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1,25-Dihydroxyvitamin D Regulation of Triacylglycerol Accumulation in Differentiated Adipocytes

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1,25-DIHYDROXYVITAMIN D REGULATION OF TRIACYLGLYCEROL ACCUMULATION IN DIFFERENTIATED ADIPOCYTES

A Dissertation

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Brienna M. Larrick

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of

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West Lafayette, Indiana

For Ronald J. Larrick.

With love,

BJ

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TABLE OF CONTENTS

Page

Page

LIST OF TABLES

LIST OF FIGURES

Figure Page

LIST OF ABBREVIATIONS

Abbreviation	Term
$1,25(OH)_2D$	1,25-dihydroxyvitamin D
25(OH)D	25-hydroxyvitamin D
AI	Adequate Intake
ATGL	Adipose triglyceride lipase
BMI	Body mass index
BODIPY	4,4-Difluoro-5,7-Dimethyl-4-Bora-3a,4a-Diaza-s-Indacene-3-Hexadecanoic Acid
cAMP	Cyclic adenosine monophosphate
CGI-58	Comparative gene identification-58
CPT-1	Carnitine palmitoyltransferase-1
DAG	Diacylglycerol
DBP	Vitamin D binding protein
DEXA	Dual-energy X-ray absorptiometry
DRI	Dietary Reference Intake
HSL	Hormone sensitive lipase
LD	Lipid droplet
LPL	Lipoprotein lipase
MARRS	Membrane-associated rapid response steroid binding protein
MGL	Monoacylglycerol
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-dephenyltetrazolium bromide
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
PBS	Phosphate buffered saline

PC	Pyruvate carboxylase
PKA	Protein kinase A
PPAR	Peroxisome proliferator-activated receptor
PTH	Parathyroid hormone
RDA	Recommended Dietary Allowance
RXR	Retinoid X receptor
shRNA	Small hairpin RNA
TAG	Triacylglycerol
TCA	Tricarboxylic acid
TNFα	Tumor necrosis factor-α
UL	Tolerable Upper Intake Level
VDR	Vitamin D receptor
VDRE	Vitamin D response element

ABSTRACT

Larrick, Brienna M. Ph.D., Purdue University, May 2016. 1,25-Dihydroxyvitamin D Regulation of Triacylglycerol Accumulation in Differentiated Adipocytes. Major Professor: Dorothy Teegarden.

Obesity is a major public health concern, both in the United States and worldwide. Therefore, identification of measures by which obesity may be prevented and reversed is of high priority. Epidemiological studies consistently demonstrate an inverse relationship between serum 25(OH)D levels, an indicator of vitamin D status, and measures of adiposity. These data suggest that vitamin D may play a role in the prevention of excessive adiposity. However, whether vitamin D impacts lipid storage and metabolism in terminally differentiated adipocytes is not yet known. The purpose of this work was to determine the impact of 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), the bioactive vitamin D metabolite, on triacylglycerol accumulation and lipid and glucose metabolism in differentiated adipocytes. To study this, 3T3-L1 adipocytes were differentiated for 9 days, followed by stimulation with $1,25(OH)_2D$ (10 nM) or vehicle for 1-7 days. Results indicate that $1,25(OH)_2D$ stimulates a 21% reduction in TAG accumulation in differentiated 3T3-L1 adipocytes after 4 days (P=0.01). This occurs despite a significant increase in fatty acid uptake (P<0.01), assessed using BODIPY FL C_{16}, and with concomitant stimulation of PKA-dependent

glycerol release (P<0.01), which is typically indicative of lipolysis. Additionally, we demonstrate that 1,25$(OH)_2$D stimulates a 2.5-fold increase in complete fatty acid oxidation (P<0.01), assessed by quantifying the production of $^{14}CO_2$ from [1-^{14}C] palmitic acid. These results suggest a novel mechanism by which 1,25$(OH)_2$D may be protective against excessive adiposity. In addition to its impact on fatty acid metabolism, the impact of 1,25$(OH)_2$D on glucose metabolism was also examined. Glucose contributes significantly to the intracellular TAG pool, serving as a substrate for both fatty acid synthesis as well as glycerol production to support TAG synthesis. The results indicate that 1,25$(OH)_2$D reduces the incorporation of D-[U-^{13}C]glucose incorporation into palmitic, palmitoleic, stearic, and oleic acids (P=0.03), determined by liquid chromatography-mass spectrometry (LC-MS). Interestingly, [$^{13}C_2$]acetate incorporation into these fatty acids was reduced by only 10% (p<0.01), suggesting that while *de novo* lipogenesis is slightly inhibited in response to 1,25$(OH)_2$D, the contribution of glucose specifically as a substrate for fatty acid synthesis is reduced. Study of glucose uptake and disposal as lactate revealed that these two processes are not impacted by 1,25$(OH)_2$D, suggesting that glucose may instead be used for the synthesis of glycerol. Indeed, inhibition of glycolysis to reduce substrate availability for glycerol synthesis completely prevented 1,25$(OH)_2$D-stimulation of glycerol release. These data suggest that rather than stimulating TAG hydrolysis, 1,25$(OH)_2$D stimulates disposal of glucose as glycerol, while reducing its utilization as a substrate for fatty acid synthesis. While the mRNA expression of pyruvate carboxylase (PC) is reduced by 40% in response to 1,25$(OH)_2$D

(P<0.01), suggesting that 1,25$(OH)_2$D may limit pyruvate entry into the TCA cycle, at this time it is not clear whether this underlies the 1,25$(OH)_2$D-stimulated changes in glucose metabolism and TAG storage. In conclusion, 1,25$(OH)_2$D stimulates fatty acid oxidation in 3T3-L1 adipocytes, and reduces the contribution of glucose to the fatty acid pool, likely by stimulating glucose disposal as glycerol. These results demonstrate that 1,25$(OH)_2$D regulates both fatty acid and glucose metabolism in differentiated 3T3-L1 adipocytes to reduce TAG storage. These novel findings demonstrate a mechanism by with 1,25$(OH)_2$D may protect against excessive fat mass accumulation, and provide support for the inverse relationship between vitamin D and obesity.

CHAPTER 1. LITERATURE REVIEW

1.1 Obesity and Associated Metabolic Disorders

Obesity has become a major public health concern, with over 36% of adults and 17% of children and adolescents in the United States considered obese (1). Individuals who are obese are at increased risk for developing chronic diseases and metabolic disorders, as well as certain types of cancer (2). Additionally, it is estimated that medical costs associated with obesity account for over 20% of all annual health care spending in the United States, and that direct and indirect costs from obesity exceed $275 billion annually (3). Because of these devastating physical, psychological, and financial costs of obesity, it is imperative that effective measures to prevent and reverse obesity are identified.

Several measures exist by which individuals may be classified according to their weight status and associated disease risk. Body mass index (BMI) is a quick and inexpensive method of weight assessment, and is positively correlated with metabolic disorders that are associated with obesity. Therefore, BMI is often used as a screening tool for categorizing individuals by weight. Body mass index is calculated by dividing an individual's weight in kilograms by the square of height in meters. While BMI is not a direct measure of adiposity, BMI does correlate with direct measures of body fat, including dual energy x-ray

absorptiometry (DEXA), bioelectrical impedance, densitometry, and skinfold thickness measurements (4, 5). For adults 20 years and older, BMI is used to categorize individuals as underweight (BMI < 18.5), normal weight (BMI 18.5-24.9), overweight (BMI 25.0-29.9), or obese (BMI >30.0) (2). Epidemiological data show a modest increased risk of mortality when BMI reaches above 25 kg/m^2 (6-8), and a 50-100% risk of all-cause mortality when BMI reaches 30 kg/m^2 (7, 8).

While BMI serves as a convenient and inexpensive measure of weight assessment, it does not distinguish between excessive adipose and muscle mass, and is therefore not diagnostic individual of health risk (9). A more accurate representation of an individual's health risk may be obtained by assessing percent body fat. The "gold standard" for measuring body fat is DEXA, but this method is expensive and not always accessible. Estimates of percent body fat may also be obtained using handheld or scale instruments that measure bioimpedance, or by measurements of skinfold (10). To avoid elevated weight-associated health risks, the recommended body fat percentage ranges for men and women are 12-20% and 20-30%, respectively (11).

There are many diseases and health conditions associated with obesity. Individuals who are obese are more likely than lean individuals to experience sleep apnea, depression, anxiety, and osteoarthritis (2, 12, 13). Further, metabolic disorders such as hypertension, dyslipidemia, coronary artery disease, and type 2 diabetes are more prevalent among those who are obese, as are certain types of cancer such as that of the breast, colon, kidney, gallbladder, and

liver (2, 14). Adipose tissue dysfunction often underlies obesity-associated metabolic disorders, and modulation of adipose tissue metabolism has become a target for combating obesity and associated chronic diseases.

1.1.1 Adipose Tissue Physiology

1.1.1.1 Characteristics of Adipose Tissue

For the vast majority of individuals, adipose tissue is the largest energy reservoir in the body (15). In animals, approximately one-third of the cells found in adipose tissue are adipocytes, while the remaining two-thirds are a combination of fibroblasts, preadipocytes, stem cells, as well as nerve tissue and vasculature (16). Historically, adipose tissue was considered to be an organ devoted mainly to energy storage and thermal regulation, while serving as a source of cushioning to protect internal organs (17). However, the complexity of this tissue has been identified in recent years, and it is now known that functions of adipose tissue also include regulation of appetite, insulin sensitivity and glucose homeostasis, lipid metabolism, inflammation and immunity, as well as angiogenesis and blood pressure (17, 18).

In humans, adipose tissue is organized in discrete anatomical depots: subcutaneous and visceral. Adipose tissue ranges from 5-60% of total body mass, with subcutaneous adipose tissue (SAT) accounting for 80% or more of the total fat mass (19). The subcutaneous adipose tissue lies under the skin and includes abdominal, gluteal, and femoral adipose depots (19). Accumulation of

subcutaneous fat mass, or peripheral obesity, is thought to be protective against metabolic disease (20-26).

On the other hand, visceral adipose tissue (VAT) is associated with digestive organs, and represents 10-20% of the total fat mass in men, and 5-10% of that in women. Visceral adipose depots include the omental, which associates with the stomach, the intestine-associated mesenteric, and the epiploic, which can be found along the colon (19). Excessive accumulation of visceral adipose tissue, termed “central obesity,” is associated with risk of metabolic disorders (20-24, 26). It is thought that VAT confers elevated risk of metabolic disorders because of its direct access to the portal vein; hepatocytes are exposed to high levels of fatty acids freed by lipolysis from VAT, which may lead to hepatic insulin resistance, hepatic glucose production and fasting hyperglycemia (27). Further, production of proinflammatory cytokines has been found to be higher in VAT than in SAT depots (28), contributing to the increased metabolic disease risk with excessive VAT accumulation.

1.1.1.2 Functions of Adipose Tissue

One of the longest-known functions of adipose tissue is maintenance of energy homeostasis. Adipocytes can store excess energy in cytoplasmic lipid droplets, in the form of triacylglycerol (TAG). Postprandially, or in response to overnutrition, this function prevents ectopic lipid deposition in tissues such as the liver, heart, and skeletal muscle, and prevents lipotoxicity in these organs (29). Conversely, during fasting, or when energy demand exceeds energy intake, TAG

stores may be hydrolyzed to release fatty acids from the cell for use by other tissues. The storage and hydrolysis of TAG in adipocytes prevents excess circulating energy substrates when food is plentiful, and ensures constant energy availability during prolonged periods of fasting (30).

While adipose tissue was originally considered an inert energy storage reservoir that also provides temperature and mechanical insulation, adipose tissue has recently recognized as an active endocrine organ (31). Adipose tissue secretes a variety of bioactive peptides, called adipokines, which participate in the regulation of feeding behavior, glucose and lipid metabolism, energy homeostasis, immunity, inflammation, adipogenesis and vascular function (32). Adipokines secreted by adipose tissue include adiponectin, leptin, resistin, adipsin, and visfatin, as well as cytokines such as IL-6 and TNFα. These factors that are secreted by adipocytes act locally, peripherally, and centrally to impact tissue metabolism and cross-talk (15).

1.1.1.3 Adipocyte Biology

While adipocytes account for approximately one-third of the cells found in adipose tissue, adipocytes constitute approximately 90% of the total tissue volume due to their large size. Three distinct types of adipocytes exist: white, brown, and beige. Phenotypically, white adipocytes are large, round, and unilocular (i.e. they contain one large lipid droplet). White adipocytes do not express uncoupling protein-1 (UCP-1), and they have a low mitochondrial density. Their main functions are to act as an energy reservoir and as an

endocrine organ. Brown adipocytes are multilocular, exhibit high levels of UCP-1 expression, and have a high mitochondrial density, which confers their brown color. Brown adipocytes also function as a source of thermogenesis. Beige, or brite, adipocytes exhibit characteristics that are intermediate to brown and white adipocytes. Beige adipocytes exhibit UCP-1 expression, and have multiple lipid droplets. The major functions and characteristics of these different types of adipocytes are still under investigation (33).

1.1.1.3.1 White Adipocytes

The white adipocyte is the most abundant type of adipocyte in the adipose tissue. Its major functions are to store and release fuel during times of energy excess and deprivation, and to contribute to the endocrine functions of adipose tissue. Lipogenesis and lipolysis within the adipocyte are regulated in the short term by hormonal signals in circulation. However, long-term maintenance of adipose tissue and changes in energy storage require changes in both size and number of adipocytes. An increase in cell number, or hyperplasia, is achieved by stimulating the differentiation of preadipocytes, which are also found in adipose tissue.

Much of what is known about adipocyte differentiation was determined using the murine 3T3-L1 preadipocyte cell line. This cell line is one of the most common, and best characterized, models used to investigate adipocyte development and function. When injected into mice, 3T3-L1 preadipocytes differentiate and form fat pads that are indistinguishable from the mouse adipose

tissue (34). Differentiation of 3T3-L1 post-confluent preadipocytes can be achieved using a differentiation cocktail consisting of insulin, a glucocorticoid, an agent that stimulates intracellular cyclic AMP (cAMP) formation, and fetal bovine serum (35). Typically, a cocktail of insulin, dexamethasone (DEX), a synthetic glucocorticoid agonist, and isomethylbutylxanthine (IBMX), a phosphodiesterase inhibitor used to stimulate intracellular cAMP accumulation, is used to stimulate adipogenesis in 3T3-L1 adipocytes (36).

Preadipocyte differentiation occurs following a temporal cascade of transcriptional events (Figure 1.1) (37). Cell-to-cell contact at confluence stimulates the expression of lipoprotein lipase (LPL) and type IV collagen, early markers of adipocyte differentiation (38, 39). Within one hour after addition of the differentiation cocktail, transient expression (lasting 2-6 hours) of c-fos, c-jun, and c-myc is observed, as well as the expression of CCAAT/enhancer binding protein (C/EBP) β and δ (40). Within 24 hours after administration of the differentiation cocktail, postconfluent mitosis and subsequent growth arrest occur, after which cells are committed to becoming adipocytes (41). Upon removal of the differentiation cocktail, expression of C/EBPβ and δ dissipate within 2-8 days (42). However, their transient expression and activity are needed for the expression of peroxisome proliferator-activated receptor γ (PPARγ) (43, 44) and C/EBPα (45, 46), which are induced 2 days after initiation of differentiation and are maximally expressed by 3-5 days following initiation of differentiation. The sustained expression of both PPARγ and C/EBPα are necessary for the subsequent expression of adipocyte- specific genes, such as those involved in

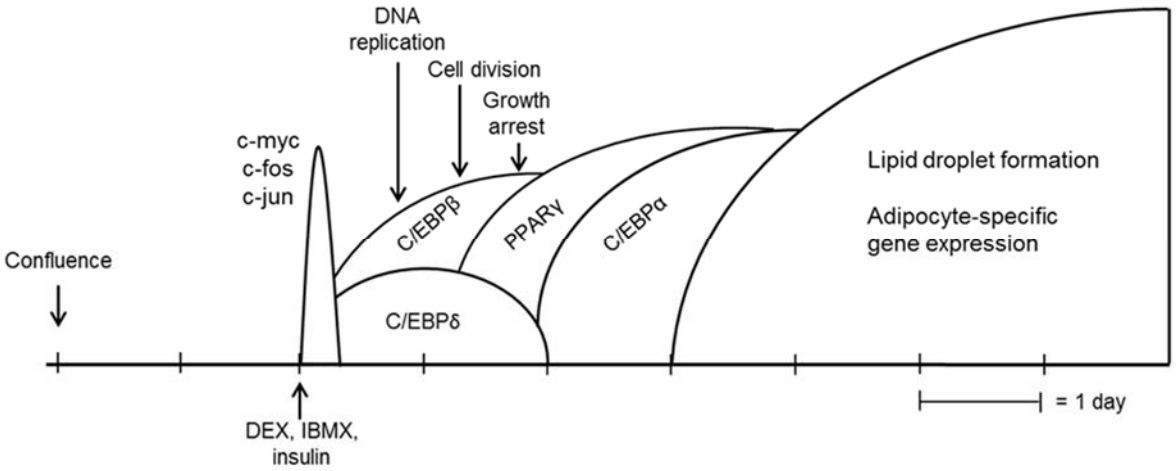


Figure 1.1. Adipocyte differentiation transcriptional cascade. C/EBP: CCAAT-enhancer-binding protein; DEX: dexamethasone; IBMX: 3-isobutyl-1-methylxanthine. Adapted from "Adipocyte differentiation and gene expression," by Ntambi JM and Young-Cheul K., *The Journal of Nutrition 2000; 130: 3122S-3126S*. Adapted with permission from the American Society for Nutrition.

lipid and glucose metabolism, and for development of the mature adipocyte phenotype (47).

1.1.1.3.2 Brown Adipocytes

There are many characteristics that distinguish brown from white adipocytes, namely, high mitochondrial density, UCP-1 expression, and the presence of many small lipid droplets (33). The high levels of mitochondrial UCP-1 expression in brown adipocytes cause uncoupling of the mitochondrial respiratory chain from ATP production, allowing energy to dissipate as heat by a process called non-shivering thermogenesis (48). This process is stimulated in response to β-adrenergic receptor stimulation, such as that which occurs when noradrenaline is released upon cold exposure (49, 50). In mice, brown adipocytes are found in inter-scapular, sub-scapular, and cervical regions (51). While originally thought to be only present in infant humans, recent studies employing ^{18}F-fluorodeoxyglucose (FDG) positron emission tomography/computed tomography (PET-CT) analysis of glucose uptake have identified BAT in adult humans (52). Further, it has been demonstrated that the abundance of BAT is inversely correlated with BMI and total adipose mass, highlighting the therapeutic potential of targeting BAT to combat obesity (52-57). Several studies suggest independent lineages for the developmental origins of white and brown adipocytes; specifically, that brown adipocytes share a common lineage with myogenic cells (58-62). However, the origins of brown and white adipocytes remain incompletely understood, and whether selectively increasing

BAT mass and/or activity in humans is achievable, in order to combat obesity, is under current investigation (33).

