and hormonal stimuli. The first rate limiting enzyme for hepatic gluconeogenesis is the cytosolic form of phosphoenolpyruvate carboxykinase (PEPCK-C), whose activity is controlled by the expression of its gene (Pck1) (31). Insulin suppresses the hepatic expression of Pck1 (32). It has been shown that RA stimulates the Pck1 expression in hepatoma cells via two RARE in its promoter (33). To understand the effects of the endogenous lipophilic molecules on the expression of insulin-regulated hepatic genes, lipophilic extracts were prepared from rat livers. The lipophilic extracts (LE) induced Pck1 expression levels and attenuated insulin-mediated reduction of its expression in primary rat hepatocytes (34). Subsequently, the active molecules in the extract were identified as ROL and RAL, and the proximal RARE in the Pck1 promoter was found to be responsible for arbitrating retinoids effects in primary rat hepatocytes (8)(30). An increase in the hepatic VA content has been observed in diabetic patients (35) as well as streptozotocin-induced diabetic rats (36) which may contribute to the alterations in
insulin-regulated gene expression. Because the primary function of ROL and RE is to serve as precursors for biosynthesis of RA, it is important to investigate the physiological consequence of this increase in VA storage. The retinoids effects on the hepatic expression of Pck1 (8) and Gck (30) demonstrate the interaction between the insulin and retinoid signaling pathways, which deserves further investigation.

2.3.2 Impact of whole-body VA status and retinoid availability on hepatic FA metabolism

The liver plays a critical role in the maintenance of hepatic lipid homeostasis, relying on a delicate balance of lipid uptake, synthesis, catabolism, and excretion. Alterations in lipogenesis, fat oxidation, and lipoprotein metabolism can favor the development of obesity and the metabolic syndrome (37). Several transcription factors cooperate in the control of hepatic lipogenesis including liver X receptor (LXR) and sterol regulatory element–binding protein 1c (SREBP-1c), a transcription factor that induces the expression of hepatic lipogenic genes (38). The expression of its gene (Srebp-1c) is induced by insulin, or activations of LXR and RXR (39). The insulin-responsive elements of Srebp-1c promoter have been identified as two LXR receptor elements (LXRE) and one sterol regulatory element (SRE) in its promoter (40). Later, it was shown that RA was capable of inducing Srebp1-c in primary rat hepatocytes and the LXREs are also RAREs (9).

A significant portion of patients with acne receiving isotretinoin (13-cis RA) treatment developed hypertriglyceridemia (41). In addition, clinical studies have indicated that the excessive VA supplementation resulted in hepatic hypervitaminosis A, which exacerbates abnormal lipid storage in the liver (42). Rats fed multiple isomeric
forms of RA all displayed hypertriglyceridemia, consistent with human observations (3). Rats on a VAD diet exhibited a lowered plasma lipid profile compared to VAS controls (43). This may be caused by the dual existence of VA deficiency and hypoinsulinemia, which results in the reduction of hepatic lipogenesis (44). The activation of RXR by its specific agonist (LG100268) induced hepatic lipogenesis, which interestingly increased insulin sensitivity in obese and diabetic rats (45). Since Sreb1-c is a critical transcription factor for lipid homeostasis (46) and RA has been shown to affect the insulin-mediated expression of Sreb1-c (9), it is reasonable to conclude that VA status and retinoids play roles in hepatic FA homeostasis.

2.3.3 VA Status in Animals and Retinoids Affect Insulin Secretion from Pancreatic β-cells

Insulin controls hepatic glucose and fatty acid metabolism in response to macronutrients. Glucose metabolism causes the rise of ATP/ADP ratios and subsequently stimulates the release of insulin from insulin granules in pancreatic β-cells (47). Glucose stimulated insulin secretion (GSIS) is impaired in VAD rats and is recovered by VA repletion (44). Additionally, VAD rats had pancreatic β-cell dysfunction which may be attributed to a reduction in fetal β-cell mass (7). In isolated rat pancreatic islets, ROL either potentiated (0.1µmol/L) or inhibited (100 µmol/L) GSIS (48). RA was capable of potentiating GSIS via induction of transglutaminase activity in INS-1 cells (49). Recently, it has been shown that pancreatic β-cells produce 9-cis RA, whose level is elevated in islets of diet-induced obesity, ob/ob and db/db mice (50). When mouse islets were treated with 9-cis RA, GSIS was reduced due to a reduction in GLUT2 and GK activities (50). In addition, lipid depletion in pancreatic β-cells caused impairment of GSIS, which can be restored in the presence of FAs (51). Additionally, RA has been
reported to induce Srebp-1c mRNA in INS-1 insulin secreting cells (9). Taken together, VA status or retinoid levels can indirectly control hepatic glucose and FA metabolism through regulating the insulin secretion from pancreatic β-cells. If RA is produced in the islets, it becomes essential to learn the mechanism for the production of the various RA isoforms to fully understand the roles of the retinoids in pancreatic β-cell function.

2.4 Impact of Vitamin A Derivatives on Lipid Metabolism

2.4.1 Pharmacological Uses of VA and Contraindications

Lipid alterations have been contraindicated for the use of VA derivatives as pharmacologic agents. Attributed to their role in cellular differentiation, VA derivatives have been universally used in the treatment of hyper- and parakeratotic skin disease, severe acne, and some lymphomas for the past three decades (52). In fact, isotretinoin (13-cis retinoic acid, or [Accutane]) is the treatment of choice for severe acne that is non-responsive to other forms of treatment. It has been reported that in 70-80% of patients, long-term remission from severe acne is achieved with this medication (53). Unfortunately, in approximately 20% of these patients, there is an observed elevation of TG-rich lipoproteins that, depending on the severity, may cause acute pancreatitis. Lipid disturbances after administration of systemic therapy with VA derivatives have provoked dermatologists to monitor fasting plasma lipid levels routinely after treatment. These alterations in lipid metabolism can vary depending on the specific ligand-receptor interactions that occur. The table below identifies the receptor-binding classes of commonly used VA derivatives.
Table 2.1 Classification and profiles of VA derivatives for systemic administration (52)

