# Role of vitamin A status and its catabolism in the regulation of glucose and lipid homeostasis in rats under physiological and disease conditions

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# Dedication

I dedicate this dissertation work to my mother Aidong Lu. Without her emphasis of my education as an important thing in my life and her endless support through these years, it will not be possible for me to complete this work. I also want to dedicate this work to my fiancé Ming Qi who supports me during the past four and half years.

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# Abstract

The increased number of individuals with metabolic diseases has become a public health concern. Vitamin A (VA, retinol) is required to maintain the general health of an individual. How VA contributes to the regulation of glucose and lipid homeostasis in normal and metabolic disease states is unclear. VA's physiological activities are mainly mediated by its metabolite, retinoic acid (RA), which activates several transcriptional factors in the nuclear receptor super family and in turn, regulates the expression of numerous genes for macronutrient metabolism. For the RA production, retinol is first oxidized into retinal and then from retinal to RA. We hypothesize that VA status and dynamic production of RA play a role in the control of glucose and fatty acid metabolism. This dissertation summarized my investigation of VA's role in metabolism: (1) We compared the expression levels of RA-producing enzymes in the liver of Zucker lean (ZL) and fatty (ZF) rats. We found that the hepatic expression levels of retinaldehyde dehydrogenase family 1 gene (*Raldh1*) mRNA and RALDH1 protein were higher in ZF rats than that in ZL rats. This elevated RALDH1 expression may cause the excessive RA production, which may enhance the hepatic lipogenesis and lead to the hepatic insulin resistance; (2) We compared the expression levels of insulin-regulated genes in the liver of VA deficient (VAD) and VA sufficient (VAS) ZL rats in response to the cycle of fasting and refeeding, a normal physiological condition. We observed that VA status and its dietary availability play a role in the expression levels of insulin-regulated gene in the rat liver; (3) We further used streptozotocin (STZ) to induce insulin-dependent diabetes mellitus in both VAD and VAS rats, a disease condition. The hepatic

expression levels of insulin-regulated genes in these animals treated with control, RA, insulin and insulin + RA were investigated. The effects of VA status and RA production on the regulation of the hepatic gene expression were also observed. We conclude that VA status and dynamic RA production contribute to the expression of hepatic genes, which in turn regulate glucose and fatty acid homeostasis.

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# List of Abbreviations

| | |
|---|---|
| ACC | acetyl-CoA carboxylase |
| ACL | ATP citrate lyase |
| AD | ad libitum |
| ADH | alcohol dehydrogenases |
| AMPK | AMP-activated protein kinase α |
| ANOVA | one-way analysis of variance |
| APL | acute promyelocytic leukemia |
| Apo B48 | apolipoprotein B48 |
| Apo B100 | apolipoprotein B100 |
| ARAT | acyl-CoA:retinol acyltransferase |
| BAT | brown adipose tissue |
| BM | body mass |
| BMI | body mass index |
| c-AMP | cyclic AMP |
| CB1 | cannabinoid receptor 1 |
| C/EBPβ | CCAAT-enhancer-binding protein β |
| CLAMS | Comprehensive Lab Animal Monitoring System |
| COUP-TFs | chicken ovalbumin upstream promoter-transcription factors |
| CRABP | cellular-retinoic acid binding protein |
| CRBP | cellular-retinol binding protein |
| CYP | cytochrome P450 |
| DM | diabetes mellitus |
| DMEM | Dulbecco's Modification of Eagle Medium |
| ER | endoplasmic reticulum |
| FA | fatty acid |
| FAS | fatty acid synthase |
| FFA | free fatty acid |
| GCK, *Gck* | glucokinase |

| | |
|---|---|
| GKA | glucokinase activator |
| GLUT2 | glucose transporter type 2 |
| GLUT4 | glucose transporter type 4 |
| GSIS | glucose-stimulated insulin secretion |
| G6P | glucose 6-phosphatase |
| *G6pc* | glucose 6-phosphatase catalytic subunit |
| HFD | high-fat diet |
| HNF4 | hepatocyte nuclear factor 4 α |
| IR | insulin receptor |
| IRS, *Insr* | insulin receptor substrate |
| IGF | insulin-like growth factor |
| IGFBP1 | insulin-like growth factor-binding protein 1 |
| IU | international unit |
| LPL | lipoprotein lipase |
| LRAT | lecithin:retinol acyltransfera |
| LSD | least significant difference |
| LXR | liver X receptor |
| MDR | medium-chain dehydrogenase/reductase |
| MODY | maturity onset diabetes of the young |
| NDDG | National Diabetes Data Group |
| NHANES | National Health and Nutrition Examination Survey |
| Ob | Obese gene |
| PEPCK-C, *Pck1* | cytosolic form of phosphoenolpyruvate carboxykinase |
| PI3K | phosphatidylinositol 3-kinase |
| PIP2 | phosphatidylinositol-4,5-bisphosphate |
| PIP3 | phosphatidylinositol-3,4,5-triphosphate |
| PKB/AKT | protein kinase B |
| PPAR | peroxisome proliferator-activated receptor |
| PML | promyelocytic leukemia |
| RA | retinoic acid |

| | |
|---|---|
| RAE | retinol activity equivalent |
| RALDH | retinal dehydrogenase |
| RAR | retinoid acid receptor |
| RARE | retinoic acid response element |
| RBP | retinol binding protein |
| RDA | recommended dietary allowance |
| RDH | retinol dehydrogenase |
| RE | retinol equivalents |
| REH | retinyl ester hydrolase |
| RER | respiratory exchange ratio |
| ROL | retinol |
| RQ | respiratory quotient |
| RXR | retinoid X receptors |
| SD | Sprague-Dawley |
| SDR | short-chain dehydrogenase/reductase |
| SES | socioeconomic status |
| SRE | sterol regulatory element |
| SREBP-1c, *Srebp-1c* | sterol regulatory element-binding protein-1c |
| STZ | Streptozotocin |
| T1DM | type 1 diabetes mellitus |
| T2DM | type 2 diabetes mellitus |
| TG | triacylglycerol |
| Th2 | T-helper type 2 |
| TIFF | Tagged Image File Format |
| TTR | transthyretin |
| UCP | uncoupling protein |
| UDP | uridine 5’-diphospho |
| VA | vitamin A |
| VAD | VA deficient |
| VAS | VA sufficient |

| | |
|---|---|
| VDR | vitamin D3 receptor |
| VLDL | very low density lipoprotein |
| WAT | white adipose tissue |
| WHO | WHO |
| ZL | Zucker lean |
| ZF | Zucker fatty |

# Chapter One

# Introduction

## 1. Vitamin A

### 1.1. The Discovery of vitamin A

The early studies of vitamin A (VA, retinol) activities can be dated back to 19th century. In 1881, Nicolai Ivanovich Lunin claimed that "a natural food such as milk must contain, besides the known principal ingredients (protein, sugar, fat, and salts), small quantities of other and unknown substances essential to life" [1]. In 1891, Carl A. Socin showed that an unknown fat-like substance in egg yolk was essential to life [2]. In 1906, Frederick Gowland Hopkins stated that "no animal can live upon a mixture of pure protein, fat, and carbohydrate, and even when the necessary inorganic material is carefully supplied the animal still cannot flourish" [3]. In 1909, Paul Knapp continued Lunin's study on the substances of milk and observed that the animals fed a diet of protein, carbohydrate, fats, and minerals developed conjunctivitis and corneal ulceration prior to death, but the eye disease could be avoided if milk was provided [4]. In 1911, Wilhelm Stepp further indicated that "certain lipid substances present in milk and soluble in alcohol-ether are indispensable for the survival of mice" [5]. In 1914, following the previous investigations, Thomas Osborne and Lafayette Mendel reported that rats fed a basal diet developed inflamed eyes and diarrhea, which could be prevented when cod liver oil or butter fat was added to the diet, suggesting a lipid derived factor that is essential to life [6]. In 1918, Elmer McCollum suggested this "factor" that supported growth and eye health be named "fat-soluble A" in distinction to "water-soluble B" [7].

In 1920, Jack Cecil Drummond named all the vitamins as “vitamin A, B, C, etc.” [8]. The chemical structure of β-carotene, precursor of VA, was described by Paul Karrer in 1931, which led to the clarification of the structure of retinol [9]. Ruth Corbet crystallized VA in 1937 [10]. Later, David Adriaan van Dorp and Jozef Ferdinand Arens synthesized VA [11] and Otto Isler developed a method for widespread production of VA [12]. The functions of VA were slowly disclosed through clinical observations including its important role in vision which was discovered by George Wald [13].

## 1.2. Sources, Digestion, and Absorption of VA

### 1.2.1. Sources of VA

Figure 1-1 shows molecules with VA activities. The nutritional need for retinol (Figure 1-1A) can be met by sufficient intake from the diet of these molecules. The dietary molecules with VA activities are in two forms, preformed VA (retinol and retinyl esters) and provitamin A (carotenoids).

Preformed VA is found primarily in animal-derived food sources, especially the liver and dairy products including whole milk, cheese, and butter as well as fish [14]. The main preformed VA in these foods is as either retinol or retinyl esters, mainly retinyl palmitate (as shown in Figure 1-1B), which can undergo oxidation if exposed to varying degrees of oxygen, heat, and some metals [14]. Retinyl palmitate, the ester form of VA (retinol conjugated with palmitic acid) is frequently used as a cosmetic ingredient [15].

Provitamin A carotenoids are abundant in many fruits and vegetables in the forms of β-carotene (Figure 1-1C), α-carotene (Figure1-1D), and β-cryptoxanthin (Figure 1-1E) [16]. Dietary β-carotene is present at high levels in carrots, and yellow and green leafy

vegetables; α-carotene is present in carrots and red palm oil; and β-cryptoxanthine is found in sweet red pepper, oranges, and papayas [16].

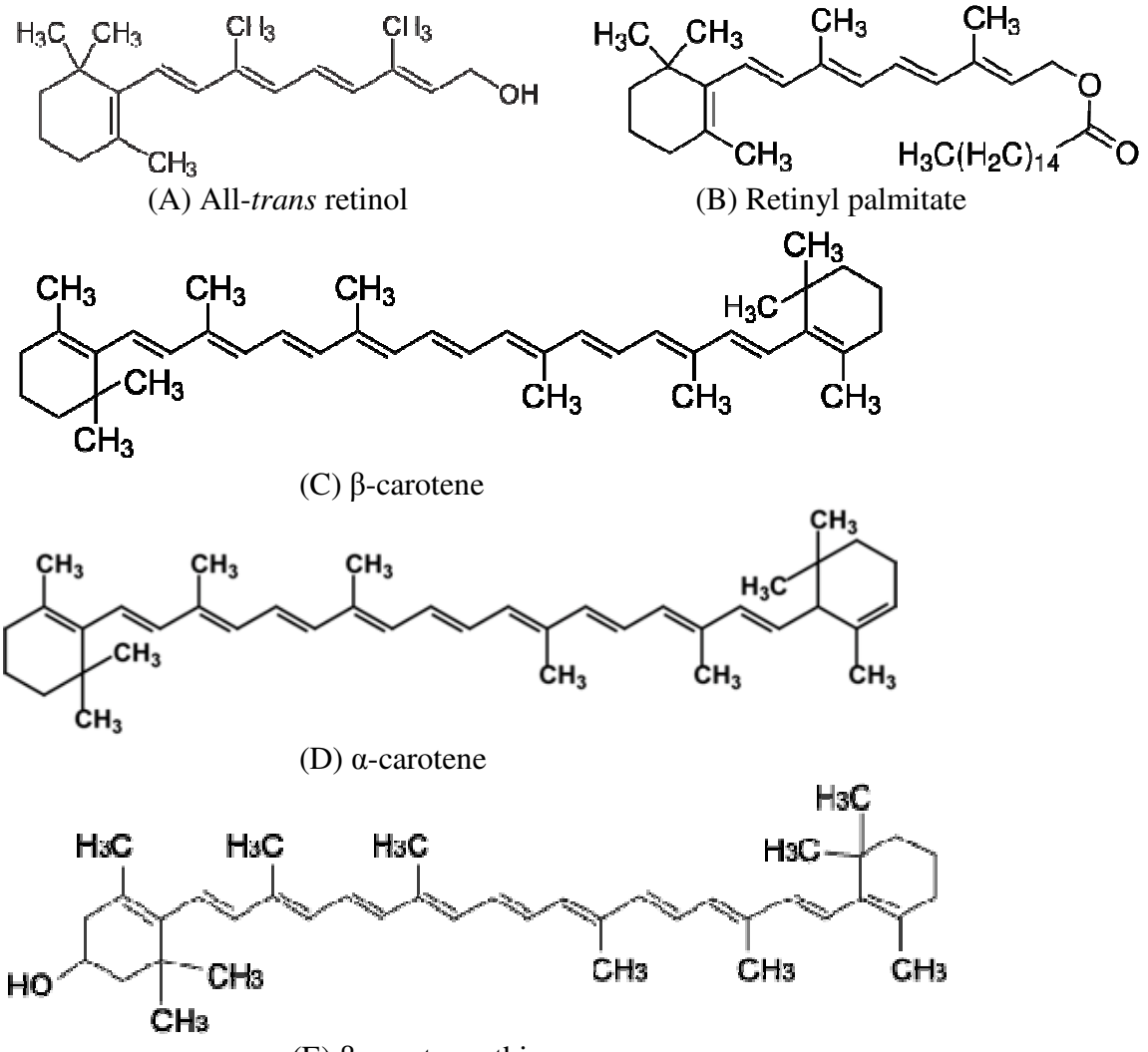



**Figure 1- 1 Structure of representatives of molecules with VA activities.**
(A) All-*trans* retinol, (B) Retinyl palmitate, (C) β-carotene, (D) α-carotene, and (E) β-cryptoxanthin.

### 1.2.2. Recommended dietary allowance of VA

The international unit (IU) had been established and used to describe VA activity as the basis for Nutrition Labeling [17]. In 1989, the unit was replaced with retinol equivalents (RE) through which VA activity was expressed based on the equivalent weight of retinol [18]. Currently, the recommended dietary allowances (RDAs) for VA are recognized as micrograms (μg) of retinol activity equivalents (RAE) to account for the different bioactivities of retinol and carotenoids [19].

1 IU = 0.3 μg retinol or 0.6 μg β-carotene or 1.2 μg other carotenoids;

1 RE = 1 μg retinol or 6 μg β-carotene or 12 μg other carotenoids;

1 RAE = 1 μg retinol or 12 μg β-carotene or 24 μg other carotenoids [20].

Current U.S. RDAs for VA are 900 μg RAE/day for adult men and 700 μg RAE/day for adult women [19].

### 1.2.3. Digestion and Absorption of VA

Dietary molecules with VA activities are typically bound to other components in the food. They have to be digested to an absorbable form. In the diet from animal source, retinol is often esterified with fatty acids to form retinyl esters, mainly retinyl palmitate. The dietary retinyl esters and carotenoids are in complexes with proteins. In the stomach, the release of retinyl esters and carotenoids from protein complexes is initiated by the action of pepsin, which digests proteins. The released retinyl esters and carotenoids coalesce to form fat globules due to their fat solubility. These fat globules are then moved into the duodenum where proteolytic enzymes further hydrolyze proteins and release any

protein-bound retinyl esters or carotenoids. In the lumen of the small intestine, retinyl esters are hydrolyzed by retinyl ester hydrolases (REH) to retinol and the corresponding fatty acids. Hydrolysis of retinyl esters requires bile salts that solubilize the retinyl esters in micellar particles and activate the hydrolyzing enzymes. The different hydrolyzing enzymes have distinct substrate specificities: pancreatic esterase preferentially cleaves short-chain retinyl esters, whereas intestinal brush-border hydrolases prefers long-chain retinyl esters.

The released free retinol and carotenoids, solubilized in micelles with other fat-soluble food components, are then absorbed by the enterocytes. Most of the retinol is passively absorbed by the enterocytes with a relatively high efficiency (70%-90%). Carotenoids are absorbed into the enterocytes by passive diffusion with a low efficiency (9%-22%). Within enterocytes, cellular retinol-binding protein (CRBP) II binds to retinol, which is then esterified by lecithin:retinol acyltransferase (LRAT) with palmitic acyl CoA to form CRBP II-retinyl palmitate. The newly formed retinyl esters are packed in chylomicrons along with triacylglycerols, phospholipids, and cholesterol esters. Chylomicrons are transported to the lymphatic system and ultimately into the blood circulation. Chylomicrons deliver retinyl esters and unesterified retinol to extrahepatic tissues, and the retinyl esters not taken up by the tissues are transported to the liver as part of the chylomicron remnants. About 70%-75% of chylomicron retinoids are cleared from circulation by the liver.

Within the enterocytes, β-carotene is either hydrolyzed by noncentral cleavage enzymes generating alcohol and aldehydes or by β-carotene 15,15'-oxygenase forming

retinal (one molecule of β-carotene forms two molecules of retinal) which is then primarily reduced to retinol and bound to CRBP II. About 60-70% of β-carotene is cleaved and the remainder is transferred into blood deposited in liver and adipose tissue. CRBP II-bound retinol is then esterified by LRAT and incorporated into chylomicrons. A small portion of retinal is oxidized to retinoic acid (RA), bound to albumin, and transported through the portal venous system [14, 16, 21, 22].

## 1.3. Storage of VA

The liver is the major organ for VA storage. Some retinyl esters are stored in the parenchymal cells, but almost 80%-95% of retinyl esters are stored in stellate cells [14]. In the parenchymal cells, retinol and fatty acids are released from retinyl esters. Free retinol and CRBP-bound retinol are esterified via acyl-CoA:retinol acyltransferase (ARAT) and LRAT to form retinyl esters. Retinyl esters are stored with lipid droplets in the stellate cells until needed. Hydrolases can release the retinol from the stores to maintain retinol homeostasis [14]. VA is transported between the parechymal and stellate cells in the form of free retinol though the mechanism is not completely understood.

## 1.4. Cellular Metabolism of Retinol

### 1.4.1. Cellular Retinol Binding Proteins

Due to the hydrophobic nature, free retinoids are mainly associated with proteins. Within the cells, retinol binds with CRBP, and RA binds with cellular retinoic acid binding protein (CRABP) [14]. Most tissues contain CRBPs, especially the liver, kidney, intestine, and male reproductive organs. Some of these tissues and the skin also contains CRABPs [23]. CRBP and CRABP are thought to function as “buffer proteins” to control

the concentrations of free or unbound retinol and RA within the cells, and as “guide proteins” to direct retinoids to specific enzymes of metabolism to prevent nonspecific oxidation [23]. Unbound CRBPs are also thought to inhibit LRAT and thereby prevent esterification of retinol for storage [14].

To date, three CRBP isoforms (CRBP I, II, and III) have been identified [24]. The roles of CRBP I and CRBP III are to facilitate the esterification of retinol to retinyl ester catalyzed by LRAT [25, 26]. Both CRBP I and CRBP III are expressed in adipose tissues with CRBP I in preadipocytes and CRBP III in adipocytes, and they are reported to play important roles in adipogenesis [27]. Lack of CRBP I led to increased adiposity [28], whereas mice lacking CRBP III developed decreased adiposity [29]. It seems that CRBP I and III play opposit roles in adipogenesis with CRBP I repressing and CRBP III maintaining adipogenesis.

### 1.4.2. The conversion of retinol to retinal

Within the liver, both hepatocytes and hepatic stellate cells are involved in retinoid metabolism. In hepatocytes, retinyl esters are hydrolyzed to retinol, which is then either oxidized to RA through a two-step reaction, or bound to retinol binding protein (RBP) and secreted out into circulation for the uptake of extrahepatic target tissues including adipose tissue [14]. In hepatic stellate cells, retinol is re-esterified to retinyl ester by LRAT and stored there [30]. The first step of retinol oxidation, from retinol to retinal (also called retinaldehyde), is reversible and is catalyzed by retinol dehydrogenases (RDHs), which belong to the cytosolic alcohol dehydrogenases (ADHs) of the medium-chain dehydrogenase/reductase (MDR) superfamily and microsomal

retinol dehydrogenases of the short-chain dehydrogenase/reductase (SDR) superfamily [31] (Figure 1-2).

Four classes of ADH, ADH1-4, exist in both mice and humans with all genes in both species localized in a single tandem cluster in the same order and same transcriptional orientation [32]. ADH4 is expressed at high levels in the stomach and esophagus but is totally absent from the liver, whereas ADH1, ADH2, and ADH3 are highly expressed in the liver [32]. The knockout studies demonstrate that ADH1, ADH3, and ADH4 function in retinol metabolism [33-35]. ADH1 functions as the primary enzyme responsible for the efficient oxidative clearance of excess retinol, providing protection and increased survival during VA toxicity [35]. ADH4 functions in metabolism of retinol to retinal in peripheral tissues to provide protection against VA deficiency [36]. ADH3 alone is adequate to catalyze the oxidation of retinol to retinal under VA sufficiency, but ADH4 overlaps its function during VA deficiency and ADH1 overlaps its function during VA toxicity [34].

At least three RDHs (RDH1, RDH2 and RDH10) are thought to be involved in the first step of RA biosynthesis. RDH1 and RDH2 recognize CRBP-bound retinol as substrate, and have higher activity with NADP than NAD as a cofactor [37, 38]. RDH1 has higher activity toward to all-*trans* retinol than to other *cis*-retinols, and its function in retinal biosynthesis starts early during embryogenesis [39]. RDH2, identified as protein p29 in another study [40], is thought to have retinal reductase activity in the presence of NADPH and the addition of proteins CYP2D1. NADPH-P450 increased RDH2 reductase activity, contributing to retinoid metabolism [41]. RDH3 is only expressed in the liver. It

has 97.8% identity with RDH1 and 82.3% identity with RDH2 [42]. RDH10 was first cloned in the retinal pigment epithelium [43] and then in retinal Muller cells [44]. RDH10 is correlated with several sites of all-*trans* RA biosynthesis in the mouse embryos [45, 46]. Both RDH1 and RDH10 are active with oxidation of all-*trans* retinol and 9-*cis* retinol [47].

### 1.4.3. The conversion of retinal to retinoic acids

The second step of RA production is retinal oxidation, from retinal to RA. It is catalyzed by cytosolic aldehyde dehydrogenases (ALDHs) [31]. To date, four RALDHs (ALDHs), RALDH1 (or ALDH1A1), RALDH2 (or ALDH1A2), RALDH3 (or ALDH1A3), and RALDH4 (or ALDH8A1), have been cloned in rats and believed to be responsible for all-*trans* RA or 9-*cis* RA production in various tissues (Table 1-1) [48-51].

The 2.3 kb mRNA of RALDH1 is highly expressed in the embryonic and adult dorsal retina as well as in the liver of adult mice [52]. RALDH1 accounts for at least 90% of the all-*trans* and 9-*cis* retinal dehydrogenase activities in adult rat/mouse liver and kidney while RALDH2, -3, and -4 contribute to the remaining activity without apparent domination by any one [51].

Overexpression of mouse RALDH1 in *Xenopus* embryo leads to premature synthesis of RA during embryogenesis, indicating its involvement in RA biosynthesis [53]. Elevated RA was thought to control its own production through the feedback inhibition of RALDH1 expression via retinoic acid receptor α (RARα) by decreasing the C/EBPβ (CCAAT/enhancer binding protein β) concentration [54]. The mechanism was

later confirmed as that all-*trans* RA increases the GADD153 and C/EBPβ interaction [55]. RALDH1 has been associated with obesity and energy balance [56]. RALDH1 expression was up-regulated in the kidney and liver of *db/db* mice with decreased levels of all-*trans* RA which may cause the compensatory increase in RALDH1 [57]. The *Raldh1*-/- mice are protected against diet-induced obesity (DIO) and insulin resistance [56]. Deficiency of *Raldh1* gene activates a brown adipose tissue (BAT)-like thermogenic program in white adipose tissue (WAT), promoting energy dissipation [58]. *Raldh1*-deficient mice also display decreased gluconeogenesis and attenuated hepatic triacylglycerol synthesis [59]. The mechanism has been thought that RALDH1 generates the major amount of all-*trans* RA used to promote adipogenesis and RALDH1 deficiency impairs all-*trans* RA production, therefore decreasing adipogenesis, specifically in visceral fat. Thus, the inhibition of RALDH1 activity may influence depot-specific fat formation [60].

The amino acid sequence of RALDH2 has the highest identity (71.5%) and similarity with RALDH1 [61]. The *Raldh2*-/- embryos can be rescued with external administration of RA, which leads to the conclusion that RA synthesized by RALDH2 is required for early embryonic development [62]. Overexpression of mouse RALDH2 in *Xenopus* embryos resulted in high levels of RA, suggesting its distinct role in retinoid signaling pathway by elevating RA biosynthesis [63]. The analysis of RALDH2 promoter indicated the presence of binding site for sterol regulatory element binding proteins (SREBPs) [64]. Cholesterol metabolite, oxysterol, was demonstrated to induce RALDH1 and RALDH2 expression through the activation of SREBP-1c and liver X receptor α/β

(LXRα/β), establishing the crosstalk between cholesterol metabolism and all-*trans* RA biosynthesis [65].

Mouse RALDH3 shares 70% sequence identity with RALDH1 and 71% sequence identity with RLAHD2 [66]. RALDH3 oxidizes all-*trans* retinal but does not show activity for either 9-*cis* or 13-*cis* retinal substrates [67]. RALDH3 expression in embryos is distinct from that of RALDH1 or RALDH2, showing its unique role in organ development [66]. The *Raldh3*-/- mutants have defects in nasal and ocular development, demonstrating its importance in nasal development [68]. Overexpression of RALDH3 reduced insulin secretion but increased glucagon secretion *in vitro*, indicating that RALDH3 disrupts the balance between insulin and glucagon, and may induce β-cell dysfunction leading to the development of type 2 diabetes mellitus (T2DM) [69].

RALDH4 is inactive for all-*trans* retinal but exhibits high activity for 9-*cis* retinal oxidation [67]. RALDH4 functions with *cis*-retinoid/androgen dehydrogenase 1 (CRAD1), CRAD3, or RDH1 to generate 9-*cis* RA, and has a unique expression pattern in kidney compared with three other ALDHs [51].

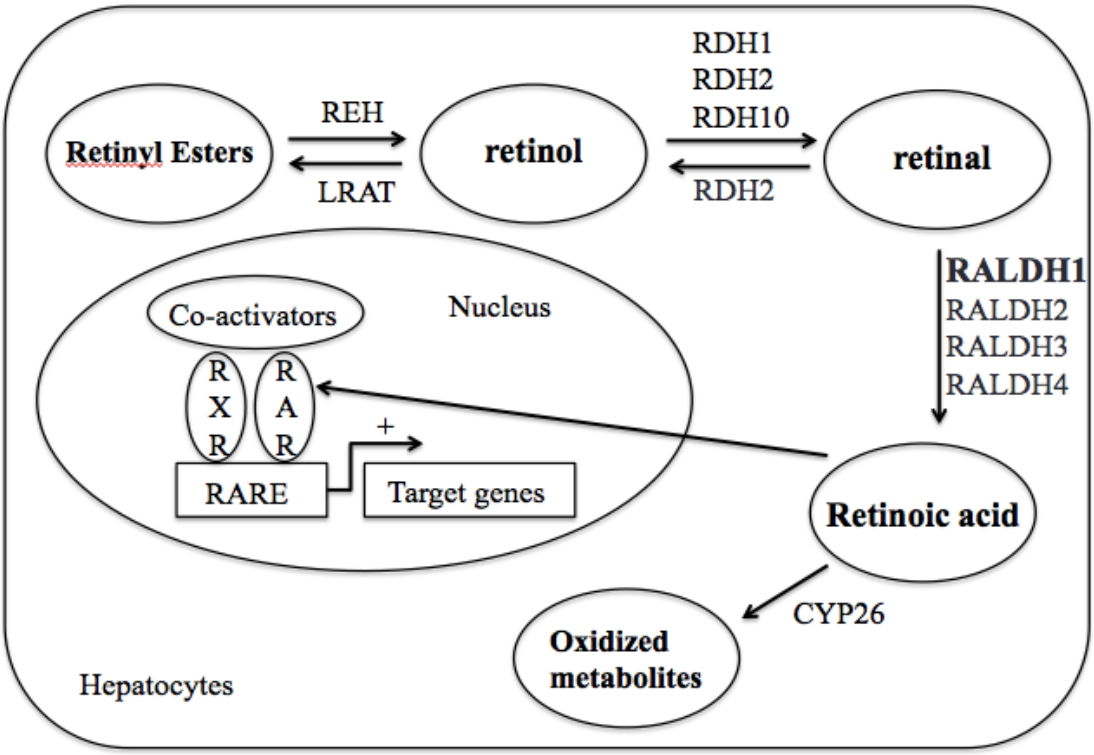



**Figure 1- 2 VA metabolism and RA signaling.**
Retinyl esters are hydrolyzed to retinol and free fatty acids after they enter hepatocytes. Retinol is reversibly oxidized to retinal by RDHs. RDH1, 2, and 10 are the major enzymes in the conversion from retinol to retinal. RDH2 has reductase activity, which is able to reduce retinal back to retinol. Retinal can be irreversibly oxidized by RALDHs (RALDH1 to 4) to RA. RALDH1 is the major enzymes catalyze the conversion from retinal to RA. RA can be modified to more hydrophilic metabolites by CYP26, especially CYP26A1. RA is the active form of VA functioning in the regulation of nuclear gene expression by binding to nuclear receptors mediating RA effects. In the presence of RA, heterodimer RAR/RXR or other nuclear receptors associated with RARE recruit co-factors to modulate target gene expression.