1.1.1.3.3 Beige Adipocytes

A third type of adipocyte is the beige, or “brite” (brown-in-white) adipocyte. These adipocytes resemble brown adipocytes in that they express UCP-1, are multilocular, and have higher mitochondrial density than white adipocytes (63, 64). Under basal conditions, beige adipocytes exhibit a phenotype similar to white adipocytes. However, in response to cold stimulation, these adipocytes are “browned,” and display characteristics similar to the brown adipocyte (33). While the primary function of beige adipocytes appears to be thermogenesis, the characterization of this cell type is under investigation.

1.1.1.4 Adipocyte Lipid Metabolism

Fatty acids serve a variety of functions within all cells, including as an energy source, as a precursor for cellular membranes, and as precursors for signaling molecules. In adipocytes, fatty acids are also stored in the form of TAG, so that during times of energy deficit they may be released for use by other tissues. As such, the adipocyte must maintain tight regulation over the processes used to take up, synthesize, store, and release fatty acids.

1.1.1.4.1 Fatty Acid Uptake

Fatty acids can be taken up as free fatty acids (FFA), which circulate bound to serum albumin, or can be obtained through the hydrolysis of TAG, which are carried in circulation by lipoproteins such as chylomicrons or very low-density lipoproteins (VLDL). Postprandially, or after a meal, fatty acids obtained from the diet are packaged by enterocytes into chylomicrons, which first enter circulation within the lymphatic system, followed by entry into the bloodstream. These chylomicrons, along with very low density lipoproteins (VLDL) secreted by the liver, transport fatty acids in the form of TAG to peripheral tissues such as adipose tissue, skeletal muscle, and the heart. Chylomicrons and VLDLs are spherical particles featuring a hydrophobic lipid core containing neutral lipids such as TAG, cholesterol esters, and sterol esters, surrounded by a polar lipid surface layer that contains phospholipids and free cholesterol, as well as apolipoproteins that collectively provide a hydrophilic interface with the aqueous surroundings in circulation (65, 66).

Chylomicrons and VLDL are relatively large in size, having diameters of 100-1200 nm and 30-80 nm, respectively (66). In order to enter the cell, TAG must be hydrolyzed to fatty acids intravascularly by lipoprotein lipase (LPL). Intravascular lipolysis removes up to 90% of TAG from the circulating lipoprotein, allowing fatty acids to be taken up by peripheral tissues. The apolipoprotein remnant is taken up by hepatocytes, for use in VLDL synthesis (67).

Uptake of fatty acids by cells can occur by several mechanisms (68). While long chain fatty acids may diffuse passively across phospholipid bilayers (69), a variety of facilitated transport mechanisms by integral or membrane-associated proteins have been identified (70). To enter the cell, the fatty acid must first dissociate from the lipoprotein or serum albumin, diffuse through the outer aqueous phase and insert into the outer plasma membrane, translocate or “flip-flop” from the outer to the inner plasma membrane, and dissociate from the inner membrane to the aqueous cytosolic phase of the cell (71). Proteins at the plasma membrane such as fatty acid transport protein (FATP), fatty acid binding protein (FABP), caveolin-1, and CD36 have been implicated in the uptake and utilization of fatty acids (68, 71). However, the precise mechanisms of FA transport across cell membranes, and the relative contributions of diffusion- and protein-mediated uptake, remain a source of interest and controversy (71, 72).

1.1.1.4.2 Fatty Acid Synthesis

In addition to the uptake of exogenous fatty acids, the adipocyte may synthesize fatty acids *de novo* from non-lipid precursors such as glucose (Figure 1.2). The key transcription factor regulating expression of genes involved in lipogenesis is sterol regulatory element-binding protein-1c (SREBP-1c), and the primary enzymes involved in fatty acid synthesis are fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) (73). When glucose is abundant, increased flux through glycolysis leads to excess citrate in the tricarboxylic acid (TCA) cycle, which is then redirected to the cytosol and converted to acetyl-CoA for

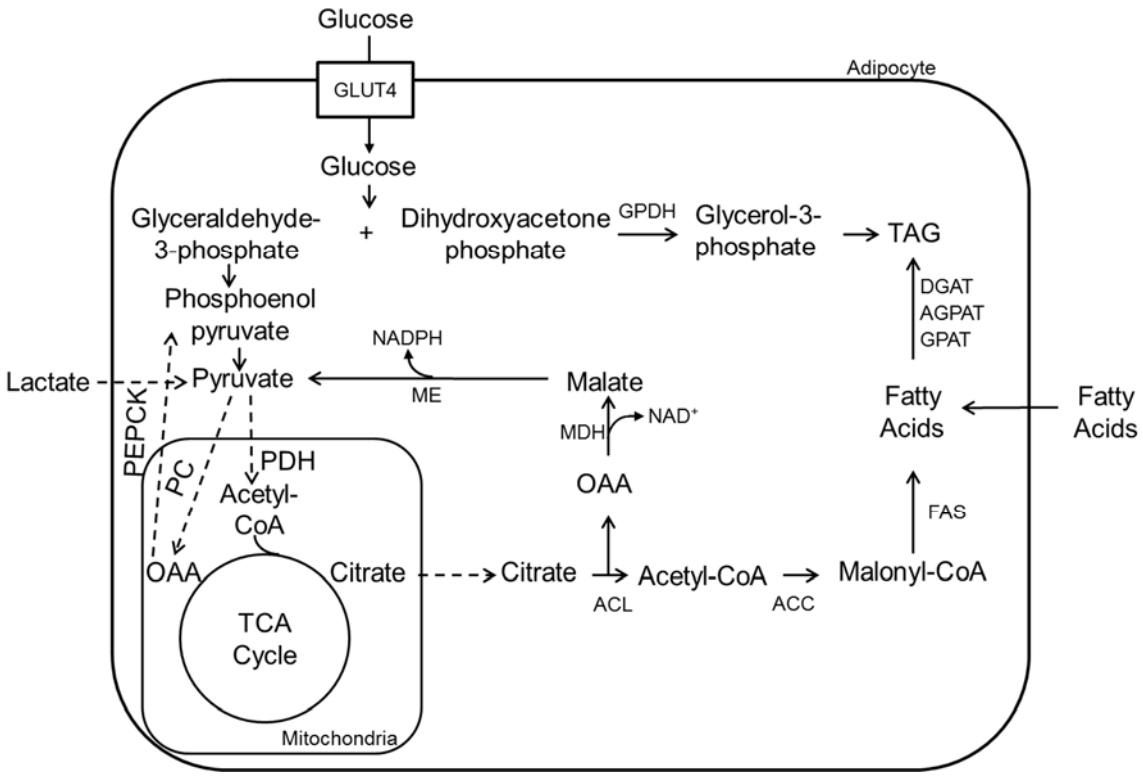


Figure 1.2. Summary of fatty acid and triacylglycerol synthesis in adipocytes. ACC: acetyl-CoA carboxylase; ACL: ATP-citrate lyase; AGPAT: 1-acylglycerol-3-phosphate acyltransferase; DGAT: diacylglycerol acyltransferase; FAS: fatty acid synthase; GPAT: glycerol-3-phosphate acyltransferase; GPDH: glycerol-3-phosphate dehydrogenase; MDH: malate dehydrogenase; ME: malic enzyme; OAA: oxaloacetate; PC: pyruvate carboxylase; PDH; pyruvate dehydrogenase; PEPCK: phosphoenolpyruvate carboxykinase.

fatty acid synthesis (74). Cytosolic acetyl-CoA is carboxylated by ACC to form malonyl-CoA. Malonyl-CoA joined to acetyl-CoA by FAS, and this hydrocarbon chain is elongated to form palmitic acid, which may then be further elongated or desaturated, and esterified with G3P to form TAG (15). In humans, *de novo* lipogenesis accounts for approximately 20% of newly formed TAG-palmitate (75), highlighting the significance of adipose tissue DNL contribution to total fat mass stores.

1.1.1.4.3 Triacylglycerol Synthesis

Whether obtained from circulation or synthesized *de novo*, intracellular fatty acids are esterified to glycerol-3-phosphate (G3P) for storage in lipid droplets as TAG. In lipogenic tissues such as adipose tissue and the liver, glycerol-3-phosphate may be derived from three sources: glycolysis, glyceroneogenesis, and glycerol kinase activity (76). During glycolysis, glucose that has been taken up into the cell is phosphorylated and through a series of reactions converted to G3P and dihydroxyacetone phosphate (DHAP). The DHAP may be converted to G3P by glycerol phosphate dehydrogenase (GPDH) to form G3P, which may then be used for TAG synthesis. In humans, approximately 20-25% of glucose taken up by adipocytes following an overnight fast is used for G3P synthesis and incorporation into TAG (77).

Alternatively, G3P may be synthesized from non-glucose precursors such as pyruvate, lactate, and amino acids by a process called glyceroneogenesis. Pyruvate is carboxylated to oxaloacetate (OAA), which then exits the

mitochondria and is decarboxylated by cytoplasmic phosphoenolpyruvate carboxykinase (PEPCK) to form phosphoenolpyruvate (PEP), the rate-limiting step of glyceroneogenesis (78). Phosphoenolpyruvate is then converted glyceraldehyde-3-phosphate followed by DHAP, which can then be converted to G3P by GPDH. While hepatocytes may use glycerol secreted by adipose during lipolysis for G3P and TAG synthesis, the activity of glycerol kinase, which phosphorylates glycerol to G3P, is negligible in adipose tissue, and glyceroneogenesis is quantitatively the largest contributor of adipocyte G3P synthesis (79).

In adipocytes, TAG is synthesized by esterification of alcoholic residues of G3P by a series of enzymes including glycerol-3-phosphate acyltransferase (GPAT), 1-acylglycerol-3-phosphate acyltransferase (AGPAT), phosphatidic acid phosphatase, and diacylglycerol acyltransferase (DGAT), found on the smooth endoplasmic reticulum (15). TAG that is synthesized in adipocytes is stored in cytosolic lipids droplets. In humans, *de novo* lipogenesis may account for up to 40% of whole-body lipogenesis (80).

1.1.1.4.4 Lipolysis

When energy demand exceeds energy intake, adipose TAG maybe hydrolyzed by a process called lipolysis, so that fatty acids and glycerol moieties may be utilized by other tissues (66) (Figure 1.3). The initial step of TAG hydrolysis is catalyzed by adipose triglyceride lipase (ATGL) (81). ATGL activity is regulated transcriptionally (65), by vesicular delivery to lipid droplets (82-84),

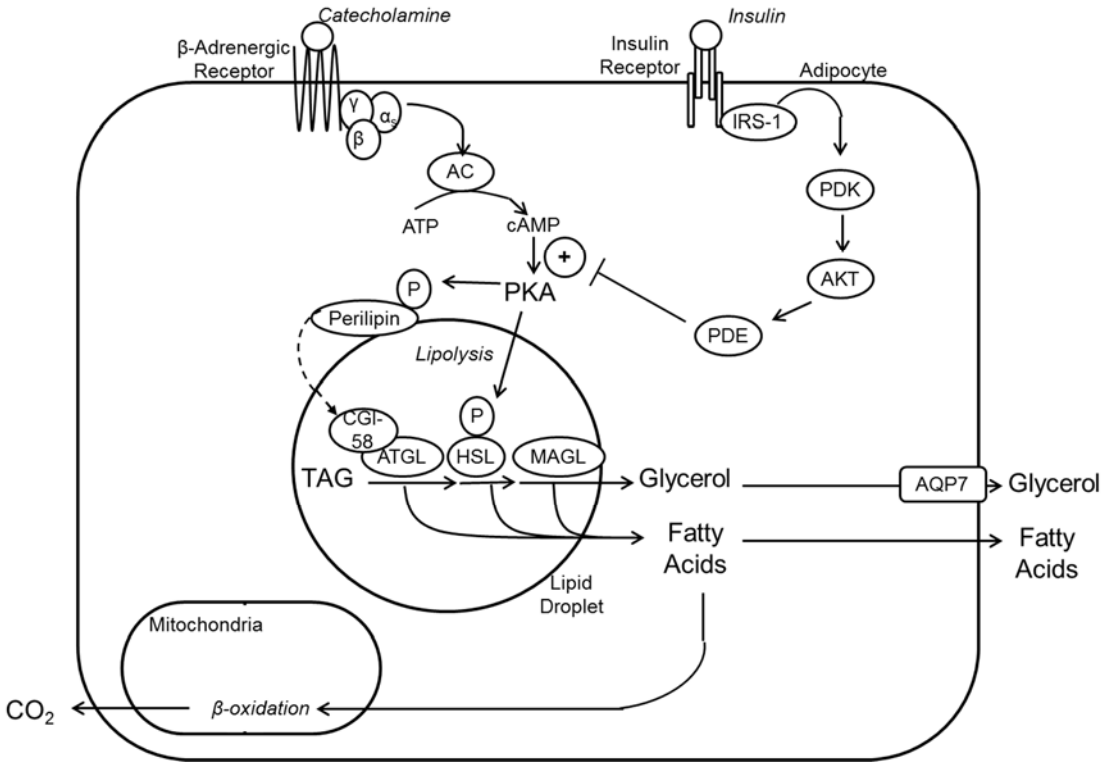


Figure 1.3. Summary of adipocyte lipolysis. AC: adenylate cyclase; ATGL: adipocyte triglyceride lipase 1; AQP7: aquaglyceroporin 7; CGI-58: comparative gene identification-58; HSL: hormone sensitive lipase; IRS-1: insulin receptor substrate-1; MAGL: monoacylglycerol lipase; PDE: phosphodiesterase E; PDK: pyruvate dehydrogenase kinase; PKA: protein kinase A.

and by activation by cofactor comparative gene identification-58 (CGI-58) (85). In the basal state, CGI-58 is associated with perilipin on the surface of the lipid droplet. In response to a lipolytic stimulus, such as β-adrenergic stimulation by a catecholamine, perilipin is phosphorylated by protein kinase A (PKA). This stimulates the dissociation of CGI-58 from perilipin, and its translocation to ATGL, stimulating ATGL hydrolase activity (86, 87). ATGL is rate-limiting for TAG hydrolysis (66).

Activation of PKA also results in the phosphorylation of hormone-sensitive lipase (HSL), the rate-limiting enzyme for diacylglycerol (DAG) hydrolysis (88). In addition to DAG, HSL can also hydrolyze fatty acids from TAG, monoacylglycerol (MAG), and cholesterol esters (66), and phosphorylation of HSL at serine 660 (Ser660) regulates HSL enzymatic activity (89). The monocylglycerol that is produced by the combined activities of ATGL and HSL is hydrolyzed to glycerol and free fatty acid by the enzyme monoglyceride lipase (MGL). MGL is expressed ubiquitously, with highest levels of expression found in adipose tissue and the kidney (66).

Hormone-stimulated lipolysis occurs when a hormone such as a catecholamine binds to the G-protein-coupled β-adrenergic receptor. This stimulates adenylate cyclase to produce cyclic-AMP (cAMP), the accumulation of which activates PKA. PKA phosphorylates perilipin and HSL, and these two phosphorylation events stimulate the translocation of CGI-58 from perilipin to ATGL, and the activation and translocation of cytoplasmic HSL to the lipid droplet, respectively. These phosphorylation and translocation events trigger the

complete hydrolysis of TAG to three fatty acids and free glycerol. The fatty acids freed by lipolysis can exit the cell to be used by other tissues, or a portion of them may be oxidized through a process called β-oxidation in the adipocyte (66). A small amount of fatty acids hydrolyzed in response to hormone-stimulated lipolysis, approximately 10-20%, are reesterified as TAG (73, 90-92).

In addition to hormone-stimulated lipolysis, adipocytes also undergo basal lipolysis. In this state, 30-100% of fatty acids hydrolyzed from the lipid droplet are reesterified *in situ* as TAG in a process termed futile TAG/FA cycling (73, 90-92). This TAG/FA cycling is essential for buffering plasma fatty acid levels and maintaining tight control over opposing metabolic fluxes (93). This cycling also plays a role in the synthesis of lipolytic signaling molecules, e.g. fatty acids and diacylglycerols (73).

1.1.1.4.5 Fatty Acid Oxidation

While the oxidation of fatty acids, or β-oxidation, has been most extensively studied in oxidative tissue such as skeletal muscle and the liver, β-oxidation plays a significant role in white adipocyte metabolism (74). During differentiation, adipocytes exhibit increased expression of mitochondrial biogenesis marker PGC1α, and increased mitochondrial oxidative capacity (73). Prior to entry into the mitochondria for oxidation, fatty acids are activated to form fatty acyl-CoAs. Following activation, these fatty acyl-CoAs, and are then transported through the outer mitochondrial membrane by carnitine palmitoyl

transferase-1 (CPT-1). This transport of fatty acyl-CoAs across the mitochondrial membrane byCPT-1 is the rate-limiting step of β-oxidation.

The coordinate regulation of lipolysis, β-oxidation, and TAG synthesis are modulated hormonally, in response to food intake and thus substrate availability (74). The purpose of lipolysis is to provide energy in the form of fatty acids, for adipose and other tissues. As glucose is the preferred energy substrate of these tissues, there is no need for lipolysis or β-oxidation when glucose is present. Insulin, released from the pancreas in response to feeding, blunts lipolysis in adipose tissue through Akt-mediated phosphorylation of phosphodiesterase 3B (PDE3B) (94). Activation of PDE3B reduces the intracellular cAMP pool, and consequently, PKA-mediated activation of ATGL and HSL. Additionally, indirect suppression of lipolysis occurs in response to insulin-stimulated glucose uptake and flux through glycolysis. Adipocytes dispose of a portion of glucose as lactate (95, 96). Once lactate exits the cell, it can bind to the G protein-coupled GPR81 receptor to inhibit cAMP synthesis, and downstream lipolytic signaling via an autocrine lactate loop (97). In the presence of glucose, the cytosolic acetyl-CoA pool that is derived from TCA cycle intermediates is converted to malonyl-CoA by ACC during *de novo* lipogenesis. ACC activity is regulated via phosphorylation by cellular energy sensory AMPK. Malonyl-CoA is a potent inhibitor of CPT-1, and prevents β-oxidation in a lipogenic state (74). Conversely, when cellular energy demands exceed substrate availability, AMPK-stimulation of cAMP synthesis by adenylate cyclase activates PKA, and consequently, stimulates hydrolysis of TAG (74).