<table>
<thead>
<tr>
<th>Chemical Components</th>
<th>Substance</th>
<th>Receptor</th>
<th>Endogenous Metabolite/Synthetic</th>
<th>Indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>All-trans RA</td>
<td>Tretinoin</td>
<td>RAR</td>
<td>Metabolite</td>
<td>Acute promyelocytic leukemia; Acne Vulgaris; Kerotosis Polaris</td>
</tr>
<tr>
<td>13-cis RA</td>
<td>Isotretinoin</td>
<td>RAR</td>
<td>Metabolite</td>
<td>Cystic Acne Vulgaris</td>
</tr>
<tr>
<td>13-cis-RA</td>
<td>Acitretin</td>
<td>RAR</td>
<td>Synthetic</td>
<td>Psoriasis, lichen planus</td>
</tr>
<tr>
<td>9-cis-RA</td>
<td>Bexarotene</td>
<td>RXR</td>
<td>Synthetic</td>
<td>Cutaneous T-cell lymphoma</td>
</tr>
<tr>
<td>9-cis-RA</td>
<td>Alitretinoin</td>
<td>RAR/RXR</td>
<td>Metabolite</td>
<td>Chronic hand dermatitis; Kaposi’s sarcoma</td>
</tr>
</tbody>
</table>

For some of these pharmacological agents, they are exclusively used to treat skin disorders when other therapeutic alternatives unavailable. For example, alitretinoin serves as the only licensed systemic treatment for chronic hand dermatitis. It becomes important to maximize its therapeutic potential by eliminating contraindications such as the undesirable effects on lipid metabolism.

**2.4.2 Proposed Mechanisms of Lipid Disturbances During VA Therapy**

The mechanism that VA derivatives employ to cause rapid disturbances in lipid metabolism remains elusive. Currently, it is believed this change in lipid metabolism is due to RAR activation resulting in a marked, reversible, dose-dependent elevation in serum lipids and VLDL particles (54). The mechanism proposed is increased production of TG-rich VLDL through inhibition of fatty acid degradation in the liver. This inhibition
causes accelerated hepatic TG production and decreased clearance of VLDL particles by LPL. Additionally, hepatic Apo C-III is increased due to RXR activation (55). Human ApoC-III is a major factor in determining plasma triglyceride levels. Elevated Apo C-III results decreased uptake of TG-rich lipoproteins from the blood, causing hyperlipidemia (4)(54).

Beyond this, there seems to be a genetic predisposition to develop pathologic serum lipid levels during VA therapy (56). Previous diagnoses for various chronic diseases such as obesity, DM, hypothyroidism, CHD, previous MI and kidney dysfunction are contraindications to VA therapy as they place the patient at risk for adverse changes in lipid profile. Furthermore, it has been proposed that VA therapy may reveal latent, familial hyperlipidemia disorders which characteristically lie quiescent during childhood and gradually apparent as an individual ages (57) (5).

2.4.3 Insulin-Mediated Apoc3 Transcriptional Regulation

It has been reported that insulin-dependent diabetes mellitus (IDDM) patients who exhibit non-compliance to insulin therapy have elevated plasma TG levels that can be normalized upon insulin treatment (58)(59)(60). These elevated TG levels have been linked to a state of insulin deficiency where LPL activity is decreased resulting in decreased TG clearance from VLDL and chylomicrons in the blood. This is possible in accordance with the human observation of increased ApoCIII activity in the insulin-deficient state, contributing to hyperlipidemia. Concomitantly, insulin’s inability to inhibit hormone-sensitive lipase (HSL) in adipose tissue causes an influx of FFA to the liver, inducing VLDL synthesis. The combined effects of these metabolic changes have been shown to contribute to increased triglyceride levels. Indeed, STZ-treated diabetic mice
showed a decrease in Apoc3 mRNA in response to insulin treatment. This is reasonable due to the presence of an insulin-response element (IRE) in the Apoc3 gene promoter (61). Therefore, it becomes important to understand the contributions of both Srebp1-c and Apoc3 in RA-induced hyperlipidemia.

2.5 Nuclear Receptors

The regulation of hepatic glucose and lipid homeostasis is attributed, at least in part, to the regulation of the expression levels of genes involved in response to hormonal and nutritional stimuli. A transcriptional network that can be impacted by lipid-soluble factors such as VA orchestrates the liver’s response to nutritional and hormonal signals (62). The impacts may be in concert with the expression profiles of those transcription factors mediating the hepatocytes differentiation and functions.

2.5.1 Chicken Ovalbumin Upstream Promoter Transcription Factor II (COUP-TF-II)

COUP-TF-II nuclear receptors comprise the most highly conserved subfamilies studied. Because of its DNA-binding ability, it can activate or inhibit gene expression depending on the presence of certain ligands, corepressors, or coactivators (63). For example, COUP-TF-II can positively regulate Cyp7a1 expression by recruiting coactivators, i.e., glucocorticoid receptors (GR) that further activate gene transcription. In contrast, COUP-TF-II acts as to repress gene transcription by the recruitment of nuclear corepressors to silence activities of other NR such as thyroid receptors (64). Targeted deletion of COUP-TF-II can be lethal and compromise processes such as angiogenesis and cardiovascular development (63). In multiple cell lines, COUP-TF-II acts constitutively but its activity can be modulated by the presence of ligands such as RA (65). Both variants are able to regulate gene transcription because of their ability to
bind 5'-AGGTCA-3' direct repeats (DR) and modulate gene transcription. It has been reported that COUP-TFII can activate Rarb (63). Furthermore, RA has been shown to release COUP-TFII from the auto-repressed conformation due to its interaction with the LBD, which may affect some of the metabolic activities orchestrated by COUP-TFII (66).

2.5.2 Hepatocyte nuclear factor 4 alpha (HNF4α)

HNF4α is a liver-enriched transcription factor responsible for regulation of genes controlling lipid and bile synthesis, gluconeogenesis, and a variety of liver-specific functions (67). It remains one of the most significantly conserved DNA-binding proteins in the liver, with an estimated 40% of transcribed genes containing an HNF4α-response element (68). Additionally, it has been indicated to direct approximately 11% of genes actively transcribed in pancreatic islets (69). Its expression is present in lower amounts in tissues such as the kidneys, intestine, and pancreas relative to the liver (70). The obligatory role it plays in hepatic lipid homeostasis is implied by the development of hepatic steatosis in HNF4α-deficient mouse livers, attributed to the accumulation of liver cholesterol (67). Inherited mutations in the human HNF4α gene results in maturity onset diabetes of the young type 1 (MODY1), which is characterized by pancreatic β-cell dysfunction evidenced by a loss of insulin secretion in response to glucose (71). Moreover, HNF4α maintains the differentiation state, phenotype, as well as directs energy metabolism in the hepatocyte (71).