**Table 1- 1 Enzymes in RA biosynthesis**

| Name | Substrates | Major expression sites | Km |
|---|---|---|---|
| **Alcohol dehydrogenases (ADH) / Retinol dehydrogenases (RDH)** | | | |
| ADH1 | | Liver, kidney | |
| ADH3 | | Ubiquitous | |
| ADH4 | | Stomach | |
| RDH1 | Oxidation: all-*trans* retinol, 9-cis retinol | Liver, kidney, lung, testis, brain | 0.9 μM |
| RDH2 | Reduction & oxidation: all-*trans*-retinol | Liver, kidney, lung, testis, brain | 2 mM |
| RDH10 | Oxidation: all-*trans* retinol, 9-cis retinol | Retina | |
| **Aldehyde dehydrogenases (ALDHs)** | | | |
| RALDH1 | Oxidation: all-*trans* retinal, 9-cis retinal | Liver, kidney, small intestine, pituitary glands | 1.4 μM |
| RALDH2 | Oxidation: all-*trans* retinal, 9-cis retinal | Liver, kidney, small intestine, testis, pituitary glands, lung, heart | 0.2 μM |
| RALDH3 | Oxidation: all-*trans* retinal | Kidney, pituitary glands | 0.2 μM |
| RALDH4 | Oxidation: 9-*cis* retinal | Liver, kidney | |

### 1.4.4. The further catabolism of RA, studies of CYP26A1

Cytochrome P450 family 26 (CYP26) enzymes have been identified as a major contributor to the oxidative metabolism of RA in various tissues [70]. Oxidation of RA by CYP26 goes through two phases, oxidation and conjugation. During phase 1, RA is oxidized by CYP26 to polar metabolites, 4-*hydroxy*-RA and 4-*oxo*-RA. They are then converted by uridine 5'-diphospho-glucuronosyl transferases to water-soluble compound, 4-O-β-glucuronide [70]. The CYP26 family includes CYP26A1, CYP26B1, and CYP26C1, and they eliminate RA in different tissue-specific patterns [70]. CYP26A1 was first discovered in zebrafish as P450RAI [71], and then the cDNA of CYP26A1 was cloned in human and several other species [72]. It has been shown that all-*trans* RA is not only the substrate of CYP26A1 but also a potent inducer of it, which is particularly evident in the liver [73]. CYP26A1 is the most sensitive liver-expressed gene in response to the dietary VA intake and exogenous RA treatment [74]. It has been widely accepted that CYP26A1 is the primary enzyme that mediate clearance of endogenous RA [75]. Hepatocytes sense the concentration of RA effectively upon RA entering into the cells, which triggers CYP26A1 and UDP-glucuronidyl transferases to convert excess RA to water-soluble compounds for their elimination from the body as biliary metabolites [70]. Thus, CYP26A1 is usually considered as an indicator of RA production due to its sensitivity to the increase in cellular RA concentration [76].

Some studies have reported an increase in CYP26A1 mRNA expression in cancer cells and tumors [77-79], suggesting a link between CYP26A1 and tumorigenicity. Therefore, suppression of the catalytic activity of CYP26A1 has been brought up as a

therapeutic approach for the treatment of some types of cancer, like leukemia. A number of RA metabolism-blocking agents (RAMBAs) have been developed to inhibit all-*trans* RA metabolism by CYP26A1 [80].

## 1.5. Isomers of retinoic acids

The molecules with part or all of VA activities share a 20-carbon structure with a methyl substituted cyclohexenyl ring and a tetraene side chain with a hydroxyl group (retinol), aldehyde group (retinal), carboxylic acid group (retinoic acid), or ester group (retinyl ester) at carbon-15 [19]. The all-*trans* and three mono-*cis* isomers, 9-*cis*, 11-*cis*, and 13-*cis*, have been reported to play specific physiological role in nature [81].

RA derivatives have been used in the treatment of a lot of diseases over a long time. As shown in Table 1-2, RA isomers include isotretinoin (13-*cis* RA), tretinoin (all-*trans* RA), acitretin, bexarotene and alitretinoin (9-*cis* RA) [82]. They have different stereo structures and regulate target gene expression through activating specific nuclear receptors, RAR or retinoid X receptor (RXR) [82]. Isotretinoin (13-*cis* RA), tretinoin (all-*trans* RA), and acitretin (9-*cis* RA) primarily bind to RAR. Bexarotene (9-*cis* RA) binds to RXR in T-cell lymphoma, and alitretinoin (9-*cis* RA) binds to both RAR and RXR in skin cells [82].

**Table 1- 2 Profiles of RA derivatives**

| Name | Receptors | Treatment |
|---|---|---|
| Tretinoin | RAR | Acute promyelocytic leukemia |
| Isotretinoin | RAR | Acne |
| Acitretin | RAR | Psoriasis, lichen planus |
| Bexarotene | RXR | Cutaneous T-cell lymphoma |
| Alitretinoin | RAR/RXR | Chronic hand dermatitis |

## 1.6. Retinol transport and distribution

### 1.6.1. Transport of retinol from the liver to other tissues

VA is transported in the blood in the form of retinol, bound to a specific protein, RBP which is synthesized by hepatic parenchymal cells. Synthesis of RBP depends on the protein, retinol, and zinc status of the body [14]. RBP was first isolated in 1968 by Masamitsu Kanai et al. [83], and its structure and physical properties have been well studied. RBP is a single polypeptide chain with a molecular weight of approximately 21 kDa, and has a single binding site for one molecule of all-*trans*-retinol [84]. In hepatocytes, retinol combines with RBP to form the retinol-RBP complex, holo-RBP, which is then released to the blood [85]. In the blood circulation, RBP interacts with another protein, transthyretin (TTR), a tetrametic protein with two identical thyroxine-binding sites, to form holo-RBP-TTR complex which is then bound to thyroxine (T4) to

form a trimolecular complex. [14]. The normal level of RBP in plasma is about 40-50 μg/ml, and that of TTR is about 200-300 μg/ml [86]. Retinol mobilization and delivery are regulated by the processes that control the rates of RBP synthesis and secretion by the liver [85]. Specific cell surface receptors that recognize RBP may be involved in the delivery of retinol to peripheral tissues [87-89]. STRA6, a multitransmembrane domain protein, was reported by Riki Kawaguchi et al. as a specific membrane receptor for RBP [90]. However, other view supports that the uptake of retinol from RBP is receptor independent [91, 92].

### 1.6.2. RBP4 and its role in insulin resistance

RBP4 is recently identified as an adipokine that is linked to adiposity, insulin resistance, and T2DM. Barbara Kahn's research group found that mice with an adipose-specific knockout of glucose transporter type 4 (GLUT4), a glucose transporter that mediates insulin-stimulated glucose uptake in adipocytes and muscle, developed insulin resistance in the muscle and liver, suggesting that a circulating factor secreted from the adipocytes that causes the observed changes [93]. They then indicated that RBP4 may be the circulating factor and showed that expression of RBP4 was elevated in adipose tissue of adipose-*Glut4* -/- mice. They also showed that serum RBP4 levels were elevated in insulin-resistant mice and human subjects with obesity and T2DM. Transgenic overexpression of human RBP4 or injection of a recombinant RBP4 in normal mice caused insulin resistance, whereas genetic deletion of *Rbp4* enhanced insulin sensitivity. Moreover, administration of fenretinide, a synthetic retinoid, normalized serum RBP4 levels of obese mice [94]. These studies demonstrate that RBP4 overexpression may

affect insulin sensitivity and contribute to T2DM. The absence of RBP4 may improve insulin sensitivity.

In clinical studies, the causality of increased serum RBP4 levels and insulin resistance is controversial. In some studies, serum RBP4 levels were reported to be higher in subjects with impaired glucose tolerance, T2DM, and to be correlated inversely with insulin sensitivity in non-diabetic subjects with family diabetes history [95, 96]. The improvement of insulin resistance after exercise training is associated with reduced serum RBP4 levels [95]. Significant weight loss after bariatric surgery resulted in decrease of circulating RBP4 levels [97]. All these results indicate that lowing circulating RBP4 levels is a potential treatment strategy to increase insulin sensitivity and improve T2DM. RBP4 seems to be an adipokine that is involved in the early phases of the development of insulin resistance and other components of the metabolic syndrome [98].

However, in other studies, such association was not observed in either healthy [99] or obese subjects [100, 101]. RBP4 gene expression in nondiabetic human subjects was not related to insulin resistance [102].

The molecular mechanism of serum RBP4 effects on insulin sensitivity has not been completely understood. Qin Yang et al. claimed that increased release of RBP4 from adipocytes interrupts insulin signaling in the muscle by reducing phosphoinositide-3-kinase (PI-3-kinase) activity and subsequent phosphorylation of insulin receptor substrate-1 (IRS-1), and increased the expression of enzyme phosphoenolpyruvate kinase (PEPCK) in liver, resulting in increased hepatic glucose output [94]. The link between RBP4 and T2DM may also be mediated through the negative effects of RBP4 on β-cell

function through preventing the binding of TTR to its receptor [101]. Several RBP4 single nucleotide polymorphisms (SNPs) and their haplotypes are likely to affect measures of insulin resistance and RBP4 mRNA levels in visceral adipose tissue in humans, which may result in T2DM [103].

The fact that RBP4 is a transport protein for retinol increases the possibility that alteration of retinol metabolism might influence the action of insulin. Thus, it is of importance to determine the effects of dietary VA intake on insulin sensitivity and plasma RBP4 levels.

## 1.7. Functions of VA

VA and its metabolites play roles in a spectrum of biological functions, including vision, immunity, cellular differentiation, and signaling.

### 1.7.1. Roles of VA in vision

Night blindness has been known since ancient Egyptian times and eating raw liver was recognized as a cure for night blindness [104]. In early 1900s, cod liver oil was discovered as a cure for night blindness and the symptom could be reversed by a diet including whole milk or butter in which the essential nutritional factor was later discovered as “fat-soluble VA” by Thomas Osborne and Lafayette Mendel [104].

A number of studies have demonstrated the link between VA and night blindness. VA is important in the visual cycle, which depends on the roles of 11-*cis*-retinal in the transduction of light into neural signals necessary for vision [105], and RA in maintaining normal differentiation of the cornea and conjunctival membranes [106]. Photons enter the eye through the cornea, then pass through the lens and the vitreous humor, and hit the

retina. The photoreceptors of the retina consist of rods, which are stimulated by weak light of a broad range of wavelengths, and cones, which are responsible for color vision under bright light. Retinal is essential to form rhodopsin, a retinal-containing pigment protein that is made up of *cis*-retinal and opsin in the rod. In the rod visual cycle, absorption of a photon by rhodopsin-bound 11-*cis*-retinal causes isomerization of 11-*cis*-retinal to form all-*trans*-retinal, and releases opsin, producing the visual signal that is sent to the brain. To regenerate rhodopsin again, released all-*trans*-retinal is reduced to all-*trans*-retinol and transported from the rods into the pigment epithelium of the retina, where it is catalyzed by LRAT to all-*trans*-retinyl esters. All-*trans*-retinyl esters are then converted to 11-*cis*-retinol, which is attached to cellular retinal-binding protein (CRALBP). This compound is oxidized to 11-*cis*-retinal, which detaches from CRALBP and transports back to rods and reattached to opsin to form rhodopsin, a process closing the visual cycle. Inadequate VA supply may result in failure or delayed recovery of vision in the dark, which results in night blindness [14, 21, 107, 108].

### 1.7.2. Roles of VA in immunity

VA also plays an important role in enhancing immune responses. RA stimulates phagocytic activity and cytokine production, and maintains natural killer cell concentrations. VA is also needed for T-lymphocyte function and for antibody response to viral, parasitic, and bacterial infections [109]. Therefore, people with VA deficiency have reduced ability of regeneration of mucosal barriers damaged by infection, impaired protective functions of neutrophils, macrophages, and natural killer cells, diminished

antibody responses to antigens that require T-helper type 2 (Th2)-mediated help, which contribute to the greater risk of infection and increased severity of disease [109].

### 1.7.3. Roles of VA in differentiation

Many cells need VA, especially RA, for cell differentiation. For example, RA maintains the normal structure and function of the epithelial cells that are part of our skin and all internal body tracts. The mechanism is that RA is one of the signals that switch on the expression levels of genes for keratin proteins, thereby directs the differentiation of immature skin cells into mature epidermal cells [23].

Additionally, VA is required for bone health through involvement with osteoblasts (bone-forming cells) and osteoclasts (bone resorption cells). VA deficiency can cause excessive deposition of bone by osteoblasts and reduced bone degradation by osteoclasts [14].

### 1.7.4. The toxicity of excess VA intake

The daily tolerable upper intake level (UL) for preformed VA for adults is 3,000 μg RAE (10,000 IU) per day [19]. Acute VA toxicity has been reported to occur following consumption of polar bear and seal liver by Eskinos and Arctic explorers [21]. Excess VA intake in a short time can result in acute hypervitaminosis A with the symptoms of nausea, vomiting, double vision, headache, dizziness, and general desquamation of the skin. Chronic VA toxicity occurs following routine intake of large doses of VA over a period of several months, and is manifested by a variety of symptoms, including anorexia, desquamating skin, hair loss, headache bone and muscle pain, increased bone fractures, and liver damage [14]. Excess oral intake of VA also

causes birth defects. Use of Accurane, an acne treatment medicine containing 13-*cis*-RA, in the early months of pregnancy resulted in a number of birth defects among the infants [14]. One mechanism is that excess VA intake rises serum retinol levels, and retinol is no longer transported by RBP but is presented to the cell membranes, releasing toxic products [14].

## 1.8. Regulation of gene expression

### 1.8.1. The nuclear receptor super family and their activation mechanism

Nuclear receptors are a family of ligand-activated transcription factors that play important roles in numerous physiological processes responding to lipophilic hormones, vitamins, dietary lipids, or other intracellular signals [110]. The nuclear receptors can be classified into three groups based on the understanding of the working mechanisms. The first group includes endocrine receptors with high affinity for fat-soluble hormones and vitamins, like RAR. The second group is named adopted orphans as their physiological ligands were not known when their cDNAs were identified based on sequence homology to endocrine receptors, like RXR, LXR, peroxisome proliferator-activated receptor (PPAR), and hepatocyte nuclear factor 4 α (HNF4α). The third group is named true orphans as the ligands or agonists for their activation are currently unknown [110].

RA regulates gene expression through activation of two families of nuclear receptors, RAR activated by all-*trans* and 9-*cis* RA and RXR activated by 9-*cis* RA [111]. Each of them has three isoforms, α, β, and γ that are encoded by distinct genes. Each isoform has multiple subtypes produced by alternative splicing or differential promoter use. RAR/RXR hetero- or RXR/RXR homo-dimer binds to the RA responsive

elements (RARE) at the promoters of their downstream targeted genes and recruits cofactors to regulate transcription of these genes in the presence of retinoids.

### 1.8.2. Retinoic acid receptors

Three isoforms of RAR, α, β, and γ, were first identified in 1987 independently by Chambon's and Evans's groups [112, 113] and they originate from three distinct genes [114]. RARs can bind to RXRs to form heterodimers and then bind to the specific RARE sequences located at the promoter region of the target genes. In the absence of RAR agonist, the RAR/RXR heterodimer recruits co-repressor proteins to prevent transcription; when RAR agonist binds to it, co-repressors are released and co-activator proteins are recruited to activate transcription [115, 116]. However, RXR agonists are unable to activate the heterodimer without RAR ligand [117].

The link between all-*trans* RA and acute promyelocytic leukemia (APL) had been established by Hugues de Thé [118], who found that a translocation occurred in APL cells in which RARα gene is fused to the promyelocytic leukemia (PML) gene, producing a fusion protein PML/RARα. Expression of this protein led to the development of APL in transgenic mice harboring *PML/ RARα* gene in myeloid cells [119]. Normally, PML acts as a tumor suppressor protein but its function is blocked by the PML/ RARα fusion protein [120]. Later, all-*trans* RA was found to down-regulate RARα in a dose- and time-dependent manner [121] and all-*trans* RA stimulates catabolism of the PML/RARα fusion protein, turning off its function [122].

### 1.8.3. Retinoid X receptors

RXR has three isoforms, RXRα, β, and γ that are encoded by three distinct genes [123]. 9-*cis* RA is a high-affinity ligand for all three RXR isoforms [123]. RXRs can function as a homodimer (RXR/RXR) or heterodimers with other nuclear receptors, such as RAR, PPAR, LXR, vitamin $D_3$ receptor (VDR), or thyroid hormone receptor (TR) [21]. PPAR and LXR are permissive partners that can be activated by agonists of both RXR and the partner receptor independently or together; RAR, TR, and VDR are non-permissive partners that cannot be activated by the agonist of RXR but only by the agonist of the partner receptor [124].

PPARγ plays a major role in the regulation of glucose and lipid metabolism, and the rexinoid LG100754 efficiently induces lower glucose levels in rats with T2DM [125, 126].

### 1.8.4. Other nuclear receptors

Other than RARs and RXRs, many nuclear receptors mediate RA responses, such as HNF4α and chicken ovalbumin upstream promoter-transcription factors (COUP-TFs) [127].

## 2. Metabolic diseases

### 2.1. Obesity

For adults, overweight and obesity ranges are determined by using an individual's weight in kilograms over height squared in meters to calculate body mass index (BMI). An adult who has a BMI between 25 and 29.9 is considered overweight. An

adult who has a BMI of 30 or higher is considered obese [128]. A child's weight status is determined using an age- and sex-specific percentile for BMI rather than the BMI categories used for adults. Overweight is defined as a BMI at or above the 85$^{th}$ percentile and lower than the 95$^{th}$ percentile while obesity is defined as a BMI at or above the 95$^{th}$ percentile for children of the same age and sex [129].

In 2010, an estimated two-thirds (67%) of U.S. adults were overweight, and more than one-third (35.7%) of adults and almost 17% of youth were obese [130]. Non-Hispanic blacks have the highest obesity rate (49.5%) compared with Mexican Americans (40.4%), all Hispanics (39.1%) and non-Hispanic whites (34.3%) [131]. Women with higher incomes or education levels are less likely to be obese than those of lower socioeconomic status (SES), whereas there is no significant association between obesity and SES among men [132]. Between 1960 and 1980, the obesity rate was relatively stable. But obesity rates for adults have doubled and rates for children have tripled regardless of sex, ethnicity, socioeconomic status, or geographic region since 1980 [133]. The rate of obesity is increasing around the world according to the data from the World Health Organization (WHO) [134]. In 2010, 35.7% of U.S. adults were obese and almost 17% of youth were obese [130]. The latest analysis of National Health and Nutrition Examination Survey (NHANES) from 2009-2010 data suggested a slowing or leveling off of the prevalence of adult obesity in U.S. [131]. However, the prevalence remains unacceptably high and obesity continues to be a serious national health concern [133].

The development of obesity results in heath and economic consequences. Overweight and obesity increase the risk of many life-threatening diseases, such as T2DM, heart disease, hypertension, respiratory problems, osteoarthritis, gallbladder disease, and certain types of cancer [133]. It is reported that overweight and obesity are linked to more deaths worldwide than underweight. 65% of the world's population live in countries where overweight and obesity kills more people than underweight [134]. Additionally, overweight and obesity decrease the quality of life due to limited physical mobility, incidence of discrimination, low self-esteem, and depression [133]. The annual medical burden of obesity and health-related problems has risen to almost 10% of all medical spending in U.S. [135].

### 2.1.1. Causes of obesity

Obesity is fundamentally caused by an imbalance between food consumption/energy intake and physical activity/energy expenditure. When energy intake is greater than energy expenditure, energy balance is positive, which leads to weight gain and increased fat stores. On the other hand, when energy consumption is less than energy expenditure, energy balance is negative, which results in reduced body fat stores. Both environmental and genetic factors contribute to the development of overweight and obesity. The environmental factors such as consumption of sugar-sweetened beverages, increased portion size, decrease in physical activity, etc, have been proposed to play a role in the obesity development. Nearly half of the added sugar in the U.S. diet is from sugar-sweetened beverages, including carbonated drinks, sweetened fruit drinks, and sports beverages [136]. A lot of studies have shown that consumption of sweetened

beverages, especially those with high-fructose corn syrup, is associated with increased energy intake and incidence of obesity [137, 138].

Genetic mutations also contribute to the development of obesity. For example, mutations of leptin gene and its receptor have been shown to cause the development of overweight and obesity [139]. The genes encoding leptin (*Ob*) and its receptors (*Obr*) were cloned in 1990s. The obese mice bearing a mutation of *Ob* gene is leptin deficiency [133]. Leptin, mainly from adipose tissues, is a circulating signal for the regulation of body weight and whole body energy expenditure [140]. Circulating leptin levels are correlated with the body fat [141] and the fasting and re-feeding cycle. The plasma leptin level falls markedly after fasting and rises promptly following the restoration of food intake [142]. When fat stores in adipocytes increase, more leptin is released as a signal to leptin receptors in the brain, resulting in a feeling of satiety and reduced food intake; when body fat stores decrease, lowered leptin levels lead to increase in food intake [143]. However, leptin deficiency is very rare in humans [144] and obese people who were given high doses of leptin only lost a small amount of weight [133]. Thus, human obesity is considered a leptin resistance state due to the elevated plasma leptin levels and reduced responses to the exogenous leptin treatment [145]. Leptin resistance could contribute to insulin resistance and the metabolic syndrome in human obesity [145].

### 2.1.2. Treatment of obesity

A combination of lifestyle, behavioral, and dietary changes is required to lose weight due to the complexity of weight control. Individuals should match the calories consumed with energy expended to maintain a healthy body weight. The general energy

requirements range from 1,600 to 2,400 calories per day for adult women and 2,000 to 3,000 calories per day for adult men [133]. In addition to calorie control and regular exercise, antiobesity drugs will enhance the effects of weight loss. Currently, sympathomimetic drugs and lipase inhibitors are the only two types of obesity medications that are approved by FDA [133]. Sympathomimetic drugs include Diethylpropion (Tenuate), Phendimetrazine (Bontril, Plegine), and Benzhetamine (Didrex) and they are only approved for short-term use (12 weeks) [133]. Orlistat (Xenical), a lipase inhibitor, is the only weight-loss medication that can be used for a long-term treatment [133]. It can inhibit the absorption for up to 30% of dietary fat at the recommended dose [133].

For people with severe obesity and chronic health conditions, bariatric surgery is the only intervention to induce significant weight loss [146]. Most bariatric surgery patients have experienced a number of attempts to lose weight by using nonsurgical methods [147]. Bariatric surgery procedures are categorized as malabsorptive, restrictive, and a combination of the two. Malabsorptive procedures reduce the food digestion and nutrient absorption of the stomach and the small intestine, which results in malabsorption [133].

## 2.2. Diabetes

### 2.2.1. Definition of diabetes mellitus

Until 2010, diabetes affects 25.8 million people, 8.3% of the U.S. population, including both diagnosed and undiagnosed people [148]. Both prevalence and incidence of diabetes are still increasing. Diabetes mellitus (DM) is characterized by abnormally

high levels of glucose in the blood due to deficiency of insulin secretion, or the resistance of the cells to the action of insulin, or to a combination of both [149]. The National Diabetes Data Group (NDDG) and the WHO classification system distinguishes four forms of DM, insulin-dependent DM/type 1 DM (T1DM), non-insulin-dependent DM/T2DM, other types of DM, and gestational DM [149].

T1DM, formerly termed juvenile-onset DM, is mostly common among children and adolescents. T1DM is the result of β-cell destruction that leads to total loss of insulin secretion [149]. Patients with T1DM are dependent on exogenous insulin to sustain life. T2DM results from insulin resistance in the muscle, liver, and adipose tissue and eventually defects in insulin secretion. T2DM is typically unrecognized for years because of the lack of symptoms [149]. The causes of other types of DM include pancreatic disease, insulin receptor abnormalities, and pancreatic injury from drugs or chemicals [149]. Gestational DM is characterized by hyperglycemia that occurs in pregnancy [149]. The diagnostic criteria for DM are shown in Table 1-3.

**Table 1- 3 Diagnostic criteria for DM**

| **Diabetes** |
| --- |
| Symptoms of diabetes and casual plasma glucose ≥ 200 mg/dL |
| Fasting plasma glucose ≥ 126 mg/dL |
| Plasma glucose ≥ 200 mg/dL at 2 hr after a 75-g oral glucose challenge |
| **Pre-diabetes** |
| Fasting plasma glucose 110-126 mg/dL |
| **Normal** |
| Fasting plasma glucose < 110 mg/dL |

### 2.2.2. The link of obesity and T2DM

T2DM accounts for approximately 90% of cases in the DM syndrome. Both genetic factors and environmental factors contribute to the development of T2DM. More than 80% of patients with T2DM are obese, but only 10% of obese subjects are diabetic. Increased food intake and decreased fat oxidation cause accumulation of TG in adipose tissues and the development of obesity. The TG storage capacity of fat cells becomes limited due to excessive accumulation of TG in them. This leads to an increase in plasma levels of TG and free fatty acid (FFA). Elevated plasma lipid levels lead to an increase of FFAs in liver and muscle cells, which inhibits the action of insulin in the skeletal muscle and liver, and causes insulin resistance and hyperinsulinemia. Impaired β-cell function is unable to compensate for insulin resistance, causing onset of T2DM [149]. Insulin

secretion eventually declines in obese subjects due to genetic reasons, which results in hyperglycemia and development of overt diabetes.

### 2.2.3. Treatment of T2DM

Dietary interventions, drugs, and surgical procedures have been used to treat T2DM. Reducing the size and increasing the frequency of carbohydrate feedings has been shown acutely to result in lower mean blood glucose and insulin levels over the day with the mechanism of slowing the rate of carbohydrate absorption [149]. Drugs, such as Metformin and Troglitazone, are the first-line drugs for the treatment of T2DM through activation of AMP-activated protein kinase (AMPK) [150] and nuclear receptor peroxisome proliferator-activated receptors (PPARs) [151], respectively. Bariatric surgery procedures have been shown to completely eliminate symptoms of associated with T2DM in human subjects [152]. New drugs have been consistently tested and pathways have been consistently explored for the intervention of T2DM. It has been shown that losing 10% of initial body weight can alleviate the symptoms of those diabetic subjects [153]. It seems to be so obvious that over consumption of nutrients contributes to the development of T2DM in human subjects. However, the roles of each micronutrient in development and treatment of T2DM have not been studied extensively.

## 2.3. Insulin resistance

### 2.3.1. Insulin secretion

It has been known that the balance between insulin and glucagon, both secreted by pancreatic islets, regulates glucose homeostasis. After a meal containing carbohydrate, elevated blood glucose level stimulates pancreatic β-cells are to secret insulin to promote

glucose uptake and inhibit glucose release by the liver. When the blood glucose drops to a low level, glucagon is secreted from pancreas to increase blood glucose via the stimulation of gluconeogenesis. Glucose-stimulated insulin secretion (GSIS) is critical to maintain normal glucose levels and the impairment of GSIS is a key feature in T2DM [154]. Insulin secretion is also regulated by factors other than glucose. Physiologically elevated plasma FFAs potentiate GSIS resulting in elevated circulating insulin levels [155]. The insulinotropic effect increases and decreases with chain length and degree of unsaturation of FFAs, respectively [156]. Impaired GSIS was observed in VA deficient rats, which was improved with repletion [157]. All-*trans* RA has been shown to potentiate GSIS in insulin secreting INS-1 cells [158].

### 2.3.2. Insulin resistance

One common characteristic of obesity and T2DM is insulin resistance. Insulin resistance is the state in which cells are unable to response to the stimulation of insulin effectively leading to hyperglycemia. It has been known that insulin resistance is associated with increases in body weight. Reduction of body weight enhances insulin sensitivity in overweight/obese population and decreases the incidence of T2DM [159, 160]. Additionally, variations in macronutrient content is also a potent factor affecting insulin action [161]. Consumption of high fat diet (HFD) impairs glucose uptake by muscle cells, which attributes to impaired GLUT4 translocation [162]. Elevated plasma FFAs has been considered the factor responsible for HFD-induced insulin resistance [163].

### 2.3.3. Insulin signal transduction

The active form of insulin consists of two chains that are linked by two disulfide bonds: the A chain contains an intrachain disulfide bridge and the B chain has a central helix [164]. Insulin receptor (IR) consists of two extracellular α-subunits and two intracellular β-subunits with tyrosine-kinase activity [164]. Insulin binds to the α-subunit of IR and activates the tyrosine-kinase activity of the β-subunit that selectively catalyzes the phosphorylation of itself and IR substrates (IRSs), which initiates the signal transduction [165-168]. Insulin signaling is transduced by multiple components in a complex network containing cascades of kinases and phosphatases [169, 170]. The insulin signaling event is further amplified *via* phosphatidylinositide 3-kinase (PI3K)/protein kinase B (PKB/Akt) pathway, and GRB2/mitogen-activated protein kinase (MAPK) pathway [171-173]. Another serine/threoine kinase that can be activated by PI3K is the atypical protein kinase C [174]. Human liver expresses several protein-tyrosine phosphatases (PTP) [175]. These components are shared by insulin-like growth factor (IGF) receptor and involved in mediating insulin and IGF signals [176].

## 2.4. Insulin-mediated hepatic gene expression

Insulin controls the expression of a variety of genes involved in glucose and lipid metabolism [177], including glycolysis, glycogenesis, gluconeogenesis, and lipogenesis. Insulin induces the expression of glucokinase gene (*Gck*) [178], the enzyme responsible for the first step of hepatic glycolysis, and suppresses the expression of phosphoenolpyruvate carboxykinase gene (*Pck1*) and glucose 6-phosphatase catalytic subunit (*G6pc*), the enzymes involved in the first and last steps of gluconeogenesis,

respectively [179]. Insulin also increases the expression levels of sterol regulatory element-binding protein 1c gene (*Srebp-1c*), a family member of SREBPs that regulate the hepatic cholesterol and fatty acid biosynthesis [180]. SREBPs induce the expression of fatty acid synthase gene (*Fas*) in hepatocytes and other tissue culture cells [181].

### 2.4.1. Glucokinase (*Gck*)

Hexokinases, found in different organisms ranging from bacteria to humans, are a family of enzymes that catalyze the initial phosphorylation of glucose to glucose-6-phosphate (G6P) for the utilization of glucose in cells [182]. The tissue-specific isoenzyme forms of hexokinases catalyzing the same reaction differ in kinetics, regulatory mechanisms, and tissue localization [21]. Hexokinase II, in the skeletal muscle and other peripheral tissues, is coordinated with GLUT4 that specifically transports glucose in response to insulin stimulation to maintain a balance between glucose uptake and glucose phosphorylation [21]. Hexokinase IV, also known as glucokinase (GK), is the predominant hexokinase in both the liver and pancreatic β-cells. GK has a high $K_m$ for glucose (10 mmol/L) and its activity is linked to GLUT2, a high $K_m$ glucose transporter [21]. In the liver, GK serves as an insulin-dependent modulator of glucose utilization. During the period of rising glucose levels after a carbohydrate-containing meal, the increase of GK activity allows the phosphorylation of glucose thus maintaining a glucose concentration deficit for its entry. During the period of fasting, GK activity falls because of the declined insulin levels, thus reducing the glucose usage in the liver. In pancreatic β-cells, GK functions as a glucose sensor that controls the rate of insulin

secretion from β-cells. GK activity in pancreatic beta-cells is not affected by insulin, but by glucose levels [183, 184].