1.1.1.5 Glucose Metabolism

While skeletal muscle is considered the primary “sink” for glucose disposal, adipose tissue plays a critical role in regulating glucose homeostasis (98). A study by Abel *et al.* demonstrated that relative to control mice, those with adipose-specific ablation of the GLUT4 glucose transporter exhibit reduced glucose utilization in adipose tissue and glucose intolerance, as well as impaired insulin signaling in the liver and skeletal mice (99). Further, glucose homeostasis in mice lacking GLUT4 in skeletal muscle can be restored by overexpressing GLUT4 in adipose tissue (100). This beneficial effect of adipose glucose disposal is dependent on the ability of adipose tissue to utilize glucose for the synthesis of fatty acids (101).

Glucose may be taken up into the adipocyte via glucose transporter type 1 (GLUT), under basal conditions, or via the GLUT4 glucose transporter in response to insulin stimulation after a meal (102). While GLUT1 is constitutively expressed on the plasma membrane of adipocytes, insulin-stimulated GLUT4 is considered the predominant glucose transporter in adipocytes (74). Under basal conditions, GLUT4 is stored in small intracellular vesicles (103). In response to insulin, the PI3K-Akt signaling cascade is triggered, stimulation the exocytosis of GLUT4-containing vesicles to the cell membrane, and within minutes increasing flux of glucose into the cell 10- to 30-fold (103, 104).

Once inside the cell, glucose is utilized in multiple cell processes. In addition to serving as a source of ATP, glucose also serves as a substrate for the

synthesis of fatty acids. Additionally, as adipocytes are unable to recycle glycerol freed by lipolysis, glucose also serves as a substrate for G3P synthesis. It is estimated that 20-25% of glucose consumed by adipocytes is used for synthesis of TAG (77).

1.1.2 Changes in Adipose Tissue During Obesity

In a lean individual, adipose tissue works with other tissues to maintain normal metabolic, inflammatory, and vascular function. In response to overnutrition, the adipocyte can store excess energy in lipid droplets by esterifying fatty acids, obtained directly from the diet or synthesized *de novo* from glucose, into triacylglycerol. This partitioning of excess energy into adipose tissue prevent ectopic lipid deposition and lipotoxicity in organs such as the liver, heart, and skeletal muscle, and preserves normal metabolic functioning in these tissues (29). However, during the development of obesity, several changes occur in adipose tissue that disrupt normal functioning.

In response to chronic overnutrition, adipose tissue must expand in order to accommodate the increased need for energy storage. To do so, the adipose tissue must increase in cell number, by a process called hyperplasia, or in cell size, by a process called hypertrophy (74). While both of these processes contribute to the maintenance of body fat mass, a study by Spalding et al. demonstrate that changes in cell size, rather than in cell number, underlie changes in adipose mass during weight loss or gain (105). In this study, it was found that adipocytes exhibit an annual turnover rate of 10%, regardless of age

and body mass index. Enlargement of adipocytes stimulates changes in adipocyte metabolism and secretory profile, and this adipocyte dysfunction plays a central role in the development of obesity-associated comorbidities, including insulin resistance, type 2 diabetes, and cardiovascular disease (32).

The expansion of adipose mass triggers a series of inflammatory events. As the adipocyte expands, a state of hypoxia develops as interstitial oxygen tension decreases within the adipose tissue (106, 107). This stimulates stabilization of HIF1α, which is rapidly degraded under normoxic conditions, and upregulation of genes containing HIF1α response elements, such as those required for angiogenesis (108). In lean individuals, macrophages found in adipose tissue express markers of the M2, or alternatively activated, state. M2 macrophages are characterized by secretion of anti-inflammatory cytokines, such as interleukin-10 (IL-10) (31). During obesity, recruitment and accumulation of M1, or classically activated, macrophages occurs. M1 macrophages characterized by increased production of pro-inflammatory cytokines tumor necrosis factor alpha (TNFα) and interleukin-6 (IL-6), which promote insulin resistance and low-grade systemic inflammation (18, 31).

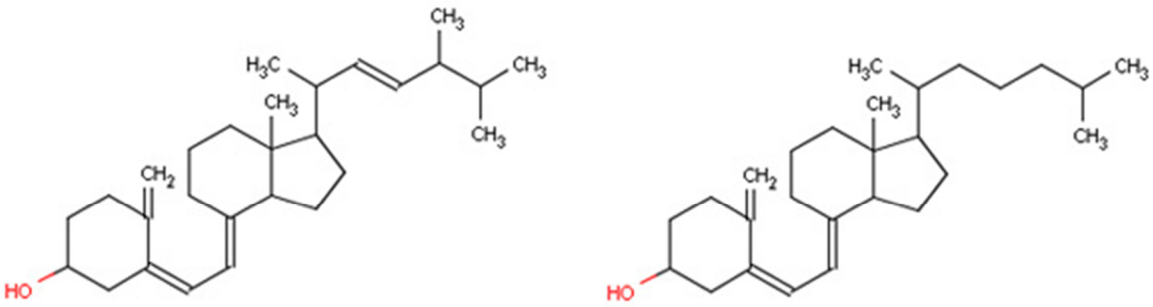
As the white adipose tissue further expands, necrosis occurs, stimulating the development of “crown-like structures” composed of the dead adipocyte surrounded by macrophages (109). The rate of cell death is positively correlated with adipocyte size in mice and in humans, and is increased up to 30-fold in obese compared to lean individuals (110). This inflammatory response is implicated in the metabolic disturbances associated with obesity.

In addition to elevated local and systemic inflammation, dysregulation of lipolysis occurs during the development of obesity (111). In obese and insulin-resistant individuals, catecholamine-induced lipolysis is blunted in subcutaneous adipose tissue, but increased in visceral adipose depots (112, 113). Further, basal (unstimulated) lipolysis is increased in obese compared to individuals (112, 114), and this uncontrolled release of fatty acids is closely associated with obesity-associated insulin resistance, independent of age or BMI (115). These obesity-induced metabolic consequences have a devastating impact on overall health and quality of life. The need to identify safe, effective measures by which the burden of obesity can be prevented and reversed is critical. This dissertation discusses the potential impact of vitamin D on obesity and obesity-associated metabolic disorders.

1.2 Role of Vitamin D in Human Health

1.2.1 Sources of Vitamin D

Vitamin D is a 9, 10 secosteroid that was first discovered by Dr. E.V. McCollum in 1922 as the factor present in cod liver oil that cured rickets in experimental beagles (116). Of the many forms of vitamin D that have been identified, only vitamins D_2 (ergocalciferol; fungal origin) and D_3 (cholecalciferol; animal origin) (Figure 1.4) are relevant to human health (117). Vitamin D_3 is a biologically inert prohormone made endogenously from 7-dehydrocholesterol in the skin upon exposure to ultraviolet light (Figure 1.5) (118). Upon irradiation,



Vitamin D_2 Vitamin D_3

Figure 1.4. Chemical structures of vitamins D_2 and D_3.

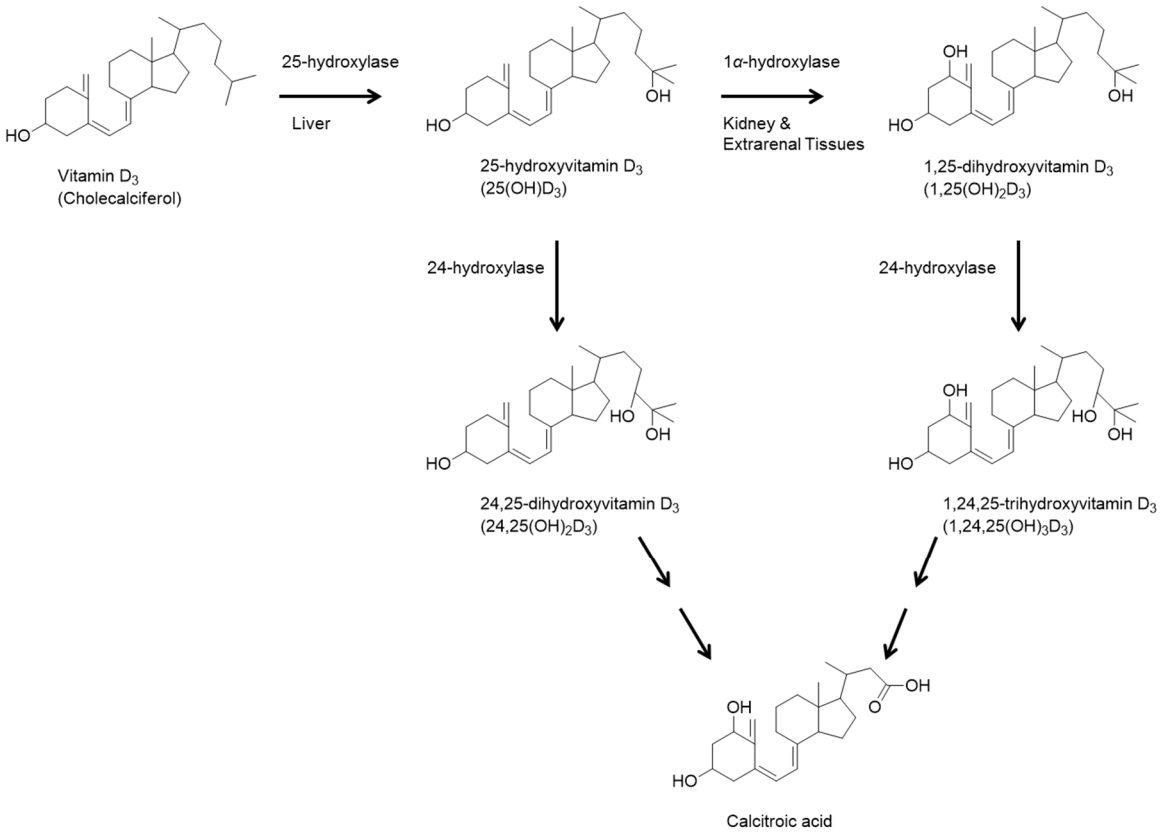


Figure 1.5. Vitamin D_3 biosynthesis and metabolism.

7-dehydrocholesterol is converted to pre-vitamin D_3 which is then converted to vitamin D_3 upon the temperature-sensitive rearrangement of three double bonds (119). Modest amounts of vitamin D_3 may also be obtained in the diet through consumption of vitamin D-fortified dairy products and fatty fish. While reports of superiority of vitamin D_3 have been made when supplemented at high doses (120), at low doses, vitamins D_2 and D_3 are nutritionally equivalent (121).

1.2.1.1 Vitamin D Metabolism

Whether obtained through the diet or synthesized endogenously, vitamin D circulates in the blood bound to vitamin D binding protein (DBP). While vitamin D itself does not have biological activity, it becomes active upon hydroxylation at two distinct sites. In the liver, vitamin D is hydroxylated C-25 by the 25-hydroxylase CYP2R1 to produce 25-hydroxyvitamin D (25(OH)D). 25-hydroxylation in the liver is not a highly regulated step (122), and serum concentrations of 25(OH)D are a universally accepted biomarker of vitamin D status (123-125).

Though 25(OH)D is the major circulating metabolite of vitamin D, conversion to the active metabolite 1,25-dihydroxyvitamin D (1α,$25(OH)_2D$; calcitriol) requires an additional hydroxylation at C-1, which occurs in the kidney. When DBP-bound 25(OH)D reaches the kidney, it is filtered by the glomerulus. Megalin, a transmembrane protein found in tubular epithelial cells, acts as a cell surface receptor for DPB, and catalyzes the uptake of 25(OH)D by endocytic internalization (126). In the proximal renal tubule, conversion of 25(OH)D to

1*α*,25(OH)$_2$D is mediated by the renal 1α-hydroxylase CYP27B1. This enzyme is under tight regulation in the kidney; it is activated by serum PTH and suppressed by 1,25(OH)$_2$D, calcium and phosphorus (127, 128). While the kidney is the main site of 25(OH)D activation under periods of low calcium or low phosphorus stress, CYP27B1 expression and 1α-hydroxylase activity have now been identified in several extra-renal tissues, including skin, colon, lung, bone, prostate, intestine, pancreatic islets, vasculature, liver, brain, muscle and adipose tissue (129-131). The extra-renal 1α-hydroxylase is not regulated by PTH (132, 133), suggesting that elevated 25(OH)D status may lead to local production of 1α,25(OH)2D independent of calcium status to yield autocrine and paracrine responses. In response to elevated circulating 1,25(OH)$_2$D levels, the renal CYP27B1 hydroxylates 1,25(OH)$_2$D, and also 25(OH)2D, at C-24 to signal for its degradation and excretion (134, 135). While concentrations vary widely based on dietary intake, studies have demonstrated that both vitamin D and 25(OH)D may be found in adipose tissue, skeletal muscle and liver, though the largest pool of 25(OH)D is found in serum (125, 136).

1.2.1.2 Classical Signaling of 1,25(OH)$_2$D through the Vitamin D Receptor

Like other steroid hormones, 1,25(OH)$_2$D functions through a nuclear receptor, in this case, the vitamin D receptor (VDR). The VDR belongs to the class II of steroid receptors, and is closely related to the thyroid hormone and retinoic acid receptors (128, 137), and is evolutionarily conserved among mammals, birds, and fish (138). Like other nuclear receptors, the VDR has a

ligand-binding domain called the E-domain, a DNA-binding domain called the C-domain, and an activating domain called the F-domain (116). The human VDR protein contains 427 amino acids, and acts by binding to vitamin D response elements (VDREs), which are typically found within 1 kilobase of the transcription start site of the vitamin D target gene. The VDREs are repeat sequences of 6 nucleotides separated by 3 nonspecific bases (116).

Upon entry into the cell, $1,25(OH)_2D$ binds to the VDR, causing a conformational change in the VDR that allows it to interact and form a heterodimer with the retinoid X receptor (RXR). This heterodimer binds to the VDRE to regulate transcription of vitamin D target genes (139). Binding of the VDR-RXR heterodimer to VDREs stimulates recruitment of transcriptional coactivators such as p160 coactivator and steroid receptor activator (SRC) (119). Once this complex forms, transcription is either stimulated or repressed, depending on the gene (116). $1,25(OH)_2D$ regulation of transcriptional events through the VDR is responsible for the regulation of calcium homeostasis (137, 140). In addition to its identification in tissues controlling calcium homeostasis, the VDR has now been identified in virtually all other tissues (141-143), as well as a variety of cancer cells including those from the breast, colon, and prostate (144-147). This broad distribution of VDR supports the existence of extraskeletal effects for $1,25(OH)_2D$ (139, 148, 149).

1.2.1.3 Rapid Signaling through the Membrane Vitamin D Receptor

Though the most well-established mechanisms of calcitriol action are through the nuclear VDR to modulate gene expression, strong evidence exists proving non-genomic actions of $1,25(OH)_2D$ as well. First documentation of rapid $1,25(OH)_2D$ action was in a 1984 study by Nemere (150), in which addition of 120 pM $1,25(OH)_2D$ to perfused deuodena of normal, vitamin D-replete chicks resulted in a significant increase of ^{45}Ca transport from the lumen to the vascular effluent within 14 minutes. This rapid response, within minutes, contrasts with genomic responses which may take hours to several days, and can be blocked by inhibitors of transcription and translation. Since this discovery, many studies have emerged supporting the existence of non-genomic actions of calcitriol in regulation of calcium transport in several cell types, including hepatocytes, the promyelocytic HC-60 cell line, renal tissue and mammary glands, among others (151).

Investigation of the mechanisms of $1,25(OH)_2D$-stimulation of rapid signaling events have revealed a novel role for the vitamin D receptor. Many ligand structure-function studies have been done to elucidate rapid responses of $1,25(OH)_2D$ bound to the classic VDR, including rapid stimulation of: calcium absorption in chick enterocytes (152, 153), insulin secretion from rat pancreatic β cells (89), rate of human endothelial cell migration (154), and MAPK and PI3K/Akt signaling cascades to regulate proliferation and differentiation in skeletal muscle (155-157). In these studies, the $1\alpha,25(OH)_2D$ agonist could be

inhibited by the rapid response antagonist 1β,25(OH)$_2$D, but not by the genomic response antagonist (23S)-25-dehydro-1α-OH-D$_3$-26,23-lactone (158). Further, these rapid responses were also induced by 6-s-*cis* locked 1α,25(OH)$_2$-lumisterol, which binds to the VDR (153). Together, these data demonstrate that the rapid responses initiated by 1,25(OH)$_2$D do in fact occur through the classical VDR, but also that rapid responses require a different ligand structure (6-s-*cis*) than that required for a 1,25(OH)$_2$D-induced genomic response (6-s-*trans*) (159).

1.2.1.4 A Non-VDR Membrane-Associated Binding Protein is Involved in Rapid 1,25(OH)$_2$D Signaling

Though there are a data to support a direct role for the VDR in non-genomic 1,25(OH)$_2$D actions, initial studies in enterocytes, chondrocytes, and osteoblasts suggested that a different 1,25(OH)$_2$D binding protein controlled rapid calcitriol-induced signal transduction pathways. Indirect evidence for a putative membrane receptor for 1,25(OH)$_2$D was obtained during the initial discovery of calcitriol rapid signaling in the intestine. In these studies, treatment of the basolateral membrane with exogenous 1,25(OH)$_2$D rapidly enhanced calcium transport, whereas exposure of the brush border failed to have this effect (150). This suggested that 1,25(OH)$_2$D receptor exists on the basolateral membrane.

Isolation and full characterization and identification of this basolateral membrane receptor in chick intestinal epithelial cells was achieved several years later, and was termed the 1,25(OH)$_2$D-membrane-associated, rapid-response steroid-binding (MARRS) protein (160). The transcript for the 1,25(OH)$_2$D-

MARRS includes a 5'-unstranslated region, a signal peptide, no transmembrane domain and two thioredoxin motifs. Analysis of the cloned chicken cDNA for the 1,25$(OH)_2$D-MARRS revealed that it is identical to the multifunctional protein ERp57 protein (also called p57/GRp58/Pdia3). The ERp57 is a thiol:protein disulphide oxidoreductase, and is a member of the protein disulfide isomerase (PDI) family (161). First identified as a luminal chaperone protein of the endoplasmic reticulum to facilitate oxidative folding of glycoproteins, ERp57 exists also in the cytoplasm and nucleus, and functions as a scaffolding protein on the cell surface (162).