It has been suggested that the metabolic syndrome and/or diabetes may contribute to deleterious effects on liver function characteristic of hepatocellular carcinoma which may be arbitrated by a disruption in HNF4α activity (67). Not surprisingly, excess energy intake is a factor contributing to non-alcoholic fatty-liver
disease (NAFLD), which is characterized by disturbed fat distribution associated with significant energy accumulation. The direct results include exacerbating conditions such as obesity and insulin resistance, creating a dangerous platform for the development of hepatocellular carcinoma (HCC), one of the most prevalent liver cancers in the United States and China. While much of the nutritional contributions to these conditions have been focused on macronutrient intake, our lab considers the effects of micronutrients as a nutritional signal that can impact this transcriptional network. This knowledge may impact our understanding of pathophysiological outcomes associated with metabolic disorders and invite new target therapies for improving prognosis.

In humans treated with VA derivatives, 1 in 10 may develop hyperlipidemia as a result of these medications. As mentioned previously, this has been attributed to the elevation of Apoc3 mRNA and ApoCIII protein expression. However, little regard has been given to the impact of Srebp1-c in the pathogenesis of RA-induced hyperlipidemia. It has been indicated that VAD rats had lower plasma TG level and hepatic lipogenic activity than VAS rats did (43), suggesting an association between dietary VA intake and changes in hepatic lipogenesis. Because Srebp1-c is a critical factor mediating hepatic lipogenesis, we want to investigate the contributions of this gene to RA-induced hyperlipidemia as well as the mechanism for changes in Apoc3 expression in response to retinoid treatment. We have identified two RAREs in the Srebp1-c promoter (9) which may be responsible for sensing VA status. Additionally, we have demonstrated that RAL and RA have the ability to synergize with insulin to induce Srebp1-c expression in primary rat hepatocytes (9).
CHAPTER III

MATERIALS AND METHODS
3.1 MATERIALS AND METHODS

3.1.1 Reagents

Reagents for primary hepatocyte isolation and culture have been published previously (34) as well as the source of LG268 (30). All other compounds ((E)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl-1-yl)-1-propenyl] benzoic acid or TTNBP CAS 71441-28-6, T1317 (N-(2,2,2-Trifluoroethyl)-N-[4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl]phenyl]benzenesulfonamide)) were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Reagents for cDNA synthesis and real-time PCR were obtained from Applied Biosystems unless otherwise indicated (Foster City, CA). Medium 199 (catalog number 11150-059), liver perfusion medium (catalog number 17701-038) and liver digest buffer (catalog number 17703-034) were obtained from Invitrogen (Life Technologies, Grand Island, NY). Dulbecco’s Modification of Eagle Medium (DMEM), fetal bovine serum (FBS), and streptomycin/penicillin (catalog number 091670249) were obtained from Fisher Scientific (Pittsburgh, PA, 15275). Trypsin-EDTA solution was obtained from Gibco® (catalog number 15050-065, Grand Island, NY14072). Primary antibodies against HNF4α, COUP-TFII, and β-actin (#3113, #6434, #4970, respectively) were purchased from Cell Signaling Technology, Danvers, MA.

3.1.2 Animals and Primary Hepatocyte Isolation

Male Sprague-Dawley (SD) rats were purchased from Harlan Breeders (Indianapolis, IN). Male Zucker lean (ZL) and Zucker fatty (ZF) rats were bred at UTK.

The animals were housed in colony cages, fed a standard rodent diet and maintained on a 12-h light/ 12-h dark cycle. The Institutional Animal Care and Use
Committee at the University of Tennessee at Knoxville approved all procedures under protocol 1642 and 1582.

For primary hepatocyte isolation, rats were euthanized with carbon dioxide. After death, the abdominal cavity was opened by excision. A 24 G catheter was inserted into portal vein and connected to a peristaltic pump for infusing liver perfusion medium. The inferior vena cava was cut open to allow the outflow of the media at flow rate of 10 ml/min. Following perfusion (~200ml), the liver was digested with 140 ml of liver digest buffer at 10 ml/min. Then, livers were excised from the rat and placed into a tissue culture plate containing 30 ml of liver digest buffer to remove connection tissues and allow the release of hepatocytes. Medium containing hepatocytes were filtered (100-microne pores, BD) and spun at 50×g for 3 minutes. The cell pellets were washed twice with 50 ml of wash medium: DMEM containing 5% FBS, 100-units/ml sodium penicillin, and 100 µg/ml streptomycin sulfate. After the final wash, cells were suspended in the wash medium and the viability of the isolated hepatocytes were determined by Tryptan blue staining. The isolated hepatocytes were plated onto collagen type I-coated 60-mm dishes (2 to 3 million cells/dish) and incubated in 4 ml of the same medium at 37°C and 5% CO2. After 3–4 hours, the attached cells were washed once with 4 ml of PBS, and incubated in medium 199 supplemented with 100 nM dexamethasone, 100 nM 3,3',5-triiodo-l-thyronine (T3), 100 units/ml penicillin, and 100 µg/ml streptomycin sulfate plus 1 nM insulin for 14–16 hours until being used for the indicated experiments.

3.1.3 Cell Culture

HL1c cells, which were stably transfected with a reporter gene construct containing a fragment of rat Pck1 promoter (72), were a kind gift from Dr. Donald K.
Scott at University of Pittsburgh. Cells were incubated in DMEM supplemented with 4.5 g/L glucose, 4% FBS, 100 U/mL of penicillin, and 100 µg/mL of streptomycin sulfate at 37°C and 5% CO₂/95% air. Cells in 60 mm culture dishes were grown to 60-70% confluence before being treated with 2 ml serum-free DMEM containing indicated reagents in the figure legends for the indicated time as shown in the figure legends. All cells were maintained at 37°C and 5% CO₂.

3.1.4 Preparation of Recombinant Adenovirus

Methods for recombinant adenovirus generation have been described previously (73). HEK293 cells were seeded into 6-well plates at a density of 1 × 10⁶ cells/well in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin sulfate, and allow to grow to 90% confluence. The cells were co-transfected with 1 µg each of pACCMV5-rHNF4α and pJM17 with Lipofectamine 2000 (Life Technologies) according to manufacturer’s instructions. Transfected 293 cells were incubated in 2 ml of DMEM containing 2% FBS at 37°C and 5% CO₂. Samples demonstrating lysis 6-10 days post-transfection were harvested for viral amplification. Crude lysates were screened for the presence of the specific genes via PCR.

For each recombinant adenovirus, confirmed original crude lysate was then used to infect HEK 293 cells grown to 80% confluence in 150 mm tissue culture plates. The ratio of the medium to crude lysate is 10 to 1 (v/v). After the lysis of the cells at around 48 h post infection, the cell culture medium (crude lysate) was collected, and stored at -80°C until being used.