The expression of GK is encoded by a single gene with two distinct cell-specific promoters: an upstream promoter regulates transcription of *Gck* in β-cells and certain neurons, and a downstream promoter controls transcription of *Gck* in the liver [178, 185]. The resulted GK proteins differ in amino acids encoded by their first exons. Thus, different *Gck* mRNAs are produced in these two tissues, resulting in two distinct GK isoforms. Therefore, they have specific tissue responses to hormonal and nutritional stimuli: β-cell GK activity is stimulated by glucose, whereas hepatic GK is induced by insulin and suppressed by cyclic AMP (cAMP) [178]. Short-term control of GK activity is regulated through binding to its regulatory protein [186], phosphorylation by protein kinase A [187], or interaction with a cytosolic dual specificity phosphatase [188]. Long-term regulation of GK activity is through transcription of its mRNA. Multiple transcriptional factor binding sites have been identified at the hepatic *Gck* promoter [189-191]. SREBP-1c has been considered as the major mediator of insulin-induced *Gck* expression in hepatocytes [192]. However, recent studies from our lab and others have demonstrated that insulin-induced *Gck* expression occurs in a SREBP-1c independent manner [193].

Mutations that alter the enzymatic activity of GK can result in maturity onset diabetes of the young (MODY) [194]. Transgenic knock-outs of *Gck* revealed that both β-cell GK and hepatic GK were essential for glucose homeostasis [195, 196]. Expression of GK in transgenic animals resulted in improved blood glucose and enhanced glucose

metabolism [197, 198]. Allosteric GK activators (GKAs) were demonstrated to increase the glucose affinity and maximum velocity (Vmax) of GK to augment GSIS and hepatic glucose metabolism [199]. Small molecule GKAs were reported to stimulate hepatic glucose metabolism by a mechanism independent of GK regulatory protein [200]. Therefore, GK is critical for the hepatic glucose metabolism and is a potential target for the treatment of diabetes.

### 2.4.2. The cytosolic form of phosphoenolpyruvate carboxyl kinase (PEPCK-C, *Pck1*)

PEPCK catalyzes from the conversion of oxaloacetate to phosphoenolpyruvate, and PEPCK-C is essential for the hepatic gluconeogenesis [21]. The expression of the PEPCK-C gene (*Pck1)* can be detected in the liver, kidney, and adipose tissues [201]. A single gene encodes the cytosolic form of PEPCK with one promoter directing transcription of the gene, which is composed of a number of DNA response elements that are regulated by nutrients, hormones, and tissue-specific transcription factors [201]. The regulation of *Pck1* gene expression is controlled by nutritional and hormonal stimuli [202]. It has been known that glucagon (acting via cAMP) and glucocorticoids induce *Pck1* gene expression [203, 204], whereas insulin inhibits its expression [205]. The regulation of *Pck1* transcription by insulin is probably by PI3K [206]. Fasting or starvation induces *Pck1* mRNA levels while refeeding of a high-carbohydrate diet reduces its mRNA levels [207].

Transgenic mice over-expressing *Pck1* developed insulin resistance and T2DM [208, 209]. Mice with the liver-specific deletion of *Pck1* can preserve euglycemia during

starvation by increasing extrahepatic gluconeogenesis [210] but developed hepatic steatosis [211]. Mice with homologous deletion of *Pck1* died with profound hypoglycemia and changes in lipid and amino acid metabolism were detected prior to death [212]. These results suggest that PEPCK-C is not only a regulator in hepatic gluconeogenesis but also impacts lipid metabolism.

### 2.4.3. Sterol regulatory element-binding protein 1 (*Srebp-1c*)

Insulin is required in hepatic utilization of glucose for lipogenesis in both normal and diabetic rats [213]. Feeding a high-carbohydrate, fat-free diet to fasted rats caused an increase in the quantity of enzymes involved in fatty acid synthesis, such as acetyl-CoA carboxylase (ACC) [214] and FAS [215]. Alloxan diabetic rats and poracaval-shunted rats had reduced levels of FAS in the liver while it returned to the normal range upon treatment of insulin [215]. The action of insulin on ACC and FAS is attributed to the changes at the mRNA level [216], which is regulated by two transcription factors, SREBP-1c and carbohydrate response element-binding protein (ChREBP) [217]. SREBPs play critical roles in lipid homeostasis due to their stimulation of the expression of more than 30 genes dedicated to the synthesis of cholesterol, fatty acids, and triacylglycerol [180]. SREBPs are generated as inactive precursors in the membrane of the endoplasmic reticulum (ER). The $NH_2$-terminal domain of each SREBPs should be released from the membrane to reach the nucleus and activate target genes [180]. During this process, SREBP cleavage-activating protein (SCAP) escorts the SREBP from the ER to the Golgi apparatus, where the two proteases, S1P and S2P, cleave the SREBP to release the $NH_2$-terminus [180]. SREBP has three isoforms, SREBP-1a, SREBP-1c, and

SREBP-2. Both SREBP-1a and -1c are derived from a single gene. SREBP-1c is a transcriptional factor for multiple genes for fatty acid synthesis, including ACC and FAS [181] while SREBP-2 preferentially activates cholesterol synthesis [192].

Studies have shown that the hepatic expression of SREBP-1c is stimulated by insulin and LXR ligands [218], and repressed by glucagon [219]. The effects of insulin on SREBP-1c are through enhancing transcription, increasing mobilization to the nucleus, and activating transcriptional activity [192]. Insulin responsive elements (IREs) in the *Srebp-1c* promoter are identified as two LXR binding elements (LXRE) and one sterol regulatory element (SRE) [220]. Maximal activation of *Srebp-1c* expression by insulin also requires specificity protein 1 (Sp1) to interact with other transcriptional factors [221]. PPAR binding element has been identified in the promoter of SREBP-1c and PPARα agonists act in cooperation with LXR or insulin to induce lipogenesis [222].

Overexpression of SREBP-1c can mimic insulin effects on gene expression in hepatocytes [223]. Deletion of *Srebp-1c* severely impaired the induction of hepatic fatty acid synthetic genes in response to a cycle of fasting and refeeding [224]. Deletion of *Srebp-1c* in the liver diminished hepatic response to a fasting and refeeding cycle and LXR agonists [225]. All these studies demonstrate the importance of SREBP-1c in hepatic fatty acid synthesis. Evidence indicates that obesity is associated with increased hepatic lipogenesis [226] and the fatty liver of insulin resistant animals is accompanied by elevation of the expression of SREBP-1c [180]. Insulin selectively increases *Srebp-1c* mRNA in the livers of diabetic rats induced by the treatment of streptozotocin (STZ) [227]. Continuous activation of SREBP-1c transcription by insulin increases lipogenic

gene expression and further enhances fatty acid synthesis [228]. Insulin-independent signaling pathways can partially compensate for insulin in the induction of SREBP-1c by feeding, but further induction by obesity or T2DM is entirely dependent on insulin [229].

## 2.5. Effects of VA on metabolism and the hepatic insulin-mediated gene expression

In recent years, researchers have focused on the roles of VA status in the development of metabolic diseases. The importance of retinoid catabolism in hepatic glucose and lipid metabolism has gradually become more apparent. As early as 1937, when VA reserve in the liver was analyzed, it was observed that its level was elevated in patients with diabetes [230]. The loss of the carcass fat was observed in VA deficient (VAD) rats [231], but cholesterol content was not affected by VA status [232]. Feeding rats with all-*trans* and 13-*cis* RA induced hypertriglyceridemia, suggesting the roles of retinoids in the regulation of plasma TG clearance or secretion from the liver [233]. Patients who received isotretinoin (13-*cis* RA) for the treatment of their acne developed isotretinoin-induced hypertriglyceridemia [234]. Systemic therapy with tretinoin (all-*trans* RA) for human also caused massive hypertriglyceridemia [82].

On the other hand, all-*trans* RA treatment in mice was observed to effectively reduce the body weight, which was probably due to decreased expression of genes involved in hepatic lipogenesis [235] and remodeling of white adipose tissue [236]. Additionally, all-*trans* RA has been shown to repress obesity and insulin resistance by activating PPARβ/δ and RAR [237]. Recently, RA was reported to inhibit adipogenesis by activating the CRABP-II/RARγ pathway in preadipose cells [238]. All these

observations demonstrated the relationship between VA and glucose and lipid metabolism.

It has been reported that all-*trans* RA induces the *Gck* expression in rat hepatocytes in the absence of insulin [239]. Recently, we have reported that retinoids synergize with insulin to induce *Gck* expression [240]. RA was reported to increase the rate of transcription of *Pck1* in primary hepatocytes with RAREs located in the *Pck1* promoter [241, 242]. We reported that retinoids induce *Pck1* expression and attenuate insulin-mediated suppression of its expression in primary rat hepatocytes [243].

VA status affects obesity development in Zucker fatty (ZF) rats and VAD rats had reduced plasma TG levels and decreased *Srebp1-c* mRNA levels [244]. RA synergizes with insulin to induce the expression of *Srebp1-c* in primary rat hepatocytes through the activation of RXR and previously identified LXREs in the *Srebp-1c* promoter are also RAREs [245]. Thus, further studies should be done to investigate the effects of RA on insulin-mediated expression of genes that are involved in hepatic glucose and lipid metabolism.

In addition to RA, the enzymes involved in RA biosynthesis are also observed to participate in lipid metabolism. The *Raldh1* knockout mice are resistant DIO and insulin resistance [56], which may attribute to the elevated retinal contents in adipose tissues that suppress the adipogenesis via retinal-inhibited activation of PPARγ and RXR [56]. RALDH1 was also observed to be up-regulated in both the kidney and the liver of diabetic mice despite decreased RA levels in association with a decrease in PPAR β/δ [57]. Feeding mice with high cholesterol diet stimulated the hepatic expression of

RALDH1 and RALDH2 through oxysterol-induced *Srebp-1c* expression, indicating the interaction of cholesterol and RA metabolism [65]. All these observations indicate the potential roles of these enzymes in the development of obesity and insulin resistance, which deserves to be investigated.

Thus, we hypothesize that VA contributes to the regulation of expression of hepatic genes in glucose and lipid metabolism. Enzymes involved in RA biosynthesis may also contribute to the regulation through the control of RA production. Whether the dietary VA from food or the stored VA in the liver plays the major role in the regulation of gene expression deserves to be investigated

# Chapter Two

## Elevated hepatic *Raldh1* expression in Zucker fatty rats and its over-expression introduced retinal-induced *Srebp-1c* expression in INS-1 cells

### 1. Introduction

The high obesity prevalence in the population of the United States [246] is predictive for type 2 diabetes mellitus (T2DM) [247], a major public health concern [248]. Genetic mutations, such as mutations of leptin and its receptor, have been shown to cause the development of obesity and diabetes [249]. Currently, the associations of a variety of genes with the development of human obesity or T2DM have been identified [250, 251]. Metabolic abnormalities are often associated with profound changes of hepatic glucose and lipid metabolism [252], which are attributed, at least in part, to the expression of genes involved in these processes [228, 253]. On the other hand, overconsumption of nutrients, such as fructose in sweetened beverages, has also been implied to play a role in the rise of obesity [138]. Dietary nutrients provide not only energy, but also vitamins and other essential factors with regulatory roles. The effects of individual micronutrients on the development of metabolic diseases remain to be revealed.

As an essential and fat-soluble micronutrient, vitamin A (VA, retinol) plays crucial roles in the general health of an individual, such as vision, tissue differentiation, and immunity [254]. The majority of the physiological actions of retinol are mediated by its active metabolite, retinoic acid (RA), which exists in multiple isomeric forms, such as all-*trans* RA and 9-*cis* RA [255]. RA regulates gene expression through the activation of

two families of nuclear receptors, retinoic acid receptors (RARα, β and γ) activated by all-*trans* RA, and retinoid X receptors (RXRα, β and γ) activated by 9-*cis* RA [256].

In an attempt to understand the effects of endogenous lipophilic molecules on the insulin-regulated gene expression in primary rat hepatocytes, we have analyzed the rat liver lipophilic extract and found that retinol and retinal in it dose-dependently induced the mRNA expression levels of *Pck1* [243], *Gck* [240], and *Srebp-1c* [245]. The proximal one of the two previously identified retinoic acid response elements (RAREs) in *Pck1* promoter [241, 257, 258] mediates the RA effect in primary hepatocytes. The previously identified two liver X receptor (LXR) binding sites mediating the insulin-stimulated *Srebp-1c* expression [220] were also RAREs in its promoter [245], demonstrating the convergence of hormonal and nutritional responses. The hepatic expression level of cannabinoid receptor 1 (CB1), the target of anti-obesity drug Rimonabant [259] can also be induced by the treatment of RA via activation of RAR-γ [260], indicating the connection between the endocannabinoid pathways and the nutritional signals. The effects of VA status on glucose and lipid metabolism have been summarized in details [111].

Retinol is reversibly oxidized into retinal by retinol dehydrogenases (RDHs). Retinal is irreversibly oxidized into RA by retinaldehyde (aldehyde) dehydrogenases (RALDHs) [256, 261, 262]. Currently, four RALDHs have been cloned and thought to be responsible for all-*trans* or 9-*cis* RA production in various tissues [49, 51, 66, 263]. RALDH1-4 proteins can be detected in some but not all hepatocytes of the mouse liver using immunohistochemistry, with RALDH1 being frequently expressed in lipid-

engorged cells [51]. *Raldh1* (also known as *Aldh1a1*) mRNA is expressed weakly in the rat liver [263], whereas *Raldh4* (also known as *Aldh8a1*) mRNA is expressed at high level in the mouse liver [51]. The mice with *Raldh1* deletion (*Raldh1*-/-) have reduced RA production in the liver 2 hr after a challenge of retinol [264]. The *Raldh1*-/- mice are resistant to diet-induced obesity (DIO) and insulin resistance [56]. The Imprinting Control Region (ICR) mice fed a high cholesterol diet had elevation of the hepatic expression of RALDH1 and RALDH2 through oxysterol-induced *Srebp-1c* expression, demonstrating the interaction of cholesterol and RA metabolism [65]. All these observations indicate the potential roles of these enzymes in the development of obesity and insulin resistance, which deserves to be further defined.

The fact that the levels of the enzymes for the hepatic retinoid metabolism can be dynamically regulated leads us to hypothesize that the change of the expression of these enzymes and the production of RA may contribute to the regulation of the expression of hepatic lipogenic genes. Here, we examined the effects of retinol and retinal on the expression of RA responsive genes in primary rat hepatocytes, rat hepatoma cells and insulinoma INS-1 cells. We observed the elevated expression levels of *Raldh1* mRNA in hepatocytes and its protein in the liver of Zucker fatty (ZF) rats. The recombinant adenovirus-mediated over-expression of RALDH1 resulted in retinal-mediated induction of *Srebp-1c* expression in INS-1 cells, which originally lack the response to retinal.

## 2. Materials and Methods

### 2.1. Reagents

The reagents for primary hepatocyte isolation and culture have been published previously [265]. Source of LG268 was reported previously as well [240]. All other compounds were purchased from Sigma (St. Louis, MO) unless described otherwise. The reagents for cDNA synthesis and real-time PCR were obtained from Applied Biosystems (Foster city, CA). Medium 199, liver perfusion medium and liver digest buffer were obtained from Invitrogen (now Life Technologies, Grand Island, NY 14072). Dulbecco's Modification of Eagle Medium (DMEM), RPI-1640 medium, fetal bovine serum, antibiotics, and trypsin-EDTA solution were obtained from Fisher Scientific (Pittsburgh, PA 15275).

### 2.2. Animals

Male Sprague-Dawley (SD) rats were purchased from Harlan Breeders (Indianapolis, IN). Male Zucker lean (ZL) and ZF rats were bred at UTK. Rats were housed in colony cages, and fed a standard rodent diet before isolation of primary hepatocytes. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Tennessee at Knoxville (Protocol numbers 1642 and 1582).

### 2.3. Cultures of primary rat hepatocytes, HL1C rat hepatoma cells, 293 human embryonic kidney cells, and INS-1 rat insulinoma cells

Methods for the primary rat hepatocyte preparation and analysis of RNA were described previously [265]. For the hepatocyte isolation and pretreatment, the rats were

euthanized with carbon dioxide. A catheter was inserted into portal vein and connected to a peristaltic pump with liver perfusion medium and liver digestive buffer. The inferior vena cava was cut open to allow the outflow of the media at 10 ml/min. After completion of the perfusion, the liver was excised and put into a tissue culture dish containing liver digestive buffer for removing the connection tissues and allowing the release of hepatocytes. The medium containing hepatocytes was filtered through a cell strainer (100 μm), and precipitated by 50×g centrifugation for 3 min. The cell pellet was washed twice with DMEM containing 5% fetal bovine serum (FBS), 100 U/ml sodium penicillin, and 100 μg/ml streptomycin sulfate after re-suspension and precipitation. After the wash, the isolated hepatocytes were plated onto 60 mm collagen type I coated dishes at $3\times10^6$ cells/ per dish and incubated in 4 ml of the same medium at 37°C and 5% CO2 for 3 to 4 hr. The attached cells were washed once with 4 ml of PBS, and incubated in medium A (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) containing 1 nM insulin for overnight (14–16 hr) until being used for the indicated experiments.

The HL1C cells [266] which was stably transfected with a reporter gene construct containing a fragment of *Pck1* promoter sequence were kindly provided by Dr. Donald K. Scott at University of Pittsburgh. They were maintained in DMEM containing 4.5 g/L glucose, 4% FBS, 100 U/ml of penicillin and 100 μg/ml streptomycin sulfate. They were incubated in 60 mm dishes with serum free DMEM containing the indicated reagents shown in the figure legends for the indicated length of time before being subjected to analysis.

The 833/15 and 834/40 clonal insulinoma INS-1 cells [267] were cultured in medium B (RPMI 1640, 2 mM L-glutamine, 1 mM Na-pyruvate, 50 μM β-mercaptoethanol, 100 U/ml of penicillin and 100 μg/ml streptomycin sulfate) containing 10% FBS as described previously. For the treatment, INS-1 cells in 60 mm dishes were incubated in medium B containing the indicated reagents shown in the figure legends for the indicated length of time before being subjected to analysis.

The HEK293 cells were cultured in DMEM containing 4.5 g/L glucose, 8% FBS, 100 U/ml of penicillin and 100 μg/ml streptomycin sulfate at 37ºC and 5% $CO_2$.

**2.4. RNA extraction and quantitative real-time PCR**

Total RNA was extracted from the treated cells on one 60 mm dish using 1 ml of RNA STAT 60 reagent (TEL-TEST, Inc, Friendswood, TX) according to the manufacture's protocol. The contaminated DNA was removed using the DNA-*free*™ kit (Applied Biosystems). First strand cDNA was synthesized from 2 μg of DNA-free RNA with random hexamer primers using cDNA synthesis kit (Applied Biosystems). The real-time PCR primer sequences for detecting *Cyp26a1* [268], *Srebp-1c* [245], and *Raldh1* [269] have been reported previously. The primers for detecting *Rdh2* (sense 5'-CAAGTTCTTCTACCTCCCCATGA-3', and anti-sense 5'-TCCAGTAGAAAAGGGCATCCA-3') were designed using software Primer Express 3.0 (Applied Biosystems). Each SYBR green based real-time PCR reaction contains, in a final volume of 14 μl, cDNA from 14 ng of reverse transcribed total RNA, 2.33 pmol primers, and 7 μl of 2× SYBR Green PCR Master Mix (Applied Biosystems). Triplicate PCR reactions were carried out in 96-well plates using 7300 Real-Time PCR System. The

conditions are 50ºC for 2 min, 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 seconds (s) and 60ºC for 1 min. The gene expression level was normalized to that of invariable control gene, *36B4*. Data are presented as either minus Δ cycle threshold $C_T$ [270] or the induction fold for which the control group is arbitrarily assigned a value of 1 using $\Delta\Delta C_T$ method as previously described [265].

## 2.5. Cloning of the rat *Raldh1* cDNA and generating Ad-Raldh1 recombinant adenovirus for the over-expression of RALDH1

To clone the rat *Raldh1* cDNA, sense 5'-AGCCAAACCAGCAATGTCTTC-3', and anti-sense 5'-CACTCTGCTTCTTAGGAGTTC-3' primers were designed based on its mRNA sequence (reference number NM_022407.3) from the National Center for Biotechnology Information. The *Raldh1* cDNA containing the entire coding sequence was amplified using SD rat primary hepatocyte cDNA as a template. The amplicon was ligated into pCR®2.1 vector through TA Cloning® Kit (Invitrogen) and processed according to the manufacture's protocol. The full-length cDNA with the correct sequence confirmed by DNA sequencing was inserted to pACCMV5 for making pACCMV5-Raldh1.

To generate Ad-Raldh1 recombinant adenovirus, plasmids pACCMV5-Raldh1 and JM17 were co-transfected into 293 cells using FuGENE®6 Transfection Reagent (Roche Applied Science, Indianapolis, IN). Transfected 293 cells were incubated in DMEM containing 2% FBS at 37ºC and 5% $CO_2$ until the formation of the plaques due to the lysis of the cells. The crude lysate was collected and stored at -80ºC. The presence of *Raldh1* cDNA sequence in the viral DNA was confirmed by PCR.

## 2.6. Preparation and purification of recombinant adenoviruses

To generate the crude lysate, HEK 293 cells grew in 100 mm tissue culture plates at 80% confluence were infected by the original crude lysate containing Ad-Raldh1. The ratio of the medium to crude lysate is 10 to 1. After the lysis of the cells at around 48 hr post infection, the cell culture medium (the crude lysate) was collected, and stored at -80ºC until being used.

For purification of the 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 for 30 min and subjected to centrifugation at 8,000 rpm and 4ºC for 15 min. The supernatant was transferred to a clean bottle, and 0.5× volume of 20% PEG8000/2.5 M NaCl was added. The preparation was shaken gently at 4ºC overnight. The resulting mixture was transferred to centrifuge bottles and spun at 13,000 rpm at 4ºC for 15 min. The precipitated pellet was re-suspended in a small volume of PBS (2-3 ml), and spun at 8,000 rpm and 4ºC 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 and 25ºC for 3 hr using Optima™ MAX-XP Ultracentrifuge (Beckman Coulter, Inc.). The corresponding band containing pure viral particles was collected in a total volume less than 1 ml for desalting. The PD-10 column Sephadex™ G-25M (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 densities (OD) at 260 nm of these fractions were determined using Spectronic® GENESYS™ 5 Spectrophotometer

(Thermo Scientific). The fractions containing significant values of absorbance (usually at around fractions 7-9) were collected and pooled. Bovine serum albumin 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*). We used that 1 OD equals to $1\times10^{12}$ *pfu*/ml. The final purified virus stock was frozen at -80ºC until being used in the indicated experiments.

### 2.7. Immuno-blot and quantification of the protein bands

INS-1 cells were infected with purified Ad-Raldh1 for 24 hr. The 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-glycerophsphate, 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). Proteins (40 μg/lane) in whole cell lysates were separated on SDS-PAGE, transferred to BIO-RAD Immuno-Blot PVDF membrane (Hercules, CA) and detected with primary antibodies to RALDH1 (for Figure 2-5, catalog #2052-1, Epitomics, Burlingame, CA 94010), RALDH1 (for Figure 2-1, catalog #AP1465a, Abgent, San Diego, CA 92121), CYP26A1 (catalog # CYP26A11-A, Alpha Diagnostics International, TX 78244), and β-Actin (#4970s, Cell Signaling Technology, Danvers, MA 01923)

according to the protocols provided by the manufacturers. Bound primary antibodies were visualized by chemiluminescence (ECL Western Blotting Substrate, Thermo Scientific) using a 1:2,000 dilution of goat anti-rabbit IgG (#7074P2, Cell signaling Technology) conjugated to horseradish peroxidase. 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). The ImageJ Software (http://rsbweb.nih.gov/ij/) was used to determine the density of a protein band by subtracting the background in an area of the same size immediately in front of protein band. The ratio of the densities of RALDH1 and β-Actin in the same sample was calculated and used for statistical analysis.

### 2.8. Statistics

Data are presented as means ± S.D. The number of experiments represents the independent experiments using hepatocytes isolated from different animals or indicated cells cultured in different days. Levene's test was used to determined homogeneity of variance among groups using SPSS 19.0 statistical software and where necessary natural log transformation was performed before analysis. An independent-samples t-test was used to compare two conditions. 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. Differences were considered statistically significant at $P < 0.05$.

## 3. Results

### 3.1. Determination of the mRNA levels of enzymes responsible for the retinoids catabolism in primary hepatocytes, and the elevated expression of *Raldh1* and its protein in hepatocytes of ZF rats

Enzymatic processes are involved in the conversions of retinol to retinal and retinal to RA [256, 261, 262]. It has been shown that the short chain dehydrogenase/reductase family 16C member 5 (SDR16C5), RDH2, RDH10, and RALDH1-4 are expressed in the liver [271]. Since RALDH4 is for the biosynthesis of 9-*cis* RA [51], we have focused on the expression levels of the rest of the enzymes in this study. The $C_T$ numbers of these genes were normalized to those of 36B4 (an invariable control gene) in the same samples. Based on $-\Delta C_T$ numbers (how close the abundance of the transcripts of those genes to that of 36B4), we found that *Rdh2,* and *Raldh1* were expressed in meaningful levels (compared with the expression level of 36B4) in both primary rat hepatocytes and HL1C cells (data not shown).

Since RDH2 catalyzes the conversion of retinol to retinal, and RALDH1 is reponsible for converting retinal to RA [271], we compared their expression levels in freshly isolated primary hepatocytes of ZL rats with those of ZF rats. The mRNA level of *Rdh2* in hepatocytes from ZF rats was similar to that from ZL rats. However, the mRNA level of *Raldh1* in hepatocytes from ZF rats was significantly higher than that from ZL rats (Figure 2-1A). The results observed in cultured primary hepatocytes of ZL and ZF rats were similar to that in fresh isolated hepatocytes. We observed that the *Rdh2* expression level was unchanged, and the *Raldh1* expression level was elevated

significantly in hepatocytes from ZF rats (Figure 2-1B). The mRNA level of *Raldh4* was detected, and was at the similar levels in hepatocytes from both ZL and ZF (data not shown).

Figure 2-1C shows that the RALDH1 protein levels in the liver samples of ZF rats were higher than that of ZL rats. Since two protein bands migrating close to each other were detected in the Immuno-blot, the lysate of INS-1 cells infected by recombinant adenovirus Ad-Raldh1 expressing RALDH1 protein was used as the control to determine the correct size of it. Based on the size of the control sample, the lower band detected by the antibody should be the correct one in the liver samples. Their densities were normalized to the densities of the β-Actin in the same samples. The quantified data were presented as the ratios of RALDH1/β-Actin as shown in Figure 2-1C. The ratios of ZF rats are significantly higher than that of ZL rats, demonstrating that the elevation of *Raldh1* mRNA corresponds with an induction of RALDH1 protein.

### 3.2. Retinoids induced the expression levels of *Cyp26a1* mRNA in primary rat hepatocytes

To demonstrate that retinoids catabolism can contribute to the regulation of gene expression in primary rat hepatocytes, the expression levels of *Cyp26a1* mRNA (a RA responsive gene) in response to retinol (ROL) and retinal (RAL) treatments were determined using real-time PCR. Figure 2-2A shows a 24-hour time course of the expression levels of *Cyp26a1* mRNA in Sprague-Dawley (SD) rat primary hepatocytes treated with 5 μM RA. More than 1000-fold induction of *Cyp26a1* mRNA level was observed at 3 hr after the RA treatment, the earliest time point checked. The *Cyp26a1*

mRNA level was further induced at 6 hr and maintained elevated at 12 hr after treatment. At 24 hr after the treatment, its level dropped comparing to that at 12 hr, but still remained significantly higher than that at time 0.

To determine the effects of retinol and retinal on the induction of *Cyp26a1* expression, primary rat hepatocytes from SD rats were treated with increasing concentrations of retinol or retinal for 6 hr. As shown in Figure 2-2B, the levels of *Cyp26a1* mRNA were induced by retinol and retinal in a dosage dependent manner. retinol and retinal started to induce *Cyp26a1* expression at 2 μM and 0.02 μM, respectively. When the induction folds of *Cyp26a1* expression in response to retinol and retinal treatments were compared, retinal groups had significantly higher values than retinol groups did at concentrations of 0.02 μM, 0.2 μM, 2 μM, and 20 μM. It suggests that retinal is more readily converted to RA than retinol to induce *Cyp26al* expression.

### 3.3. Inhibition of RAR activation blocked the RAL-mediated induction of *Cyp26a1* expression in primary rat hepatocytes

We have shown that the activation of RAR or/and RXR can induce *Cyp26a1* expression [272]. To confirm the involvement of RAR activation in the RAL-mediated induction of *Cyp26a1* expression, primary SD rat hepatocytes were treated with increasing concentrations of Ro 41-5253, a specific antagonist for RAR activation [273]. As shown in Figure 2-2C, retinal at 2 μM dramatically induced the *Cyp26a1* expression, further confirming the result shown in Figure 2-2B. This induction was dose-dependently inhibited with increasing concentrations of Ro 41-5253. When the concentration of Ro 41-5253 reached 10 μM or higher, the induction of *Cyp26a1* expression by retinal was

completely suppressed. This reverse association between the concentration of Ro 41-5253 and the induction of *Cyp26a1* expression by retinal demonstrated that the inhibition of RAR activation disrupted the retinoid-mediated induction of *Cyp26a1* expression in primary hepatocytes, indicating the important role of RAR activation in this induction.