1,25$(OH)_2$D-MARRS mediates several 1,25$(OH)_2$D-induced effects in various cell types, including rapid (seconds to minutes) phosphate (163, 164) and calcium (165, 166) uptake in intestinal epithelial cells and perfused duodena, modulation of growth inhibitory activity of 1,25$(OH)_2$D in breast cancer cells (167), regulation of growth plate physiology in chondrocytes and matrix vesicle models (168), and initiation of 1,25$(OH)_2$D signaling pathways in osteoblast differentiation (169).

1.2.1.5 Classical Roles of Vitamin D in Calcium Metabolism and Bone Health

Historically, the chief functions of vitamin D are those related to bone mineralization (170). It is well established that the rachitic skeleton can be mineralized by infusion of calcium and phosphorus to raise their serum levels into normal ranges (171), indicating that a direct role for vitamin D in bone mineralization is not likely. However, vitamin D does contribute to bone health by

maintaining serum calcium and phosphorus concentrations within ranges necessary to support bone mineralization. The control of serum calcium occurs through events occurring at a three-tissue axis of bone, intestine, and kidney. When dietary calcium is insufficient, vitamin D is converted to its hormonal form $1,25(OH)_2D$, that acts on the enterocyte to activate active transport of calcium and also phosphorus, on the osteoclast to mediate bone resorption, and on the kidney to increase renal reabsorption of calcium in the distal tubule. All of these events contribute to maintaining plasma calcium concentrations within a very narrow range (116, 123).

1.2.1.6 Extraskeletal Functions of Vitamin D

In addition to those related to maintaining serum calcium and bone mineralization, several extraskeletal functions of vitamin D have been identified. Meta-analysis of randomized clinical trials have demonstrated that vitamin D status is inversely correlated with cancer-related mortality, suggesting that vitamin D may act to inhibit the progression of certain cancers (119). In particular, evidence exists demonstrating vitamin D inhibition of prostate, breast, and colon cancer progression. Additionally, observational studies demonstrate an inverse relationship between vitamin D status and risk of cardiovascular disease, and *in vitro* studies have demonstrated $1,25(OH)_2D$ regulation of cell proliferation and differentiation, as well as regulation of immune function (119, 172).

1.2.1.7 Recommendations for Dietary Intake of Vitamin D

Current recommendations for serum 25(OH)D are made based on the impact that vitamin D has on skeletal health (173) (Table 1.1). Serum 25(OH)D levels of 50 nmol/L (20 ng/mL) are considered adequate to normalize bone and calcium metabolism in at least 97.5% of the population. The Institute of Medicine (IOM) defines “insufficiency” as any serum 25(OH)D concentration < 50 nmol/L, and “deficiency” as levels of serum 25(OH)D levels at which bone health is compromised, i.e. <30 nmol/L (12 ng/mL) (173). The Recommended Dietary Allowances (RDAs) for vitamin D, the average daily level of intake sufficient to meet vitamin D requirements of 97%-98% of healthy individuals, are listed in Table 1.2 (121).

While rare, it is possible to achieve vitamin D toxicity, particularly with long-term use of extreme vitamin D supplementation. Symptoms of vitamin D toxicity include anorexia leading to weight loss, heart arrhythmias, and polyuria. In more serious cases, vitamin D toxicity may lead to elevated blood calcium levels, resulting in vascular and tissue calcification, and consequently, cardiac, renal, and vascular damage (121). The occurrence of vitamin D toxicity is rare, however, and it is not possible to achieve toxicity from foods or sunlight exposure (124).The Tolerable Upper Intake Levels (ULs) for vitamin D, or the highest average daily intake level likely to pose no risk of adverse health outcomes, are listed in Table 1.3 (121).

Table 1.1 Recommended serum 25-hydroxyvitamin D [25(OH)D] concentrations

nmol/L*	ng/mL	Health Outcome
<30	<12	Vitamin D deficiency, leading to rickets and osteomalacia in children and adults
30 to <50	12 to <20	Generally considered adequate for bone health in healthy individuals
≥50	≥20	Generally considered adequate for bone health in healthy individuals
>125	>50	Risk of adverse effects is increased when serum 25(OH)D reaches >125 nmol/L
*1 nmol/L = 0.4 ng/mL		

Table 1.2 Recommended Dietary Allowances (RDAs) for vitamin D

Age	Male	Female	Pregnancy	Lactation
0-12 months*	400 IU (10 µg)	400 IU (10 µg)		
1-13 years	600 IU (15 µg)	600 IU (15 µg)		
14-18 years	600 IU (15 µg)	600 IU (15 µg)	600 IU (15 µg)	600 IU (15 µg)
19-50 years	600 IU (15 µg)	600 IU (15 µg)	600 IU (15 µg)	600 IU (15 µg)
51-70 years	600 IU (15 µg)	600 IU (15 µg)		
>70 years	800 IU (20 µg)	800 IU (20 µg)		
*Adequate Intake (AI)				

Table 1.3 Tolerable Upper Intake Levels (ULs) for vitamin D

Age	Male	Female	Pregnancy	Lactation
0-6 months	1,000 IU (25 µg)	1,000 IU (25 µg)		
7-12 months	1,500 IU (38 µg)	1,500 IU (38 µg)		
1-3 years	2,500 IU (63 µg)	2,500 IU (63 µg)		
4-8 years	3,000 IU (75 µg)	3,000 IU (75 µg)		
≥9 years	4,000 IU (100 µg)	4,000 IU (100 µg)	4,000 IU (100 µg)	4,000 IU (100 µg)

However, the above described definitions of vitamin D adequacy, insufficiency, and deficiency are not universally used, and some strongly oppose these current IOM recommendations (174). Recent evidence from some studies evaluating thresholds for serum 25(OH)D concentrations in relation to bone as well as several extraskeletal endpoints suggest that the most advantageous serum 25(OH)D concentrations begin at 75 nmol/L (30 ng/mL), and are optimal between 90 and 100 nmol/L (36-40 ng/mL) (175). Whether there is sufficient evidence to conclude that optimal serum 25(OH)D levels are higher than current IOM levels remains a matter of debate.

The prevalence of vitamin D deficiency is widespread, both in the U.S. (176) and worldwide (177). It is estimated that approximately one-third of the U.S. population is vitamin D deficient or insufficient, with certain ethnic groups at particularly high risk of low vitamin D status, including non-Hispanic blacks (>70%) and Hispanics/Mexicans (>40%) (178). This raises concern, as mounting evidence demonstrates the existence of many biological functions of vitamin D, in addition to the classical functions of vitamin D related to regulation of calcium and phosphate homeostasis and bone metabolism.

Additionally, vitamin D status has been implicated in the development of obesity-associated metabolic disorders (179). Human cross-sectional studies demonstrate that vitamin D deficiency is association with hyperglycemia (180, 181), hyperinsulinemia (182), reduced pancreatic beta-cell function (183), and measures of insulin resistance (183). Further, early studies utilizing vitamin D-deficient rats found that pancreatic insulin secretion was impaired compared to

that of vitamin D-replete rats (184, 185). The relationship between vitamin D supplementation and glycemic control remains controversial. *In vitro* studies demonstrate that $1,25(OH)_2D$ enhances insulin sensitivity in skeletal muscle (186), a major contributor to global insulin sensitivity. However, recent meta-analyses of randomized controlled trials designed to assess the impact of vitamin D supplementation on insulin sensitivity failed to show a positive effect of vitamin D supplementation on homeostatic model assessment of insulin resistance (HOMA-IR), or on 2-hour plasma glucose following an oral glucose tolerance test (OGTT) (187, 188). One meta-analysis did find small yet statistically significant reductions in fasting blood glucose and $HbA1_c$ levels in individuals with prediabetes who received vitamin D supplementation (187). The relationship between vitamin D and insulin sensitivity remains incompletely understood

1.3 Vitamin D Plays a Role in Adipose Tissue Physiology

1.3.1 Relationship between Vitamin D Status and Obesity

The inverse relationship between vitamin D status and adiposity is well-established: serum 25-hydrovyvitamin D [25(OH)D] levels are inversely correlated with measures of adiposity including fat mass, body mass index (BMI), and waist circumference (189-194). Several hypotheses have been studied regarding the nature of this relationship. One such hypothesis is that vitamin D deficiency plays a causative role in the development of obesity, which resulted in the execution of studies designed to determine the impact of vitamin D

supplementation on weight loss and obesity-associated comorbidities. The existing evidence however does not support this causal relationship (179). Analysis of intervention studies having a duration of 1 year and achieving serum 25(OH)D of >85 nmol/L found no significant changes in BMI (195, 196). Consistent with these findings, participants given 2,000 IU/day vitamin D in a short randomized controlled trial experienced significant increases in serum 25(OH)D and $1,25(OH)_2D$ with no changes in energy expenditure or adipose tissue gene expression (197). Taken together, these studies indicate that vitamin D supplementation alone is not an effective means by which to induce weight loss.

The inverse relationship between vitamin D deficiency and adiposity may be attributed to lifestyle factors that promote both of these conditions. Examples would include a diet poor in vitamin D, and little outdoor exercise (and thus reduced solar ultraviolet radiation and endogenous production of vitamin D) (191, 198). Alternatively, it has been postulated that obese individuals may have reduced circulating 25(OH)D due to sequestration of vitamin D and its metabolites in adipose tissue. The evidence is most compelling for the latter of these hypotheses.

1.3.1.1 Sequestration of Vitamin D in Adipose Tissue

It has been established that adipose tissue is a storage site of vitamin D (199-201). In rats, adipose tissue accounts or the majority of vitamin D storage, half stored as unmetabolized vitamin D and the other half stored as vitamin D

metabolites, esters, and other unidentified compounds (200). The inverse relationship between vitamin D status and adiposity may be explained by sequestration of the nutrient in adipose tissue. An investigation led by Wortsman et al. demonstrated that the increase in serum vitamin D_3 following whole-body irradiation was 57% lower in obese than in age-matched lean controls (202). This occurred despite no significant difference in the cutaneous vitamin D precursor 7-dehydrocholesterol between these two groups, and no difference in the conversion of 7-dehydrocholesterol to previtamin D_3. Further, an inverse correlation was found between BMI and peak serum vitamin D_2 concentrations following oral administration of 50,000 IU vitamin D_2, suggesting that obesity-associated vitamin D deficiency is likely due to its reduced bioavailability resulting from sequestration in adipose tissue depots (202). A study by Vimaleswaran et al. arrived at a similar conclusion upon employing a Mendelian randomization approach for determining directionality and causation of the association between vitamin D status and BMI (203). The results from this study reveal that a higher BMI was causally linked to reduced serum 25(OH)D levels, but the findings did not suggest that raising serum 25(OH)D levels will have a BMI lowering effect. While these studies clearly demonstrate that excessive adiposity underlies obesity-associated vitamin D deficiency, it is not yet known whether direct action of vitamin D on adipose tissue plays a role in this relationship.

1.3.1.2 Role of 1,25$(OH)_2$D and the VDR in Adipogenesis

Expansion of adipose tissue occurs as a result of the enlargement of adipocyte size (hypertrophy) and an increase in adipocyte number (hyperplasia). Both of these processes occur during adipocyte differentiation, in a process called adipogenesis. The vitamin D receptor, a nuclear receptor that heterodimerizes with RXR, is expressed in adipose tissue and is acutely upregulated during adipogenesis *in vitro* (204). Many *in vitro* studies using established cell lines have demonstrated 1,25$(OH)_2$D regulation of preadipocyte differentiation.

In mouse 3T3-L1 preadipocytes, differentiation is inhibited by 1,25$(OH)_2$D via downregulation of C/EBPα and PPARγ expression and sequestration of RXR, resulting in downregulation of C/EBPβ (205-208). Further, upregulation of C/EBPβ corepressor eight twenty-one (ETO) by 1,25$(OH)_2$D further reduces C/EBPβ activity and inhibits adipogenesis. More recently, Lee et al. demonstrate that in 3T3-L1 preadipocytes, 1,25$(OH)_2$D promotes maintenance of WNT10B and nuclear β-catenin expression, suppressing PPARγ activity and preserving the preadipocyte phenotype (207). Additionally, it has been demonstrated that VDR knockdown inhibits adipogenesis in 3T3-L1 preadipocytes (206), and that VDR null mice exhibit an extremely lean phenotype with resistance to diet induced obesity (209-211).

However, 1,25$(OH)_2$D regulation of preadipocyte differentiation appears to be species-dependent. In porcine mesenchymal stem cells,1,25$(OH)_2$D

stimulates differentiation towards an adipocyte phenotype by stimulating PPARγ, lipoprotein lipase (LPL), and aP2 expression (212). In human subcutaneous preadipocytes, differentiation is stimulated by 1,25$(OH)_2$D, resulting in increased expression of FABP4 and LPL (213). Narvaez et al. conducted genomic profiling of primary cultures of human adipose-derived mesenchymal progenitor cells to define the role of 1,25$(OH)_2$D and its VDR in adipogenesis, and found that 1,25$(OH)_2$D promoted lipid accumulation and enhanced expression of FABP4, FASN, and PPARγ (214). The discrepancies observed between studies utilizing human versus mouse 3T3-L1 preadipocytes may be due to methodological differences, or due to differences in the roles of adipose tissue in energy balance between the two species. Regardless, 1,25$(OH)_2$D appears to play a multifaceted role in the regulation of adipose tissue physiology.

1.3.1.3 Regulation of Inflammation by 1,25$(OH)_2$D

During obesity, expansion of adipose tissue results in insufficient blood flow to the hypertrophic tissue, stimulating hypoxia, macrophage infiltration, and inflammation. In this state, secretion of adiponectin and other anti-inflammatory adipokines are reduced, while secretion of pro-inflammatory cytokines such as interleukin-6 (IL-6), TNFα, resistin, and MCP-1 are increased (215). While vitamin D regulation of adipose tissue inflammation remains incompletely defined, several *in vitro* studies have demonstrated that 1,25$(OH)_2$D does act at several levels to modulate this system. Studies by Zemel et al. utilizing 3T3-L1 and human adipocytes have found that 1,25$(OH)_2$D exerts a pro-inflammatory effect,

inhibiting anti-inflammatory cytokine expression while stimulating expression of pro-inflammatory cytokines. In these studies, treatment of adipocytes with $1,25(OH)_2D$ increased expression of TNFα, IL-6, IL-8, macrophage colony-stimulating factor (M-CSF), and macrophage inflammatory factor (MIF) (216-218). Expression of M-CSF and MIF were ameliorated by calcium-channel antagonism with nifedipine, indicating that $1,25(OH)_2D$ operates via a calcium-dependent mechanism (218).

However, not all studies have revealed consistent findings. In contrast to the pro-inflammatory properties identified by Zemel and colleagues, several recent investigations utilizing human preadipocytes and mature human adipocytes have identified an anti-inflammatory role of $1,25(OH)_2D$ in adipocyte physiology. An investigation of the effects of high vitamin D and calcium intake in a Western diet on energy metabolism and inflammation revealed that mice on a Western diet, as expected, experienced reduced energy expenditure and excess fat accumulation compared to mice fed a control diet. Interestingly, mice fed a Western diet plus vitamin D and calcium experienced significantly higher fat deposition in AT and in the liver compared to mice fed a Western diet low in these nutrients. However, the Western diet-induced increase in circulating inflammatory markers IL-1β and MCP-1 were ameliorated by high vitamin D and calcium (219). *In vitro* studies have yielded similar findings. Investigations by Marcotorchino et al. found reductions in protein and mRNA expression of TNFα, IL-6, MCP-1, and IL-1β with $1,25(OH)_2D$ treatment of human and 3T3-L1 adipocytes (220). In human preadipocytes, $1,25(OH)_2D$ reduced MCP-1, IL-6 and

IL-8 secretion under basal and proinflammatory conditions (221). These and additional studies provide evidence to support that the anti-inflammatory effects of $1,25(OH)_2D$ on adipose tissue are via inhibition of the NFκB signaling pathway (220-223).

Though many studies do demonstrate vitamin D regulation of adipose tissue inflammation *in vitro* and *in vivo*, this system does not appear to be as highly regulated by vitamin D in humans. Clinical intervention studies designed to examine the effect of vitamin D supplementation on markers of inflammation have failed to see improvements in systemic and AT inflammation. A double-blind intervention study of 52 obese (BMI > 30 kg/m^2), vitamin D-deficient (plasma 25(OH)D <50 nM) individuals failed to see an effect of 26 weeks of 7,000 IU vitamin D_3/day on any of the examined circulating inflammatory markers, including Hs-CRP, IL-6, MCP-1, adiponectin, leptin, MMP-9, and PAI-1, despite achieving plasma 25(OH)D concentrations of 110.2 ± 21.2 nM by the end of the intervention period (224). Further analysis by the same group also confirmed no change in AT markers of inflammation in these subjects following oral vitamin D supplementation (225). While $1,25(OH)_2D$ appears to have largely anti-inflammatory effects *in vitro* and *in vivo*, more studies are needed in order to determine the physiological relevance of these findings in humans.

1.3.1.4 Vitamin D Regulation of Energy Metabolism

Vitamin D, or more specifically the vitamin D receptor, also appears to modulate energy metabolism. Recent investigations utilizing VDR knockout

($VDR^{-/-}$) mice have revealed that ablation of the VDR leads to reduced body weight, hyperphagia with resistance to diet-induced obesity, hypolipidemia, and hypoleptinemia (209-211). VDR null mice also exhibit increased UCP-1 expression, energy expenditure, oxygen consumption and basal metabolism compared to wild-type (WT) mice (210). These findings of increased energy expenditure and resistance of VDR-null mice to diet-induced obesity has led to investigations of the role of the VDR in adipocyte energy and lipid metabolism (210, 226).

An additional study by Wong et al. utilized the aP2 gene promoter to target the expression of human (h) VDR in adipocytes in mice (227). In contrast to the VDR-null mice, aP2-hVDR Tg mice exhibited reduced locomotive activity and energy expenditure, and increased accumulation of fat mass compared to WT mice. Adipose tissue of the transgenic mice exhibited reduced fatty acid β-oxidation and expression of genes involved in the regulation of fatty acid transport, thermogenesis, and lipolysis. Collectively, these findings suggest a role for VDR regulation of energy metabolism in adipocytes, and it has been speculated that vitamin D storage within adipose tissue increases local vitamin D concentration and thus activation of the VDR, negatively affecting energy expenditure and exacerbating fat mass accumulation. However, the applicability of these findings in humans has yet to be determined.