For purification of each recombinant adenovirus, NP-40 was first added into the crude lysate to reach the final concentration at 0.5%. The mixture was shaken gently at
room temperature (RT) for 30 min and subjected to centrifugation at 8,000 rpm and 4°C for 15 min. The supernatant was transferred to a clean bottle, and 0.5 × volume of 20% PEG8000/ 2.5 NaCl was added. The preparation was shaken gently at 4°C overnight. The resulting mixture was transferred to centrifuge bottles and spun at 12,000 rpm at 4°C for 15 min. The precipitated pellet was resuspended in a small volume of PBS (2-3 ml), and spun at 12,000 rpm and 4°C for 10 min to remove insoluble matters. Solid CsCl was added to the supernatant until its final density reached 1.34 g/ml. The mixture was spun at 90,000 rpm at 25°C for 3 h using OptimaTM MAX-XP Ultracentrifuge (Beckman Coulter Inc., Brea, CA). The corresponding band containing pure viral particles was collected in a total volume less than 1 ml for desalting. The PD-10 column SephadexTM G-25 M (Amersham Pharmacia Biotech AB, Sweden) was equilibrated with 5 ml PBS. The purified virus in CsCl solution was loaded onto the column and eluted with 5 ml PBS. The flow through was collected into ten fractions. The optical density (OD) of each of these fractions at 260/nm was determined after 1 to 50 dilution in water using Spectronic® GENESYSTM 5 Spectrophotometer (Thermo Scientific Inc., Pittsburgh, PA). The fractions containing significant values of OD were collected and pooled. Bovine serum albumin (BSA) and glycerol were added to the pooled solution to make the stock viral solution with the final concentrations of them at 0.2% and 10%, respectively. After the filtration of the stock solution for sterilization, its OD was determined to estimate the plaque forming units (pfu) assuming 1 OD equals $1 \times 10^{12}$ pfu/ml. The final purified virus stock was frozen at -80°C until being used in the indicated experiments. Recombinant adenovirus expressing human COUP-TFII (Ad-COUP-TFII) was provided by Mireille Vasseur-Cognet (74).
3.1.5 RNA Extraction and genomic DNA removal

One milliliter of RNA STAT 60 was added directly to each 60 mm dish containing cells treated with indicated reagents. The plate with the reagent was allowed to shake at RT for at least 10 minutes. The lysate was transferred to a 1.5 ml micro centrifuge tube. Then, the samples were stored at room temperature for 5 minutes, at which time 0.2 ml of chloroform was added to each sample. After tightly sealing, the sample was shaken vigorously for 15 seconds and left at room temperature for 2 minutes. The samples were then centrifuged at 13,000 rpm for 30 minutes at 4°C, resulting in two phases, including lower DNA/protein containing red phenol chloroform phase and colorless aqueous upper phase containing RNA. The aqueous phase (about 0.5 ml) was transferred to a fresh tube and mixed with 0.5 ml of isopropanol. Samples were spun immediately at 13,000×g for 20 minutes at 4°C. The precipitated total RNA formed a white pellet at the bottom of the tube. The supernatant was removed and RNA pellet was washed with 1 ml per tube of 70% ethanol in diethylpyrocarbonate (DEPC) water. The sample was vortexed and spun at 7,500xg for 5 minutes at 4°C. RNA was air-dried briefly without complete drying in order to preserve solubility. The pellet in each tube was dissolved in 0.88 ml of DEPC water and kept at room temperature for 30 minutes.

For the removal of contaminated genomic DNA in RNA samples, the Ambion® DNA-free™ DNase Treatment & Removal Reagents were used. Each RNA sample was treated with 4 units of rDNase 1 at 37°C for 30 minutes in a final volume of 100 µL containing 100 mM Tris, 25 mM MgCl₂, and 5 mM CaCl₂ at pH 7.5. Following incubation, 10 µL of resuspended DNase Inactivation reagent was added, mixed well with samples, and incubated at room temperature for 2 minutes. Samples were
centrifuged at 10,000×g for 2 minutes and the supernatant (RNA) was transferred to a fresh tube and stored at -80°C until further use.

3.1.6 **cDNA synthesis and real-time polymerase chain reaction (PCR)**

For RNA quantification, 2 µl of each sample was diluted in 998 µl of H₂O for the measurement of OD at 260 nm using a spectrophotometer (Thermo Scientific Spectronic Genesys 5 UV Spectrophotometer model no: 336001). In brief, the 1 mL diluents were placed in 1 ml spectrophotometer cuvette and OD was determined at 260 nm. For cDNA synthesis, 2 µg of DNA-free total RNA was used in a final volume of 100 µl reaction containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 20 mM DTT, 2 mM dNTP, 50 units RNase inhibitor (Life Technology, Ca #: N8080119), and 2.5 µM random hexamers (N6). The mixture was incubated at 25°C for 10 minutes, 48°C for 30 minutes, and 95°C for 5 minutes. First strand cDNA samples were stored at -20°C until further use for real-time PCR analysis. Each real-time PCR reaction in a final volume of 14 µl includes cDNA from 14 ng of reverse transcribed total RNA, 2.33 pmol primers set for the indicated gene, and 7 µl of 2 × SYBR Green PCR Master Mix. Triplicate PCR reaction was carried out for each cDNA samples in 96-well plates using the ABI 7300 Real-Time PCR System. The conditions are 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95°C for 15 s and 60 °C for 1 min. The relative amounts of all mRNAs were calculated using the comparative C_T method with 36B4 as the invariant control. The real-time primer sets for Cyp6a1(75), Srebp1-c (9), Pepck (30), Rarb, Apoc3 and Hnf4α have been previously reported. The oligo nucleotide sequences of real-time PCR primer sets are summarized in Table 3.1. The primer set for Coup-tfii (forward- 5'-
TGCCTGTGGTCTGTCTGATG-3’ and reverse- 5’-GGAAGGGAGACGAAGCAAAA-3’ ) was designed using Primer Express Software (Applied Biosystems).

3.1.7 Immunoblotting and Protein Band Quantification

After indicated treatments in the figure legends, HL1c cells in 60 mm dishes were washed once with 3 ml PBS and scrapped from the dish into 400 µl of whole-cell lysis buffer (1% Triton X-100, 10% glycerol, 1% IGEPAL CA-630, 50 mM Hepes, 100 mM NaF, 10 mM EDTA, 1 mM sodium molybdate, 1 mM sodium β-glycerophosphate, 5 mM sodium orthovanadate, 1.9 mg/ml aprotinin, 5 µg/ml leupeptin, 1 mM benzamide, 2.5 mM PMSF, pH 8.0). The lysates were placed on ice for at least 20 min before they were subjected to centrifugation at 13,000 rpm for 20 min. The protein concentration in the supernatant was determined with PIERCE BCA protein assay kit (Rockford, IL). This was achieved by first mixing 50 parts of Reagent A to 1 part Reagent B to make working reagent (WR), provided by assay kit. 25 µL of each standard and each unknown sample was then pipetted into appropriately labeled tubes. 200 µL of the WR was then added to each sample and mixed well. The tubes were then covered and incubated at 37°C for 30 minutes. All tubes were then cooled to room temperature.