### 3.4. Retinoids induced the expression levels of *Cyp26a1* mRNA and CYP26A1 protein in HL1C rat hepatoma cells through the activation of RAR and RXR

To screen for cultured cell lines which we can study the effects of retinoid metabolism on the expression of RA responsive gene, we analyzed the expression of *Cyp26a1* mRNA and CYP26A1 protein in response to ROL, retinal and RA treatments in HL1C rat hepatoma cells, a cell line that responds to insulin stimulation [274]. Figure 2-3A shows the expression levels of *Cyp26a1* mRNA in HL1C cells treated with the indicated concentrations of retinoids. ROL, retinal and RA started to induce *Cyp26a1* mRNA expression at 20 μM, 2 μM, and 0.2 μM, respectively, demonstrating the different potencies of these retinoids. At 2 μM or 20 μM, the fold induced by retinal was comparable to that by RA. The highest induction fold of *Cyp26a1* mRNA mediated by RA in HL1C cells was near 300-fold which was much lower than that in primary hepatocytes (1000-fold) as shown in Figure 2-2. The effects of retinal and RA on *Cyp26a1* mRNA expression in HL1C cells over a 24-hour time period were also examined as shown in Figure 2-3B. Both retinal and RA induced *Cyp26a1* mRNA expression as early as 3 hr. At 6 hr after the treatment, the elevated level of *Cyp26a1* mRNA began to drop in comparison to its level at 3 hr. At 9, 12, and 24 hr time points, its

level further declined, but remained significantly higher than that at time 0. The induction was still elevated at 24 hr after treatment.

To examine whether the activation of RAR or RXR was sufficient for the induction of *Cyp26a1* mRNA expression, HL1C cells were treated with TTNPB, LG268, and T1317, agonists for RAR, RXR and LXR activation, respectively. As shown in Figure 2-3C, the induction folds of the TTNPB, LG268 and T1317 groups are 183.0 ± 60.0, 8.0 ± 3.8, and 1.0 ± 0.4. These results demonstrated that the activation of RAR and RXR, but not LXR, induced the *Cyp26a1* mRNA expression in HL1C cells, indicating that RA activated both RAR and RXR to induce *Cyp26a1* mRNA expression. The induction fold of TTNPB group is much higher than that of LG268 group, similar to that observed in primary hepatocytes [245].

Figure 2-3D shows the expression levels of CYP26A1 protein in HL1C cells treated with retinal or RA for 12 and 24 hr. The equal loading of protein samples was confirmed by the similar levels of β-Actin protein among the samples. This result demonstrated that the induction of *Cyp26a1* mRNA resulted in significant elevation of CYP26A1 protein at both time points. All our results suggest that retinol and retinal may be converted to RA, which causes the RAR activation and the subsequent induction of *Cyp26a1* mRNA and CYP26A1 protein levels in HL1C cells.

### 3.5. RA, but not RAL, induced the *Srebp-1c* expression through the activation of RXR, but not RAR, in INS-1 rat insulinoma cells

We have screened the cultured cell lines in which retinal does not induce *Srebp-1c* expression for a tool to study the conversion of retinal to RA, and shown that RA, but

not RAL, induced the *Srebp-1c* expression dose-dependently in 833/15 INS-1 cells [245]. To further confirm that INS-1 cells are an ideal tool to study the conversion of retinal to RA, we analyzed the effects of retinal and RA on *Srebp-1c* expression in 834/40 INS-1 cells, another clonal INS-1 cells [267]. Figure 2-4A shows that RA started to significantly induce the *Srebp-1c* expression at 0.1 μM while retinal did not change its expression at any concentration tested. Figure 2-4B shows the induction of the *Srebp-1c* expression by RA in 834/40 INS-1 cells over a 24-hour time period. RA, but not RAL, induced the *Srebp-1c* expression as early as 3 hr and peaked at 6 hr. At 9, 12, and 24 hr, the induction folds declined in comparison to that at 3 and 6 hr, but still maintained significantly higher than that at 0 hour. These results and the results shown previously [245] indicate that INS-1 cells may lack the enzymatic activities that convert retinal to RA.

To determine whether the activation of RAR or RXR mediates the effects of RA on the *Srebp-1c* expression, INS-1 cells were treated with TTNPB (1 μM), LG268 (1 μM), or TTNPB + LG268 for 6 hr. Figure 2-4C shows that LG268 and LG268 + TTNPB induced the *Srebp-1c* expression while TTNPB alone did not induce it. Thus, RXR activation mediates the RA-induced *Srebp-1c* expression in INS-1 cells. All these results demonstrated that the RA-mediated activation of RXR caused the induction of *Srebp-1c* in INS-1 cells, which makes INS-1 cell lines an ideal tool to study the role of RA production in the regulation of lipogenic gene expression.

### 3.6. The over-expression of RALDH1 resulted in the RAL-mediated induction of *Srebp-1c* in INS-1 cells

To further study the effects of the elevated *Raldh1* mRNA expression in ZF hepatocytes, its full-length cDNA was cloned and inserted it into pACCMV5 for the generation of recombinant adenovirus Ad-Raldh1. As shown in Figure 2-5, INS-1 cells infected with Ad-Raldh1 had the over-expression of *Raldh1* mRNA (Figure 2-5A) and RALDH1 protein (Figure 2-5B). The equal loading of the protein samples was confirmed by the similar levels of β-Actin protein among the samples. In INS-1 cells infected with Ad-Raldh1 for overnight (~18 hr), but not in those infected with Ad-β-gal, retinal started to significantly induce the *Srebp-1c* expression at both 0.3 μM and 1 μM (Figure 2-5C). On the other hand, RA induced the *Srebp-1c* expression in cells infected by either Ad-β-gal or Ad-Raldh1 (Figure 2-5D). In those cells with the over-expression of RALDH1, the induction folds of *Srebp-1c* by retinal treatment were comparable to that by RA. These results indicate that the over-expression of RALDH1 introduced the RAL-mediated induction of *Srebp-1c* in INS-1 cells, indicating the successful conversion of retinal to RA.

## 4. Discussion

In insulin resistant animals, hyperinsulinemia is associated with elevated hepatic gluconeogenesis and lipogenesis, which should be respectively suppressed and stimulated by insulin [275]. Our lab has shown that retinoids regulate the expression levels of genes involved in hepatic glucose and fatty acid metabolism. These regulations contribute to the

modulation of insulin-mediated expression of these genes in primary hepatocytes [240, 243, 245]. The implication of these observations is that the dynamic regulation of RA production may be responsible for hepatic insulin resistance.

To confirm that the treatments of retinol and retinal can regulate gene expression presumably through the production of RA, we analyzed the expression levels of *Cyp26a1*, an RA responsive gene [268], in primary hepatocytes (Figure 2-2). Retinoids induced the expression of *Cyp26a1* in a dose- and time-dependent manner. This induction can be blocked in the presence of RAR antagonist, indicating the requirement of RAR activation. This result and the fact that retinal induced the *Cyp26a1* expression indicate that RA production occurs.

To find the cultured cell lines that can be used as tools for further studies of the retinoid catabolism, we have analyzed the retinoid-mediated gene expression in HL1C rat hepatoma cells (Figure 2-3). Retinol, retinal, and RA also induced the expression levels of *Cyp26a1* mRNA in HL1C cells in a dose- and time-dependent manner, which is similar to the results obtained in primary hepatocytes. This induction is mediated by the activation of RAR and RXR. The induction of *Cyp26a1* mRNA results in the elevated levels of CYP26A1 proteins in HL1C cells. However, the elevated levels of CYP26A1 protein are not as high as that of *Cyp26a1* mRNA at 12 and 24 hr (Figure 2-3B and D), indicating the potential roles of protein translation and/or stability in the determination of the CYP26A1 protein levels in HL1C cells. The maximal induction folds by reintoids are higher in primary hepatocytes than in HL1C cells. Retinol and retinal respectively induce the expression of *Cyp26a1* mRNA at 2 and 0.02 μM in primary hepatocytes and at 20 and

2 μM in HL1C cells. These results indicate that primary hepatocytes seem to be more readily to convert retinol to retinal and then to RA. Alternatively, HL1C cells are more readily to convert retinal to retinol so that less RA is generated. Whether it is true or not deserves further investigation. It also indicates that HL1C cells can be used as a tool to study the metabolism of retinoids and its effects on the regulation of the expression of RA responsive genes.

It has been shown that the short chain dehydrogenase/reductase family 16C member 5 (SDR16C5), RDH2, RDH10, RALDH1-4 are expressed in the liver [271]. Since *Raldh4* mRNA (encoding RALDH4) is for the production of 9-*cis* RA [51], we focused on *Rdh2* and *Raldh1*. Their mRNA levels in both freshly isolated and cultured primary hepatocytes of ZL and ZF rats were compared. The mRNA level of *Raldh1*, but not that of *Rdh2*, is higher in ZF than that in ZL rat hepatocytes. In addition, the hepatic RALDH1 protein level is also higher in ZF rats than in ZL rats (Figure 2-1). The elevated expression levels of *Raldh1* mRNA and RALDH1 protein levels may be resposible for the altered hepatic lipogenesis in ZF rats. To test the hypothesis, we made recombinant adenovirus Ad-Raldh1 to over-express RALDH1 protein in INS-1 rat insulinoma cells. We have shown previously [245] and here (Figure 2-4) that RA, but not RAL, induced *Srebp-1c* expression in INS-1 cells. It seems that INS-1 cells lack the enzymatic activities converting retinal to RA. This makes INS-1 cells an ideal tool to test our hypothesis that the elevated expression of RALDH1 in ZF rats causes the increase of RA production, and in turn, the induction of RA responsive gene expression. After the over-expression of *Raldh1* mRNA and RALDH1 protein in INS-1 cells, retinal started to induce the *Srebp-*

*1c* expression as RA did. This indicates that retinal was oxidized to RA by RALDH1 before the induction of *Srebp-1c*. The elevated levels of hepatic *Raldh1* mRNA and RALDH1 protein in ZF rats may contribute to the regulation of hepatic lipogenesis.

It has been shown that the mRNA level of *Raldh1* is elevated in the kidney of *db/db* mice [57]. Both *db/db* mice and ZF rats develop obesity due to the mutation of leptin receptor [249]. It has been shown that ICR mice fed high cholesterol diet have the elevated expression levels of *Raldh1* and *Raldh2*. This is attributed to the oxysterol-induced expression of SREBP-1c which directly binds to the sterol regulatory response elements (SREs) at the proximal promoter regions of *Raldh1* and *Raldh2*, and induces their expression [65]. Since the expression of *Srebp-1c* is induced by insulin [227] and RA [245] in primary hepatocytes, the regulation of *Raldh1* expression becomes a converge point for a feed-forward mechanism by which VA status regulates lipogenesis in the liver. It is reasonable to hypothesize that in hepatocytes of ZL rats, RA derived from retinol synergizes with insulin to induce the expression of *Srebp-1c* mRNA (Figure 2-6A). The mature SREBP-1c protein supports the expression of RALDH1, which promotes the production of RA to maintain the homeostasis of *Srebp-1c* expression in the liver of ZL rats (Figure 2-6A). ZF rats are hyperphagic due to the defect of leptin receptor, which leads to the development of obesity, insulin resistance, hyperinsulinemia and hyperlipidemia [227, 276]. We have shown that the ZF rat hepatocytes have elevated expression levels of *Srebp-1c* and *Fas* mRNA [270]. We think that the excessive supply of dietary VA caused by hyperphagia and hyperinsulinemia in ZF rats probably work together to trigger an elevation of RA production and an induction of *Srebp-1c*

expression in their hepatocytes, respectively. The elevation of the mature SREBP-1c protein induces the expression of *Raldh1* mRNA, which leads to more RA production, and further enhances the expression levels of *Srebp-1c* and its down-stream lipogenic genes. This creates a feed-forward mechanism by which the *Srebp-1c* expression is maintained at a higher level in the liver of ZF rats (Figure 2-6B). Whether the proposed feed-forward mechanism is true or not, and what mechanism is responsible for the up-regulation of *Raldh1* expression in the ZF rat liver deserve further investigation.

In summary, the results shown here suggest that retinol and retinal are metabolized into RA and regulate gene expression in rat primary hepatocytes and hepatoma cells. In primary rat hepatocytes, the responsible enzymes are most likely RDH2 and RALDH1. The expression of *Raldh1* mRNA is higher in primary hepatocytes from ZF rats than that from ZL rats, which leads to the elevated RALDH1 protein levels in the liver of ZF rats. Over-expression of RALDH1 introduces the RAL-mediated induction of *Srebp-1c* in INS-1 cells. Thus, we hypothesize that the change of RA production from the over-supply of dietary VA due to the hyperphagia of ZF rats results in higher *Srebp-1c* expression in ZF hepatocytes. The elevated SREBP-1c expression can further induce *Raldh1* expression to create a feed-forward mechanism that could be one of the reasons responsible for the increased lipogenesis in the liver of ZF rats.

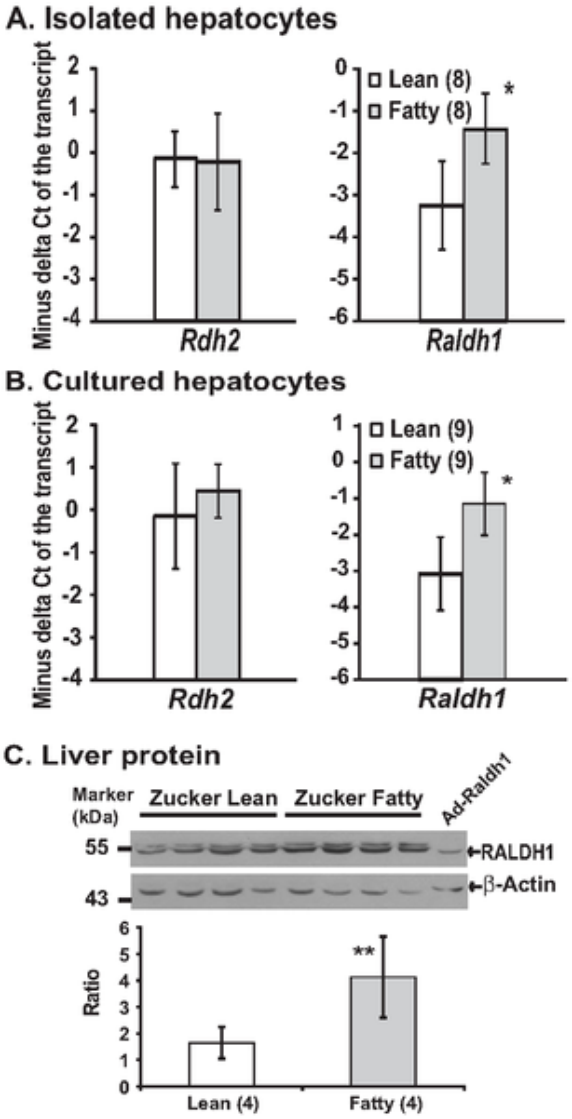
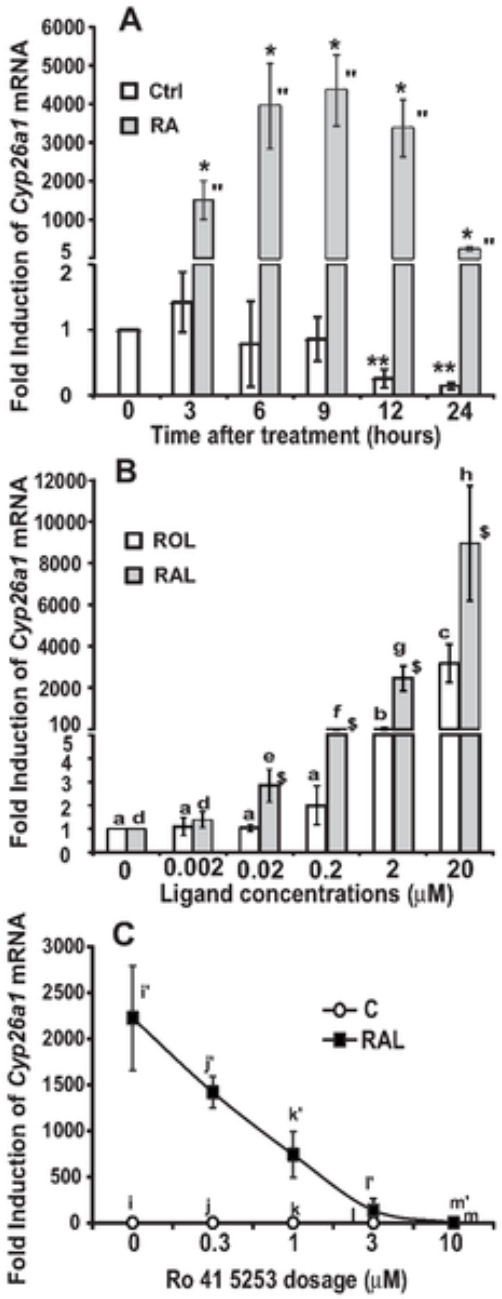



**Figure 2- 1 The mRNA levels of *Rdh2*, and *Raldh1* in (A) freshly isolated and (B) cultured primary hepatocytes, and (C) the RALDH1 protein levels in the liver of *ad libitum* ZL and ZF rats.**

(A) and (B) Total RNA was extracted from hepatocytes and subjected to real-time PCR analysis. Results were presented as means ± SD of the $-\Delta C_T$ (the $C_T$ of 36B4 - the $C_T$ of indicated transcript) from the indicated different numbers of hepatocyte isolations. * $P<0.05$ for comparing the values of ZL and ZF groups of the indicated transcripts. (C) Whole liver tissues lysates were isolated and subjected to immono-blot analysis: (100 μg/lane) of ZL (lanes 1-4) and ZF (lanes 5-8) rats, and whole cell lysates of INS-1 cells infected by Ad-Raldh1 (50 μg, lane 9). The ratio of RALDH1/β-actin were calculated and presented as means ± SD of ZL and ZF rats. ** $p<0.04$ for comparing the ratios of ZL rats with that of ZF rats.

**Figure 2- 2 The expression levels of *Cyp26a1* mRNA in response to RA treatment (A) over a 24-hour time period, (B) to different dosages of retinol or RAL, and (C) to an antagonist of the RAR activation.**

Primary hepatocytes were isolated and pre-treated as described in the Materials and Methods. Total RNA was isolated, and subjected to real-time PCR analysis. (A) A 24-hour time course of *Cyp26a1* mRNA expression induced by RA (5 μM) in primary rat hepatocytes. * for comparing the vehicle control with the RA groups at the indicated time points, and ** and " for comparing the different time points of vehicle control or RA group, all $p<0.05$. (B) The expression levels of *Cyp26a1* mRNA in primary hepatocytes treated with increasing concentrations of retinol or retinal for 6 hr. $ for comparing the fold induction of retinol group with that of retinal group at the indicated concentrations, c > b > a, h > g > f > e > d for comparing the different dosages of retinol or RAL, all $p<0.05$. (C) Inhibition of the RAR activity disrupted the induction of *Cyp26a1* by retinal treatment. Primary hepatocytes were incubated in medium A without or with 2 μM retinal in the absence or presence of increasing concentrations of Ro 41–5253 for 6 hr. i/j > l, i'/j' > k' > l' > m' for comparing the different dosages of Ro 41–5253 in the absence or presence of RAL, all $P<0.05$. Results were presented as means ± SD of 3 independent hepatocyte isolations.


A
Fold Induction of Cyp26a1 mRNA
Ctrl
RA
Time after treatment (hours)
B
Fold Induction of Cyp26a1 mRNA
ROL
RAL
Ligand concentrations (μM)
C
Fold Induction of Cyp26a1 mRNA
C
RAL
Ro 41 5253 dosage (μM)

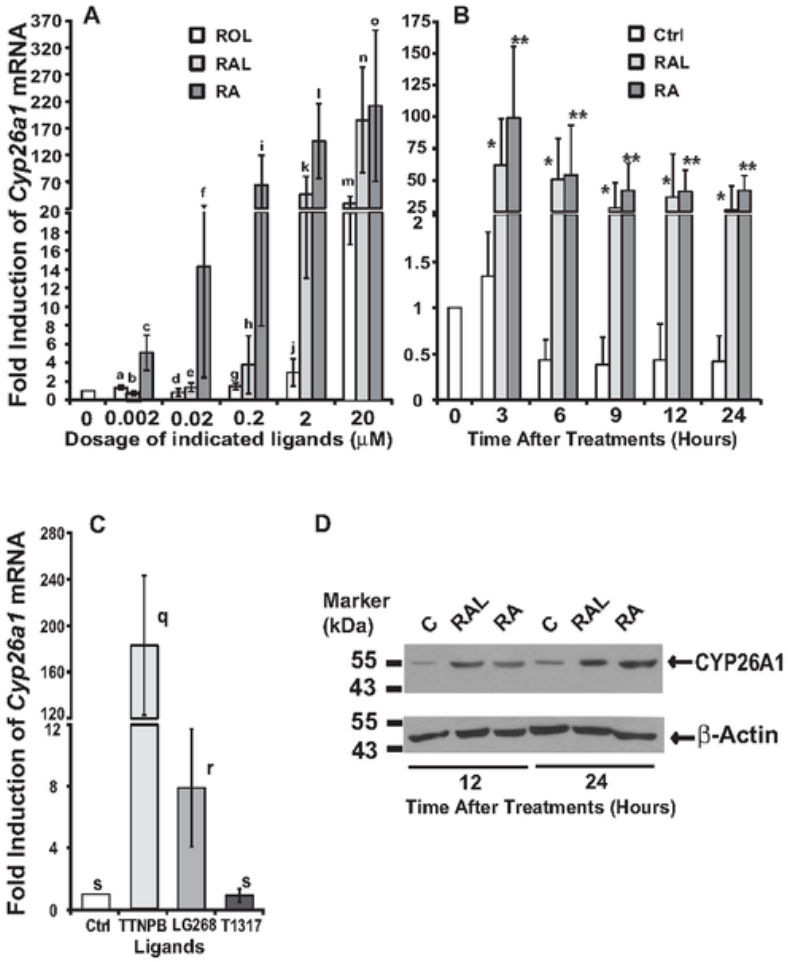



**Figure 2- 3 The effects of retinoids on the expression levels of *Cyp26a1* mRNA and CYP26A1 protein in HL1C cells.**

(A) The dose-dependent induction of *Cyp26a1* by ROL, RAL, and RA. HL1C cells were treated with ROL, RAL, or RA at the doses of 0, 0.002 μM, 0.02 μM, 0.2 μM, 2 μM, and 20 μM for 6 hr. a/b < c, d/e < f, g/h < i, j < k/l, m < n/o, a/d/g/j < m, b/e/h < k < n, c/f < i/l/o for comparing ROL, retinal and RA at the indicated concentrations, and for comparing each ligand at the different dosages, all $p<0.05$. (B) The expression levels of *Cyp26a1* mRNA overtime (3, 6, 9, 12, and 24 hr) in HL1C cells treated with 5 μM retinal or RA. * or ** for comparing retinal or RA with vehicle control groups at the indicated time points, all $p<0.05$. (C) The expression levels of *Cyp26a1* mRNA in HL1C cells treated with RAR (TTNPB), RXR (LG268), and LXR (T1317) agonists. HL1C cells were treated with 1 μM TTNPB, LG268, and T1317 for 6 hr. Total RNA was extracted and subjected to real-time PCR analysis. Data were presented as mean ± SD of 3 independent treatments. q > r>s, all $p<0.05$. D. Whole cell lysates (50 μg/sample) of HL1C cells treated the vehicle control, 5 μM retinal or RA for 12 (lanes 1–3) and 24 (lanes 4–6) hr were separated in 8% SDS PAGE gels, and transferred to the PVDF membranes.

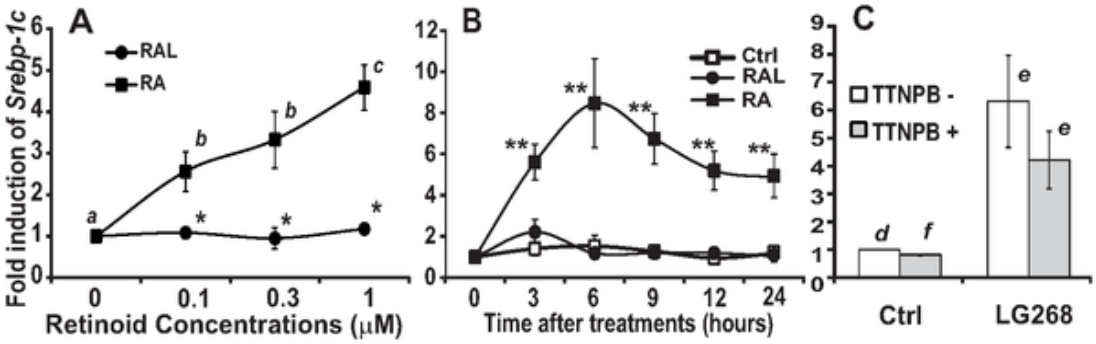



**Figure 2- 4 The *Srebp-1c* mRNA levels in INS-1 cells treated with retinoids or ligands.**

(A) 834/40 INS-1 cells were treated with increasing concentrations of retinal or RA for 6 hr. (B) 834/40 cells were treated with 2 μM of retinal or RA for indicated time. (C) 833/15 cells were treated with 1 μM of specific ligands of RXR (LG268), RAR (TTNPB) or in combination. Total RNA was extracted from INS-1 cells and subjected to real-time PCR analysis. Results were presented as means ± SD of fold inductions. c > b > a for comparing the different dosages of RA-treated groups, and e > d > f for comparing control with LG 268 in the absence or presence of TTNPB. * for comparing retinal with RA groups at the indicated dosages, and ** for comparing RA with retinal or vehicle control groups at the indicated time points, $n = 3$, all $P<0.05$.

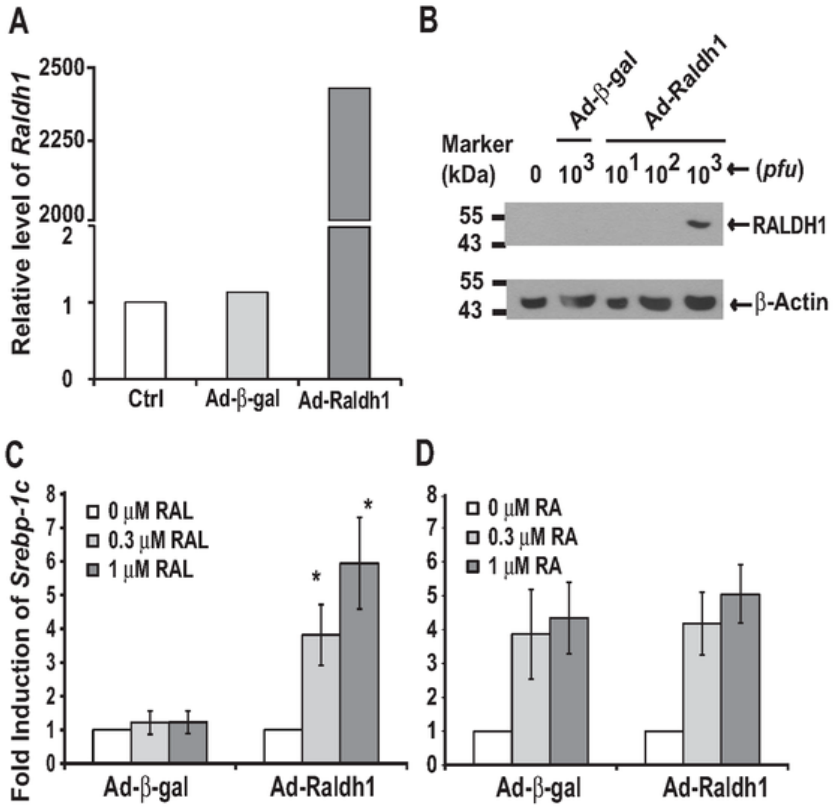



**Figure 2- 5 The over-expression of RALDH1 resulted in the RAL-mediated induction of Srebp-1c in 833/15 INS-1 cells.**

(A) The adenovirus-mediated *Raldh1* mRNA expression. (B) Immuno-blot of the over-expression of RALDH1 protein in INS-1 cells: whole cell lysates (50 μg/sample) of the control cells (lane 1), cells infected by the indicated *pfu* of Ad-β-gal (lane 2), and Ad-Raldh1 (lanes 3–5). (C) Retinal only induced *Srebp-1c* expression in cells over-expressing RALDH1, but not β-gal. D. RA induced *Srebp-1c* expression in cells over-expressing either β-gal or RALDH1. Results were presented as means ± SD of fold inductions. * for comparing the different dosages of retinal in cells infected by Ad-Raldh1, $n = 3$, all $p<0.05$.

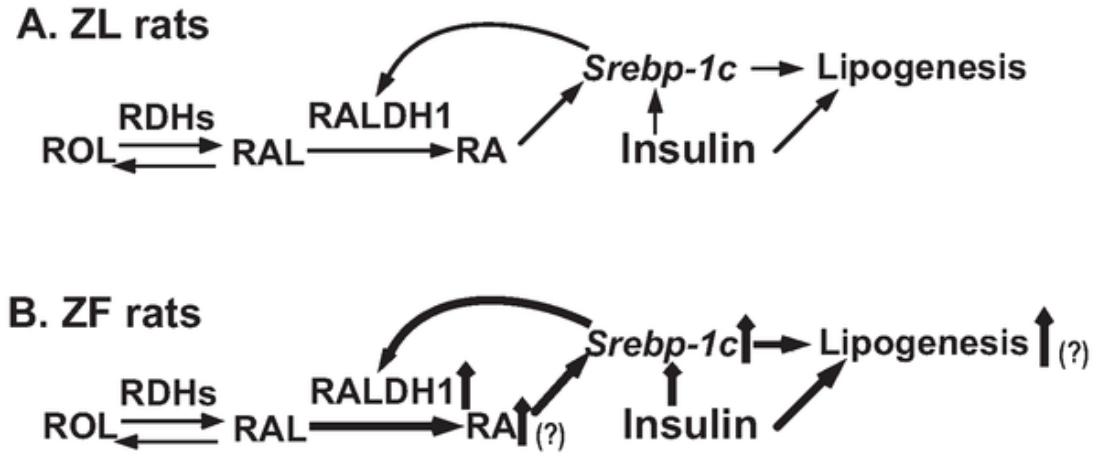



**Figure 2- 6 The hypothesized role of the RA production in the feed-forward induction of the expression of *Srebp-1c* and its downstream lipogenic genes in the liver of ZL (A) and ZF (B) rats.**

(A) In ZL rats, the hepatic expression of *Srebp-1c* is controlled by the RA production and insulin stimulation. The SREBP-1c also regulates RA production via the induction of *Raldh1* expression until a homeostasis is reached. (B) The hyperphagia of ZF rats due to the leptin receptor deficiency causes the over-supply of dietary VA, and hyperinsulinemia. The possible elevation of RA production in combination with insulin stimulation leads to higher expression of hepatic *Srebp-1c* in ZF rats, which disrupts the homeostasis. The expression of *Raldh1* mRNA is further induced by SREBP-1c. Therefore, the hepatic expression of *Srebp-1c* in ZF rats is maintained at a higher level than that in ZL rats. The consequence is the elevated hepatic lipogenesis in ZF rats. The up arrows next to the texts indicate induction. The intensified weight of the lines indicates the induction of that part of the pathway. The question marks indicate the steps remained to be confirmed in this hypothesis.