1.3.1.5 $1,25(OH)_2D$ May Regulate Adipocyte Lipid Metabolism

While limited data exist regarding direct effects of $1,25(OH)_2D$ on adipocyte lipid metabolism, the hormone may modulate lipid metabolism indirectly via its relationship with calcium metabolism. It was previously noted that a high calcium diet suppressed renal $1,25(OH)_2D$ production in aP2-agouti transgenic mice, leading to decreased intracellular Ca^{2+} ($[Ca^{2+}]i$), stimulation of lipolysis, inhibition of lipogenesis, and reduced adiposity (228, 229). To determine whether this modulation of adipocyte lipid metabolism is a direct effect of inhibition of $1,25(OH)_2D$-induced $[Ca^{2+}]i$, differentiated adipocytes from a human adipocyte cell line and primary-cultured human adipocytes were treated with $1,25(OH)_2D$ for 48 hours, and intracellular $[Ca^{2+}]i$, rate of lipolysis, and expression and activities of lipid metabolism enzymes examined. Adipocytes exhibited a $1,25(OH)_2D$ dose-responsive (1-50 nM) increase in $[Ca^{2+}]i$ ($P<0.01$), a 50-100% increase in FAS expression and activity ($P<0.02$), a 61% increase in GPDH activity ($P<0.01$), and 80% inhibition of isoproterenol-stimulated lipolysis ($P<0.001$) (230). Using an agonist ($1\alpha,25$-dihydroxylumisterol$_3$) and antagonist ($1\beta,25$-$(OH)_2D_3$) of the putative membrane vitamin D receptor (mVDR), the authors demonstrated that $1,25(OH)_2D$ elicits a nongenomic response in adipocytes, resulting in stimulation of $[Ca^{2+}]_i$ and corresponding modulation of lipid metabolism. Further study by the same group demonstrated that treatment of human adipocytes for 48 hrs with 1 nM $1,25(OH)_2D$ simulated a 50% reduction in UCP2 mRNA and protein levels ($P<0.002$), and blocked isoproterenol- or fatty

acid-stimulated increases in UCP2 expression (231). Collectively, the authors conclude that the anti-obesity effects of dietary calcium are, at least in part, via inhibition of $1,25(OH)_2D$ production.

Some investigations have been conducted to examine this relationship between calcium, vitamin D, and lipolysis in humans. Four studies have reported that obese individuals have high serum concentrations of $1,25(OH)_2D$ compared to non-obese individuals (189, 190, 232, 233), and it has been suggested that $1,25(OH)_2D$, via its ability to increase intracellular Ca^{2+} concentrations particularly in adipocytes, may exacerbate fat mass accumulation in those predisposed to obesity (228, 234). However, these studies employed very small cohorts of obese subjects, all of whom appeared to have been Caucasian. To address this, a subsequent study was conducted by Parikh et al., in which the relationships between calciotropic hormones and body adiposity were examined in 154 healthy obese (BMI = 37.3 ± 5.8 kg/m^2) and 148 healthy non-obese (BMI 25.6 ± 2.9 kg/m^2) individuals of Caucasian (62.9%), African-American (27.8%), and other (9.3%) race/ethnicities (235). In both Caucasian and African-American adults, it was found that serum $1,25(OH)_2D$ was significantly lower in obese subjects relative to nonobese subjects (105.7 ± 41.1 *vs.* 124.8 ± 36.7pmol/liter; $P < 0.0001$). Serum intact PTH was positively correlated with both BMI ($r = 0.42$; $P<0.0001$) and body fat mass ($r = 0.37$; $P < 0.0001$), while serum 25(OH)D and $1,25(OH)_2D$ were negatively correlated with BMI (25(OH)D: $r = 0.4$, $P < 0.0001$; $1,25(OH)_2D$: $r = 0.26$; $P < 0.0001$) and body fat mass (25(OH)D: $r = 0.41$; $P < 0.0001$; $1,25(OH)_2D$: $r = 0.25$; $P < 0.0001$). These relationships existed

independent of age, sex, or race, and do not support the hypothesis that $1,25(OH)_2D$ exacerbates fat mass accumulation in the obese.

While the effect of vitamin D on adipocyte differentiation, inflammation, and energy metabolism have been studied, the impact of vitamin D on lipid metabolism in adipocytes that have completed terminal differentiation is incompletely understood. The studies described within aim to address this knowledge gap.

CHAPTER 2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Dulbecco's Modification of Eagle's Medium (DMEM) was obtained from Corning (Manassas, VA). Bovine calf serum was obtained from Thermo Fisher Scientific (Pittsburgh, PA). 1,25$(OH)_2$D was obtained from Enzo Life Sciences (East Farmingdale, NY). Fetal bovine serum, trypsin, and penicillin/streptomycin were obtained from Life Technologies, Gibco-BRL (Rockville, MD). Protease inhibitors cocktail, essentially fatty acid-free bovine serum albumin (FA-Free BSA),trypan blue solution (0.4%), insulin (bovine), dexamethasone, 3-Isobutyl-1-methylxanthine were obtained from Sigma-Aldrich (St. Louis, MO). Rosiglitazone was obtained from Cayman Chemical (Ann Arbor, MI). PKA inhibitor H-89 dihydrochloride was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies for HSL, phospho-HSL (Ser565 and Ser660), perilipin, and GAPDH were purchased from Cell Signaling Technology, Inc. (Danvers, MA).

2.2 Cell Culture

3T3-L1 fibroblasts were obtained from American Type Culture Collection (ATCC CL-173; Manassas, VA), and cultured in DMEM containing 10% (vol:vol) FCS with 100 U/mL penicillin and 100 µg/mL streptomycin at 37ºC and 5% CO_2.

Adipocyte differentiation was initiated 2 days post-confluence (referred to as *day 0*) using DMEMcontaining 10% (vol:vol) FBS, antibiotics, and a differentiation cocktail consisting of 1.0 µg/mL insulin, 0.5 mM isobutylmethylxanthine, 1.0 µM dexamethasone, and 2.0 µM rosiglitazone (236). On *day 2*, this medium was replaced with DMEM containing 10% FBS, antibiotics, and 1.0 µg/mL insulin. Beginning on *day 4*, cells were maintained in DMEM containing 10% (vol:vol) FCS and antibiotics, and the medium was changed every 2 days. Adipocytes were differentiated for 9 days, to allow for complete differentiation, and lipid accumulation (Figure 2.1). On *day 9*, 1,25$(OH)_2$D (10 nM) was delivered to cells for the times indicated in 100% ethanol at a final ethanol concentration of <0.1%. For experiments lasting longer than 24 hours, cell medium containing 1,25$(OH)_2$D or vehicle was refreshed every 24 hours. Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and trypan blue exclusion assays, according to manufacturer protocol.

2.3 Triacylglycerol Quantification

Following 1,25$(OH)_2$D treatment, neutral lipids were extracted using a 3:1 hexane:isopropanol solution, and dried under nitrogen gas. Neutral lipids were resuspended in chloroform containing 1% (vol:vol) triton X-100. Lipids were again dried under nitrogen gas, and resuspended in dH_2O containing 2% (vol:vol) triton X-100. Triacylglycerol accumulation was assessed using a spectrophotometric assay kit from Wako Diagnostics (Richmond, VA), according to manufacturer

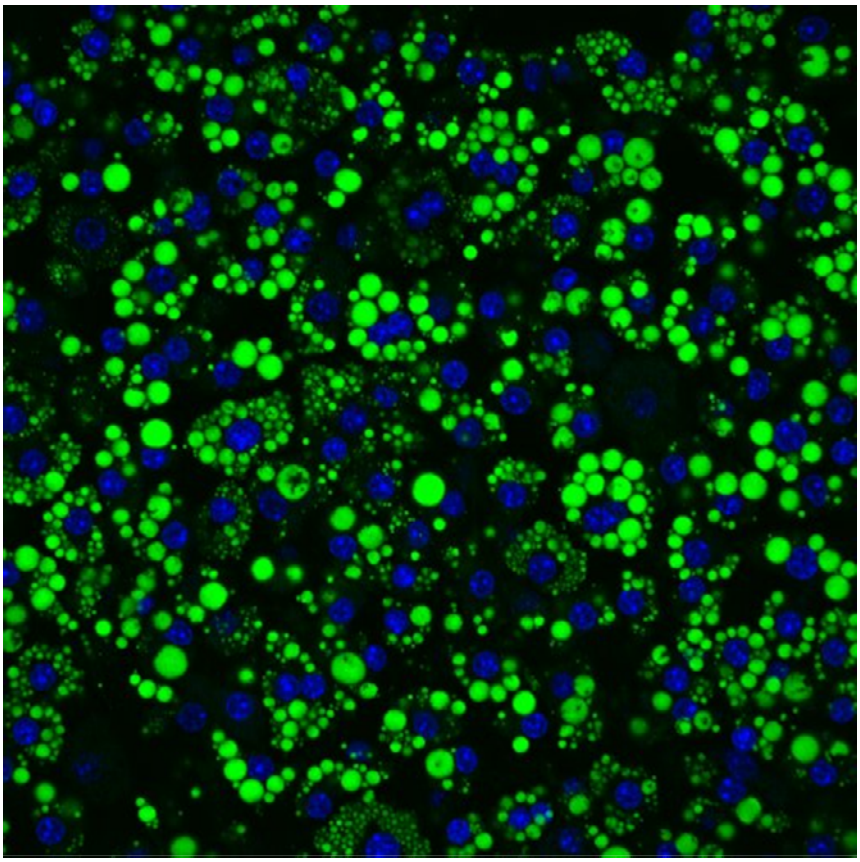


Figure 2.1. BODIPY staining of neutral lipids in differentiated 3T3-L1 adipocytes. Differentiated adipocytes were fixed with paraformaldehyde, and stained with BODIPY 493/503 (green) and DAPI (blue) to stain neutral lipids and nuclei, respectively. Imaging was performed using a Nikon A1R_MP confocal microscope.

protocol. Results are normalized against quantity of protein per well, which was quantified using the bicinchoninic acid assay (BCA assay: Pierce; Rockford, IL).

2.4 Glycerol, Non-Esterified Fatty Acid, and Lactate Release

Spent medium samples were collected after 24 hours of treatment and used for analysis of glycerol, NEFA, and lactate. Glycerol and NEFA were quantified using spectrophotometric assay kits from Sigma-Aldrich (St. Louis, MO) and Wako Diagnostics (Richmond, VA), respectively. For PKA inhibition studies, following 48 hours of treatment with $1,25(OH)_2D$ (10 nM) or vehicle, cells were pre-treated for two hours with PKA inhibitor H-89 (75 μM), with $1,25(OH)_2D$ as indicated. Following pre-treatment, the medium was replaced with serum-free DMEM containing vehicle or $1,25(OH)_2D$ and H-89 as indicated for an additional 4 hours, after which the medium was collected for determination of glycerol release. Lactate secretion was determined using a spectrophotometric method based on the principle that in the presence of NAD^+, lactate in spent media samples can be converted to pyruvate by lactate dehydrogenase. Hydrazine is added to destroy the pyruvate produced, allowing the reaction to run to the complete oxidation of all lactate molecules. The amount of NADH formed by the reaction is proportionate to the amount of lactate in the media, and is measured spectrophotometrically at 340 nm. All results are normalized against quantity of protein per well, which was quantified using the bicinchoninic acid assay (BCA assay: Pierce; Rockford, IL).

2.5 Intracellular Cyclic AMP Accumulation

Cells were harvested into lysis buffer as described above. Intracellular cyclic AMP accumulation was determined using a commercially available kit from Enzo Life Sciences (East Farmingdale, NY), according to the manufacturer's protocol.

2.6 Fatty Acid Uptake

Cells were washed with calcium/magnesium-free phosphate buffered saline (CMF-PBS, pH=7.4), incubated with 10 μM BODIPY® FL C_{16} (4,4-Difluoro-5,7-Dimethyl-4-Bora-3a,4a-Diaza-*s*-Indacene-3-Hexadecanoic Acid; Life Technologies, Carlsbad, CA) in 0.1% FA-Free BSA in Hanks' balanced salt solution (HBSS; Gibco – Life Technologies, Carlsbad, CA) for 1 minute, and then washed twice with an ice-cold solution of 0.2% FA-Free BSA in HBSS. Fluorescence was measured using a Synergy H1 Multi-Mode Reader (BioTek Instruments, Inc.; Winooski, VT), and analyzed using Gen5™ Data Analysis Software (BioTek Instruments, Inc.). The relative BODIPY FL C_{16} uptake is expressed as fluorescence intensity per well normalized to the total amount of protein per well. In separate experiments, cells were incubated in serum-free DMEM containing 1 mM palmitic acid (500,000 DPM/mL) in 3% BSA for 1 minute, and then washed twice with an ice-cold solution of 0.2% FA-Free BSA in HBSS. Cells were lysed by incubating with lysis buffer (0.1 M NaOH, 0.2% (w:v) SDS) at 37°C for two hours. Cell lysates were transferred to a scintillation vial,

and assessed for radioactivity in a liquid scintillation counter (Tri-Carb 1600 TR Liquid Scintillation Analyzer, PerkinElmer, Waltham, MA).

2.7 Fatty Acid Oxidation

Fatty acid oxidation (FAO) was assessed by quantifying the production of $^{14}CO_2$ from [1-^{14}C] palmitic acid as previously described (237). Cells were plated in 35 mm dishes, differentiated as described above, and incubated with 10 nM 1,25$(OH)_2$D for 4 days. To measure FAO, dishes were placed and sealed in wide-mouth jars with screw-top lids, and cells were incubated in serum-free DMEM containing 1 mM palmitic acid (500,000 DPM/mL) in 3% BSA for 3 hours at 37°C and 5% CO_2. After 3 hours, the reaction was terminated by injection of 5 N perchloric acid to the cell culture dish. To trap $^{14}CO_2$, 2-phenethylamine was injected into well inserts containing Whatman 1 filter papers located inside the jar. After 1 hour, filter papers were placed in vials with scintillation fluid and assessed for radioactivity in a liquid scintillation counter (Tri-Carb 1600 TR Liquid Scintillation Analyzer, PerkinElmer, Waltham, MA). Oxidation values were normalized to the protein content of treatment-matched control dishes.

2.8 De Novo Lipogenesis:

Cells were differentiated in 6-well tissue culture dishes as described above, and incubated with 10 nM 1,25$(OH)_2$D for 4 days During the last 24 hours of treatment, 3T3-L1 adipocytes were incubated with 10 mM [$^{13}C_2$]acetate (Sigma-Aldrich, St. Louis, MO). Cells were harvested into 300 uL lysis buffer

containing 25 mM HEPES, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100. Lipid hydrolysis, extraction, derivatization and analysis by high pressure liquid chromatography/electrospray ionization-mass spectrometry (HPLC/ESI-MS/MS) were performed as previously described (238). Briefly, cell lysates were heated at 90°C in a solution of acetonitrile:HCl (37%) (4:1, v/v) to hydrolyze fatty acids. After cooling to room temperature, fatty acids were extracted with hexane and dried under nitrogen gas. Fatty acids were resuspended in acetone, derivatized with 2-bromo-1-methylpyridinium iodide and 3-carbinol-1-methylpyridinium iodide to form 3-acyloxymethyl-1-methylpyridinium iodide (AMMP), and subjected to HPLC/ESI-MS/MS analysis. Substrate incorporation into 16:0, 16:1, 18:0, and 18:1 fatty acids was assessed, and data are expressed percent of fatty acid with ^{13}C label, relative to vehicle. To determine the contribution of glucose as a substrate for *de novo* lipogenesis, in separate experiments, 3T3-L1 adipocytes were incubated in glucose-free DMEM containing a 1:1 mixture of D-[U-^{13}C]glucose (Sigma-Aldrich, St. Louis, MO) and non-labeled D-glucose, at a final glucose concentration of 4.5 g/L. Incorporation of [U-^{13}C]glucose into fatty acids during the last 24 hours of treatment was assessed as described above.

2.9 Glucose Uptake and Consumption

Basal and insulin-stimulated glucose uptake were assessed using the 2-[1,2-$^{3}H(N)$]-deoxy-D-glucose uptake assay. Cells were washed with calcium/magnesium free phosphate buffered saline (CMF-PBS, pH=7.4), and incubated with serum- and glucose-free DMEM for 2 hours at 37°C and 5% CO_2.

Subsequently, cells were incubated in the presence or absence of insulin (100 nM) for an additional 20 minutes at 37°C, before the 2-[1,2-^{3}H(N)]-deoxy-D-glucose uptake assay was initiated by the addition of serum- and glucose-free DMEM containing insulin (or vehicle), 2-deoxyglucose (100 µM, Sigma-Aldrich, St. Louis, MO) and 2-[1,2-^{3}H(N)]-deoxy-D-glucose (1.0 µCi/mL, Perkin Elmer, Oak Brook, IL). Cells were incubated for a further 10 minutes, and the reaction was terminated by washing 3 times with ice-cold CMF-PBS containing phloretin (0.2 mM). Nonspecific uptake, determined in the presence of phloretin (0.2 mM, Sigma-Aldrich, St. Louis, MO), was subtracted from all values (239). Cells were lysed by incubating with lysis buffer (0.1 M NaOH, 0.2% (w:v) SDS) at 37°C for two hours. Cell lysates were transferred to a scintillation vial, and assessed for radioactivity in a liquid scintillation counter (Tri-Carb 1600 TR Liquid Scintillation Analyzer, PerkinElmer, Waltham, MA). Glucose uptake values were normalized to the protein content of treatment-matched control dishes. Glucose concentrations in spent media samples were measured using a commercially available kit (Cayman Chemicals, Ann Arbor, MI), utilizing the glucose oxidase-peroxide reaction. Glucose consumption was calculated using the difference between spent and fresh media samples, and data normalized to total protein.

2.10 Western Blotting

Cells were washed with calcium/magnesium free phosphate buffered saline (CMF-PBS, pH=7.4) and harvested on ice into lysis buffer containing 25 mM HEPES, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 1% each of

protease and phosphatase inhibitor cocktails (Sigma-Aldrich, St. Louis, MO). Cells were briefly sonicated and cell debris was removed by centrifugation at 12,000 RPM for 15 min at 4°C. Protein concentration was determined using the BCA assay. Proteins (20–30 µg) were resolved by SDS-PAGE on 7.5%, 10% or 12% polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA). Resolved proteins were transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Inc.), and probed with specific antibodies against proteins as indicated. Antigen-antibody complexes were detected using the Lumiglo Reagent (Cell Signaling Technology, Inc.). Densities of immunoreactive bands were assessed using UN-SCAN-IT gel analysis software (Silk Scientific, Orem, UT). Densities of the bands were in the linear range of detectability. Results are expressed as fold change of respective protein/GAPDH, or phosphorylated protein over total.