For the standard curve, protein concentration was measured using a plate reader set at 562 nm. The average absorbance of the blank standard replicates at 562 nm was then subtracted from the 562 nm measurements of all other individual standard and unknown sample replicates. A standard curve was prepared by plotting the average blank-corrected 562 nm measurement for each BSA standard vs. its concentration in µg/mL. The standard curve with a formula was then created and used to determine the protein concentration of each unknown sample.
To make the resolving gel, 2 mL 30:0.8% w/v acrylamide:bisacrylamide, 3 mL 1.0M Tris-Cl pH 8.8, 38 µL 20% SDS, and 2.43 mL dH₂O were mixed together and 36 µL of 10% APS and 5 µL of TEMED to reach a final volume of 7.5 ml. To make the stacking gel 660 µL of 30:0.8% w/v acrylamide:bisacrylamide, 30 µL 1.0M Tris-Cl pH 6.8, 25 µL of 20% SDS and 3.6m mL of dH₂O were mixed. After mixing, 25 µL 10% APS and 5 µL TEMED was added immediately before pouring for a total of 5 mL. Proteins (40 µg/lane) in whole cell lysates were separated on an 8% SDS-PAGE gel. It was then transferred (1.5 hr) to BIO-RAD Immuno-Blot PVDF membrane (Hercules, CA), and blocked for 1 hr at RT in TBS plus 0.05% TWEEN® 20 (TBST), 5% non-fat dry milk, and incubated overnight at 4°C in primary antibodies against HNF4α, COUP-TFII, and β-actin diluted 1:1000 in TBST and 1% BSA, according to the protocols provided by the manufacturers. After this, blots were washed with TBST twice for 5 minutes and twice for 10 min. at RT and incubated at RT for 1 hr using a 1:2,000 dilution of goat anti-rabbit IgG (#7074P2, Cell Signaling Technology) conjugated to horseradish peroxidase. Bound primary antibodies were visualized by chemiluminescence (ECL Western Blotting Substrate, Thermo Scientific) and membranes were exposed to X-ray films (Phenix Research Products, Candler, NC) for protein band detection. The films were scanned using an HP Scanjet 3970 (Palo Alto, CA 94304) and stored as Tagged Image File Format (TIFF) at 300 dpi.

### 3.1.8 Statistical Analysis

Cumulative data is presented as means ± standard errors (S.D.). Levene's test was used to determined homogeneity of variance among groups using SPSS 21.0 statistical software (IBM, Armonk, NY) and where necessary natural log transformation
was performed before analysis. Multiple comparisons were analyzed by one-way analysis of variance (ANOVA) using least significant different (LSD) when equal variance was assumed, and Games-Howell test was used when equal variance was not assumed. The independent sample t-test was used to compare two conditions. Differences were considered statistically significant at $P < 0.05$.

**Table 3.1 Primer sequences used for qPCR**

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<th>Gene</th>
<th>Primer (oGC-)</th>
<th>Sequence (5'-3')</th>
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</thead>
<tbody>
<tr>
<td>36B4</td>
<td></td>
<td>TTCCCACTGGCTGAAAAGGT</td>
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<td></td>
<td></td>
<td>CGCAGCCGCAAATGC</td>
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<td>Rarβ</td>
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<td>TGCACTGACACCAACCGGT</td>
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<td>Srebp1-c</td>
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<td></td>
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CHAPTER IV

RESULTS AND DISCUSSION
4.1 RESULTS AND DISCUSSION

4.1.1 Elevated \textit{Srebp1-c} in ZF rats hepatic tissue and isolated hepatocytes may contribute to RA-induced hyperlipidemia

ZF rats (76) have been universally accepted as model systems to study the obesity development due to hyperphagia and its effects on related metabolic disorders due to an autosomal Mendelian recessive trait affecting all leptin receptor isoforms (77)(78). We used this model to analyze the mRNA levels of genes involved in hepatic lipid metabolism in isolated primary hepatocytes from ZF and ZL rats in comparison to liver tissue. It has been reported that VAD rats display decreased plasma TG levels and hepatic lipogenic activity than VAS rats (43). This observation may be a result of reduced \textit{Srebp1-c} activity, a critical gene for fatty acid biosynthesis (46). We have shown that retinoids are capable of synergizing with insulin to induce \textit{Srebp1-c} expression via LXR binding sites (9). Consistent with this hypothesis, in both the liver tissue and isolated hepatocytes of ZF rats, \textit{Srebp1-c} mRNA amounts were significantly higher than ZL counterparts, as shown in Figure 4.1. This is expected as hepatic lipid accumulation and \textit{de novo} lipogenesis is increased in obese rats while SREBP-1c acts as a central transcriptional regulator of these alterations in hepatic lipid metabolism (79). Consistent with our overall hypothesis that excessive RA activation contributes to obesity, \textit{Rarb} mRNA, a RA-responsive gene (80), was significantly higher in ZF rats in liver tissue and isolated hepatocytes. If we consider the current model of RA-induced hyperlipidemia (5), we would expect \textit{Apoc3} mRNA to be induced in both liver tissue and isolated hepatocytes from ZF rats due to the activity of RA-signaling indicated by \textit{Rarb} induction. In liver tissue from both ZL and ZF rats, \textit{Apoc3} mRNA was not significantly
different. Interestingly, Apoc3 expression in isolated hepatocytes from ZF was significantly higher than that from ZL rats. We believe this is a result of the development of insulin resistance, which has been documented in ZF rats (81). It also suggests that other cells in the liver probably also contributed to the Apoc3 gene expression. Obese ZF rats become hyperinsulinemic and exhibit significant hepatic and peripheral insulin resistance (81). Therefore, it is reasonable to conclude that ZF isolated hepatocytes are no longer responsive to insulin-mediated suppression of Apoc3 and express significantly different levels compared with livers from ZL rats. Similar differences were observed in Cyp7a1 expression, whereas, ZF liver tissue exhibited higher amounts of Cyp7a1 mRNA, which may again be the result of a change in insulin-mediated transcriptional activity. The mRNA levels of various transcription factors, including Hnf4a, Rxrg, Hmgcr, and Couptf-ii, were not significantly different among groups in hepatocytes or liver tissue. We conclude from this data that the elevated expression of Srebp1-c in the liver of ZF rats may be a major contributor of RA-induced hyperlipidemia.
Figure 4.1 Comparison of mRNA levels in liver tissue and isolated hepatocytes of ZL and ZF male rats. Liver tissue and primary hepatocytes were obtained and isolated from ZL and ZF rats fed ad libitum. The total RNA was extracted and subjected to real-time PCR analysis. Results are presented as means ± SD of –ΔCt (against 36B4) from the indicated numbers (in parenthesis) of liver samples or hepatocyte isolations for ZL and ZF rats (*all P<0.05, for comparing the –ΔCt values of the indicated transcripts in liver or hepatocytes from ZL rats with those from ZF rats using independent-samples t test). Abbreviations: Hnf4α-hepatocyte nuclear factor 4 alpha, Srebp1-c-sterol regulatory element binding protein 1-c, Rarb-retinoic acid receptor beta, Apoc3-apolipoprotein C-III, Cyp7a1-cytochrome P450 7a1, Rxrg-retinoic acid receptor gamma, Nr2f2-nuclear receptor subfamily 2, group F, Hmgcr-3-hydroxy-3-methylglutaryl-coenzyme A.
4.1.2 RAL treatment induced \textit{Rarb}, but not \textit{Apoc3}, mRNA in primary rat hepatocytes