# Chapter Three

## Effects of vitamin A status on the regulation of hepatic genes in the liver of Zucker lean rats during the normal cycle of fasting and refeeding

### 1. Introduction

The high obesity prevalence indicates the future increase of patients with metabolic diseases such as type 2 diabetes mellitus (T2DM) [247]. This epidemic has become a public health concern [248]. The interactions of genetic and environmental/nutritional factors contribute to the development of obesity and its associated metabolic diseases. Generally, profound changes of glucose and lipid metabolism are often associated with the development of metabolic diseases. However, despite the obvious link between overnutrition and the development of metabolic diseases, the roles of individual micronutrients have not been elucidated.

The liver is essential for metabolic homeostasis, which is partially attributed to the regulation of the expression levels of hepatic genes for glucose and lipid metabolism in response to nutritional and hormonal stimuli [253]. As an anabolic hormone, insulin regulates the expression of a variety of hepatic genes involved in glycolysis, glycogenesis, gluconeogenesis, and lipogenesis [177]. These regulations contribute to the transition of catabolic and anabolic physiological states such as the cycle of fasting and refeeding, respectively. To control the glucose and lipid homeostatis, insulin induced glycolysis and lipogenesis, and suppresses gluconeogenesis in hepatocytes [277]. For instance, to control the hepatic glucose metabolism, insulin induces the expression of glucokinase gene (*Gck*) [178], the first enzyme of hepatic glycolysis, and suppresses the

expression of cytosolic form of phosphoenolpyruvate carboxykinase gene (*Pck1*) and glucose 6-phosphatase catalytic subunit (*G6pc*), the enzymes involved in the first and last steps of gluconeogenesis, respectively [179]. To regulate the hepatic lipid metabolism, insulin increases the expression level of sterol regulatory element-binding protein 1c (*Srebp-1c*), a family member of SREBPs that regulate the hepatic cholesterol and fatty acid biosynthesis [180], which in turn induces the expression of lipogenic genes, such as fatty acid synthase gene (*Fas*) [181]. The hepatic expression levels of all aforementioned genes are regulated in the cycles of fasting and refeeding, and have been considered to be part of the insulin's actions to modulate the transition of the body's metabolic states. How insulin signals are integrated with nutritional signals in transitions of physiological states is still an open question.

As an essential micronutrient, vitamin A (VA, retinol) plays crucial roles in the general health of an individual, such as vision, tissue differentiation, immunity and etc [254]. The majority of the physiological actions of retinol are mediated by its active metabolite, retinoic acid (RA), which exists in multiple isomeric forms, such as all-*trans* RA and 9-*cis* RA [255]. In hepatocytes, retinyl esters are hydrolyzed to retinol, which is then either oxidized to RA through a two-step reaction or bound to retinol binding protein (RBP) and secreted to the blood circulation for the use of the extrahepatic target tissues [14]. The first step of retinol oxidation, from retinol to retinal (also called retinaldehyde), is reversible and is catalyzed by retinol dehydrogenases [31]. The second step is irreversible and is catalyzed by cytosolic aldehyde dehydrogenases [31]. RA can be further modified into more hydrophilic products for disposal by cytochrome P450 family

26 (CYP26) enzymes, such as CYP26A1 [70]. RA regulates gene expression mainly through the activation of two families of nuclear receptors, RA receptors (RARα, β and γ) activated by all-*trans* RA, and retinoid X receptors (RXRα, β and γ) activated only by 9-*cis* RA [256]. In the presence of RA, RAR/RXR heterodimer is activated and recruits transcriptional factors to induce the expression of its target genes [278, 279]. Other nuclear receptors have been indicated to play a role in mediating the RA signaling [127].

We have shown recently that that retinoids synergize with insulin to induce the expression of *Gck* [240] and *Srebp-1c* [245] and attenuate insulin-suppressed *Pck1* expression [243] in primary rat hepatocytes. In addition, the expression levels of *Raldh1* mRNA and RALDH1 protein are elevated in the liver of Zucker fatty (ZF) rats [280]. RALDH1 is the main enzyme responsible for the conversion of retinal to RA in the liver [51]. Overexpression of RALDH1 introduced retinal-mediated *Srebp-1c* expression in INS-1 cells, indicating the role of the elevated RALDH1 expression in the increased lipogenesis in the liver of ZF rats [280]. We hypothesize that the dynamic catabolism of VA may contribute to the regulation of the expression of hepatic genes for glucose and fatty acid metabolism in response to physiological conditions, such as the cycle of fasting and refeeding.

Here, we report that VA status altered the regulation of plasma glucose and TG levels in response to the fasting and refeeding cycle. The products of VA catabolism may modulate the expression of hepatic genes for glucose and lipid metabolism during this cycle. Cellular RA pool, either from the diet or hepatic VA stores, contributes to the

regulation of the expression of insulin-mediated genes, *Gck*, *Pck1*, and *Srebp-1c,* in the liver of rats.

## 2. Materials and Methods

### 2.1. Reagents

Plasma concentrations of glucose, triacylglycerol (TG), and cholesterol were determined using kits from Stanbio Laboratory (Boerne, TX). Insulin and leptin RIA kits were purchased from Millipore Corporation (Billerica, MA). Reagents for cDNA synthesis and real-time PCR were obtained from Applied Biosystems (Foster city, CA).

Antibodies to acetyl-CoA carboxylase (ACC) (Cat #3676, Cell Signaling Technology, Danvers, MA), fatty acid synthase (FAS) (Cat #3180s, Cell Signaling Technology, Danvers, MA), ATP citrate lyase (ACL) (Cat #4332s, Cell Signaling Technology, Danvers, MA), cytosol form of phosphoenolpyruvate carboxykinase (PEPCK-C) (#10004943, Cayman Chemical, Ann Arbor, MI), glucokinase (GK) (sc-7908, Santa Cruz Biotechnology, Inc., Dallas, TX), and β-Actin (Cat #4970s, Cell Signaling Technology, Danvers, MA) were obtained from the perspective vendors as indicated, and used according to the protocols provided by the manufacturers.

### 2.2. Animals and dietary manipulations

Male Zucker lean (ZL, wild type and heterozygous) and fatty (ZF) rats were bred in the Department of Nutrition at the University of Tennessee at Knoxville. Zucker rats at weaning (3 weeks old) were fed a VA sufficient (VAS, #5755, TestDiet) or an isocaloric VA deficient (VAD, #5822, TestDiet) diet. Both diets contain the same amount of

calories and other nutrients except for VA. The fasting and refeeding experiments were implemented when the rats fed the VAD diet stopped gaining body mass (BM). This is the most repeatable sign of VA deficiency observed by McCollum and Davis originally [281].

For the dietary manipulation during the cycle of fasting and refeeding, rats were divided into four groups upon the development of VA deficiency in the VAD rats. The VAS or VAD rats were respectively fed *ad libitum* (VAS-AD or VAD-AD), fasted for 48 (VAS-Fasted or VAD-Fasted), or fasted for 48 hr and then refed either a VAS (VAS-Refed-VAS or VAD-Refed-VAS) or a VAD (VAS-Refed-VAD or VAD-Refed-VAD) diet for 6 hr. At the end of the dietary manipulations, the plasma and tissue samples of these animals were collected and subjected to further analysis. BM and tail tip blood glucose level were recorded during the fasting and refeeding cycle. Whole blood glucose level from the tail tip was measured using OneTouch® UltraMini® Blood Glucose Meter (LifeScan, Inc., Milpitas, CA). All procedures were approved by the Institutional Animal Care and Use Committee at the University of Tennessee at Knoxville (Protocol numbers 1256 and 1899).

### 2.3. Indirect calorimetry

To measure the indirect calorimetry, ZL rats were housed individually in metabolic cages of Comprehensive Lab Animal Monitoring System (CLAMS, Columbus Instruments, Columbus, OH 43204). The system was turned on at least 6 h before use to warm up and then it was calibrated using the reference gas (0.4910 % CO2 and 20.50% O2 balanced with N2) from Airgas Mid America (St. Louis, MO). During calibration, the

percentage of $CO_2$ was adjusted to 0.50% and the percentage of $O_2$ was adjusted to 20.50%. The BM of each rat was measured before they were placed into the system. The data recording of the CLAMS system generally started at 2 pm and stopped at 4 pm the next day to get at least 24-hour data that covered 12-hour light and 12-hour dark phases. Calorimetric measurements were conducted weekly from the 1st week when the weaning rats started to receive a VAS or a VAD diet to the 6th week when animals showed signs of VA deficiency. In the CLAMS system, the rats had free access to water and their perspective diets *ad libitum*. Respiratory exchange ratio (RER) and accumulated heat production were calculated by the system based on the following formulas.

$$RER = VCO_2/VO_2$$

$$Heat = CV \times VO_2, CV \text{ (calorific value)} = 3.815 + 1.232 \times RER$$

**2.4. Plasma analysis**

Accumulated venous blood was collected using the vacuum blood collection tube with K3 EDTA coated on the wall (BD, Franklin Lakes, NJ) to block coagulation and placed on ice before further processing. Whole blood samples were centrifuged at 3,000× rpm for 30 min at 4ºC shortly after they were collected. The supernatant (plasma) was transferred to a new tube and stored at -80ºC for later use. The liver was collected after the accumulated venous blood was drained up. Liver weight was recorded before frozen in liquid nitrogen. The pads of epididymal white adipose tissue (WAT) were removed carefully from both sides and were weighed before frozen. All the samples were stored in -80ºC freezer for further analysis.

Plasma glucose, TG, and cholesterol levels were respectively measured using the commercial kits Glucose Standard (Ref #1072-030), Triglycerides Standard (Ref #2103-030), and Cholesterol Standard (Ref #1012-030) from Stanbio Laboratory (Boerne, TX) following the manufacture's manual. Plasma insulin and leptin concentrations were determined with the Insulin RIA kit (Cat #RI-13K) and Leptin RIA kit (Cat #RL-83K) from Millipore Corporation according to the manufacture's manual, respectively.

### 2.5. RNA extraction and Quantitative Real-Time PCR

Total RNA was extracted from about 100 mg liver sample using 1 ml of RNA STAT 60 reagent (TEL-TEST, Inc., Friendswood, TX) according to the manufacture's manual. The contaminated DNA was purified and synthesized to cDNA as described previously [280]. The real-time PCR primer sequences for detecting insulin receptor (*Insr*) [244], insulin-like growth factor-binding protein 1 (*Igfbp1*) [243], *Gck* [282], *Pck1* [265], *Srebp-1c* [240], and *Fas* [245] have been reported previously. The gene expression level was normalized to that of invariable control gene, 36B4. $-\Delta C_T$ values were obtained by subtracting the $C_T$ value of 36B4 (an invariable control gene) from the $C_T$ values of the indicated genes. It indicates difference between the expression levels of 36B4 and enzymes.

### 2.6. Protein extraction and immuno-blot

About 100 mg liver tissue was homogenized using a tissue homogenizer (Tissue Master 240, OMNI International, Kennesaw, GA) within 1 ml of tissue 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-glycerophsphate, 5 mM sodium

orthovanadate, 1.9 mg/ml aprotinin, 5 μg/ml leupeptin, 1 mM benzamide, 2.5 mM PMSF, pH 8.0). The total liver 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 of the supernatant was determined using PIERCE BCA protein assay kit (Rockford, IL). Proteins (50 μg/lane) in whole tissue lysates were separated on an 8% or 10% SDS-PAGE gel, transferred to a BIO-RAD Immuno-Blot PVDF membrane (Hercules, CA), and detected with primary antibody to ATP citrate lyase (ACL), acetyl-CoA carboxylase (ACC), FAS, PEPCK-C, GK, or β-Actin according to the protocols provided by the manufacturers. Bound primary antibodies were visualized by chemiluminescence (ECL Western Blotting Substrate, Thermo Scientific) using a 1:2,000 dilution of goat anti-rabbit IgG (#7074P2, Cell signaling Technology) conjugated to horseradish peroxidase. Membranes were exposed to X-ray films (Phenix Research Products, Candler, NC) for protein band visualization. The films were scanned using an HP Scanjet 3970 (Palo Alto, CA 94304) and stored as Tagged Image File Format (TIFF). The ImageJ Software (http://rsbweb.nih.gov/ij/) was used to determine the densitometry of the protein bands. The ratio of the densities of the detected protein and β-actin in the same sample were calculated and used for quantification.

### 2.7. Statistics

Data are presented as means ± SEM. The number represents the plasma or liver samples from different animals. Levene's test was used to determined homogeneity of variance among groups using SPSS 21.0 statistical software and where necessary natural log transformation was performed before analysis. An independent-samples t-test was

used to compare two conditions. Multiple comparisons were analyzed by one-way ANOVA using Least Significant Different (LSD) when equal variance was assumed, or Games-Howell test and nonparametric test Mann-Whitney U when equal variance was not assumed. Differences were considered statistically significant at $P < 0.05$.

## 3. Results

### 3.1. VA status affects RER in ZL rats

Figure 3-1A shows that ZL rats fed a VAS diet started to have higher percentage gain of BM than those fed a VAD diet did at week 4. The gain of BM in VAD rats stopped at week 6, whereas VAS rats continued to gain BM throughout the experimental period These results demonstrated the role of VA in the somatic growth of rats and confirmed our previous observations [244].

In order to determine the effects of VA status on metabolism, we measured RER and accumulated heat production of the rats weekly for 6 weeks. As shown in Figure 3-1B and C, during week 1-3, the RERs of rats fed the VAD diet in both light and dark phases were not different from that of the rats fed the VAS diet except for the dark phase RER at the end of week 3. The RER values of this period of time were at around 0.85. Starting from the end of week 4, the RER values of the rats fed the VAS diet in both light and dark phases elevated toward 1.0, while those fed the VAD diet remained at around 0.85. Compared to the values of light phase RER that were in the range of 0.8-0.95, the values of dark phase RER were slightly higher and in the range of 0.85-1.0. The accumulated heat productions of the rats fed the VAS diet during both light (Figure 3-1D)

and dark phases (Figure 3-1E) were greater than that fed the VAD diet at week 5 and 6. However, after normalized to BM, the adjusted accumulated heat productions of VAS rats were not different from those of VAD rats during the 6-week time period (data not shown), demonstrating the consequences of the reduced BM of the rats fed the VAD diet.

### 3.2. The effects of VA status on the body compositions of ZL rats in the cycles of fasting and refeeding

To determine the roles of the dynamic VA catabolism in the modulation of the expression levels of genes for glucose and lipid metabolism in the liver, the VAS or VAD rats were subjected to the cycle of fasting and refeeding. The ratios of the liver to BM (liver/BM) and the epididymal WAT to BM (WAT/BM) were calculated to determine the effects of VA status on the body compositions of VAS or VAD ZL rats in the cycle. As shown in Figure 3-2A, the liver/BM ratio of VAS rats was not different from that of VAD rats. On the other hand, the WAT/BM ratio of VAS-AD rats was significantly higher than that of VAD rats (Figure 3-2B). All these data demonstrate that VA status does not affect liver/BM ratio, but affects WAT/BM ratio, confirming our previous published results [244].

### 3.3. VA status affects plasma levels of glucose and TG of the ZL rats in the cycle of fasting and refeeding

To investigate the effects of VA status on glucose and lipid metabolism, we compared the plasma levels of glucose, TG, and cholesterol of those rats in the fasting and refeeding cycle. Figure 3-2C shows that the plasma glucose levels of VAD-Refed-VAS and VAD-Refed-VAD rats were respectively lower than that of VAS-Refed-VAS

and VAS-Refed-VAD rats, demonstrating that VA deficiency results in a reduction in plasma glucose level. Compared to that of VAS-AD rats, the glucose levels of VAS rats (white bars) decreased after fasting (VAS-Fasted) and increased upon refeeding of either a VAS or VAD diet (VAS-Refed-VAS or VAS-Refed-VAD) (Figure 3-2C). In the contrast, the glucose levels of VAD rats did not return upon refeeding of a VAD diet (VAD-Refed-VAD) (Figure 3-2C).

The plasma TG levels of VAD-Refed-VAS and VAD-Refed-VAD rats were respectively lower than that of VAS-Refed-VAS and VAS-Refed-VAD rats (Figure 3-2D). Compared to that of VAS rats fed *ad libitum* (VAS-AD), the plasma TG levels of VAS rats reduced after fasting (VAS-Fasted) and elevated upon refeeding of either a VAS (VAS-Refed-VAS) or a VAD diet (VAS-Refed-VAD). The plasma TG levels of VAD rats elevated upon refeeding in those rats fed a VAS diet (VAD-Refed-VAS) but not in those fed a VAD diet (VAD-Refed-VAD rats). These results reveal that VA status affects the plasma glucose and TG levels in response to the fasting and refeeding cycle, a normal physiological phenomenon.

We also measured the plasma levels of cholesterol among those rats, but no significant difference was observed between VAS rats and VAD rats during the fasting and refeeding cycle (data not shown).

### 3.4. VA status impacts the responses of plasma insulin and leptin levels to the cycle of fasting and refeeding

Figure 3-2E shows that the plasma insulin levels of VAD-AD, VAD-Refed-VAS and VAD-Refed-VAD rats were significantly lower than that of VAS-AD, VAS-Refed-

VAS and VAS-Refed-VAD rats, respectively, demonstrating that VA deficiency reduces plasma insulin levels as we have shown previously [244]. The plasma insulin levels of VAS rats decreased after fasting (VAS-Fasted) and increased when these animals were refed either a VAS (VAS-Refed-VAS) or a VAD diet (VAS-Refed-VAD) as compared to that of VAS rats fed *ad libitum* (VAS-AD rats) (white columns). The plasma insulin levels of VAD rats elevated upon refeeding of a VAS diet (VAD-Refed-VAS) rats but not a VAD diet (Figure 3-2E). These results indicate that the dietary VA may directly affects plasma insulin levels in VAD rats.

Figure 3-2F shows that compared to that of VAS-AD rats, the leptin levels of VAS rats decreased after fasting (VAS-Fasted) and increased upon refeeding (VAS-Refed-VAS and VAS-Refed-VAD). However, the leptin levels of VAD rats did not change significantly throughout the cycle (grey columns), probably due to the reduction of body fat mass. These results indicate that VA status may affect the plasma leptin levels via the control of adiposity in rats, and the dietary VA does not affect the plasma leptin level in VAD rats.

### 3.5. Dynamic catabolism of VA affects the responses of the mRNA expression levels of hepatic genes for glucose and lipid metabolism to the cycle of fasting and refeeding

To confirm whether the dynamic VA catabolism in the liver contributes to the regulation of the hepatic glucose and fatty acid metabolism, we compared the hepatic mRNA levels of *Insr*, *Igfbp1*, *Gck*, *Pck1*, *Srebp-1c*, and *Fas* in rats during the cycle of fasting and refeeding. Figure 3-3A shows that the hepatic expression levels of *Insr* of

VAD rats were not different from that of VAS rats in all feeding groups. In addition, the cycle of fasting and refeeding did not cause any change in the hepatic *Insr* mRNA levels of both VAD and VAS rats. The hepatic expression levels of *Igfbp1* were elevated in all VAD groups respectively in comparison to that in VAS groups (Figure 3-3B). Among the VAS groups, the hepatic *Igfbp1* mRNA levels of VAS-Fasted rats were similar to that of VAS-AD rats. However, the *Igfbp1* mRNA levels were reduced upon refeeding of a VAS (VAS-Refed-VAS), and further decreased after refeeding of a VAD diet (VAS-Refed-VAD). On the other hand, the hepatic expression levels of *Igfbp1* of VAD groups remained high and unchanged throughout the cycle.

The hepatic of *Gck* mRNA levels of VAD-AD and VAD-Refed-VAD rats were significantly lower than that of VAS-AD and VAS-Refed-VAD rats, respectively (Figure 3-3C). As compared with that of VAS-AD rats, the hepatic expression levels of *Gck* dropped after fasting (VAS-Fasted) and restored after refeeding of either a VAS (VAS-Refed-VAS) or a VAD (VAS-Refed-VAD) diet. On the other hand, fasting did not significantly reduce the hepatic expression levels of *Gck* of VAD rats (VAD-Fasted) in comparison to that of the VAD rats fed *ad libitum* (VAD-AD). However, refeeding of a VAS diet (VAD-Refed-VAS) but not a VAD diet (VAD-Refed-VAD) dramatically induced the hepatic expression levels of *Gck*, indicating that the catabolism of dietary VA contributes to the induction of hepatic *Gck* expression in the cycle of fasting and refeeding.

The hepatic expression levels of *Pck1* of VAS-Refed-VAD rats were significantly lower than that of VAD-Refed-VAD rats (Figure 3-3D). Compared to that of VAS-AD

rats, the hepatic expression levels of *Pck1* was reduced by refeeding of either a VAS diet (VAS-Refed-VAS) or a VAD diet (VAS-Refed-VAD), which may be caused by the suppressive effect of the elevated plasma insulin. The hepatic *Pck1* mRNA levels of VAD-Refed-VAS rats were slightly lower than that of VAD-Refed-VAD rats probably due to the slightly increase in insulin level after refeeding of the VAS diet to the rats (Figure 3-2E). These results demonstrate that VA catabolism contributes to the regulation of the expression of *Gck* and *Pck1,* the representative genes for the hepatic glucose metabolism.

*Srebp-1c* and *Fas* are representative genes of the hepatic lipid metabolism. As shown in Figure 3-3E, the hepatic expression levels of *Srebp-1c* of VAD-AD and VAD-Refed-VAD rats were respectively lower than that of VAS-AD and VAS-Refed-VAD rats. Compared to that of VAS-AD rats, the expression levels of *Srebp-1c* were decreased after fasting (VAS-Fasted) and restored upon refeeding of either a VAS (VAS-Refed-VAS) or a VAD diet (VAS-Refed-VAD). Among the VAD groups, compared to that of VAD-AD rats, the hepatic expression levels of *Srebp-1c* were not significantly decreased after fasting (VAD-Fasted) but decreased upon refeeding of a VAD diet (VAD-Refed-VAS) (grey columns). This result indicates that the catabolism of the dietary VA contributes to the induction of *Srebp-1c* in VAD-Refed-VAS rats. Among the VAS rats, the hepatic expression levels of *Fas* were dramatically reduced after fasting (VAS-Fasted) and restored upon refeeding of either a VAS diet (VAS-Refed-VAS) or a VAD diet (VAS-Refed-VAD) in comparison to that of VAS-AD rats (Figure 3-3F). Interestingly, although the hepatic expression levels of *Fas* in VAD-Fasted rats dropped

after fasting, it was still higher than that of VAS-Fasted rats, which deserves further investigation.

### 3.6. Dynamic catabolism of VA affects the responses of the protein levels of hepatic genes for glucose and lipid metabolism to the cycle of fasting and refeeding

To confirm that the changes of mRNA level reflect the changes of protein level in the same liver samples, we analyzed the hepatic expression levels of ACL, ACC, FAS, PEPCK-C, and GK proteins using immune-blot. The comparison was conducted in two ways. The first one was designed to compare the changes of the hepatic protein levels of the rats with the same VA status in response to the cycle of fasting and refeeding (Figure 3-4). The second one was designed to compare the protein levels between the VAD and VAS groups (Figure 3-5).

As shown in Figure 3-4A, the expression levels of ACL, ACC, FAS, and GK proteins in the liver of VAS rats dropped after fasting (VAS-Fasted) and slightly restored upon refeeding of a VAS diet (VAS-Refed-VAS) in comparison to that of VAS-AD rats (Figure 3-4A). But the protein levels of those refed a VAD diet had not been restored probably due to that the time when we collected the protein samples, 6-hour refeeding, was too early to see the expression at the protein level. The hepatic expression levels of PEPCK-C protein of VAS rats were not changed throughout the feeding cycle. As shown in Figure 3-4B, the feeding cycle only influenced the expression levels of GK, the changes of which were consistent with that of the *Gck* mRNA levels.

Next, we compared the protein levels between VAS rats and VAD rats. In comparison to that in the VAS-AD rats, we observed reductions in the hepatic expression levels of ACL and GK in the VAD-AD rats (Figure 3-5A). We also observed decreased expression levels of ACL and GK in the VAD-Fasted rats as compared to that in the VAS-Fasted rats, (Figure 3-5A). The expression levels of FAS of the VAD-Refed-VAS rats were lower than that of the VAS-Refed-VAS rats (Figure 3-5B), while the protein levels of ACL, FAS, and GK of the VAD-Refed-VAD rats were lower than that of the VAS-Refed-VAD rats (Figure 3-5B).

## 4. Discussion

Here, we demonstrate that VA status and its dynamic catabolism contribute to the regulation of macronutrient metabolism in ZL rats. The RER results obtained from the indirect calorimetry demonstrate that the VAS ZL rats are mainly in the anabolic state as evidenced by their elevated RER values towards 1.0 after week 4. This indicates that VAS rats used carbohydrate as the primary energy source and synthesized fatty acids. On the contrary, the RER values of rats fed the VAD diet did not rise, and remained at about 0.85, which was lower than that of VAS rats after week 4. This suggests that the VAD ZL rats used a mixture of carbohydrate and fatty acids as the energy source, and the fatty acid synthesis in the VAD rats was low. It is worth to note that the percentage gain of BM of VAD ZL rats was reduced at week 4 as well, indicating the development of VA deficiency. The RER data indicate that VA status plays a role in the regulation of the carbohydrate and fatty acid metabolism, and in turn, the anabolic status in rats. The lower

RER value of VAD rats suggests that the anabolic rate of rats seems to require the presence of VA. In another word, VA contributes to the maintenance of anabolic state in rats.

The accumulated heat production of VAS rats after week 4 was higher than that of VAD rats, which is reasonable because VAS rats had larger body size that may produce more heat. After we normalized the heat production to BM (data not shown), the adjusted heat production of VAS rats was no longer different from that of VAD rats. We have shown previously and in this study that VAD rats have less body fat than VAS rats do. This indicates that VAD rats may have relatively higher lean BM to total BM ratio than VAS rats have. Theoretically, muscle cells produce more heat than fat cells do. To maintain the same adjusted heat production rate, the heat production rate per unit of muscle mass of VAD rats might be lower than that of VAS rats. It has been shown that RA induces the expression of uncoupling protein 3 in muscle cells [283, 284]. It will be interesting to determine the roles of VA status in muscle metabolism and heat production, an ongoing project in our lab.

It has been shown that glucose-stimulated insulin secretion is impaired in islets or pancreas isolated from VAD rats, which can be restored after the replenishment of VA status in those animals [157]. In our study, we observed that VA deficiency altered the normal response of plasma insulin level to the fasting and refeeding cycle, and led to a significant reduction of it [244]. The depletion of VA storage may lead to hypoinsulinemia in VAD rats, indicating the important role of VA storage in maintaining normal plasma insulin levels. The increase in insulin level of VAD-Refed-VAS rats may

be attributed to the dynamic usage of the dietary VA. But this increase was not as high as that in VAS-Refed-VAS rats, which may suggest that dynamic VA usage or RA production from the diet alone is not sufficient to completely restore insulin secretion in the current experimental settings. The stored and dietary VA may be integrated into the intracellular VA pool to maintain the normal insulin release from pancreatic β-cells.

It is interesting to note that the response of plasma leptin levels to the fasting and refeeding cycle is also affected by VA status. Leptin is secreted from WAT, and its circulating level is closely related to fat mass [141] and calorie intake [285]. Normally, plasma leptin level falls during fasting and increases upon refeeding [286] as we observed in VAS rats. But the response of leptin to the cycle of fasting and refeeding was significantly blunted in VAD rats according to our observation. This may be attributed to the loss of WAT caused by the VAD status. Indeed, the ratio of WAT/BM of VAD rats was significantly lower than that of VAS rats (Figure 3-2B), which may limit the leptin produced from WAT in response to refeeding.

Insulin induces *Gck* expression [287] and suppresses *Pck1* expression [288] in the liver, which has been considered a signature event in the cycle of fasting and refeeding. Insulin also inhibits the hepatic expression of *Igfbp1* that prevents hypoglycemia caused by insulin-like growth factor 1 [289]. In our study, the elevated expression level of *Igfbp1* in VAD rats is probably due to the reduction in plasma insulin level. However, it is interesting to note that the hepatic expression levels of *Igfbp1* of VAS-AD rats were similar to that of VAS-Fasted rats. It suggests that the decrease in plasma insulin level of

VAS-Fasted rats may not be sufficient to affect the *Igfbp1* mRNA levels, indicating the involvement of other factors in the control of *Igfbp1* expression in *ad libitum* state.

The decrease in *Gck* mRNA levels of VAD rats seems to be the combined effects of decreased insulin levels and VA deficiency. The *Gck* expression levels of VAD-Refed-VAS rats were not as low as that of VAD-Refed-VAD rats probably due to the relatively higher levels of insulin of VAD-Refed-VAS rats. We have reported that retinoids synergize with insulin to induce *Gck* expression in primary rat hepatocytes [240]. Here, the *in vivo* data also demonstrate that dynamic RA production from diet synergizes with insulin to induce *Gck* expression in VAD-Refed-VAS rats. The expression of GK at the protein level is consistent with that at the mRNA level. Thus, RA production, from either VA storage or dietary source, modulates the insulin-induced *Gck* expression in the liver. We have reported that retinoids attenuate insulin-mediated suppression of *Pck1* in primary rat hepatocytes [243]. In the current study, the hepatic expression of *Pck1* does not seem to be influenced by the RA pool.

On the other hand, insulin controls hepatic lipogenesis via stimulating the expression of *Srebp-1c* and its downstream targeted gene *Fas* [180]. VA deficiency led to the reduced expression levels of *Srebp-1c*, which is due to the combined effects of decease in insulin levels and RA production. The higher mRNA levels of *Srebp-1c* in VAD-Refed-VAS rats compared to that in VAD-Refed-VAD rats suggest that dynamic RA production from diet synergizes with insulin to induce *Srebp-1c* expression, which is consistent with our previous observation in primary rat hepatocytes [245]. The change of mRNA levels of *Fas* was consistent with that of *Srebp-1c* except for the one after fasting.