2.11 RNA Isolation and Analysis

RNA was isolated using TriReagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocol. Reverse transcription of total RNA was performed using MMLV reverse transcriptase (Promega, Madison, WI). Real-time quantitative PCR was performed using the Ultra-Fast SYBR® Green QPCR Master Mix (Agilent Technologies, Santa Clara, CA). The mRNA abundance of target genes were determined using the threshold cycle (Ct) value. Data are normalized to 18S expression, and expressed as fold change relative to vehicle. Primers used are shown in Table 4.

2.12 Transfection of 3T3-L1 Adipocytes

The pyruvate carboxylase (PC) plasmid was synthesized and obtained through OriGene Technologies (Rockville, MD). The PC transcript variant 1 clone was synthesized, and inserted into the pCMV6-Neo vector downstream of the cytomegalovirus (CMV) immediate-early promoter. The plasmid was transiently transfected into differentiated 3T3-L1 adipocytes using Lipofectamine 3000 (Invitrogen, Carlsbad, CA), according to manufacturer protocol.

2.13 Statistical Analysis

Values are presented as mean ± SEM. Results are expressed compared to vehicle by the Student's t-test (LSD) or by analysis of variance (ANOVA), with $P<0.05$ considered statistically significant. All experiments were performed at least three times.

Table 2.1 Primers used in the QPCR analysis of gene expression

Gene	Primer information	
ACC1	Forward: 5'-	GCGTCGGGTAGATCCAGTT-3'
	Reverse: 5'-	CTCAGTGGGGCTTAGCTCTG-3'
ACC2	Forward: 5'-	ACTGTCCTGAGATCCCCCTC-3'
	Reverse: 5'-	GGACCCAGTCCTTCAGCTTC-3'
AQP7	Forward: 5'-	TGTCGCTAGGCATGAACTCC-3'
	Reverse: 5'-	CAGGAGATCCCAAGGAGTGG-3'
CPT-1α	Forward: 5'-	CTGCAGACTCGGTCACCACT-3'
	Reverse: 5'-	ACACCCACCACCACGATAAG-3'
FAS	Forward: 5'-	ACCACTGCATTGACGGCCGG-3'
	Reverse: 5'-	GGGTCAGGCGGGAGACCGAT-3'
GLUT1	Forward: 5'-	GTGACGATCTGAGCTACGGG-3'
	Reverse: 5'-	GAGAGACCAAAGCGTGGTGA-3'
GLUT4	Forward: 5'-	GTGACTGGAACACTGGTCCTA-3'
	Reverse: 5'-	CCAGCCACGTTGCATTGTAG-3'
hPC	Forward: 5'-	ATGTTGCCCACAACTTCAGCAAGC-3'
	Reverse: 5'-	AGTTGAGGGAGTCAAACACACGGA-3'
PC	Forward: 5'-	TCCTCTATCACGTCCTCTGTGT-3'
	Reverse: 5'-	ACATTTGGGGAGGCAACAGG-3'

CHAPTER 3. 1,25-DIHYDROXYVITAMIN D REGULATES TRIACYLGLYCEROL ACCUMULATION IN 3T3-L1 ADIPOCYTES

3.1 $1,25(OH)_2D$ Regulates Lipid Metabolism in 3T3-L1 Adipocytes

3.1.1 $1,25(OH)_2D$ Reduces Triacylglycerol Accumulation in 3T3-L1 Adipocytes

Previous studies have demonstrated the ability of $1,25(OH)_2D$ to inhibit the onset of adipogenic gene expression, and consequent lipid accumulation, during 3T3-L1 preadipocyte differentiation (206, 208, 240). However, whether $1,25(OH)_2D$ impacts lipid metabolism in adipocytes that have completed terminal differentiation is not known. To determine the impact of $1,25(OH)_2D$ on lipid metabolism in mature adipocytes that have completed terminal differentiation, 3T3-L1 preadipocytes were first differentiated for 9 days. By Day 9, cells had achieved complete differentiation, and cells contained large lipid droplets. To determine whether $1,25(OH)_2D$ modulates triacylglycerol (TAG) accumulation in mature adipocytes, differentiated cells were stimulated with $1,25(OH)_2D$ (10 nM) for 4 or 7 days, and total triacylglycerol levels assessed. Following 4 days of treatment, triacylglycerol accumulation was 21% lower in $1,25(OH)_2D$-treated cells compared to vehicle ($p=0.01$) when normalized to protein content (Figure 3.1). The TAG-lowering effect of $1,25(OH)_2D$ persisted following 7 days of treatment, demonstrating achievement of a stable state of reduced lipid storage

(Figure 3.1). Cell viability, assessed using the MTT (Figure 3.2A) and trypan blue exclusion assays (Figure 3.2B), was not affected following 4 days of $1,25(OH)_2D$ treatment. These data indicate that $1,25(OH)_2D$ acts on mature adipocytes to stimulate a reduction in lipid storage in the absence of cytotoxicity.

3.1.2 $1,25(OH)_2D$ Stimulates Glycerol Release in 3T3-L1 Adipocytes

Triacylglycerol accumulation in adipocyte lipid droplets is a balance between storage of fatty acids that are taken up by the cell or synthesized *de novo*, and release of fatty acids that are hydrolyzed from TAG by lipolysis. Hydrolysis of stored TAG in adipocytes by lipolysis allows for the release of fatty acids from the cell, so that they may be used as an energy source for other tissues such cardiac and skeletal muscle. Lipolysis is a highly regulated process, and disturbances in the regulation of this process underlie several metabolic disorders, including dyslipidemia and insulin resistance (74). To determine whether $1,25(OH)_2D$ enhances rates of basal lipolysis, glycerol and non-esterified fatty acid (NEFA) release in response to $1,25(OH)_2D$ were assessed. When triacylglycerol stores are completely hydrolyzed to provide energy substrates for use by other tissues, glycerol and non-esterified fatty acid moieties are released from the lipid droplet at a 3:1 molar ratio. While fatty acids may be re-esterified, oxidized, or released from the cell upon hydrolysis from the lipid droplet, glycerol kinase activity is negligible in white adipose tissue, meaning that glycerol released during lipolysis cannot be phosphorylated and used for TAG

synthesis (76). Therefore, release of glycerol from the cell is frequently used as an indicator of lipolysis.

Glycerol release was stimulated by 1,25$(OH)_2$D following 1-4 days of treatment, with a maximal increase of 66% occurring after 2 days ($p<0.001$) (Figure 3.3). No change in glycerol release was observed within 1-8 hours of treatment (data not shown), suggesting genomic or indirect action of 1,25$(OH)_2$D rather than activation of a rapid signaling pathway. Consistent with an increase in glycerol release, mRNA expression of the glycerol transporter aquaporin 7 (AQP7) was increased by 27% by 1,25$(OH)_2$D after 2 days ($P<0.05$) (Figure 3.4).

However, despite a 66% increase in glycerol release with 1,25$(OH)_2$D treatment, NEFA accumulation in the cell medium was below the limit of detection in both vehicle- and 1,25$(OH)_2$D-treated cells, and did not change dramatically with 1,25$(OH)_2$D (data not shown). High rates of fatty acid re-esterification occur during lipolysis in WAT; 30-100% of fatty acids in the basal state, and 10-20% of those freed in response to lipolytic stimuli may be re-esterified to TAG in a process termed futile TAG/FA cycling (73, 90-92). These data suggest that under basal and 1,25$(OH)_2$D-stimulated conditions, either the majority of fatty acids that are released are either being re-esterified as TAG or the glycerol that is being released in response to 1,25$(OH)_2$D is not of lipolytic origin.

To explore the potential impact of 1,25$(OH)_2$D on TAG hydrolysis, regulators of lipolytic action were investigated. Chemical inhibition of protein kinase A (PKA) with H-89 dihydrochloride completely prevented 1,25$(OH)_2$D

stimulation of glycerol release (Figure 3.5A), demonstrating that PKA activity is necessary for 1,25$(OH)_2$D-stimulated lipolysis or release of glycerol. Additionally, phosphorylation of hormone sensitive lipase (HSL) at PKA phosphorylation site Ser660 was significantly increased with 1,25$(OH)_2$D treatment (Figure 3.5B), while no change in phosphorylation level was observed at the AMPK phosphorylation site Ser565 (data not shown). Further, despite a reduction in ATGL protein expression, the protein expression of ATGL cofactor CGI-58 is significantly increased in response to 1,25$(OH)_2$D treatment (Figure 3.5C).

Given these 1,25$(OH)_2$D-induced changes in the lipolysis signaling pathway, that the glycerol released in response to 1,25$(OH)_2$D is due to hydrolysis of TAG remains a possibility. However, not all data are consistent with 1,25$(OH)_2$D stimulation of TAG hydrolysis. First, the reduction in TAG accumulation after 4 days in response to 1,25$(OH)_2$D cannot quantitatively sustain the increase in glycerol release that is stimulated by 1,25$(OH)_2$D. As described above, no increase in NEFA release was observed with 1,25$(OH)_2$D stimulation. Further, after two days of treatment, during maximal 1,25$(OH)_2$D-stimulated glycerol release, intracellular accumulation of cAMP was not altered by 1,25$(OH)_2$D (Figure 3.6). PKA activation, which occurs during TAG hydrolysis, is stimulated by intracellular accumulation of cyclic AMP (cAMP) (241). Lack of intracellular cAMP accumulation and NEFA release are not consistent with PKA-mediated TAG hydrolysis, and collectively, these data suggest that the glycerol released in response to 1,25$(OH)_2$D is not primarily due to hydrolysis of TAG.

There are two alternate potential sources of glycerol that could be contributing to the pool of free glycerol released from the adipocytes. First, glycerol may be synthesized from glucose during glycolysis. Second, glycerol may be synthesized from glucose-derived pyruvate or lactate, by a process called glyceroneogenesis (76). In response to $1,25(OH)_2D$, whether the released glycerol is of lipolytic or of glycolytic/glyceroneogenic origin is not yet clear. The possibility of $1,25(OH)_2D$ stimulation of glycerol release that is of glycolytic/glyceroneogenic origin is explored further in Chapter 3.2.

3.1.3 $1,25(OH)_2D$ Stimulates Fatty Acid Uptake and Oxidation in 3T3-L1 Adipocytes

Another mechanism that can influence TAG accumulation is uptake of fatty acids. To determine whether $1,25(OH)_2D$ impacts the cells' ability to take up exogenous fatty acids, uptake of BODIPY FL C_{16}, a fluorescent C16:0 fatty acid, was assessed following 1, 2, 3, and 4 days of $1,25(OH)_2D$ stimulation. Interestingly, despite a net reduction in TAG storage achieved following 4 days of $1,25(OH)_2D$ stimulation, BODIPY fatty acid uptake was significantly increased in response to $1,25(OH)_2D$ at 24 hours ($P<0.05$), and remained elevated throughout the 4 days of treatment (Figure 3.7). These data indicate that $1,25(OH)_2D$ does not impair the cells' ability to take up exogenous fatty acids.

In addition, fatty acid balance and changes in TAG accumulation are influenced by fatty acid oxidation. To determine whether $1,25(OH)_2D$ alters fatty acid oxidation in 3T3-L1 adipocytes, rates of β-oxidation were measured in

vehicle- and 1,25(OH)$_2$D-treated cells. To quantify complete oxidation of fatty acids, $^{14}CO_2$ production was assessed in response to stimulation by 1 mM palmitic acid, as described in Materials and Methods. Compared to vehicle-treated cells, which exhibited a $^{14}CO_2$ production rate of 0.624 nmol·hr^{-1}·mg protein^{-1}, those stimulated with 1,25(OH)$_2$D for four days exhibited a $^{14}CO_2$ production rate of 1.55 nmol·hr^{-1}·mg protein^{-1}, an increase of 148% ($p=0.002$) (Figure 3.8A). This 2.5-fold increase in FAO is interesting, as 1,25(OH)$_2$D reduces the mRNA expression of the FAO rate-limiting enzyme carnitine palmitoyltransferase I (CPT-1) by 56% after 4 days ($p<0.01$), while increasing the mRNA expression of acetyl-CoA carboxylase 2 (ACC2) by 48% ($p=0.02$) (Figure 3.8B). When glucose is available, this isoform of ACC is responsible for the production of malonyl-CoA from glucose-derived acetyl-CoA. Production of malonyl-CoA inhibits CPT-1, and consequently, oxidation of fatty acids. Expression of FAO-related genes in response to 1,25(OH)$_2$D are therefore not indicative of FAO activity in 3T3-L1 adipocytes.

After 4 days of treatment, BODIPY FL C_{16} uptake was increased by approximately 1.8-fold in 1,25(OH)$_2$D- vs vehicle-treated cells. At this same time point, oxidation of exogenously supplied palmitic acid was increased approximately 2.5-fold by 1,25(OH)$_2$D. To determine if the increase in FAO is a consequence of increased ability to take up fatty acids, measurement of fatty acid uptake under the same conditions as those used for FAO experiments was assessed. In response to 1 mM palmitic acid, [1-^{14}C] palmitic acid uptake was not significantly different in 1,25(OH)$_2$D- compared to vehicle-treated cells (Figure

3.8C). A possible explanation for the discrepancy between the impact of $1,25(OH)_2D$ on the uptake of palmitic acid vs. that of BODIPY FL C_{16} is that $1,25(OH)_2D$ may alter the Km of fatty acid transporters, allowing regulation of fatty acid uptake to be observed under conditions where fatty acid uptake in response to increasing extracellular fatty acids is linear. If this is the case, such regulation may not be observed in response to relatively high fatty acid concentrations such has the 1 mm palmitic acid used to measure fatty acid oxidation. Regardless, these data demonstrated that $1,25(OH)_2D$ stimulates fatty acid oxidation in 3T3-L1 adipocytes, independently of its effect on fatty acid uptake. This increase in fatty acid oxidation may contribute to the reduction in TAG accumulation that is observed with $1,25(OH)_2D$ stimulation.

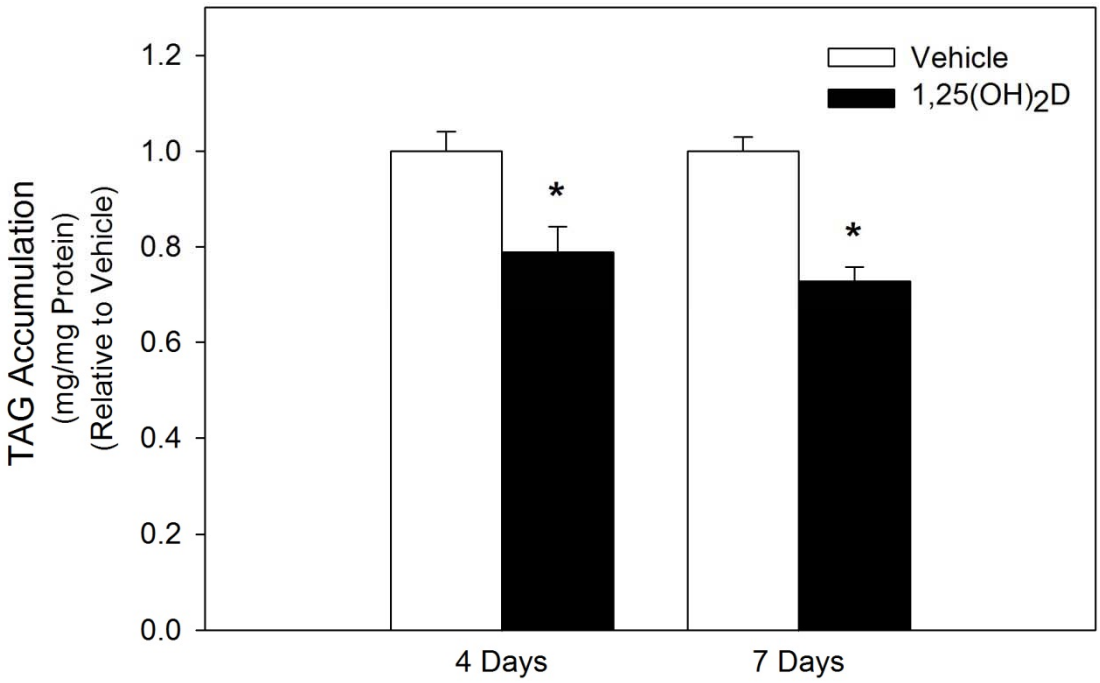


Figure 3.1. $1,25(OH)_2D$ reduces triacylglycerol accumulation in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were treated with vehicle or $1,25(OH)_2D$ (10 nM) for 4 or 7 days before analysis as indicated. Triacylglycerol accumulation in adipocytes was assessed using a commercially available kit. *P<0.05 compared to vehicle at the same time point (n=3-6/group).

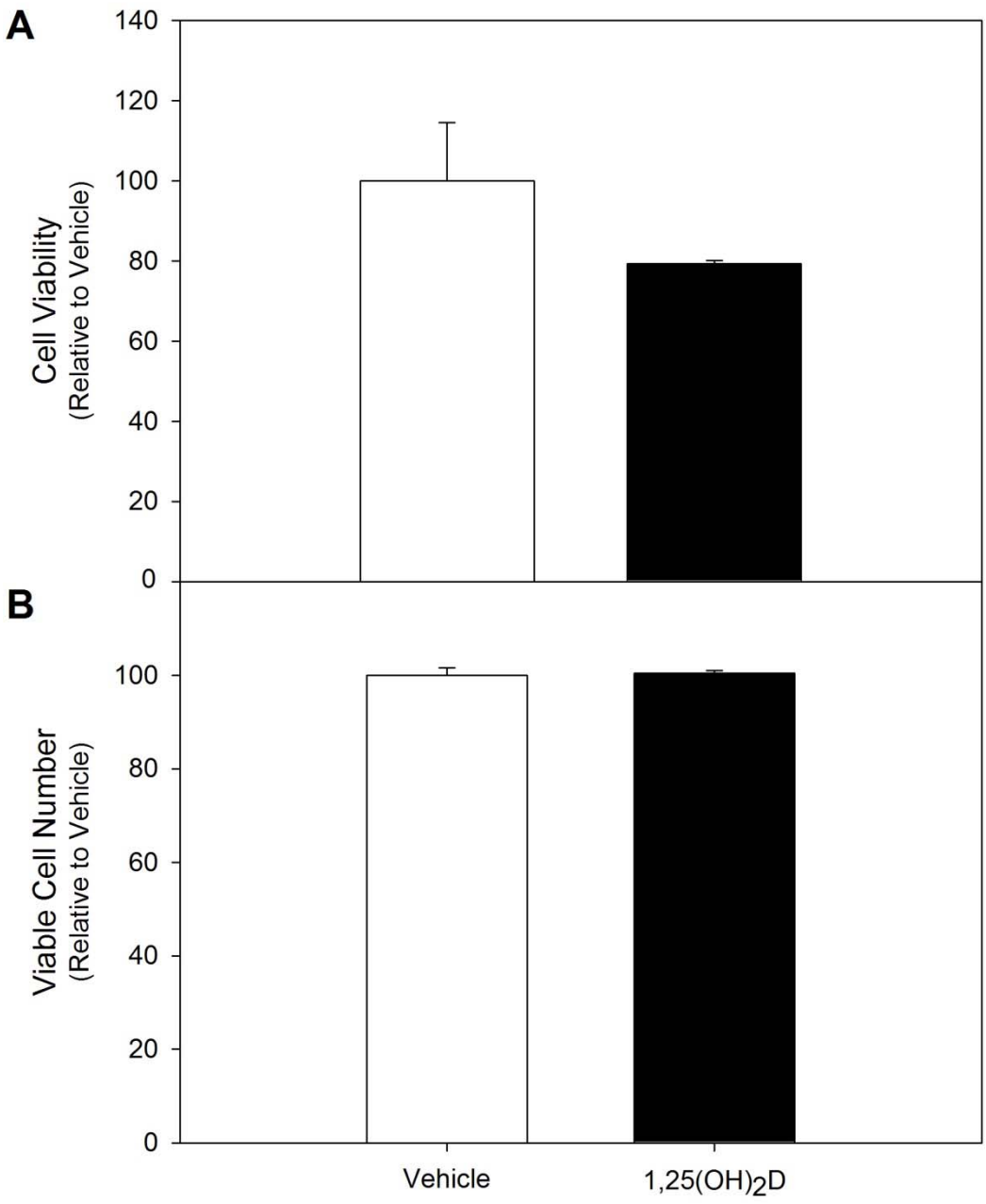


Figure 3.2. $1,25(OH)_2D$ does not affect cell viability. 3T3-L1 adipocytes were treated with $1,25(OH)_2D$ (10 nM) for 4 days, and cell viability was assessed using the MTT (A) and Trypan Blue Exclusion (B) assays (n=6-8/group). No significant difference in cell viability was observed between vehicle- and $1,25(OH)_2D$-treated cells.