It has been reported that RA treatment directly induces the \textit{Apoc3} mRNA levels probably via activation of RXRs in HepG2 hepatoma cells and human primary hepatocytes derived from healthy organ donors (82). We have identified RAL as one of the active lipophilic molecules from our original study that is capable of synergizing with insulin to induce \textit{Pck1} expression (34)(8). Since we have shown that RA can be dynamically synthesized from RAL in primary rat hepatocytes to induce the expression of genes involved in glucose and lipid metabolism (2), primary rat hepatocytes were treated with increasing concentrations of RAL in the absence or presence of 1 nM insulin to determine \textit{Rarb}, \textit{Hnf4a}, and \textit{Apoc3} mRNA levels in response to retinoid treatment. In the absence of insulin, \textit{Rarb} mRNA, as a positive gene for retinoid treatment, was induced by RAL at 0.2 $\mu$M in a dose-dependent manner. Insulin treatment inhibited the basal, but not RAL-induced \textit{Rarb} mRNA expression. \textit{Hnf4a} mRNA level was not affected by RAL and insulin treatments, except for the RAL at 0.02 $\mu$M, which induced \textit{Hnf4a} expression. The expression level of \textit{Apoc3} mRNA was not affected by RAL or insulin treatment.
Figure 4.2. Effects of RAL on the mRNA levels of *Rarb* (A), *Apoc3* (B), and *Hnf4a* (C) in primary rat hepatocytes. Hepatocytes were incubated in medium A with or without increasing concentrations of RAL in the absence or presence of 1 nM insulin for 6 h. Total RNA was extracted and subject to real-time PCR analysis. The mRNA level of the respective gene in ethanol vehicle control group was assigned a value of 1 (mean ± SD, n = 3, * P<0.05 for comparing the group in the absence of insulin with the groups in the presence of insulin at the indicated RAL dosage).
4.1.3 Endogenous *Apoc3* expression is not significantly affected in response to retinoid treatment in rat hepatoma cells.

Since retinoids also regulate gene expression in HL1C rat hepatoma cells (3), endogenous responses of *Apoc3* to retinoids as analyzed. HL1c cells were treated with increasing dosages of ROL and RAL and the changes of *Rarb*, *Pck1* and *Apoc3* mRNA levels were determined. RA has been shown to bind *Rarb*, mediating its cellular signaling. Therefore, we used *Rarb* and *Pck1*, known target genes of RA-signaling, as a reflection of retinoid activity in this cell. There was a dose-dependent, significant increase of *Rarb* and *Pck1* mRNA expression levels in response to RAL treatment compared to the vehicle control (Figure 4.3). Additionally, *Rarb* mRNA levels were significantly lower in cells treated with ROL than those with RAL. This is expected as ROL has been shown to be less potent than RAL on gene expression because of its required metabolism to more active forms (73). For ROL at 2 µM, *Pck1* mRNA was significantly lower than RAL groups at the same concentration. However, *Apoc3* mRNA presented no significant changes among all groups, regardless of retinoid potency and concentration. Therefore, we concluded that retinoids do not directly affect endogenous *Apoc3* transcription in rat HL1C hepatoma cells.
Figure 4.3. The mRNA expression levels of *Rarb*, *Pck1*, and *ApoC3* in response to retinoid treatment in rat hepatoma cells. HL1c cells were treated with ROL and RAL at indicated conditions for gene expression studies. Cells were harvested 6 hours after treatment and total RNA was isolated and subjected to reverse transcription to generate cDNA. *Rarb*, *Pck1*, and *ApoC3* expression was detected using RT-PCR relative to an internal control gene, *36B4*. Results are presented as a means of ± SD of three independent experiments. (All P<0.05; a<b, c<d<e using one-way ANOVA; * for comparing ROL with RAL groups using independent sample t-test)
4.1.4 Specific agonists activating RAR and RXR are not sufficient to induce Apoc3 mRNA in rat hepatoma cells.

RA regulates gene expression through activation of transcription factors, namely RXRs and RARs (83). Activation of RAR antagonized the induction of Srebp-1c mediated by RXR activation in primary rat hepatocytes (6). Additionally, it has been shown that HNF4a directly binds to human Apoc3 promoter sequences (7) and mediates glucose-induced Apoc3 expression in primary rat hepatocytes (8).

Subsequently, we measured the expression levels of Cyp26a1, Pck1, Hnf4a and Apoc3 mRNA in those cells. It has been reported that pharmacological activation of LXR can elevate VLDL synthesis (84). Furthermore, we have shown that RA is capable of inducing Srebp1-c expression via LXREs in its promoter to induce hepatic lipogenic activities (9). LXRs have been shown to dimerize with RA-specific nuclear receptors to direct the actions of lipid metabolism within the cell including lipoproteins (85). In order to understand the roles of these nuclear receptors in VLDL synthesis coupled with RA-induced hyperlipidemia, ApoC3 mRNA was measured along with reported target genes of RA-specific receptors such as Pck1 and Cyp26a1 (73). To determine if activation of RAR, RXR or LXR alone plays a role in the regulation of Apoc3 in rat hepatoma cells, were treated with TTNBP (1 µM), a specific panagonist of RARs; LG268 (1 µM), an RXR-specific agonist; and T1317 (1 µM), a synthetic agonist for LXR activation in the absence or presence of insulin.