The expression levels of *Fas* in VAD-Fasted rats were higher than that of VAS-Fasted rats, indicating that factors other than *Srebp-1c* may regulate *Fas* expression after prolonged fasting. The immune-blotting results of FAS of fasted rats are not consistent to that at the mRNA level, suggesting that protein stability may play a role in this phenomenon.

Theoretically, RA is dynamically produced and rapidly turned over inside a cell. At any moment, the hepatocyte RA pool may be derived from two sources: one is produced directly from dietary VA; the other one is from stored VA in liver stellate cells. VAS rats have adequate VA stores in the liver whereas VAD rats should have depleted stores of hepatic VA. The rats refed the VAS diet for 6 hr should be able to use RA derived from both the stored VA, if they had, and the dietary VA. However, the rats refed the VAD diet could only use the stored RA, if they had. Our results obtained from the VAD rats indicate that the dietary VA plays an important role in the regulation of the hepatic expression of genes involved in glucose and fatty acid homeostasis. After the refeeding, the dietary VA is probably channeled immediately to the VA pool in the hepatocytes and utilized for RA production. Therefore, dynamic RA production contributes to the regulation of hepatic glucose and lipid metabolism in normal physiological conditions.

In summary, we have demonstrated that VA is essential in maintaining the normal responses of plasma levels of glucose, TG, insulin, and leptin to the cycle of fasting and refeeding. VAD rats had reduced levels of plasma glucose and TG, which is attributed to the altered plasma levels of hormones (insulin and leptin) and changes of expression

levels of genes involved in hepatic glucose and lipid metabolism. We believe that cellular VA pool plays important roles in the modulation of insulin-mediated gene expression in the liver. Both sources of the dietary VA and hepatic stored VA contribute to the formation of cellular VA pool. Therefore, we conclude that cellular VA, either from the dietary or stored source, contributes to the regulation of the expression of genes in hepatic glucose and lipid metabolism.

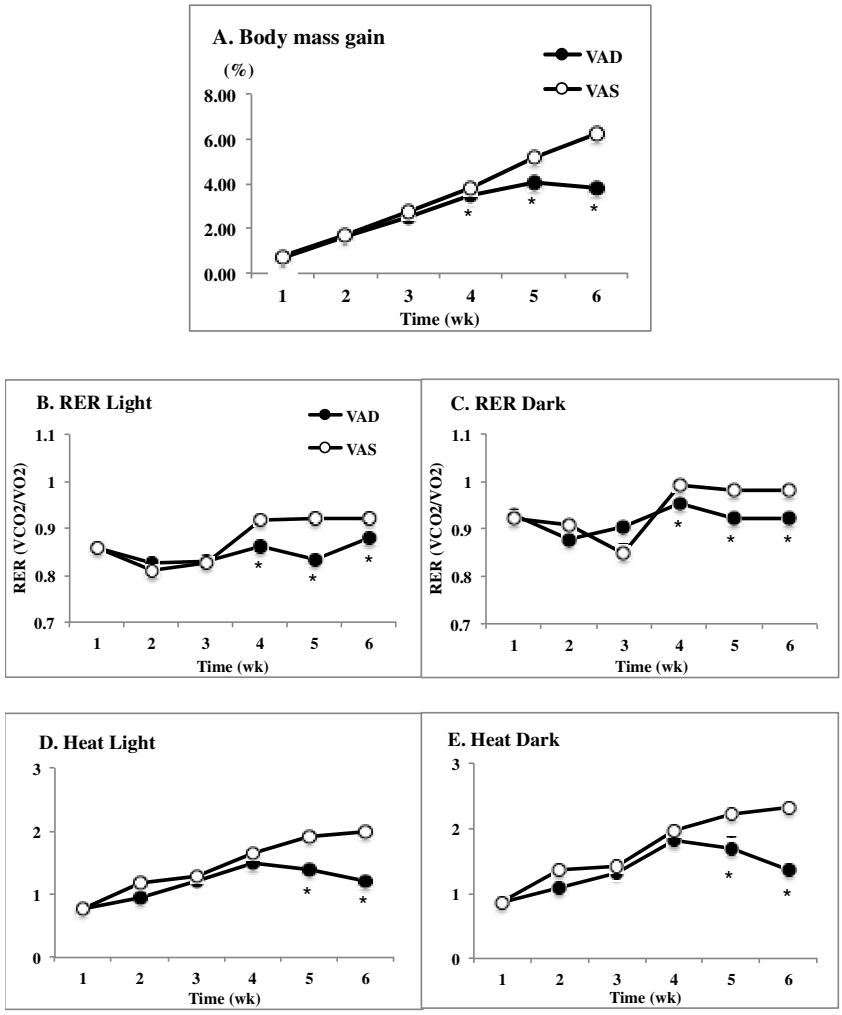



**Figure 3- 1 Body mass gain ratio and weekly RER and accumulated heat of VAS and VAD rats before the fasting and refeeding cycle.**

Body mass gain ratio = (weekly body mass – initial body mass) / initial body weight. Body mass of VAS and VAD rats were recorded weekly. Results were represented as means ± SEM of 6 independent experiments. $VCO_2$ and $VO_2$ (RER) of VAS and VAD rats were measured weekly using the indirect calorimetry system. RER ($VCO_2/VO_2$) during (B) light and (C) dark and accumulated heat production during (D) light and (E) dark were calculated automatically by the system. Results were represented means ± SEM of 6 independent experiments. * for comparing the control VAS rats and VAD rats; all $P<0.05$.

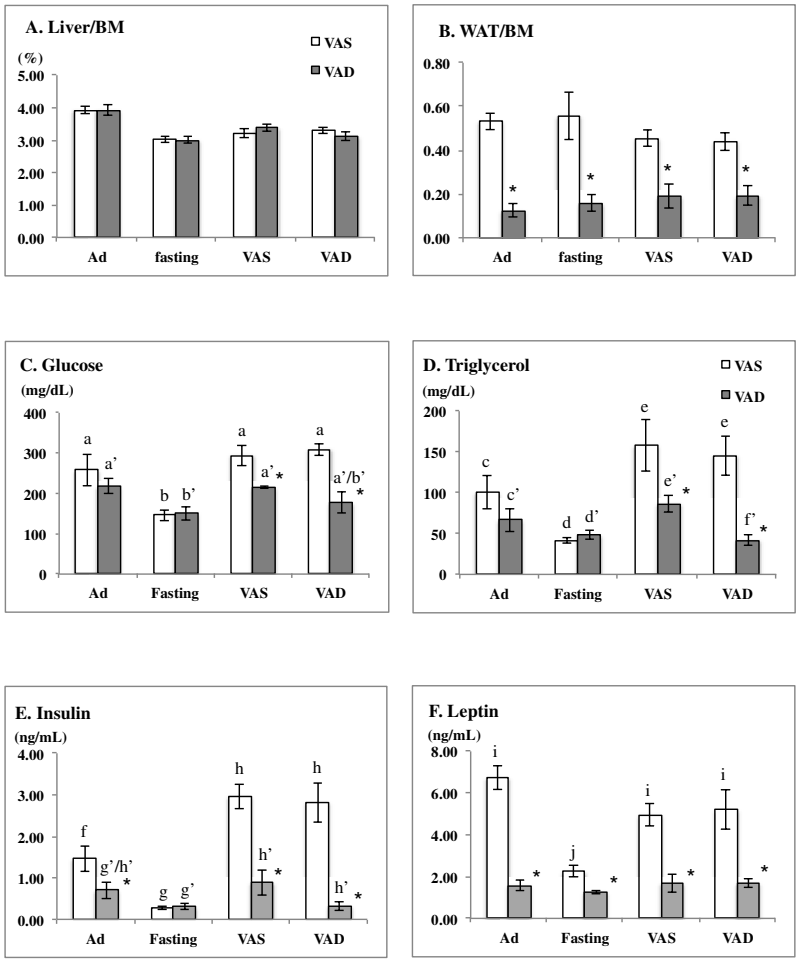



**Figure 3- 2 Ratios of (A) liver/BM and (B) WAT/BM and plasma levels of (C) glucose, (D) TG, (E) insulin, and (F) leptin of VAS and VAD rats after the fasting and refeeding cycle.**

The ratios of liver/BM and WAT/BM were calculated and compared between VAS and VAD rats. Data were represented means ± SEM of 9 independent experiments. * for comparing the control VAS rats and VAD rats at the indicated groups; all $P<0.05$. Accumulated venous plasma samples were collected at the end the feeding cycle. Plasma glucose, TG, insulin, and leptin levels were measured using the available commercial kits. Results were represented means ± SEM of 6 independent experiments. * for comparing the control VAS rats and VAD rats at the indicated groups, a>b, a'>b', d<c<e, c'>f', e'>d'/f', g<f<h, g'<h', i>j, all $P<0.05$. Ad=*ad libitum*, Fast=fasting, VAS=refed VAS, VAD=refed VAD.

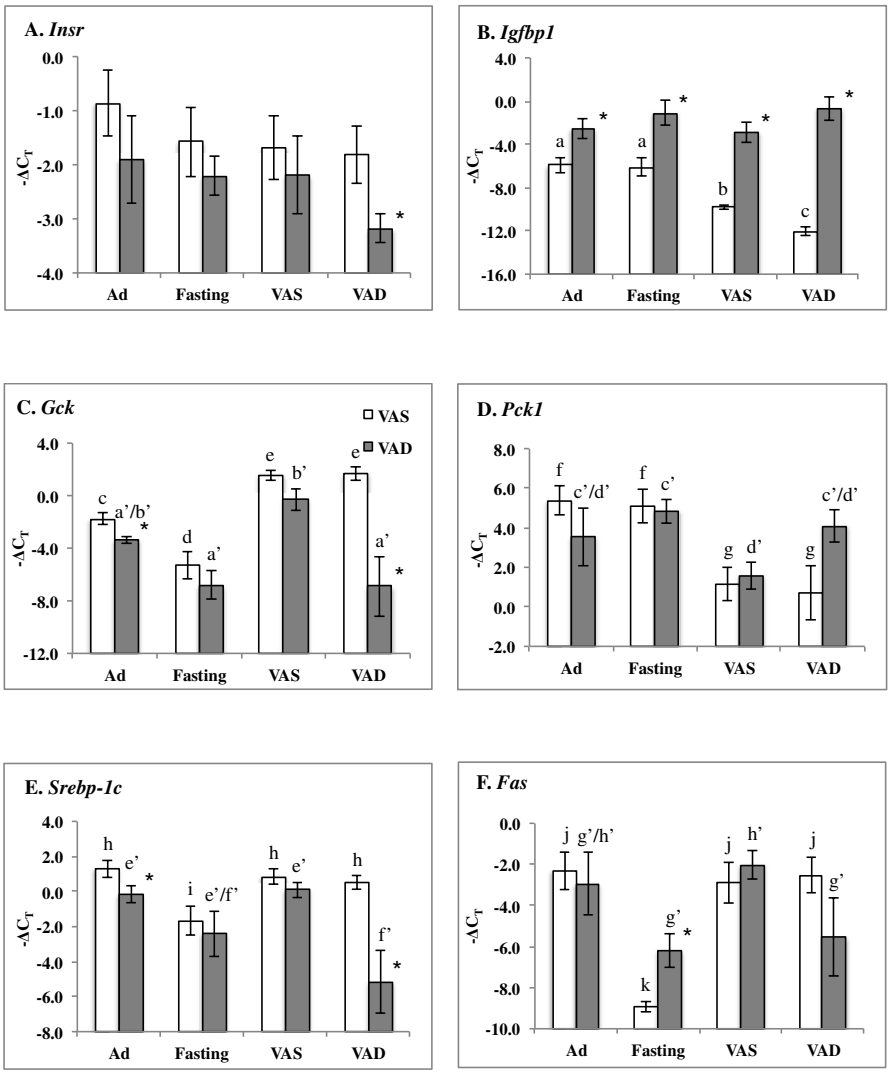
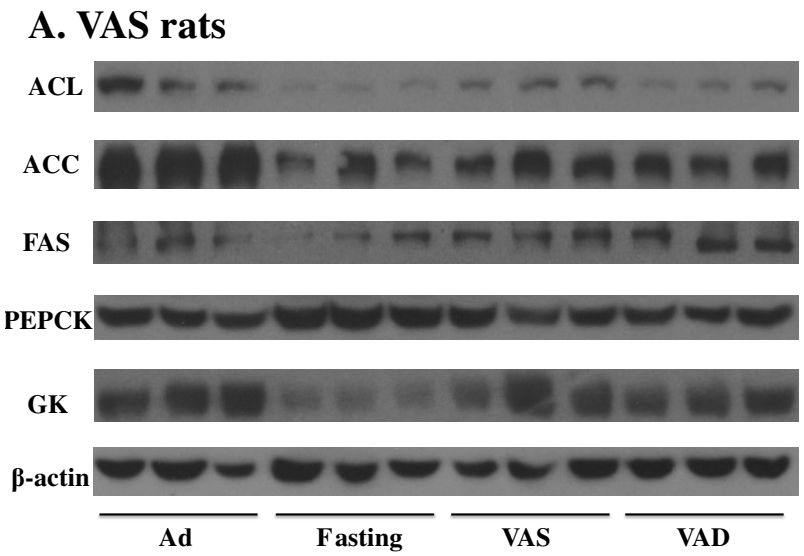



**Figure 3- 3 The expression levels of (A) *Insr*, (B) *Igfbp1*, (C) *Gck*, (D) *Pck1*, (E) *Srebp-1c*, and (F) *Fas* of VAS and VAD rats after the fasting and refeeding cycle.**
Liver tissue samples were collected at the end of the feeding cycle. Total RNAs were extracted and subjected to real-time PCR analysis. Results were presented as means ± SEM of the $-\Delta C_T$ (the $C_T$ of 36B4 - the $C_T$ of indicated transcript) from 4 independent experiments. * for comparing the control VAS rats and VAD rats at the indicated groups, a>b>c, d<c<e, a'<b', f>g, c'>d', h>i, e'>f', j>k, g'<h', all $P<0.05$. Ad=*ad libitum*, Fast=fasting, VAS=refed VAS, VAD=refed VAD.

**Figure 3- 4 The immono-blot of ACC, FAS, ACL, PEPCK, and GK in the liver of (A) VAS rats and (B) VAD rats after the fasting and refeeding cycle.**
Liver samples were collected at the end of the feeding cycle. Proteins (50 μg/lane) in whole cell lysates were isolated and subjected to immono-blot analysis. The ImageJ Software was used to determine the densitometry of a protein band. Results were presented as means ± SEM of the ratio of the measured protein to β-actin from 3 independent experiments. a>c>b, d>e, f>h>i>g, j>k, a'<b', $p<0.05$.


A. VAS rats
ACL
ACC
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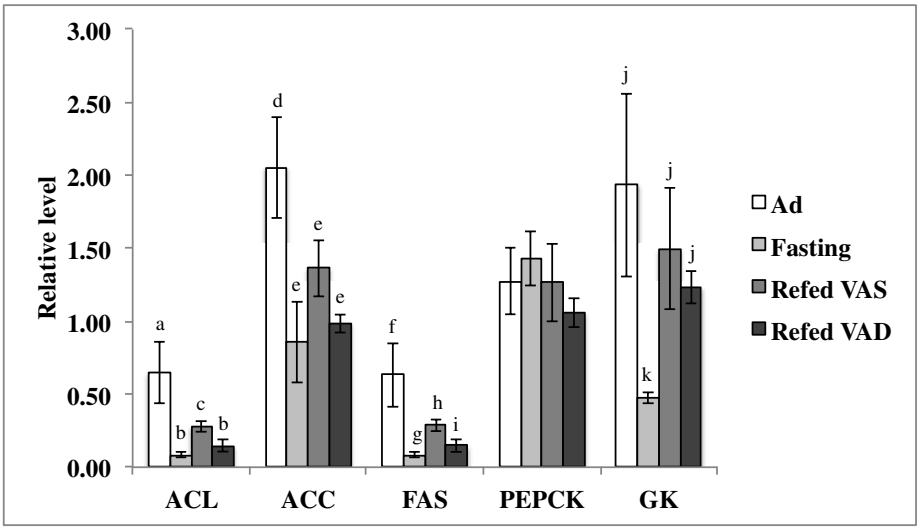
Relative level
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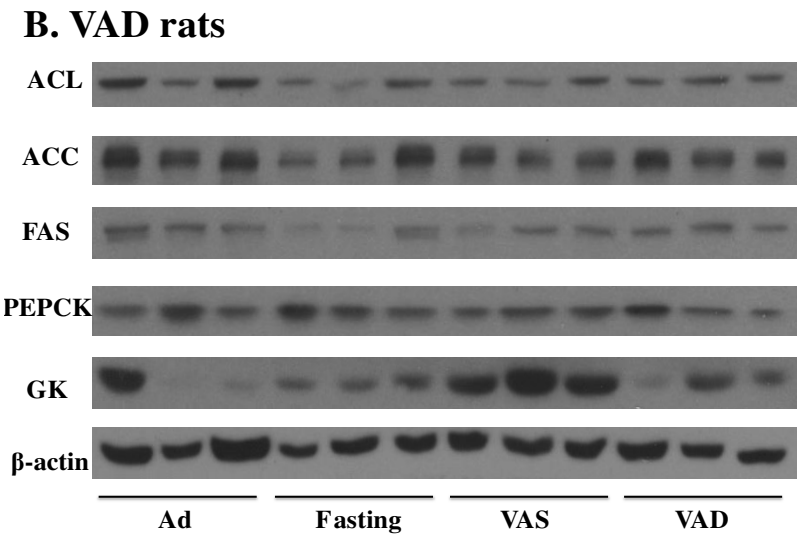
B. VAD rats
ACL
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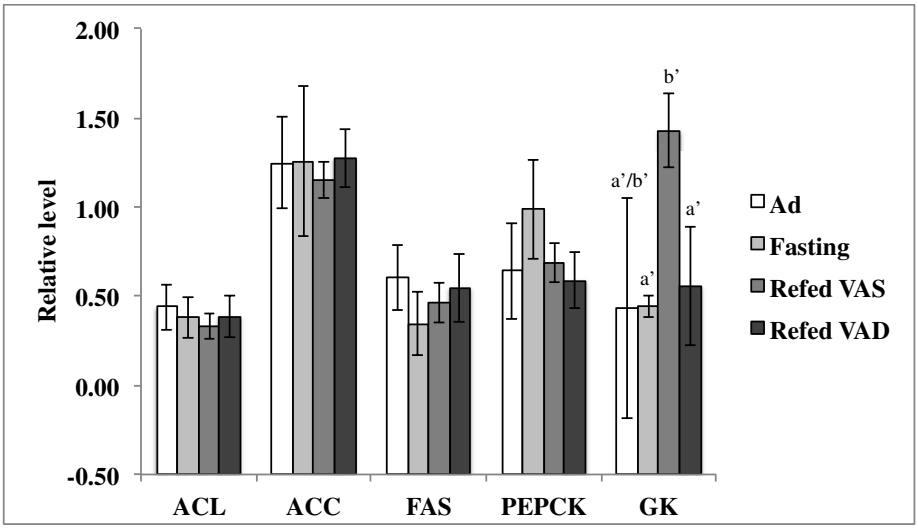
Ad
Fasting
VAS
VAD


2.00
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Relative level
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Fasting
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Refed VAD

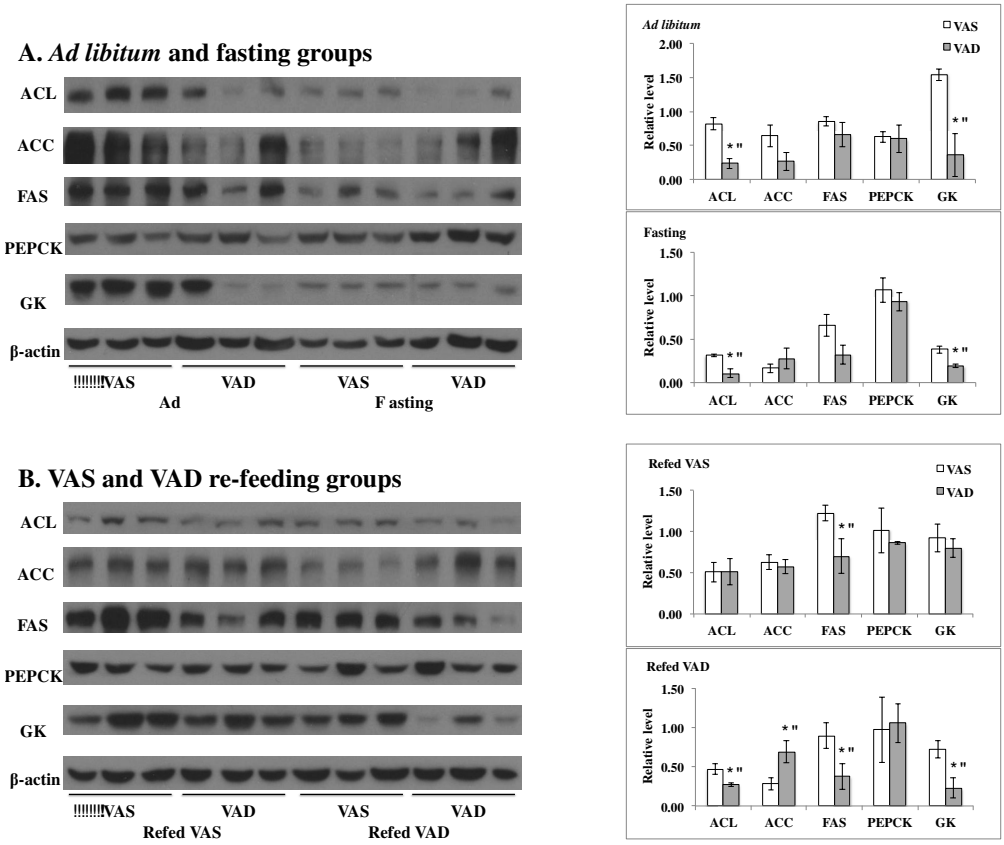



**Figure 3- 5 Comparison of the protein levels in (A) *ad libitum* and fasting states and in (B) VAS refeeding and VAD refeeding states.**

Liver samples were collected at the end of the feeding cycle. Proteins (50 μg/lane) in whole cell lysates were isolated and subjected to immono-blot analysis. The ImageJ Software was used to determine the densitometry of a protein band. * for comparing the different ratios of VAS rats and that of VAD rats, all $p<0.05$.

# Chapter Four

## Effects of vitamin A status on the regulation of hepatic genes in the liver of STZ-induced diabetic Zucker lean rats

### 1. Introduction

Metabolic homeostasis is achieved through the coordinative actions of nutritional and hormonal signals. Insulin is the most important one as the lack of its action leads to the development of diabetes mellitus. Insulin exerts it activities at least in part through the regulation of the expression of genes for the hepatic glucose and lipid metabolism. As an anabolic hormone, insulin is responsible for the expression of a variety of hepatic genes involved in glycolysis, glycogenesis, gluconeogenesis, and lipogenesis [177]. For example, to control the hepatic glucose metabolism, insulin is required for the induction of glucokinase gene (*Gck*) [178], the first enzyme of hepatic glycolysis, and suppression of cytosolic form of phosphoenolpyruvate carboxykinase gene (*Pck1*) and glucose 6-phosphatase catalytic subunit (*G6pc*), the enzymes involved in the first and last steps of gluconeogenesis, respectively [179].

It has been shown previously that insulin is also required for the hepatic fatty acid synthesis in the cycle of fasting and refeeding. The hepatic fatty acid synthesis rises sharply and continuously for more than 20 hr after rats were fasted for 48 hr and then refed a fat-free diet [290]. The amount and activity of fatty acid synthase (FAS), the enzyme responsible for fatty acid synthesis, are increased in normal rats refed a high carbohydrate diet after fasting and in diabetic rats after insulin injection [215]. However, in insulin-deficient rats, the induction of FAS after refeeding is lost until insulin is

injected, demonstrating the essential role of insulin in the induction of FAS activities in a refeeding condition [291]. This induction of FAS activity is attributed to the induction of its gene (*Fas*) transcripts [216, 292]. This has been attributed to insulin-induced expression of sterol regulatory element-binding protein 1c (*Srebp-1c*), a family member of SREBPs that regulate the hepatic cholesterol and fatty acid biosynthesis [180], which in turn induces the expression of lipogenic genes, such as *Fas* [181].

Vitamin A (VA, retinol) is an essential micronutrient that plays crucial roles in vision, cell differentiation, immunity and etc [254]. The majority of VA's physiological functions are mediated by its active metabolite, retinoic acid (RA). RA exists in multiple isomeric forms, such as all-*trans* RA and 9-*cis* RA [255]. RA regulates gene expression mainly through the activation of two families of nuclear receptors, RA receptors (RARα, β and γ) activated by all-*trans* RA, and retinoid X receptors (RXRα, β and γ) activated only by 9-*cis* RA [256]. RAR/RXR heterodimer, located at RA responsive element (RARE) in the promoter region of RA responsive genes, is activated upon binding of RA and recruits transcriptional factors to induce the target gene expression [278, 279]. Other nuclear receptors have been indicated to play a role in mediating the RA signaling [127].

Experimental results from our lab have shown that VA status plays a role in the regulation of plasma glucose and triacylglycerol (TG) levels, and expression of genes for the hepatic glucose and lipid metabolism. Retinoids synergize with insulin to induce *Gck* expression via the activation of RAR/RXR in primary rat hepatocytes [240]. Additionally, decreased *Gck* mRNA levels and activity were observed in the liver of VA-deficient (VAD) rats as compared to those of VA-sufficient (VAS) rats [240]. Retinoids

induce *Pck1* expression and attenuate insulin-mediated suppression in primary rat hepatocytes [243]. VA deficiency only reduces hepatic *Pck1* mRNA levels in Zucker lean (ZL) rats but not in Zucker fatty (ZF) rats [244]. Retinoids synergize with insulin to induce *Srebp-1c* expression in primary rat hepatocytes [245]. In addition, the elevated expression levels of retinaldehyde dehydrogenase 1 gene (*Raldh1*) and RALDH1 protein in the liver of ZF rats may contribute to the increased lipogenesis in them [280].

Since both retinoids and insulin work together to regulate the expression of several hepatic genes involved in glucose and fatty acid metabolism, the open question is whether VA is needed for insulin-regulated hepatic gene expression under physiologically relevant conditions, such as type 1 diabetes mellitus (T1DM). Streptozotocin (STZ) has been used frequently to induce T1DM in lab animals since it was reported to be diabetogenic [293]. STZ enters pancreatic β-cells through glucose transporter 2 (GLUT2) [294], causes damages of β-cells [295], and leads to the development of hyperglycemia and T1DM [296]. The STZ-induced diabetes is a desirable model for us to investigate the roles of retinoids in the modulation of the expression of insulin-regulated genes.

Here, we used STZ to induce T1DM in VAS and VAD ZL rats. The animals with T1DM were treated with saline (control), insulin, RA or insulin + RA. The mRNA and protein levels of representative insulin responsible genes in the liver of those animals were analyzed. We demonstrate here that VA status affects the expression of insulin-mediated hepatic genes for glucose and fatty acid metabolism in diabetic rats.

## 2. Materials and Methods

### 2.1. Reagents

Plasma glucose, TG, and cholesterol levels kits were obtained from Stanbio Laboratory (Boerne, TX). Insulin, leptin, and glucagon RIA kits were purchased from Millipore Corporation (Billerica, MA). Reagents for cDNA synthesis and real-time PCR were obtained from Applied Biosystems (Foster city, CA). STZ, insulin, and RA were obtained from Sigma (St. Louis, MO). STZ was prepared in sodium citrate solution at 50mM (pH 4.5) freshly before each injection. Insulin was dissolved in in saline and stored at 4°C before use. 2.5 mg RA was dissolved in 5 ml PBS with 100 μl ethanol and 10 μl Tween 80 reaching the final concentration of 0.5 mg/ml and stored at -20°C before injection. Antibodies to ATP citrate lyase (ACL, Cat #4332s), acetyl-CoA carboxylase (ACC, Cat #3676), FAS (Cat #3180s), and β-Actin (Cat #4970s) were obtained from Cell Signaling Technology (Danvers, MA). Antibodies to cytosol form of phosphoenolpyruvate carboxykinase (PEPCK-C, #10004943) and glucokinase (GK, sc-7908) were from Cayman Chemical (Ann Arbor, MI) and Santa Cruz Biotechnology Inc. (Dallas, TX), respectively.

### 2.2. Animals and treatments of reagents

Male ZL (wild type and heterozygous) rats at weaning (3 weeks old) were fed a VA sufficient (VAS, #5755, TestDiet) or an isocaloric VA deficient (VAD, #5822, TestDiet) diet. Both diets contain the same amount of calories and other nutrients except for VA. Treatments of STZ and insulin were implemented according to a protocol

described by Lakshmanan [291] after rats fed the VAD diet stopped gaining body mass (BM) and showed signs of VA deficiency.

Rats were fasted for 24 hr, and received a single injection of STZ (65 mg/kg BM) via the tail vein. After that, the VAS or VAD diet was provided *ad libitum* to the VAS or VAD rats, respectively. Tail whole blood glucose was measured 24 hr after the STZ injection using OneTouch® UltraMini® Blood Glucose Meter (LifeScan, Inc., Milpitas, CA) to confirm the successful induction of STZ-induced diabetes (glucose level > 250 mg/dL). Then the VAS or VAD rats with STZ-induced diabetes were divided into four groups. They were injected with saline (control, 100 μL, intramuscularlly), insulin (0.5 IU/100 g BM, intramuscularly), RA (100 μg in PBS, intraperitonealy), or insulin + RA (intramuscularly and intraperitonealy, respectively). BM and tail vein blood glucose were recorded during the treatment of reagents. Rats were fed *ad libitum* for 3-4 hr and then euthanized for tissue sample collection. The procedures were approved by the Institutional Animal Care and Use Committee at the University of Tennessee at Knoxville (Protocol numbers 1256 and 1899).

### 2.3. Tissue sample collection and analysis of plasma parameters

Accumulated venous blood was collected using the vacuum blood collection tube with K3 EDTA coated on the wall (BD, Franklin Lakes, NJ) to block the coagulation. Whole blood samples were centrifuged at 3,000×rpm for 30 min at 4ºC shortly after they were collected and stored on ice. The supernatant (plasma) was transferred to a new tube and stored at -80ºC for further analysis. The liver was collected after the accumulated venous blood was drained up. Liver weight was recorded before frozen in liquid nitrogen.