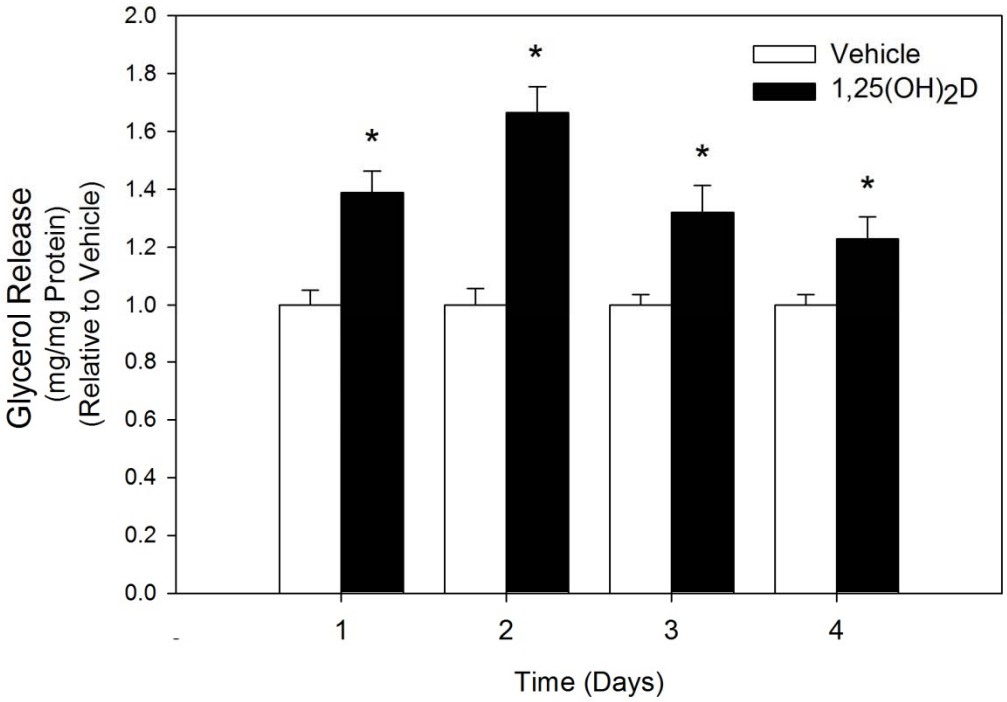


Figure 3.3. $1,25(OH)_2D$ stimulates glycerol release in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were treated with vehicle or $1,25(OH)_2D$ (10 nM) for 1-4 days as indicated. Glycerol release was assessed in spent media samples by quantitative enzymatic determination of free glycerol using a commercially available kit. *P<0.05 compared to vehicle at the same time point (n=3-6/group).

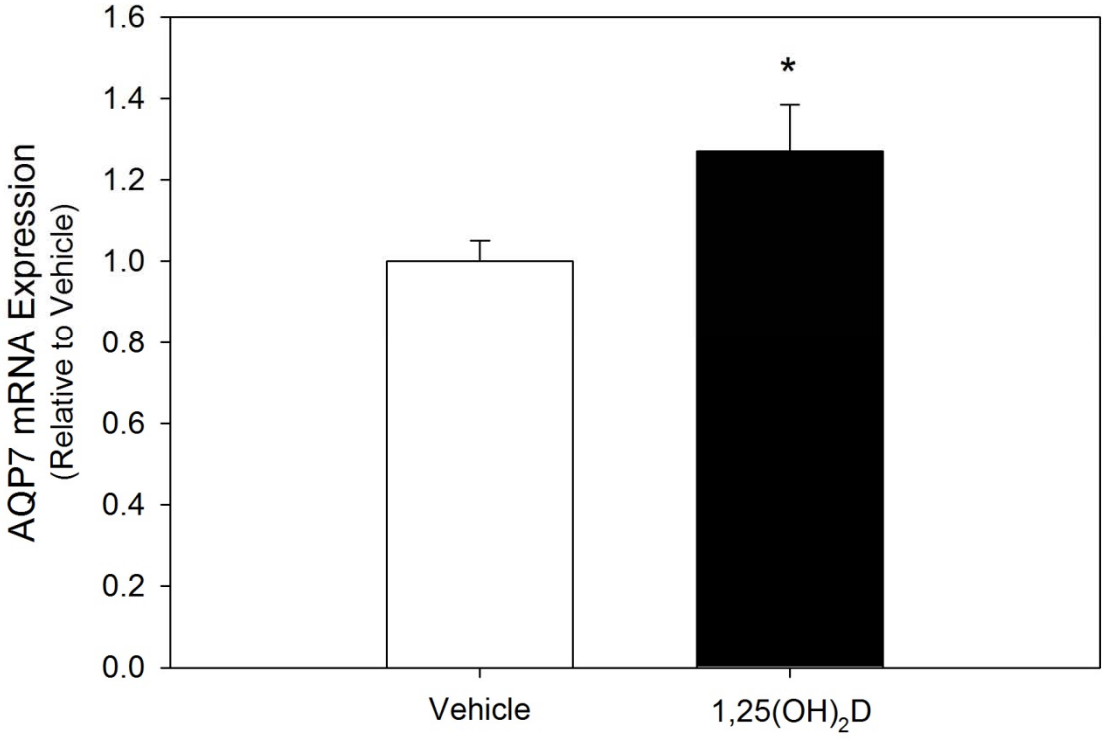


Figure 3.4. Aquaglyceroporin mRNA expression is upregulated by $1,25(OH)_2D$. Differentiated 3T3-L1 adipocytes were treated with vehicle or $1,25(OH)_2D$ (10 nM) for 2 days. The mRNA expression of glycerol transporter AQP7 is expressed relative to vehicle. *P<0.05 compared to vehicle (n=3/group).

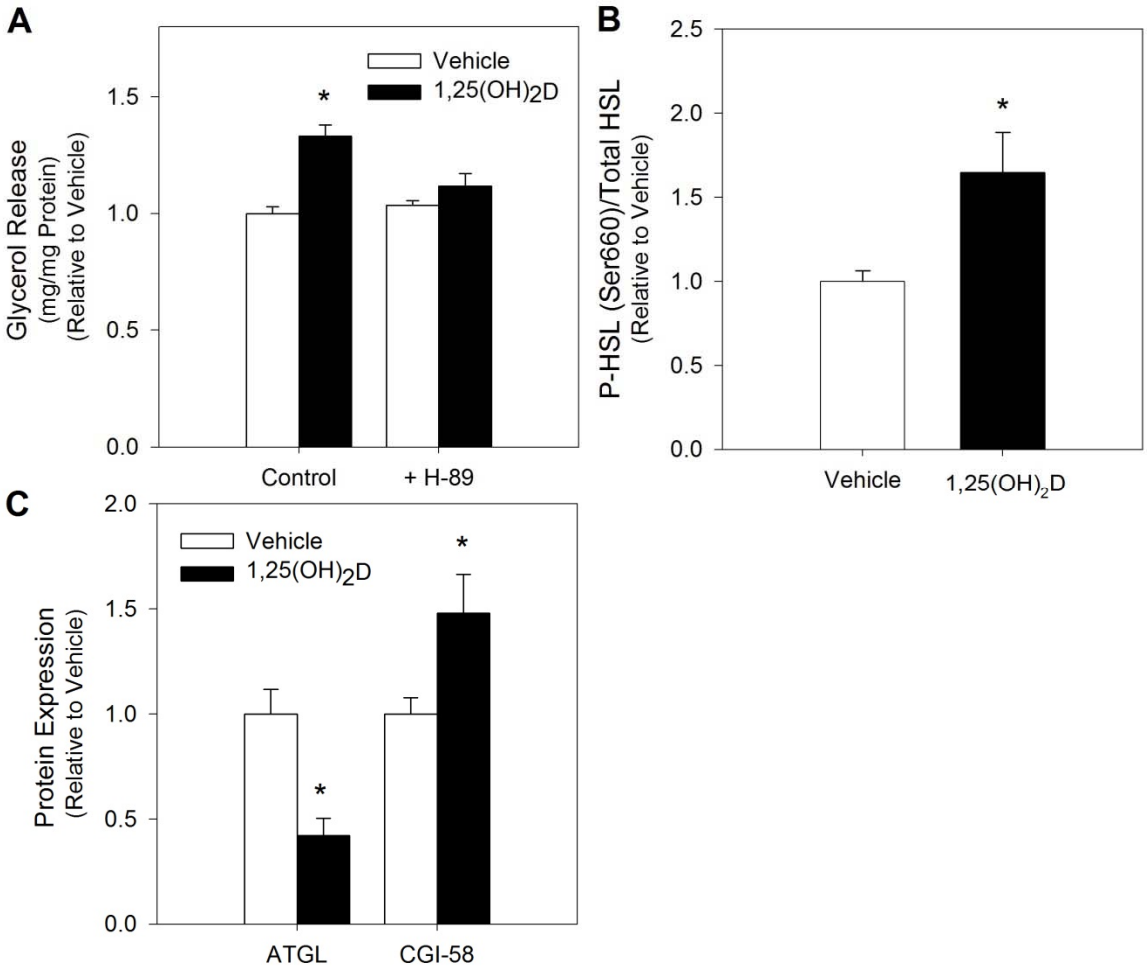


Figure 3.5. 1,25(OH)$_2$D-induced glycerol release is PKA-dependent. Differentiated adipocytes were treated with 1,25(OH)$_2$D (10 nM) or vehicle for 2 days, and then harvested in lysis buffer. A) Inhibition of PKA prevents 1,25(OH)$_2$D-induced glycerol release (n=3/group). B) 1,25(OH)$_2$D stimulates phosphorylation of HSL at PKA phosphorylation site Serine 660. Phosphorylation of HSL at the residues indicated was determined by Western blot as described in Materials and Methods. Data are expressed relative to total HSL protein expression (n=3/group). C) 1,25(OH)$_2$D regulates CGI-58 and ATGL protein expression. Data are normalized to expression of actin (n=3/group). *P<0.05 compared to vehicle.

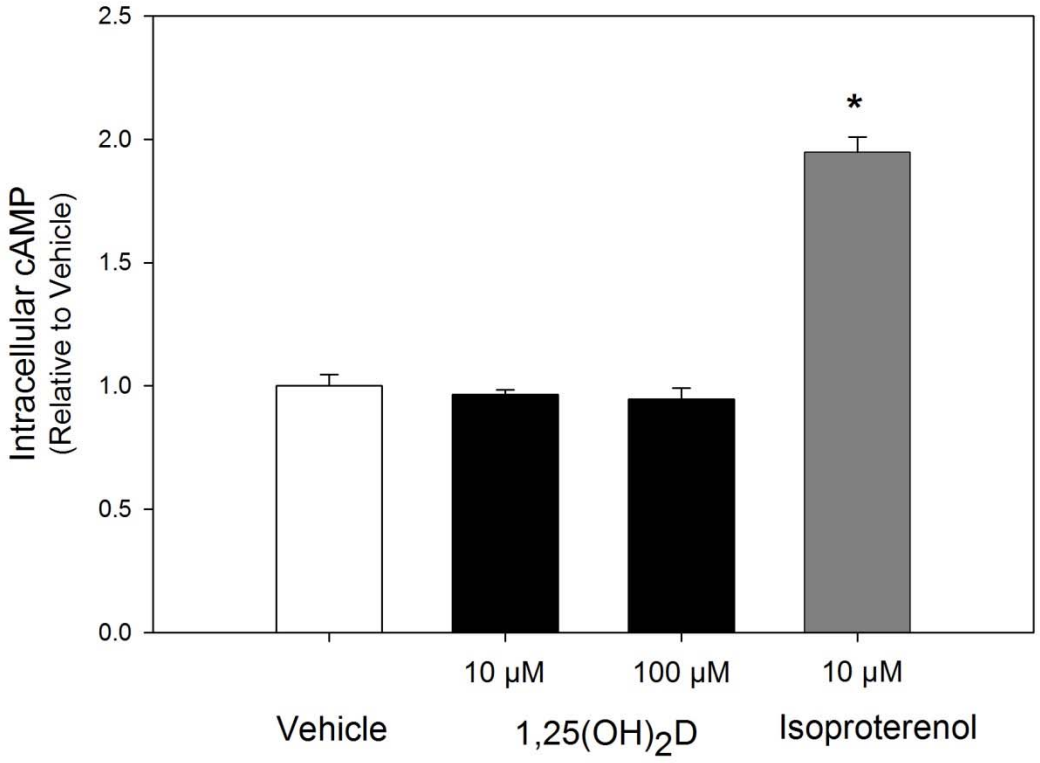


Figure 3.6. $1,25(OH)_2D$ does not stimulate intracellular cAMP accumulation. Differentiated 3T3-L1 adipocytes were treated with $1,25(OH)_2D$ (10 nM) or vehicle for 48 hours. As a positive control, some cells were stimulated with isoproterenol (10 µM) for two hours prior to harvest. Intracellular cAMP accumulation was determined as described in Materials and Methods. Data are normalized to total protein. *P<0.05 compared to vehicle (n=3-6/group).

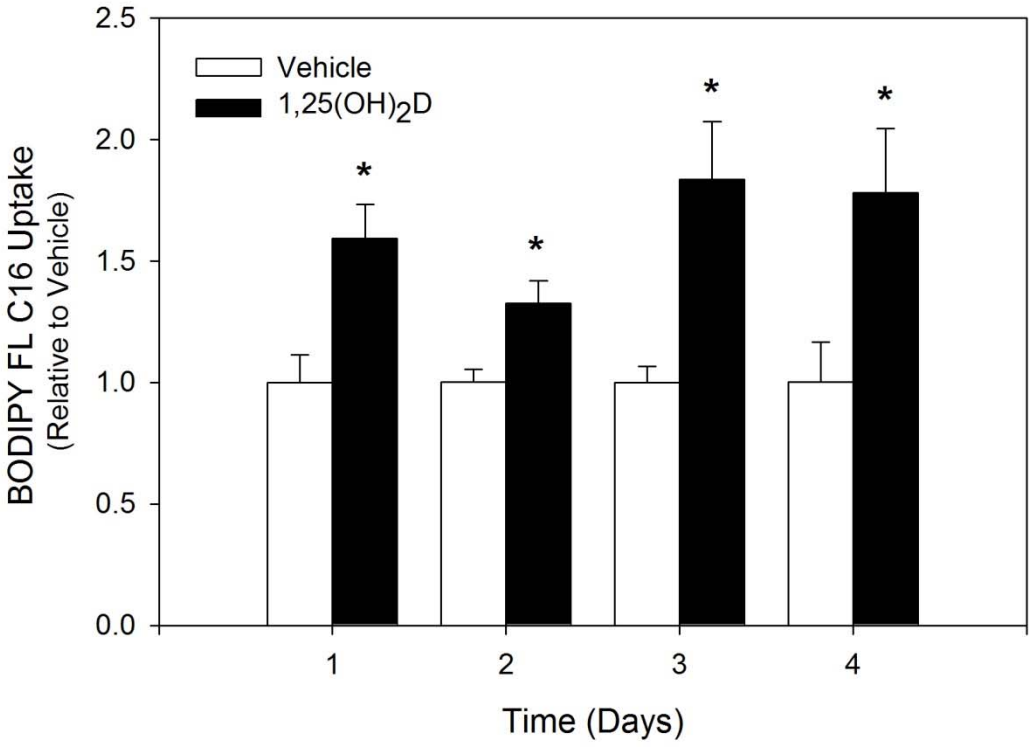


Figure 3.7. $1,25(OH)_2D$ stimulates fatty acid uptake in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were treated with $1,25(OH)_2D$ (10 nM) or vehicle for 1-4 days as indicated. Fatty acid uptake was determined following a 1-minute incubation with BODIPY FL C_{16} (10 μM) as described in Materials and Methods. The relative BODIPY FL C_{16} uptake is expressed as fluorescence intensity per well, normalized to the total amount of protein. *P<0.05 compared to vehicle at the same time point (n=4/group).