TTNPB, a potent RAR agonist, significantly induced Cyp26a1 expression, an RA-responsive gene (75) up to 150-fold in the presence and absence of insulin (1nM) (Figure 4.4A). Activation of RXR by LG268 caused a modest induction of Cyp26a1 in
the presence or absence of insulin, while LXR activation by T1317 caused suppression of Cyp26a1 in comparison to RXR/RAR activation. This suggests that RAR can be activated in HL1C cells in the presence of a specific agonist.

RAR activation can induce Pck1 expression and attenuate insulin-mediated suppression of its expression in primary rat hepatocytes (8). Similarly, a robust induction of Pck1 expression is observed upon RAR activation with concomitant attenuation of insulin-mediated suppression in Figure 4.4B. RXR activation alone did not significantly induce Pck1 and insulin-mediated suppression of its expression was not affected, in comparison to control. Although RAR activation was sufficient to induce Cyp26a1 and Pck1, no significant changes resulted in Hnf4a or Apoc3 mRNA in response to RAR, RXR or LXR activation in the presence or absence of insulin (Figure 4.4C and 4.4D). Interestingly, Apoc3 mRNA expression was significantly lower in the vehicle control group treated with insulin which is consistent with previous reports of insulin’s regulation on Apoc3 mRNA expression in diabetic mice (61). However, this reduction was not observed in primary hepatocytes (Figure 4.2). Insulin’s ability to suppress Apoc3 gene transcription was ablated when RAR, RXR, and LXR activation occurred, as shown in Figure 4.4D. These results indicate that activation of these three nuclear receptors in HL1c cells is not sufficient to induce Apoc3 mRNA. The observation of RA-induced transcription factor’s ability to attenuate insulin-mediated suppression of Apoc3 may contribute to increased plasma triglyceride levels. However, the major contribution to increased lipogenesis in response to retinoids must be employed by another gene, such as Srebp1-c, which deserves further investigation.
Figure 4.4 The expression levels of Cyp26a1 (A), Pck1 (B), Hnf4a (C), and Apoc3 (D) in HL1c cells treated with RAR, RXR, and LXR agonists. HL1c cells were treated with vehicle, TTNBP (1 μM), LG268 (1 μM), and T1317 (1 μM) in the absence and presence of insulin (1nM) for 6 h. The expression level of the vehicle control group was arbitrarily assigned a value of 1. Results are presented as a means of ± SD of three independent experiments. (All P<0.05; a<c<b, a'<c'<b', d<e, d'<e' using one-way ANOVA; # lower compared to the control group without insulin; * comparing two groups in the indicated treatments)
4.1.5 The over-expression of HNF4α or COUP-TFI respectively induced or inhibited the Apoc3 mRNA level in rat hepatoma cells.

HNF4α constitutes a family of transcription factors that direct the expression of several genes in lipid metabolism including the apolipoprotein promoters, thus making it capable of influencing plasma triglyceride levels (86). It is possible that RA acts in concert to induce or inhibit a coactivator in the regulation of transcriptional activity for genes involved in hepatic lipid metabolism, such as Apoc3. The current model of RA-induced hyperlipidemia considers the induction of Apoc3 expression as the major contributor to elevated plasma triglyceride levels. The hormone-response elements (HREs) of the ApoC-III promoter can be bound by members of the orphan nuclear receptor family as well as ligand-dependent nuclear receptors (86). Because Apoc3 is a classical target gene of HNF4α, molecules that can change the binding activity of HNF4α may be an indirect result of a change in the activity of the transcription factors involved in Apoc3 gene expression. Additionally, COUPTFII-ARP-1 serves as one of the most evolutionarily conserved orphan nuclear receptors among all species and plays a role in various biological processes including metabolic homeostasis (66). Much like HNF4α, COUP-TFI seems to be constitutively active and may be transactivated by the presence of various coactivators or corepressors (66). For example, it has been reported that RXR and COUPTF-II compete antagonistically in vivo for binding to DR-1 such that COUPTF-II has the ability to completely repress RXR-induced transcription activation in various cell lines (87). Because these along with RA-inducible transcription factors play a key role in directing the liver’s metabolic activities it becomes important to understand their regulation of ApoC3, a major contributor to plasma triglyceride levels.
We hypothesized that retinoids do not directly influence \textit{Apoc3} expression in rat hepatoma cells and primary rat hepatocytes. Rather, a change in \textit{Apoc3} expression is a result of altered HNF4\textalpha and COUPTF-II levels in rat hepatoma cells and hepatocytes. It is reasonable to propose this idea as the activity of these transcription factors have been reported to cause a change in the presence of ligand-activated receptors such as RXR in rat hepatocytes.

As shown in Figure 4.5, the mRNA and protein levels of HNF4\textalpha and COUPTF-II were dramatically induced when HL1C cells were infected with the recombinant adenoviruses. The mRNA levels of \textit{Hnf4a} and \textit{COUP-TFI\textalpha} expression were induced by ~153 and ~24 fold, respectively. This is associated with a dramatic elevation of their corresponding proteins detected in the whole cell lysates (Figure 4.6A-C). The viral dosage that we used was successful to induce over-expression of HNF4\textalpha (Figure 4.6D).

Figure 7A shows the expression of \textit{Apoc3} mRNA in HL1C cells treated with no virus, Ad-\textbeta-gal or Ad-HNF4\textalpha virus in the absence or presence of RAL. The over-expression of HNF4\textalpha significantly induced \textit{Apoc3} mRNA (~2-fold higher) compared to the control. The over-expression of Ad-COUPTF-II significantly repressed \textit{ApoC3} expression (~50%), characteristic of its ability to act as a repressor of other transcription factors (88). The addition of RAL (1\muM) did not affect on \textit{Apoc3} gene expression.