Epididymal white adipose tissues (WAT) were cut carefully from both sides and were weighed before frozen. All samples were stored in -80ºC freezer for further analysis.

Plasma glucose, TG, and ketone levels were respectively measured using the commercial kits Glucose Standard (Ref #1072-030), Triglycerides Standard (Ref #2103-030), and β-Hydroxybutyrate/TDM Linearity Standard (Ref #2450-604) from Stanbio Laboratory (Boerne, TX) following the manufacture's manual. Plasma insulin, leptin, and glucagon concentrations were determined with the Insulin RIA kit (Cat #RI-13K), Leptin RIA kit (Cat #RL-83K), and Glucagon RIA kit (Cat #GL-32K) from Millipore Corporation (Billerica, MA), respectively.

### 2.4. RNA extraction and Quantitative Real-Time PCR

Total RNA was extracted from about 100 mg liver sample using 1 ml of RNA STAT 60 reagent (TEL-TEST, Inc., Friendswood, TX) according to the manufacture's protocol. The contaminated DNA was removed, and cDNA was synthesized as described previously [280]. The real-time PCR primer sequences for detecting *Cyp26a1* [268], *G6pc* [243], *Gck* [282], *Pck1* [265], *Srebp-1c* [240], and *Fas* [245] have been reported previously. The gene expression levels were normalized to that of invariable control gene, 36B4. Minus $\Delta C_T$ values were obtained by subtracting the $C_T$ value of 36B4 (an invariable control gene) from the $C_T$ values of the indicated genes. This indicates the difference between the expression level of 36B4 and that of the measured transcript.

### 2.5. Protein extraction and immuno-blot

About 100 mg liver tissue was homogenized using a tissue homogenizer (Tissue Master 240, OMNI International, Kennesaw, GA) within 1 ml of tissue 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-glycerophsphate, 5 mM sodium orthovanadate, 1.9 mg/ml aprotinin, 5 μg/ml leupeptin, 1mM benzamide, 2.5 mM PMSF, pH 8.0). The total liver 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). Proteins (50 μg/lane) in whole tissue lysates were separated on an 8% or 10% SDS-PAGE, transferred to a BIO-RAD Immuno-Blot PVDF membrane (Hercules, CA) and detected with primary antibodies to acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), ATP citrate lyase (ACL), cytosol form of phosphoenolpyruvate carboxykinase (PEPCK-C), glucokinase (GK), CYP26a1 and β-Actin according to the protocols provided by the manufacturers. Bound primary antibodies were visualized by chemiluminescence (ECL Western Blotting Substrate, Thermo Scientific) using a 1:2,000 dilution of goat anti-rabbit IgG (#7074P2, Cell signaling Technology) conjugated to horseradish peroxidase. Membranes were exposed to X-ray films (Phenix Research Products, Candler, NC) for protein band visualization. The films were scanned using an HP Scanjet 3970 (Palo Alto, CA 94304) and stored as Tagged Image File Format (TIFF). The ImageJ Software (http://rsbweb.nih.gov/ij/) was used to determine the densitometry of the protein bands. The ratio of the densities of the detected protein and β-actin in the same sample were calculated and used for quantification.

### 2.6. Statistics

Data are presented as means ± SEM. The number of each group represents the total number of different animals received the indicated treatment. SPSS 21.0 statistical software (IBM Corporation, Armonk, NY) was used for statistical analysis. Levene's test was used to determine homogeneity of variance among groups and the necessary natural log transformation was performed before analysis. An independent-samples t-test was used to compare two conditions. Multiple comparisons were analyzed by one-way ANOVA using least significant different (LSD) when equal variance was assumed, and Games-Howell test and nonparametric test Mann-Whitney U were used when equal variance was not assumed. Differences were considered statistically significant at $p < 0.05$.

## 3. Results

### 3.1. Effects of VA status on the body compositions of STZ-induced diabetic rats

To induce VA deficiency, ZL rats were fed on a VAD diet at weaning. BM was recorded weekly to monitor the growth of the rats. Figure 4-1A shows that BM of VAD rats started to drop in week 5 and 6 compared to that of VAS rats. At the same time, we observed early signs of VA deficiency, such as dryness of the cornea, on those rats fed the VAD diet.

The liver/BM ratio and WAT/BM ratio of VAS and VAD rats were compared to evaluate the effects of VA status on the body compositions of STZ-induced diabetic rats. Figure 4-1B shows that the liver/BM ratios of VAD rats were not different from that of

VAS rats. However, the WAT/BM ratios of VAD rats were significantly lower than that of VAS rats (Figure 4-1C). These results were consistent with the results in our previous studies [244].

### 3.2. Effects of VA status on plasma levels of glucose, TG, and cholesterol in STZ-induced diabetic rats

The levels of peripheral tail whole blood glucose were recorded after fasting, STZ injection, and the treatment to monitor the changes of blood glucose throughout the experimental process. As shown in Table 4-1, a single STZ treatment was sufficient to induce the plasma glucose levels to reach more than 400 mg/dL in both VAS and VAD rats, indicating the successful development of T1DM. Saline or RA did not cause any change of plasma glucose levels. On the other hand, treatments of insulin or insulin + RA reduced the levels of tail tip whole blood glucose in both VAS and VAD rats. These results indicate that the amount of insulin and the time chosen were enough for us to observe the insulin action to lower peripheral whole blood glucose in STZ-induced diabetic rats. This setting allowed us to study the effects of insulin and RA on plasma levels of parameters and the expression levels of hepatic genes.

To investigate the effects of VA status on the homeostasis of glucose and lipid in diabetic rats, we analyzed the plasma parameters in plasma derived from the inferior vena cava. Figure 4-2A shows that the RA treatment alone did not affect the glucose levels of both VAS and VAD rats compared to that in the control groups. At the time of tissue collection, the accumulated venous plasma glucose levels in the *ad libitum* VAS rats treated with insulin or insulin + RA tended to be lower, but did not reach statistical

significance, than the ones treated with saline, suggesting the limitation of one injection of insulin to control the blood glucose in *ad libitum* VAS rats for 3 hr. On the other hand, insulin or insulin + RA treatment significantly reduced the glucose levels in VAD rats similarly. The results indicate that VA status seems to affect the control of plasma glucose in diabetic rats after a single insulin injection [291]. The amount of insulin we injected to rats was sufficient to control the plasma glucose levels in VAD rats for more than 3 hr, which might not be enough for the VAS rats.

The plasma levels of TG in VAS diabetic rats treated with RA, insulin, or insulin + RA were comparable to that in the control rats, probably due to the large variation of the control group (Figure 4-2B). RA treatment alone did not change the TG levels in VAD rats, but insulin treatment significantly decreased the TG levels of VAD rats. Treatment of insulin + RA slightly but not significantly reduced the TG levels in VAD rats. Thus, plasma levels of TG in diabetic rats are more sensitive to the treatment of insulin in the absence of VA.

The plasma levels of ketone in both VAS and VAD rats were reduced by the treatments of insulin or insulin + RA in comparison to that in the control groups (Figure 4-2C), indicating the effectiveness of insulin treatment.

### 3.3. Effects of VA status on plasma levels of plasma insulin, leptin, and glucagon in STZ-induced diabetic rats

Figure 4-2D shows that rats treated with saline (control) or RA had extremely low levels of insulin, indicating no endogenous insulin secretion due to the loss of the pancreatic β-cells caused by STZ treatment. Treatment of insulin or insulin + RA

increased the plasma levels of insulin in both VAS and VAD rats as compared to that in the control groups, suggesting the successful injection of insulin to the rats.

The plasma levels of leptin of VAS rats were not affected by the treatment of RA or insulin (Figure 4-2E). However, markedly induction of plasma leptin levels were observed in VAD rats treated with RA, insulin, or insulin + RA. Thus, VA status affects the response of leptin levels to the indicated treatments in diabetic rats. The plasma leptin levels in VAD diabetic rats are more sensitive to the treatment of insulin.

RA or insulin treatment alone did not affect the plasma levels of glucagon in VAS rat compared to the control group, but insulin + RA treatment decreased its levels (Figure 4-2F). Treatment of RA alone did not change the plasma levels of glucagon of VAD rats. However, treatment of insulin or insulin + RA significantly reduced the glucagon levels in VAD rats. These results suggest that the plasma levels of glucagon are affected by VA status. Insulin treatment decreases its levels in diabetic VAD rats.

### 3.4. VA status affects the responses of the expression levels of hepatic genes for glucose and lipid metabolism to RA and insulin treatments

To confirm the successful injection of RA, we compared the hepatic mRNA levels of *Cyp26a1* after the indicated treatments. As shown in Figure 4-3A, the expression levels of *Cyp26a1* in rats treated with RA or insulin + RA were higher than that in the saline (control) or insulin group, demonstrating that the amount of RA injected was sufficient to induce gene expression.

To investigate the effects of VA status and RA treatment on the expression levels of hepatic genes for glucose and fatty acid metabolism, we determined the mRNA levels

of *Gck*, *Pck1*, and *Srebp-1c* in the liver of STZ-induced diabetic rats after the indicated treatments. Figure 4-3B shows that the hepatic expression levels of *Gck* in VAS rats were induced after the treatment of insulin or insulin + RA as compared to that in the control group. The *Gck* mRNA levels in VAD rats were increased upon the treatment of RA and its levels were further increased by the treatment of insulin and even further elevated by the treatment of insulin + RA. These results demonstrate that VA status affects the expression levels of *Gck* after the treatment of insulin. VAD rats without any storage of VA are more sensitive to the RA treatment. In VAD rats, RA alone is sufficient to induce the hepatic expression of *Gck*, and RA also synergizes with insulin to further induce its expression.

As shown in Figure 4-3C, the hepatic expression levels of *Pck1* in VAS rats were reduced after the injection of insulin or insulin + RA in comparison to that in the control group. The mRNA levels of *Pck1* in VAD rats were also decreased after the treatment of insulin or insulin + RA, and the reductions (Δ Ct of 3.94 and 4.19) were more significant than that in the VAS rats (Δ Ct of 2.63 and 3.95). Thus, VA status may also affect the expression levels of *Pck1* after the treatment of reagents. The *Pck1* expression levels in VAD diabetic rats are more sensitive to the treatment of insulin or insulin + RA, suggesting that the stored VA and its metabolism may play a role in the attenuation of insulin-mediated suppression of *Pck1* expression.

Figure 4-3D shows that the treatment of insulin or insulin + RA induced the mRNA levels of *Srebp-1c* in VAD rats as compared to the control group, but not that in VAS rats probably due to the big variation in the control group of VAS rats. This

demonstrates that VA status contributes to insulin-mediated induction of *Srebp-1c* expression. The *Srebp-1c* mRNA levels of VAD diabetic rats are more likely to be affected by the treatment of insulin.

We also compared the expression of CYP26A1, ACC, FAS, PEPCK, and GK at the protein level. ACC and FAS are the enzymes for the productions of malonyl-CoA and palmitate, which are essential for de novo fatty acid biosynthesis. Figure 4-4 shows that treatment of insulin + RA induced the expression of CYP26A1 as compared to that of the control groups. As shown in Figure 4-5A, the expression levels of PEPCK in VAS rats were significantly reduced upon the treatment of RA, insulin, or insulin + RA as compared to that in the control group. The 3-hour treatment of insulin or RA did not influence the expression of ACC and FAS in VAS rats. The insulin-mediated expression of GK in VAS rats is increased by the treatment of insulin + RA. In Figure 4-5B, treatment of insulin or insulin + RA induced the protein levels of ACC and FAS but dramatically suppressed the expression of PEPCK in VAD rats. Treatment of insulin induced GK expression and insulin + RA treatment further increased its protein level, which was consistent to the real-time PCR result.

## 4. Discussion

Stored VA and dynamic RA production may be integrated into the intracellular VA pool to maintain the normal insulin release from pancreatic β-cells. In this study, all the ZL rats were induced to develop T1DM via injection of STZ. They had no endogenous insulin secretion. The STZ-induced diabetic rats treated with RA had

exogenous RA that contributes to the intracellular RA pool but did not have endogenous insulin secretion. This is a desirable model to study the roles of VA in the regulation of hepatic expression of genes for glucose and lipid metabolism. Rats treated with insulin had exogenous insulin supply to regulate insulin-mediated gene expression. Rats treated with insulin + RA had both insulin and RA, which is an ideal model to study the combined effects of insulin and RA on hepatic gene expression. Rats were fed with either a VAS diet or a VAD diet to investigate whether VA status or stored VA affects the hepatic gene expression and plasma levels of nutrients and hormones in response to the treatments of RA and insulin.

The plasma results had demonstrated that injection of insulin or insulin + RA successfully increased the plasma insulin levels (Figure 4-2D). The results of real-time PCR had proven that the amount of RA injected was sufficient to induce gene expression at the mRNA level (Figure 4-3A). The plasma data show that VA status affects the plasma levels of nutrients and hormones in response to RA and insulin treatments. It seems that VAD rats were more sensitive to these treatments. We did observe that tail tip whole blood glucose levels in both VAS and VAD rats decreased after treatments of insulin or insulin + RA as shown in Table 4-1. However, the accumulated venous plasma glucose levels in VAS rats were not reduced significantly by insulin or insulin + RA treatment. It seems that the insulin responses in the liver and peripheral tissues are not equal or nonconcurrent in VAS rats. This phenomenon could be attributed to the higher amount of insulin needed to control the hepatic gluconeogenesis or more less amount of insulin required for the peripheral glucose disposal. Given the data obtained from VAD

rats, it indicates that VA status affects this phenomenon. This deserves further study regarding the efficient amount of insulin for reducing accumulated venous plasma glucose levels.

The clearance of plasma TG-rich lipoprotein particles, such as very low-density lipoproteins (VLDL), is dependent on lipoprotein lipase (LPL) the activity of which is mainly regulated by insulin [297]. Insulin deficiency is associated with low adipocyte LPL activity in diabetic rats, which is increased by insulin treatment [298, 299]. In the presence of insulin, the activity of LPL is increased leading to clearance of TG. Insulin also reduces VLDL secretion from the liver by affecting the assembly of VLDL particles in rodent hepatocytes [300]. We observed decreased TG levels only in VAD rats after the treatment of insulin, which indicates the role of VA in the regulation of TG metabolism. These results demonstrate the altered TG metabolism in VAD rats. The underlying mechanism deserves further investigation.

Insulin is required to maintain the normal plasma level of leptin. Markedly reduced leptin levels had been observed in rats with insulin-deficient diabetes and treatment of insulin restored its levels [301]. Our observation that insulin treatment induced leptin levels in VAD rats is consistent with this study. However, we did not observe increased leptin levels in VAS rats treated with insulin. This is probably attributed to that VAS rats had more WAT leading to more leptin production than VAD rats did. Theoretically, VAS rats tend to have higher basal leptin levels than that of VAD rats. The short-term treatment (3-4 hr) in the current experimental setting may not be sufficient to change the expression and secretion of leptin protein. Thus, the effects of

insulin treatment for 3-4 hr on leptin levels in VAS rats may not be as efficient as that in VAD rats. It seems that RA can increase leptin levels based on our observation that RA treatment alone induced leptin levels in both VAS and VAD diabetic rats. This may partially explain why insulin treatment restored leptin levels in VAD rats but not in VAS rats. Stored VA may also contribute to the elevation of leptin levels in diabetic rats. So a dramatic induction of leptin levels by insulin treatment cannot be easily observed in VAS rats. The leptin levels in VAD rats that had no stored VA are more sensitive to the insulin treatment.

We have shown that VAS ZL rats had higher hepatic mRNA levels of *Gck* than VAD ZL rats did [244] and RA alone induces or synergizes with insulin to induce *Gck* expression in primary rat hepatocytes [240]. This can explain that RA treatment only induced *Gck* expression in VAD rats but not that in VAS rats since VAS rats had higher *Gck* mRNA levels than VAD rats. Therefore, the anticipated RA-induced *Gck* expression in VAS rats may be masked or desensitized by endogenous RA production. The mRNA levels of *Gck* in VAD rats were more sensitive to the treatment of RA due to the lack of VA storage for RA production. Insulin treatment induced the *Gck* expression in both VAS and VAD rats, and the induction was more dramatic in VAD rats, probably due to the low basal level in the control rats. Treatment of insulin + RA significantly increased the mRNA levels of *Gck* of both VAS and VAD rats, and the increase in VAD rats was even higher than those with insulin treatment. These observations are matching our previous results [244]. RA treatment alone or in combination with insulin induces *Gck* expression in VAD diabetic rats.

We have observed elevated *Srebp-1c* expression levels in VAS rats compared to that in VAD rats [244], and RA synergizes with insulin to induce *Srebp-1c* expression in primary rat hepatocytes [245]. We did not observed higher *Srebp-1c* mRNA levels in VAS rats treated with insulin in this study, which may due to the short term of insulin treatment. We have shown that the insulin-induced *Srebp-1c* expression only becomes significant after 6 hr in primary rat hepatocytes. The immono-blot results of PEPCK and GK were consistent with the real-time PCR results. They also demonstrate that VA status affects the expression of ACC and FAS, which deserves further investigation.

In summary, VA status affects the responses of nutrients and hormones in rats with STZ-induced diabetes to RA and insulin treatments. This indicates that VA status contributes to the regulation of expression of hepatic genes for glucose and lipid metabolism. VAD diabetes rats seem more sensitive to the treatment of RA or insulin. Dynamic RA production alone or in combination with insulin induces the expression of *Gck* and other genes for glucose and fatty acid homeostasis in VAD diabetic rats. The roles of VA in the regulation of gene expression in other tissues of diabetic rats need to be studied to comprehensively understand how it contributes to the glucose and lipid metabolism. We will also investigate the effects of dynamic RA production on the action of insulin on hepatic gene expression.

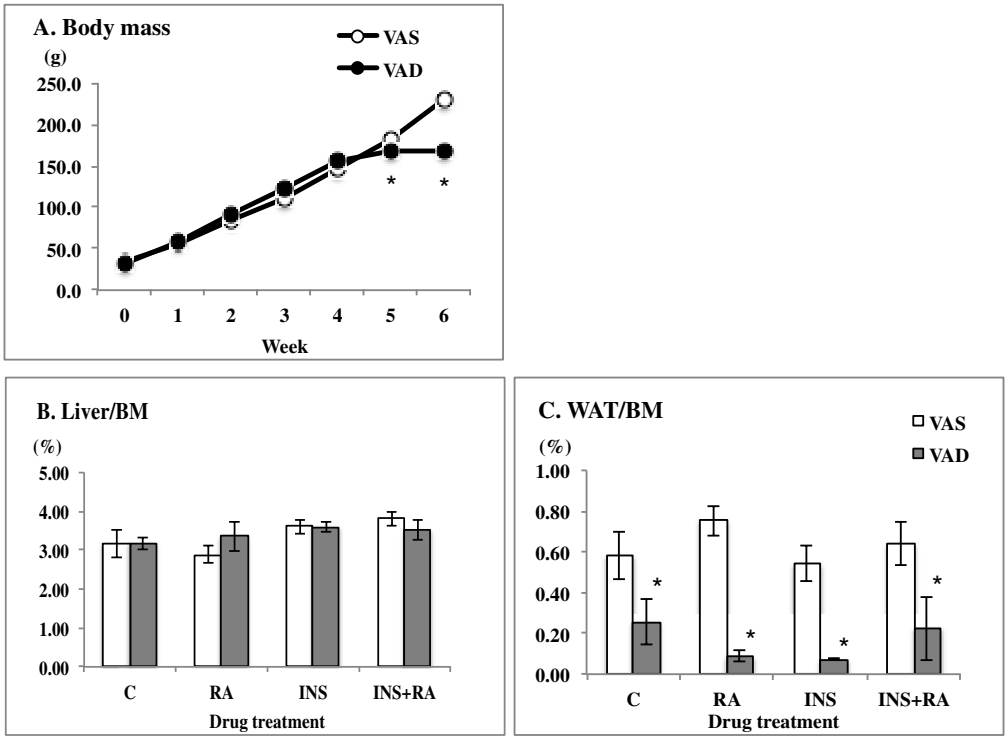



**Figure 4- 1 Body mass and body compositions of VAS and VAD rats.**
(A) Body mass of STZ-treated Zucker lean rats were recorded weekly. Results were represented means ± SEM of 16 independent experiments. * for comparing the control rats fed a VAS diet and rats fed a VAD diet; all $P < 0.05$. The ratios of (B) liver/BM and (C) WAT/BM were calculated and compared between VAS and VAD rats. Data were represented means ± SEM of 4 independent experiments. * for comparing the control VAS rats and VAD rats at the indicated groups; all $P<0.05$.

**Table 4- 1 Tail tip whole blood glucose levels of VAS and VAD rats after fasting, after STZ injection, and after treatment of saline, RA, INS, or INS + RA.**

| | | C | RA | INS | INS+RA |
|---|---|---|---|---|---|
| VAS | After fasting | 94 (±4) | 96 (±2) | 85 (±7) | 66 (±9) |
| | After STZ injection | 424 (±71) | 415 (±17) | 539 (±16) | 437 (±53) |
| | After treatment | 518[a] (±39) | 547[a] (±40) | 77[b] (±20) | 178[c] (±49) |
| VAD | After fasting | 81 (±5) | 78 (±3) | 74 (±4) | 69 (±4) |
| | After STZ injection | 403 (±15) | 491 (±45) | 490 (±29) | 467 (±25) |
| | After treatment | 489[d] (±65) | 494[d] (±46) | 116[e] (±39) | 122[e] (±37) |

Tail tip whole blood glucose levels of STZ-treated Zucker lean rats were measured after 48-hour fasting, 24 hr after STZ injection, and 3-4 hr after treatment of saline, RA, INS, or INS + RA. Results were represented means ± SEM of 4 independent experiments. a>c>b, d>e, $P<0.05$.

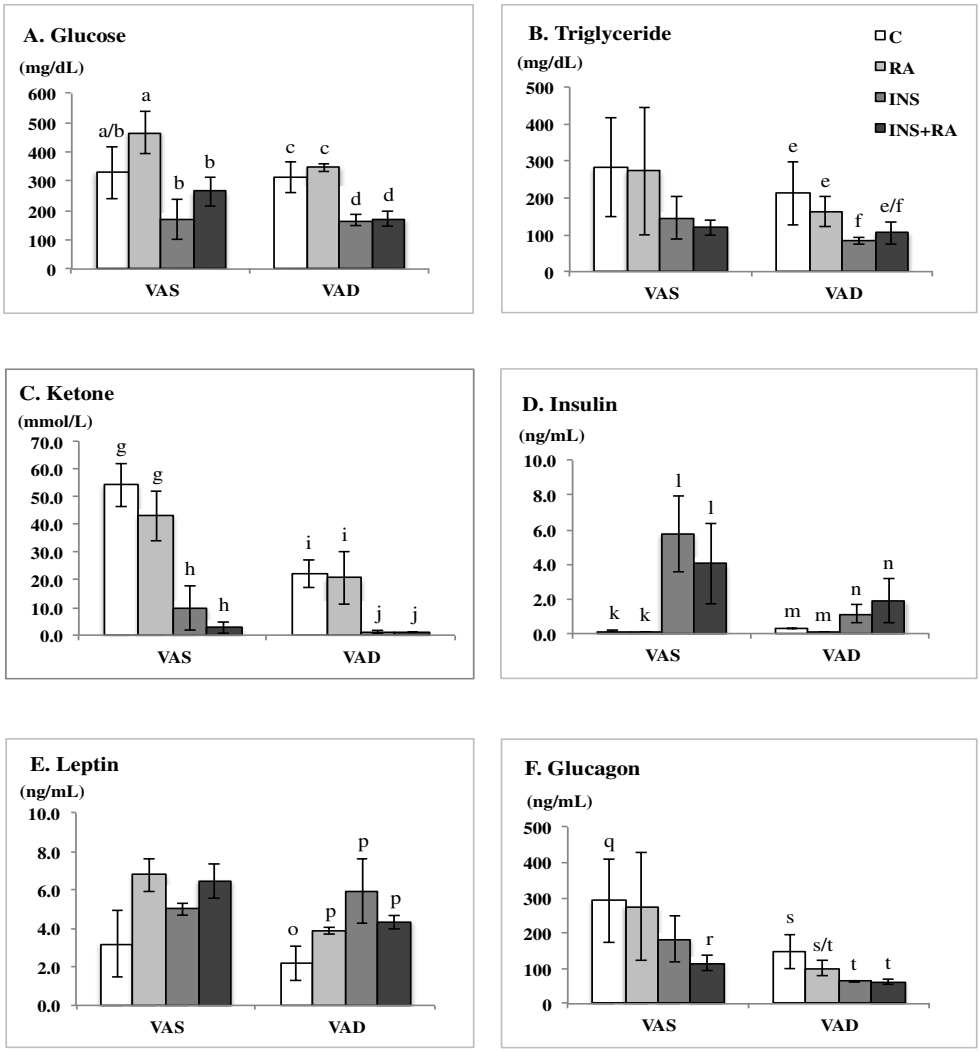



**Figure 4- 2 Plasma levels of (A) Glucose, (B) TG, (C) Ketone, (D) Insulin, (E) Leptin, and (F) Glucagon of VAS and VAD rats after the reagent treatment.**

Accumulated venous plasma samples of STZ-treated Zucker lean rats were collected 3-4 hr after the treatment of saline, RA, INS, or INS + RA. Glucose, TG, ketone, insulin, leptin, and glucagon levels were measured using the available commercial kits. Results were represented means ± SEM of 4 independent experiments. * for comparing the control VAS rats and VAD rats at the indicated groups; a>b, c>d, e>f, g>h, i>j, k<l, m<n, o<p, q>r, s>t, all $P<0.05$.

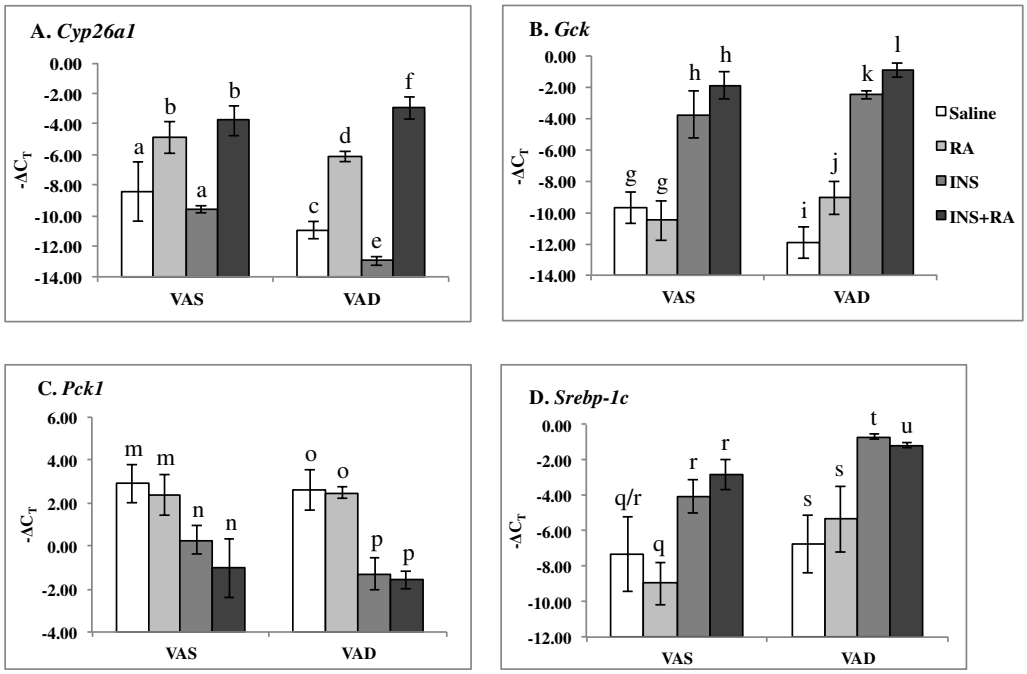



**Figure 4- 3 Comparison of mRNA levels of (A) *Cyp26a1*, (B) *Gck*, (C) *Pck1*, and (D) *Srebp-1c* of VAS and VAD rats in response to the reagent treatment.**

Liver tissue samples were collected 3-4 hr after the reagent treatment. Total RNAs were extracted and subjected to real-time PCR analysis. Results were presented as means ± SEM of the $-\Delta C_T$ (the $C_T$ of 36B4 - the $C_T$ of indicated transcript) from 4 independent experiments. a<b, e<c<d<f, g<h, i<j<k<l, m>n, o>p, q<r, s<u<t, $P<0.05$.