Retinoids induce \textit{Pck1} in primary rat hepatocytes (8). In order to show that RAL was effective in the cells, basal \textit{Pck1} mRNA expression level was significantly reduced and increased by over-expression of HNF4\textalpha and COUPTF-II, respectively (Figure 4.7B). RAL treatment significantly induced the \textit{Pck1} (~5-fold). These data indicate that
RA derived from RAL treatment can induce the expression of *Pck1*, a RA responsive gene, but not *Apoc3* expression in HL1C cells. We conclude from these data that retinoids do not directly induce *Apoc3* mRNA in short term. The hyperlipidemia in patients treated with RA derivatives may be, in part, caused by the induction of *Srebp1-c*, which acts to increase hepatic lipogenesis, and contributes to the elevated plasma triglyceride levels.
Figure 4.5. The adenovirus-mediated overexpression of Ad-HNF4α and Ad-COUPTF-II in rat hepatoma cells. HL1C cells were maintained in DMEM containing 4.5-g/L glucose, 4% FBS, 100 U/ml of penicillin and 100 U/ml streptomycin sulfate and incubated in 60 mm dishes. HL1C cells were infected at 1,000 pfu/cell for 18 hours with indicated Ad-β-gal, Ad-HNF4α, and Ad-COUPTF-II. Cells were then harvested, total RNA isolated, and subjected to RT-PCR analysis. The mRNA level of each transcript in the cells infected with Ad-β-gal and the vehicle control was arbitrarily assigned a value of 1.
Figure 4.6. Immuno-blot of adenovirus-mediated overexpression of nuclear receptors in rat hepatoma cells.

HL1c cells were infected with Ad-β-gal, Ad-HNF4α, or Ad-COUP-TFII at 100 PFU/cell for 18 h. Western blot of proteins (40µg/lane) in whole cell lysates showing relative nuclear receptors and β-actin as the loading control.
Figure 4.7 The expression levels of HNF4α and COUPTF-II in rat hepatoma cells regulate Apoc3 and Pck1 gene expression. HL1C cells were maintained in DMEM containing 4.5-g/L glucose, 4% FBS, 100 U/ml of penicillin and 100 µg/ml streptomycin sulfate and incubated in 60 mm dishes. Cells were infected 18 hours before treatment with indicated adenovirus, then treated with serum-free medium for 2 hours containing vehicle control or RAL at 1 µM. Cells were then harvested, total RNA isolated, and subjected to RT-PCR analysis. All P<0.05; b>a>c, b'>a'>c', g>e>f, e'<f' using one-way ANOVA
4.2 Summary and Future Directions

RA-induced hyperlipidemia has historically been attributed to the increase of Apoc3 mRNA in hepatocytes, which leads to elevation of plasma TG levels due to the inhibitory effect of ApoC3 on LPL. This mechanism has been used to explain studies on individuals treated with synthetic VA analogs whose therapy was contraindicated by the development of severe secondary hyperlipidemia, which puts them at risk for cardiovascular accident. Although this has been a generally accepted belief, the emerging role of retinoids in obesity development and energy homeostasis has prompted us to gain more insight into the mechanism of RA-induced hyperlipidemia.

In our current study, we observed the elevation of Srebp1-c and Rarb mRNA in ZF rats, which is consistent with our previous observation of RA-mediated induction of hepatic lipogenic activities (73). Excessive activation of Srebp1-c has been associated with hypertriglyceridemia (89) and RA with insulin to induce Srebp1-c (9). In our current study, the higher expression of Rarb in ZF compared to ZL suggests RA may be mediating the induction of Srebp1-c, and thus contributing to increased hepatic lipogenesis. Although actively produced in these cells, we could not observe the induction of Apoc3 mRNA in liver tissues. The induction of Apoc3 in isolated hepatocytes leads us to believe that insulin resistance in ZF rats compromised insulin-mediated suppression of this gene (61). It also suggests that other cells may be involved in regulating Apoc3 expression.

Our current study was designed to investigate the effects of RA on the expression of Apoc3 mRNA in primary hepatocytes and hepatoma cells. We observed that the mRNA levels of Apoc3 could not be directly induced by retinoid treatment in rat
hepatoma cells or primary rat hepatocytes, suggesting this observation is not limited to cell lines. Instead, the presence of transcription factors such as HNF4α and COUP-TF-Ii could induce or inhibit Apoc3 transcription, respectively in rat hepatoma cells. RA can dynamically regulate metabolic activities by interacting with various transcription factors to direct changes in gene expression. More specifically, nuclear receptors mediating RA-response such as RAR/RXR might participate in the hyperlipidemic effect during VA therapy. RAR, RXR, or LXR activation via synthetic analogs was not sufficient to induce Apoc3 mRNA in HL1c cells. Interestingly, 1nM of insulin was able to suppress Apoc3 by as much as 30% and this suppression was attenuated by RAR, RXR, and LXR activation. This might be caused by the variations of Apoc3 mRNA levels in the samples treated with the ligands of these three nuclear receptors or by the reorganization of transcription machinery in response to those ligands. Further studies are needed to find out the underlying mechanisms.

Taken together, we conclude that the expression levels of HNF4a and COUP-TFII in hepatoma cells can positively and negatively regulate Apoc3 mRNA levels, respectively. However, the dynamic production of RA from the RAL could not affect Apoc3 expression in the same cells. Since we only measured the acute and direct effects of retinoids on Apoc3 gene expression, we could not exclude the possibility that RA indirectly regulates Apoc3 mRNA levels via a dynamic change in the activity of transcription factors present such as HNF4a and COUP-TF-II, rather than a direct induction. In Hep3B cells, the RAREs in the promoter of HNF4α genes mediate the reduction of Hnf4a mRNA (90) and HNF4α protein levels after the RA treatment for 3 days (91). The ligand-binding of COUP-TFII is in an autorepressed conformation. At
high concentrations, RAs are able to promote COUP-TFIi to recruit coactivators and activate a COUP-TF reporter construct (66). It seems that further studies are needed to reveal the precise mechanism. Based on previously published data and ones presented here, it is sufficient to conclude that RA-induced hyperlipidemia may be, at least in part, attributed to the elevation of Srebp-1c expression.

Our overall hypothesis that excessive RA synthesis and activation contributes to obesity and associated co-morbidities such as hyperlipidemia is evident in the data presented here. By furthering our understanding of RA-induced hyperlipidemia, it may provide a novel target therapy for combating obesity and metabolic disorders.
LIST OF REFERENCES


Meredith Lee Howell was born July 11th, 1988 to James Thomas Howell and Nancy Hanks Howell in Jackson, Tennessee. She attended Jackson Christian School (K-8) and received her high school diploma from Trinity Christian Academy in Jackson, Tennessee in May of 2006. She completed her B.S degree in Nutrition and Food Science at Middle Tennessee State University under the advisory of Dr. Lisa Sheehan-Smith in December 2010. She was then accepted into the combined Graduate Degree/Dietetic Internship program at the University of Tennessee, Knoxville. In August 2011, she entered as a Master’s candidate in Dr. Guoxun Chen’s lab to study the role of vitamin A in hepatic glucose and lipid metabolism. She defended her thesis on April 26th, 2013. During her time at UT, she served as a Graduate Teaching Assistant for an introductory nutrition course and worked as a tutor in the Thornton Athletics Student Life Center for introductory nutrition and nutritional biochemistry.