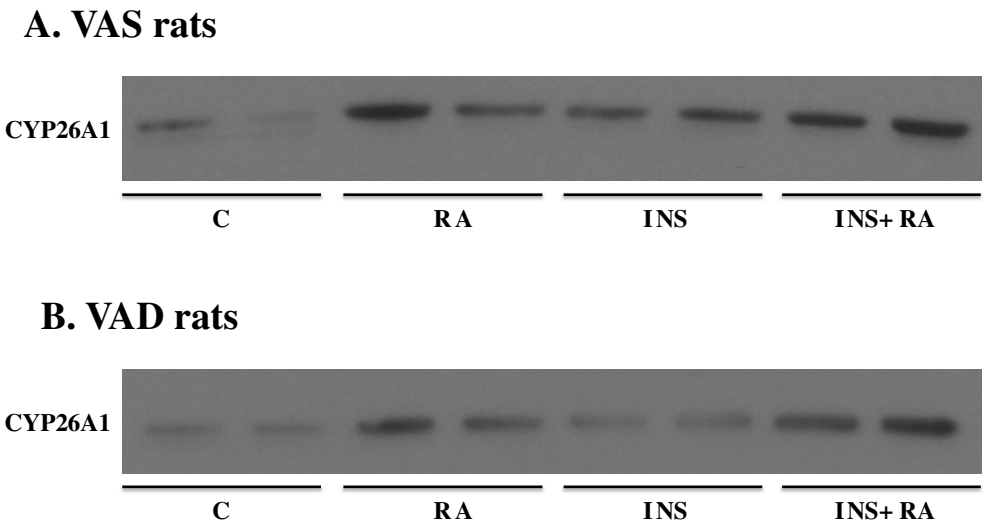
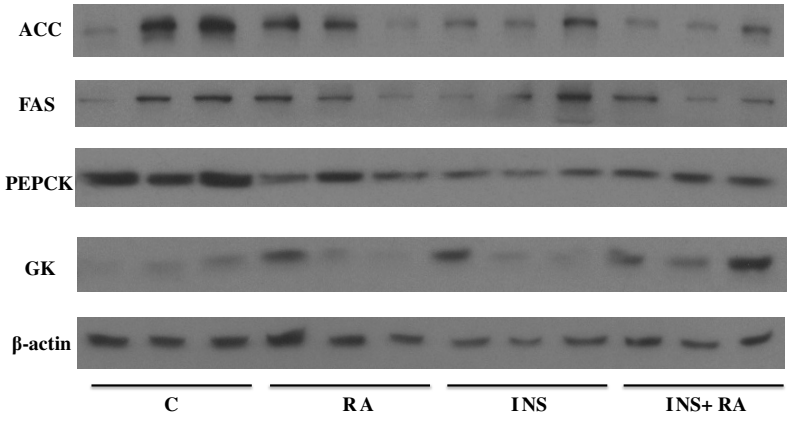


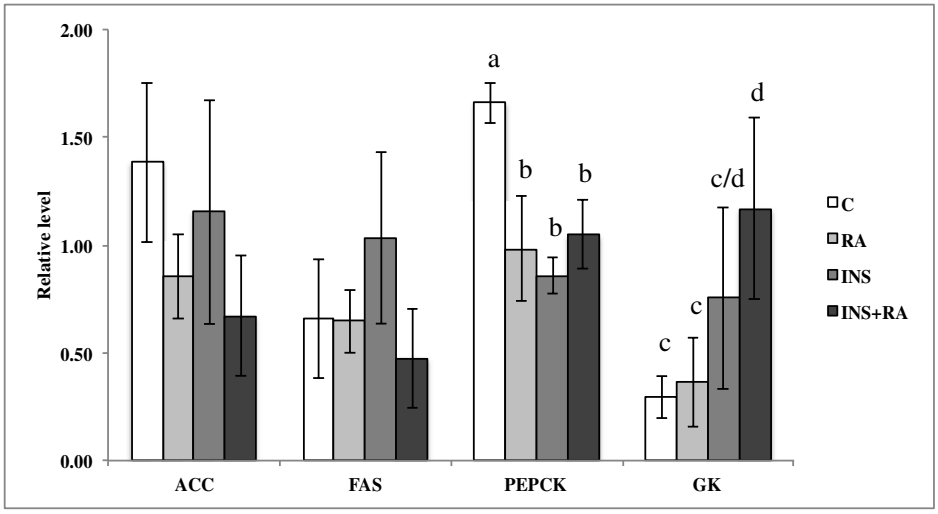

**Figure 4- 4 Comparison of the expression of CYP26A1 of STZ-treated Zucker lean (A) VAS rats and (B) VAD rats.**
Proteins (50 μg/lane) in whole cell lysates were isolated and subjected to immono-blot analysis.

**Figure 4- 5 Comparison of the expression levels of protein of STZ-treated Zucker lean (A) VAS rats and (B) VAD rats in response to the treatment of saline, RA, INS, or INS + RA.**

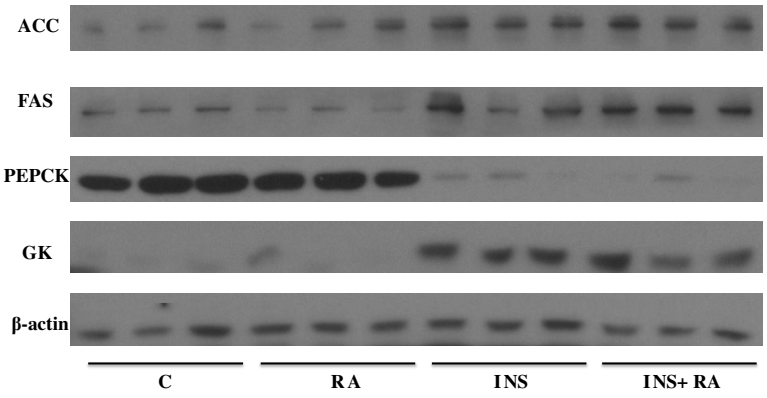
Proteins (50 μg/lane) in whole cell lysates were isolated and subjected to immono-blot analysis. The ImageJ Software was used to determine the density of a protein band. Results were presented as means ± SEM from 3 independent experiments. a>b, c<d, a'<b', d'<c'<e', f'>g', h'<i'<j', all $P<0.05$.

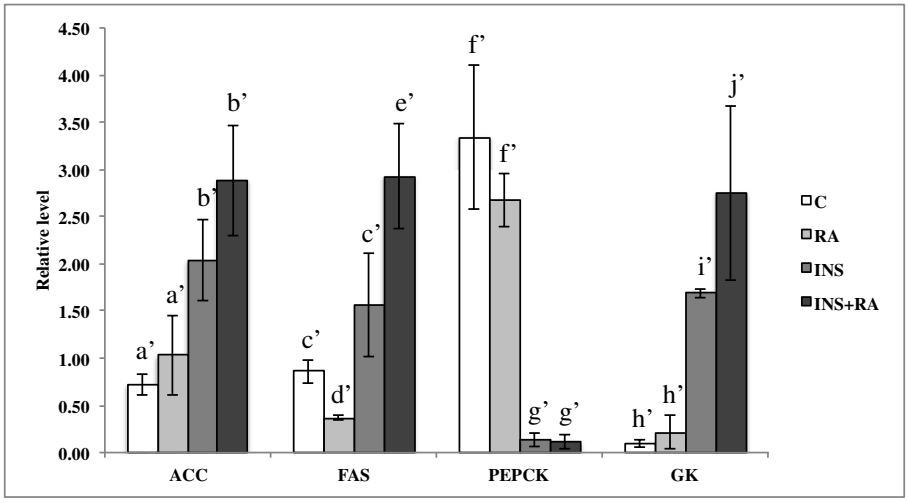
**A. VAS rats**


ACC
FAS
PEPCK
GK
β-actin
C
RA
INS
INS+ RA


2.00
1.50
1.00
0.50
0.00
Relative level
a
b
b
b
c
c
c/d
d
ACC
FAS
PEPCK
GK
C
RA
INS
INS+RA

**B. VAD rats**


ACC
FAS
PEPCK
GK
β-actin
C
RA
INS
INS+ RA


Relative level
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
a'
a'
b'
b'
c'
d'
c'
e'
f'
f'
g'
g'
h'
h'
i'
j'
ACC
FAS
PEPCK
GK
C
RA
INS
INS+RA

# Chapter Five

# General Discussion and Future Directions

## 1. Roles of dynamic VA catabolism in the regulation of hepatic glucose and lipid gene expression

### 1.1. The change of VA metabolism in obesity

Given essential roles of VA in a variety of physiological functions, we set up experiments to determine the effect of VA metabolism in the regulation of hepatic glucose and lipid metabolism. Experimental data presented through this dissertation indicate that rapid and dynamic metabolism of VA seems to contribute significantly to the expression of genes involved in hepatic glucose and fatty acid metabolism in rats. This contribution also includes the modulation of the insulin's action on gene expression.

The intracellular VA in the liver comes from two sources: stored VA in hepatic stellate cells, and VA in the diet. VA is stored in stellate cells in the form of retinyl esters and is released as retinol as needed for use. VAS rats, with adequate stores of VA in the liver, can maintain constant plasma VA level when the diet is not sufficient. VAD rats that show early signs of VA deficiency have depleted hepatic VA stores and they are dependent on the dynamic VA supply from the diet. In this study, I compared the gene expression of VAS and VAD rats in response to different physiological conditions, the fasting and refeeding cycle and the Streptozotocin (STZ)-induced diabetes, to investigate the effects of VA status and dynamic VA catabolism on expression of hepatic genes for glucose and lipid metabolism.

We first compared the expression levels of hepatic genes for the generation of RA in the liver of insulin-sensitive ZL and -resistant ZF rats. ZF rats develop obesity due to the mutation of leptin receptor [249], which is associated with profound changes of hepatic gene expression. The comparison allows us to determine whether any of these changes can be attributed to the changes of catabolism of VA in the liver of ZF rats. Based on our observations demonstrated in Chapter 2, the expression levels of both *Raldh1* mRNA and RALDH1 protein are higher in the liver of ZF than that of ZL rats. RALDH1 is the primary enzyme that converts retinal to retinoic acid (RA). We believe that the elevated expression of retinaldehyde dehydrogenase 1 (RALDH1) in the liver of Zucker fatty (ZF) rats contributes to increased lipogenesis through the induction of sterol regulatory element binding protein 1c (*Srebp-1c*). The hyperphagia of ZF rats can lead to the excessive intake of dietary VA and development of hyperinsulinemia. Excessive intake of dietary VA and elevated protein level of RALDH1 of ZF rats may trigger an increase in RA production that synergizes with insulin to induce *Srebp-1c* expression. The elevation of the mature SREBP-1c protein also induces the expression of *Raldh1* [65], which leads to more RA production, and further enhances the expression of *Srebp-1c* and its downstream lipogenic genes. This creates a feed-forward mechanism by which the *Srebp-1c* expression is maintained at a higher level in the liver of ZF rats than that of Zucker lean (ZL) rats [280].

## 1.2. Roles of the VA status in the control of plasma glucose level and hepatic glucose homeostasis

Glucose and lipid homeostasis is achieved in part by the regulation of the expression of hepatic genes involved in the process. Given the roles of VA in the regulation of the hepatic gene expression, it is anticipated that plasma glucose and lipid levels respond to VA status. This is what we have observed and presented in Chapters 3 and 4.

In ZL rats, we observed lower levels of plasma glucose, triacylglycerol (TG), insulin, leptin, and epididymal white adipose tissue (WAT) to body mass (BM) ratio of VA deficient (VAD) rats than that of VA sufficient (VAS) controls, which is consistent with the results in our previous studies [244]. We also observed reduced mRNA levels of *Gck* and *Srebp-1c*; and elevated I*gfbp1* mRNA levels in the liver of VAD ZL rats in comparison to that of VAS rats, matching our previous observations [244].

Glucose is the major source of energy for our body, and it is phosphorylated by hexokinases to generate glucose 6-phosphate for the use in cells. Hexokinase D, glucokinase (GK), is essential for glycolysis in the liver and pancreatic β-cells for the regulation of glucose homeostasis in the body. The *Gck* mRNA is induced by insulin and suppressed by glucagon in rat liver [302, 303], and the fasting and refeeding cycle changes the hepatic *Gck* expression [304]. We have shown that RA alone or synergizes with insulin to induce *Gck* expression in primary rat hepatocytes [240]. In this study, lower levels of hepatic *Gck* mRNA were observed in VAD rats as compared to that in VAS rats, demonstrating the involvement of VA in the regulation of *Gck* expression in

the liver. The VAD-refed-VAS rats had significantly higher hepatic expression levels of *Gck* mRNA than that of VAD-refed-VAD rats, which indicates that dynamic RA production contributes to the control of hepatic *Gck* expression.

In STZ-induced diabetic rats, the hepatic *Gck* expression levels in VAD rats were more sensitive to the treatment of exogenous RA or insulin. This is probably due to the fact that VAD rats had comparatively lower basal levels of *Gck* mRNA than VAS rats so the induction of *Gck* expression by the treatment of RA or insulin in VAD rats was more dramatic than that in VAS rats. It seems that RA synergizes with insulin to induce the hepatic *Gck* expression in insulin-dependent diabetic rats. Thus, dynamic VA catabolism affects the hepatic *Gck* expression levels in STZ-induced diabetic rats. This might be a potential target for treatment of diabetes and deserves further investigation.

Gluconeogenesis plays an essential role to maintain plasma glucose homeostasis during fasting. The cytosolic form of phosphoenolpuryvate carboxykinase (PEPCK-C) is the first rate-limiting enzyme of gluconeogenesis that converts oxaloacetate into phosphoenolpruvate in the presence of GTP. In the liver, insulin suppresses *Pck1* expression while glucagon induces its expression [305] so the liver can produces glucose in the fasting state. In our previous studies, we demonstrated that RA induced *Pck1* expression and attenuated insulin-mediated suppression of its expression in primary rat hepatocytes [243]. We did not observe any difference between the *Pck1* mRNA levels of VAD rats and that of VAS rats, indicating that additional factors may contribute to the regulation of *Pck1* mRNA. It is interesting to note that VAD-refed-VAD rats had higher expression levels of hepatic *Pck1* mRNA than VAD-refed-VAS rats did. This is probably

due to the relatively lower levels of insulin in VAD-refed-VAD rats so the suppression of *Pck1* expression by insulin is not as strong as that in VAD-refed-VAS rats. On the other hand, the consumed dietary VA or dynamic RA production in this case may not be sufficient to affect the insulin-mediated suppression of *Pck1*.

In STZ-induced diabetic rats, the hepatic *Pck1* mRNA levels were decreased upon the treatment of insulin or insulin + RA in both VAS and VAD rats. It is interesting to note that the reductions of hepatic *Pck1* mRNA expression upon insulin treatment in VAD rats were more dramatic than that in VAS rats. This phenomenon may be attributed to that the catabolism of the stored VA attenuates the insulin-mediated suppression of *Pck1* expression in STZ-induced VAS diabetic rats. It seems that VA status does not affect the Pck1 expression (Figure 4-3C). In addition, exogenous RA injection did not induce the Pck1 mRNA acutely.

The hypoglycemia in VAD rats may be attributed to the reduced supply of glucose from glycogenolysis in the liver due to the depletion of hepatic glycogen content. It may also result from increased utilization of glucose as energy because of the reduced availability of fatty acids (FAs) from lipolysis. The VAD rats have depletion of their white adipose tissues. In addition, the VAD rats have reduction of food intake. These cause that the VAD animals have less resources available for gluconeogenesis, anabolism and energy production. Given glucose can be used by all cells in the body, it becomes an available energy source. Therefore, its plasma level drops in the VAD status. The hypoglycemia probably also contributes to the hypoinsulinemia in the VAD rats.

### 1.3. Roles of the VA status in the control of plasma TG level and hepatic fatty acid metabolism

Fatty acid (FA) and TG homeostasis is regulated by the liver and adipose tissues in response to dietary and stored energy [306]. In the postprandial stage, the dietary and de novo synthetic FAs are stored in adipose tissues as TG [307]. Lipoprotein lipase (LPL) hydrolyzes TG in chylomicrons and very low-density lipoprotein (VLDL) into free FAs (FFAs), monoglycerides, and diglycerides to enter adipose tissues [307]. During fasting or starvation, FFAs are released from adipose tissues via lipolysis and used as energy by the liver, heart, and skeletal muscle. Insulin induces lipogenesis and inhibits lipolysis [291]. The action mediated by insulin is through the stimulation of the expression of lipogenic genes, a process mainly controlled by SREBP-1c [227].

We have reported that RA alone or synergizes with insulin to induce *Srebp-1c* expression in primary rat hepatocytes [245]. In this study, we observed reduced hepatic expression levels of *Srebp-1c* in VAD rats as compared to VAS rats. Thus, VA status affects the expression of *Srebp-1c* mRNA. The reduction of expression of hepatic lipogenic genes, such as *Srebp-1c*, may be responsible for the lower plasma TG levels and hepatic FA biosynthesis of VAD rats, which could also explain the reduced WAT/BM ratio of VAD rats. Another explanation is that RA may reduce the LPL activity in adipose tissue and skeletal muscle to decrease the clearance of TG leading to higher TG levels in VAS rats.

The VAD-refed-VAS rats had markedly higher hepatic expression levels of *Srebp-1c* than VAD-refed-VAD rats did. The hepatic mRNA levels of FA synthase gene

(*Fas*) in VAD-refed-VAS were also higher than that in VAD-refed-VAD rats. This demonstrates that the dynamic RA production contributes to the insulin-mediated regulation of *Srebp-1c* expression and the expression of its downstream gene *Fas*. In STZ-induced diabetic rats, treatment of insulin or insulin + RA induced the hepatic *Srebp-1c* expression levels in both VAS and VAD rats, but the inductions in VAD rats were more significant than that in VAS rats. This is probably due to the lower basal level of Srebp-1c in the liver of STZ-induced VAD rats. RA synergizes with insulin to induce *Srebp-1c* expression in STZ-induced diabetic rats. Thus, both stored VA and dynamic VA catabolism contribute to the insulin-mediated *Srebp-1c* expression.

### 1.4. Possible roles of VA catabolism in the development of insulin resistance in the liver

We have demonstrated in this dissertation that VA status and dynamic VA catabolism, with the combination of insulin, regulate expression of hepatic genes for glucose and lipid metabolism in different physiological conditions, such as the cycle of fasting and refeeding and STZ-induced diabetes. Dynamic RA production is primarily controlled by enzymes responsible for the synthesis of retinal and then RA. In this process, the expression of RALDH1 probably is critical as it converts retinal to RA irreversibly.

A common feature of obesity and type 2 diabetes mellitus (T2DM) is insulin resistance, which a given amount of insulin produces less than normal physiological responses. Insulin resistance is always associated with profound changes of expression levels of genes for the hepatic glucose and fatty acid metabolism. Generally, the liver of

an insulin-resistant animal loses the response to insulin regarding insulin-regulated glucose metabolism, but still retains the elevated lipogenesis. This results in a vicious cycle, which may accelerate the development of T2DM. ZF rats are obese and insulin resistant. We think that VA status and RA product play a role in the development of the hepatic insulin resistance in them. The increased lipogenesis in ZF rats can be explained by the feed-forward mechanism we mentioned above. As shown in Figure 5-1, dietary or endogenous VA enters into an intracellular pool, which is used for the RA product through a two-step oxidation process. In normal physiological condition, RA produced via this process is controlled by the expression levels and activities of RALDHs. The dynamic amount of RA contributes to the regulation of the expression of insulin-regulated genes in hepatocytes. In obese or insulin resistant animals, this homeostasis is disrupted. In the presence of excessive intake or catabolism of VA results in the over-production of RA, which alters the expression of RA and insulin-regulated genes for glucose and lipid metabolism in the liver. This may lead to the development of hepatic insulin resistance at the gene expression level.

**2. Roles of VA in the regulation of energy balance of whole body**

Given the strong effects of VA status and its catabolism in the control of the hepatic glucose and lipid homeostasis, it is reasonable to hypothesize that whole body energy balance can be affected by VA status. Using the CLAMS system, we monitored the energy expenditure of rats along with their body mass change in the course of development of VA deficiency.

We have observed that VA status contributes to the energy usage and balance of the whole body. Respiratory exchange ratio (RER) is a parameter that indicates the relative production of CO2 derived from carbohydrate and fatty acids in the oxidation process for the production of energy. RER reflects the respiratory quotient (RQ), an indicator of which fuel is being metabolized to supply the body with energy. The data presented in Chapter 3 demonstrate that RER of rats fed the VAS diet is higher than that of rats fed the VAD diet starting from week 4 after the ZL rats were fed their perspective diet. The data show that the ZL rats fed the VAS diet are using carbohydrate as the primary energy source and synthesized FAs as evidenced by their elevated RER values towards 1.0 after week 4. This indicates that ZL rats fed the VAS diet were in the anabolic state at week 4, an energy demanding process during which gluconeogenesis, glycogenesis, and lipogenesis occur. On the contrary, the RER values of ZL rats fed the VAD diet remained at about 0.85, which was lower than that of VAS rats after week 4. This suggests that ZL rats fed the VAD diet used a mixture of carbohydrate and FAs as the energy source and they were in the catabolic state, an oxidative process during which glycolysis, glycogenolysis, and β-oxidation occur. The RER data suggest that VA status plays a role in the regulation of the fuel metabolism in rats. VA is required to maintain the anabolic state in rats.

VA probably affects RER through multiple mechanisms. First, VA contributes to the hepatic glucose usage and fatty acid biosynthesis. We have shown that VA synergizes with insulin to induce the hepatic expression of *Gck* and *Srebp-1c*. This induction clearly promotes the conversion of glucose to fatty acids, indicating an anabolic process. Second,

VA is needed for the storage of glucose into glycogen. It has been shown that glycogenesis is impaired in VAD rats [244, 308], indicating the need of VA for glycogenesis. Third, VA is needed to maintain the presence of adipose tissue. We and others have shown that VA deficiency causes rats to lose their body fat, and reduction of plasma TG level. Forth, VA is needed for the proper development of pancreas, and secretion of insulin and glucagon in rats [157]. It has been shown that glucose-stimulated insulin secretion (GSIS) of VAD rats from pancreatic β-cells was impaired [157], which may contribute to the hypoinsulinemia in VAD rats. Insulin as the anabolic hormone is needed for the induction fatty acid biosynthesis, and accumulation of TG in adipose tissue. Without insulin action, the body will tend to be in a catabolic state. Additional experiments are needed to test the roles of any one or all of these pathways in the VA's effects on RER.

Other than the RER, we also measured the accumulated heat production in rats fed the VAD or VAS diet. Our data demonstrate that the accumulated heat production of rats fed the VAS diet for 4 weeks was higher than that fed the VAD diet. This result is probably due to the larger body size of VAS rats that produces more heat. After we normalized the heat production to BM, the adjusted heat production in rats fed the VAS diet was no longer different from that in rats fed the VAD diet. Since VAD rats had lower WAT/BM ratio than VAS rats did, it suggests that the VAD rats may have relatively higher lean BM to total BM ratio. Theoretically, muscle produces more heat than fat does. To maintain the same adjusted heat production rate, the heat production rate per unit of muscle mass of VAD rats might be lower than that of VAS rats. This phenomenon

suggests that VA status may also affect the muscle metabolism. Indeed, it has been shown that RA can induce the expression levels of uncoupling proteins in the culture skeletal muscle cell lines [309]. The elevated expression levels of uncoupling protein (UCP) will increase the dissipation of energy in the form of heat. This phenomenon seems to support our conclusion, which VA deficiency might reduce the heat production in the skeletal muscle. Further studies are needed to investigate the role of VA status in muscle metabolism and heat production.

## 3. Future perspective

This dissertation demonstrates that VA status and its metabolism play a critical role in the fuel homeostasis and energy utilization. It also raises more questions regarding the underlying molecular mechanisms of the observations. We believe that the following research areas deserve to be investigated further.

### 3.1. The contribution of RALDH1 in the development of hepatic insulin resistance at gene expression level

We hypothesize that SREBP-1c regulates the expression of *Raldh1* mRNA and dynamic RA production mediated by RALDH1 affects the action of insulin. To further study whether alteration of RALDH1 expression will affect the expression of insulin-mediated genes, and whether SREBP-1c regulates its expression in the hepatocytes or the liver of rats, we can first knockdown the expression of *Raldh1* in rat hepatocytes and analyze the insulin-regulated gene expression. In addition, we can overexpress and knockdown the expression of *Srebp-1c* in rat hepatocytes and analyze the expression

levels of *Raldh1* and *Cyp26a1*, an indicator of RA production. In such a way, we will be able to demonstrate the existence of a feed-forward cycle between RALDH1 and SREBP-1c, and in turn to understand the molecular mechanism of the hepatic insulin resistance at gene expression level.

### 3.2. The role of VA in normal and disease conditions

We demonstrate that VA is essential for maintaining the normal action of insulin in the liver of rats, and VA status may affect the insulin-mediated gene expression in the liver of ZL rats. An open question is whether VA is essential for the development of insulin resistance and obesity in high-fat diet (HFD)-induced obesity. As a fat-soluble vitamin, VA intake can be elevated with the intake of HFD. Therefore, we can set up experiment to determine the role of VA in the development of HFD-induced obesity in rats. ZL or other stain of rats will be fed either a high-fat diet (HFD) with sufficient amount of VA or a HFD without VA diet for 8 weeks after they are weaned. The growth and development of obesity will be analyzed during this course. Tissues and plasma samples will be collected at the end of the experiment. They will be compared between the VAD and VAS groups to determine the role of VA in the HFD-induced obesity and insulin resistance.

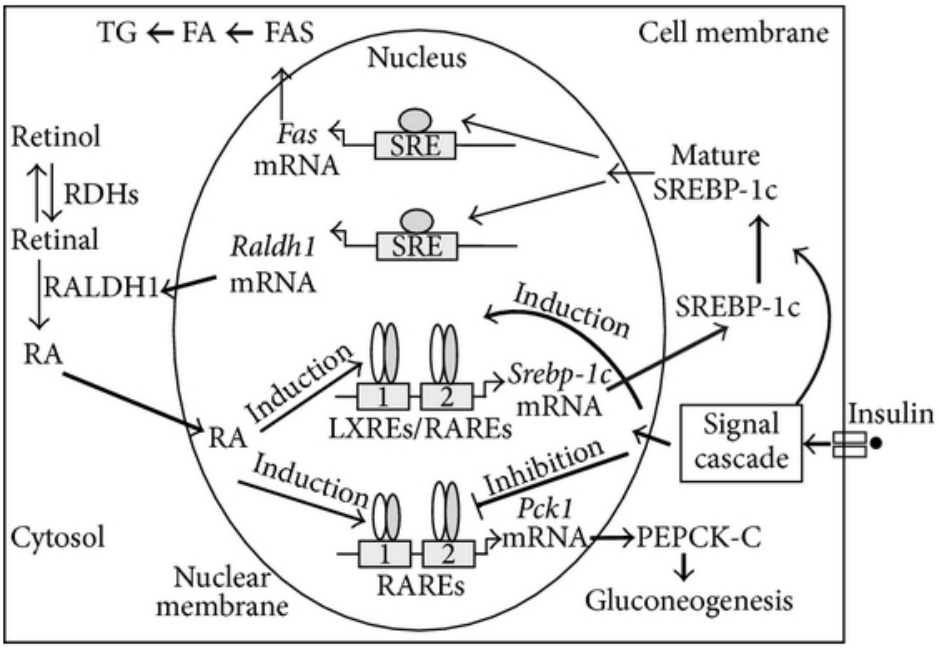
### 3.3. The mechanism by which VA affects the whole body energy usage.

RA has been reported to induce the expression of thermogenic UCP-1 in primary rat brown adipocyte [310] and stimulate UCP-3 in the muscle of mice [309]. Thus, we believe that VA status affects the whole body energy expenditure by the modulation of expression the expression of genes involved in thermogenesis. Brown adipose tissue

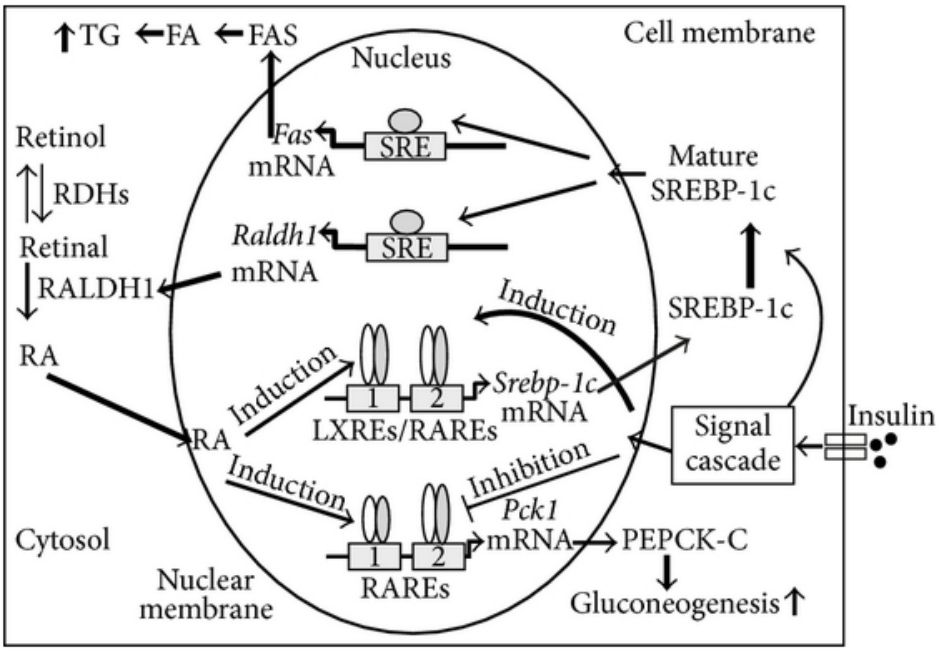
(BAT) and muscle from VAD and VAS rats will be collected and subjected to analysis. The expression levels of UCP-1 will be analyzed in the BAT of VAS and VAD ZL rats. The expression levels of enzymes in glucose and fatty acid expression, and thermogenesis such as UCP-3 in the muscle of VAS and VAD ZL rats will be compared. These data will be combined with the RER and the accumulated heat production data to disclose the role of VA in whole body energy usage and production.

**Figure 5- 1 The effects of RA on the insulin-regulated *Srebp-1c* and *Pck1* expression levels, the protein levels of SREBP-1c, the expression levels of *Raldh1* and Fas, and the fatty acid synthesis and gluconeogenesis in insulin-sensitive (a) and insulin-resistant (b) hepatocytes.**

(A) In insulin-sensitive hepatocytes, RA derived from retinal coordinates with insulin to regulate the expression of *Srebp-1c* and *Pck1*. The SREBP-1c protein is processed to mature form, which enters into the nucleus and induces the mRNA levels of *Raldh1* and *Fas*, which are, respectively, transcribed into RALDH1 and FAS. RALDH1 catalyzes the conversion of retinal to RA. FAS catalyzes the synthesis of palmitate, which is esterified into TGs or elongated to longer FAs. The *Pck1* mRNA is used to translate into PEPCK-C protein, which contributes to glucogenogenesis. (B) In the insulin-resistant hepatocytes, the elevation of Raldh1expression due to leptin deficiency or induction of SREBP-1c by high-cholesterol feeding induces the production of RA. The elevated RA levels will stimulate the transcription of *Srebp-1c* and *Pck1*. This leads to more production of SREBP-1c, and its mature form, in the presence of higher insulin levels in the insulin-resistant state. The induction of SREBP-1c further enhances the transcription of *Raldh1* and *Fas*, and their corresponding proteins for the production of more RAs and FAs, respectively. The RA production and SREBP-1c expression form a feed-forward cycle, which causes more TG and RA production in the insulin-resistant liver. At the same time, more of the PEPCK-C protein is generated due to the elevated Pck1transcription by RA stimulation, which is not responsive to insulin inhibition. This leads to elevated gluconeogenesis in the insulin-resistant liver. Eventually, the phenotypes appear as if insulin no longer suppresses gluconeogenesis while it still stimulates FA synthesis in the insulin-resistant state.




(A) Insulin-sensitive hepatocytes.




(B) Insulin-resistant hepatocytes.

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## Vita

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