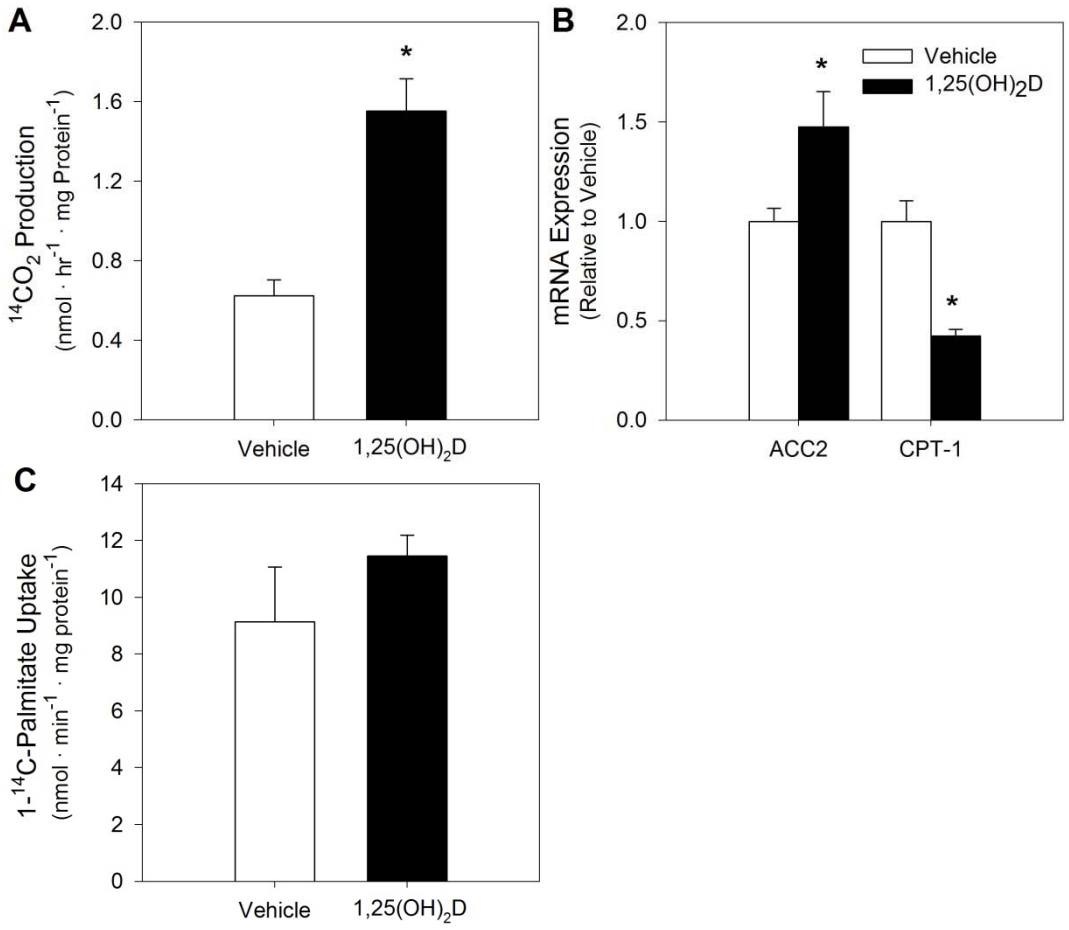


Figure 3.8. 1,25(OH)$_2$D stimulates fatty acid oxidation in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were stimulated with 1,25(OH)$_2$D (10 nM) or vehicle for 4 days. A) Complete oxidation of [1-^{14}C] palmitic acid. Fatty acid oxidation was assessed as described in Materials and Methods (n=4/group). B) 1,25(OH)$_2$D regulates carnitine palmitoyltransferase I (CPT-1) and acetyl-CoA carboxylase 2 (ACC2) mRNA expression (n=3/group). C) Uptake of [1-^{14}C] palmitic acid. *P<0.05 compared to vehicle (n=3/group).

3.2 $1,25(OH)_2D$ Regulates Glucose Metabolism and Utilization in 3T3-L1 Adipocytes

3.2.1 $1,25(OH)_2D$ Reduces Glucose and Acetate Incorporation into Fatty Acids in 3T3-L1 Adipocytes:

The previously described studies demonstrate that $1,25(OH)_2D$ stimulates PKA-dependent glycerol release and oxidation of fatty acids. While it remains unclear whether the observed glycerol release is of TAG or glyceroneogenic origin, it is likely that these alterations in lipid metabolism, particularly the elevated fatty acid oxidation, increase cellular energy expenditure and contribute to the reduction in TAG accumulation that is stimulated by $1,25(OH)_2D$. The studies described within examine the impact of $1,25(OH)_2D$ on glucose metabolism and utilization in 3T3-L1 adipocytes.

Glucose uptake by adipocytes plays a critical role in the maintenance of glucose homeostasis (74). In addition to glucose being a source of ATP for the adipocyte, it is estimated that 20-25% of the glucose consumed by adipocytes is used for the synthesis of fatty acids (77). To determine whether $1,25(OH)_2D$ reduces glucose utilization for the synthesis of fatty acids, differentiated 3T3-L1 adipocytes were stimulated with $1,25(OH)_2D$ or vehicle for four days. During the final 24 hours of $1,25(OH)_2D$ treatment, cell medium was replaced with

glucose-free DMEM, with D-glucose and D-[U-^{13}C]glucose added at a 1:1 ratio to a final glucose concentration of 4.5 g/L. Lipid extraction and hydrolysis of fatty acids were performed as described in Materials andMethods, and incorporation of the ^{13}C isotope into palmitic, palmitoleic, stearic, and oleic acids was assessed using HPLC/ESI-MS/MS. These four fatty acids comprise approximately 80% of the cellular fatty acid pool (242). Compared to vehicle-treated cells, 1,25(OH)$_2$D stimulated a 28% reduction in ^{13}C incorporation into these fatty acids ($p<0.001$) (Figure 3.9), demonstrating a reduced contribution of glucose as a substrate for *de novo* lipogenesis. This shift away from glucose utilization for *de novo* lipogenesis may contribute to the reduction in TAG that is stimulated by 1,25(OH)$_2$D.

To determine whether 1,25(OH)$_2$D reduces glucose incorporation into fatty acids by decreasing the rate of fatty acid synthesis in adipocytes, differentiated 3T3-L1 adipocytes were treated as described above, and during the last 24 hours of treatment cells were incubated with 10 mM [$^{13}C_2$]acetate was added to the cell culture medium. Cytosolic acetate serves as a substrate for fatty acid synthesis, and its incorporation into fatty acids serves as an indicator of lipogenesis. Lipid extraction and hydrolysis of fatty acids were performed as described above, and [$^{13}C_2$]acetate incorporation into palmitic, palmitoleic, stearic, and oleic acids was assessed using HPLC/ESI-MS/MS. Compared to vehicle-treated cells, those stimulated with 1,25(OH)$_2$D exhibited a 9% reduction in acetate incorporation into fatty acids ($p=0.026$) (Figure 3.10). This reduction in acetate incorporation into fatty acids suggests that the enzymatic activity of acetyl CoA carboxylase 1

(ACC1), the rate-limiting step of lipogenesis, or of fatty acid synthase (FAS), is inhibited by 1,25$(OH)_2$D. While the mRNA expression of acetyl-CoA carboxylase 1 (ACC1), the ACC isoform responsible for supplying the malonyl-CoA pool for the synthesis of fatty acids, is not significantly different in vehicle- compared to 1,25$(OH)_2$D-treated adipocytes (Figure 3.11). However, fatty acid synthase (FAS) mRNA expression is significantly reduced by 1,25$(OH)_2$D (Figure 3.11). Regardless, the relatively small magnitude of change in acetate compared to glucose incorporation into fatty acids suggests additional regulation of processes upstream of fatty acid synthesis by 1,25$(OH)_2$D, at the level of glucose uptake or oxidation.

3.2.2 1,25$(OH)_2$D Impacts Basal and Insulin-Stimulated 2-deoxglucose Uptake in 3T3-L1 Adipocytes:

To determine whether 1,25$(OH)_2$D reduces glucose incorporation into fatty acids by altering glucose uptake in 3T3-L1 adipocytes, glucose uptake was assessed after 4 days of 1,25$(OH)_2$D treatment using the 2-deoxyglucose uptake assay as described in Chapter 2 (Materials and Methods). Compared to vehicle-treated cells, those stimulated with 1,25$(OH)_2$D exhibited a 62% increase in basal 2-deoxyglucose uptake ($p<0.01$) (Figure 3.12A). Basal glucose uptake is under the control of the facilitated diffusion glucose transporter 1 (GLUT1), which is encoded by the SLC2A1 gene. While the highest levels of GLUT1 expression are observed in the brain, blood-brain barrier, erythrocytes, and fetal tissues, it is

expressed ubiquitously in tissues and cell cultures (102, 243). The mRNA expression of GLUT1 was not changed with 1,25$(OH)_2$D treatment (Figure 3.13).

While basal glucose uptake was significantly higher in 1,25$(OH)_2$D-compared to vehicle-treated cells, insulin-stimulated 2-deoxyglucose uptake was not changed with 1,25$(OH)_2$D treatment (Figure 3.12A). Thus, the fold increase in 2-deoxyglucose uptake with acute insulin stimulation, a measure of insulin sensitivity, was significantly reduced by 28% in response to 1,25$(OH)_2$D ($p=0.04$) (Figure 3.12B). Insulin-stimulated glucose uptake in skeletal muscle and adipose tissue is under the control of the GLUT4 glucose transporter, encoded by the SLC2A4 gene (102, 243). Consistent with a reduction in insulin sensitivity, mRNA expression of the insulin-responsive GLUT4 glucose transporter was reduced by 36% in response to 1,25$(OH)_2$D ($p=0.01$) (Figure 3.13).

In addition to acute basal and insulin-stimulated 2-deoxyglucose uptake, consumption of glucose and lactate secretion over a 24-hour period were also assessed. Though rates of basal 2-deoxyglucose uptake were elevated in response to 1,25$(OH)_2$D, glucose consumption (Figure 3.14A) and lactate secretion (Figure 3.14B) over 24 hours was not affected. The discrepancy between the acute 2-deoxyglucose uptake and 24-hour glucose consumption data may be explained by the substrate concentrations used in each of these two studies. Under experimental conditions where substrate availability is less than the saturation concentration for a transporter, such as the 100 μM 2-deoxyglucose used for glucose uptake experiments, alterations in the transporter Km (half-saturation concentration) may be observed. However, under

experimental conditions where substrate availability exceeds the saturation concentration for a transporter, such as the 25 mM D-glucose used in glucose consumption studies, facilitated diffusion of glucose across the membrane is at maximal capacity under basal conditions. In this case, alterations in GLUT1 Km, if applicable, would not be detected.

3.2.3 1,25$(OH)_2$D Reduces Pyruvate Carboxylase mRNA Expression

Our data indicate that 1,25$(OH)_2$D reduces the utilization of glucose as a substrate for *de novo* lipogenesis, without altering 24-hour glucose consumption. Previous studies from our laboratory demonstrate that 1,25$(OH)_2$D reduces glycolysis and glucose flux into the TCA cycle in Harvey-*ras* oncogene transfected MCF10A cells, a model of early breast cancer progression, via negative regulation of the pyruvate carboxylase (PC) gene (unpublished data). In these transformed breast epithelial cells, the mRNA expression of PC was reduced by 24% as early as 24 hours, and protein expression was reduced by 25% following 48 hours of 1,25$(OH)_2$D stimulation. Mutation analysis of the PC promoter suggested the presence and functionality of a negative VDRE, suggesting that 1,25$(OH)_2$D regulates glucose metabolism via regulation of the PC gene. These data, along with the those obtained from our current studies, raised the question of whether 1,25$(OH)_2$D regulates pyruvate carboxylase metabolism, and thus lipid accumulation, in 3T3-L1 adipocytes.

It has previously been demonstrated that perturbed pyruvate metabolism impacts triacylglycerol accumulation in 3T3-L1 adipocytes (244). In these studies,

chemical inhibition of pyruvate carboxylase by 5 and 10 mM phenylacetate reduced TAG accumulation by 21 and 34%, respectively, after 8 days. Consistent with inhibition of glycolysis, release of glycerol and lactate were significantly increased by PC inhibition. Flux estimates indicated that reduced *de novo* fatty acid synthesis contributed to the reduction in TAG accumulation. Cytosolic acetyl-CoA, derived from cleavage of citrate that is exported from the mitochondria, is required for the synthesis of fatty acids. When *de novo* fatty acid synthesis is occurring, the mitochondrial citrate pool becomes drained. The anaplerotic function of PC is to supply oxaloacetate to the mitochondria TCA cycle intermediate pool. Therefore, inhibition of PC reduces the amount of substrate availability for the synthesis of fatty acids. These studies support the critical role of PC in regulating TAG accumulation.

In our studies, 1,25$(OH)_2$D reduces glucose utilization as a substrate for fatty acid synthesis. Additionally, 1,25$(OH)_2$D stimulates a 41% reduction in pyruvate carboxylase mRNA expression in 3T3-L1 adipocytes after 2 days ($p<0.01$) (Figure 3.15). It has been demonstrated that PC enzymatic activity directly correlates with mRNA expression (245). We therefore hypothesized that 1,25$(OH)_2$D reduces glucose incorporation into fatty acids by reducing flux into the TCA cycle via inhibition of PC enzymatic activity.

To test this hypothesis, the human PC gene was overexpressed in differentiated 3T3-L1 adipocytes, and glucose incorporation into fatty acids was assessed. A 6.6-fold increase in PC mRNA abundance was achieved with transfection of the PC plasmid (Figure 3.16). As expected, overexpression of PC

significantly increased glucose incorporation into the cellular pool of palmitic, palmitoleic, stearic, and oleic acids (Figure 3.17), while reducing secretion of glycerol (Figure 3.18A) and lactate (Figure 3.18B). If $1,25(OH)_2D$ reduces glucose incorporation into fatty acids by reducing PC enzymatic activity, there would be no effect of $1,25(OH)_2D$ in PC-overexpressing cells. However, in cells overexpressing PC, $1,25(OH)_2D$ significantly reduced glucose incorporation into fatty acids, to levels similar to those in control $1,25(OH)_2D$-treated cells (Figure 3.17). Moreover, in PC-overexpressing cells, $1,25(OH)_2D$ significantly increased glycerol (Figure 3.18A) and lactate (Figure 3.18B) secretion, to levels similar of those observed in control cells. Collectively, these data suggest that $1,25(OH)_2D$ does not regulate glucose metabolism and subsequent triacylglycerol accumulation by altering PC enzymatic activity.

3.2.4 Inhibition of Glycolysis Blunts $1,25(OH)_2D$-Stimulation of Glycerol Release

Previous studies have demonstrated that in the presence of high glucose concentrations, adipocytes dispose of glucose as lactate and glycerol, potentially as a defense mechanism against excess energy substrate availability (95, 96). To determine whether $1,25(OH)_2D$ induces glycerol release by stimulating hydrolysis of TAG or by stimulating synthesis of glycerol through glycolysis/glyceroneogenesis, 3T3-L1 adipocytes were stimulated with $1,25(OH)_2D$ in the presence or absence of 2-deoxyglucose (2-DG, 20 mM), an inhibitor of glycolysis (246, 247). By inhibiting glycolysis, the substrate availability for synthesis of glycerol via glycolysis and glyceroneogenesis becomes limited.

Observation of the impact of 1,25$(OH)_2$D under these conditions will help elucidate whether 1,25$(OH)_2$D stimulates hydrolysis of TAG, or synthesis of glycerol via glycolysis/glyceroneogenesis.

In vehicle-treated cells, 2-DG stimulated a 3.4-fold increase in glycerol release within 24 hours ($p<0.01$) (Figure 3.19). This glycerol is likely derived from TAG hydrolysis in response to limited ATP availability from glucose. If 1,25$(OH)_2$D stimulates glycerol release by stimulating hydrolysis of TAG, an additive effect of 1,25$(OH)_2$D on glycerol release would be observed. However, 1,25$(OH)_2$D has no further effect on 2-DG-stimulatd glycerol release after 24 hours, and similar patterns of glycerol release were observed following 2-4 days of 2-DG and 1,25$(OH)_2$D treatment (Figure 3.19). Collectively, these data are consistent with 1,25$(OH)_2$D stimulation of glycerol synthesis via glycolysis and/or glyceroneogenesis, rather than stimulation of TAG hydrolysis.

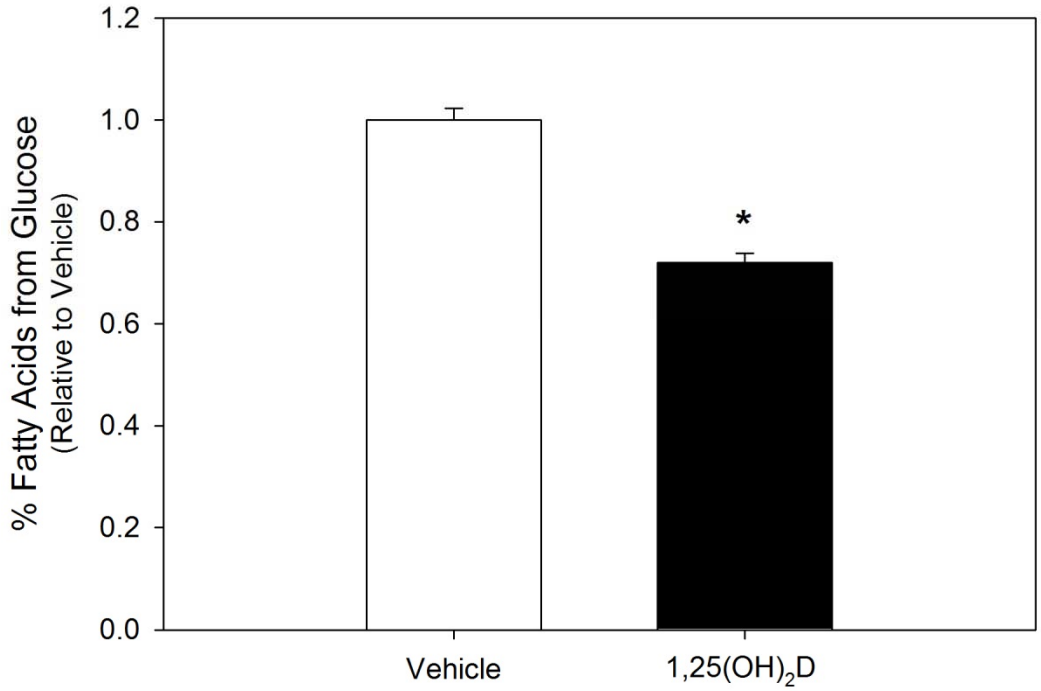


Figure 3.9. $1,25(OH)_2D$ reduces glucose incorporation into fatty acids. Differentiated 3T3-L1 adipocytes were stimulated with $1,25(OH)_2D$ (10 nM) or vehicle for 4 days. Cells were incubated with D-[U-^{13}C]glucose during the last 24 hours of treatment as described in Materials and Methods, and substrate incorporation into the cellular pool of palmitic, palmitoleic, stearic, and oleic acids was assessed using HPLC/ESI-MS/MS. Data are as the percent of labeled fatty acids containing ^{13}C, relative to vehicle. *P<0.05 compared to vehicle (n=6/group).

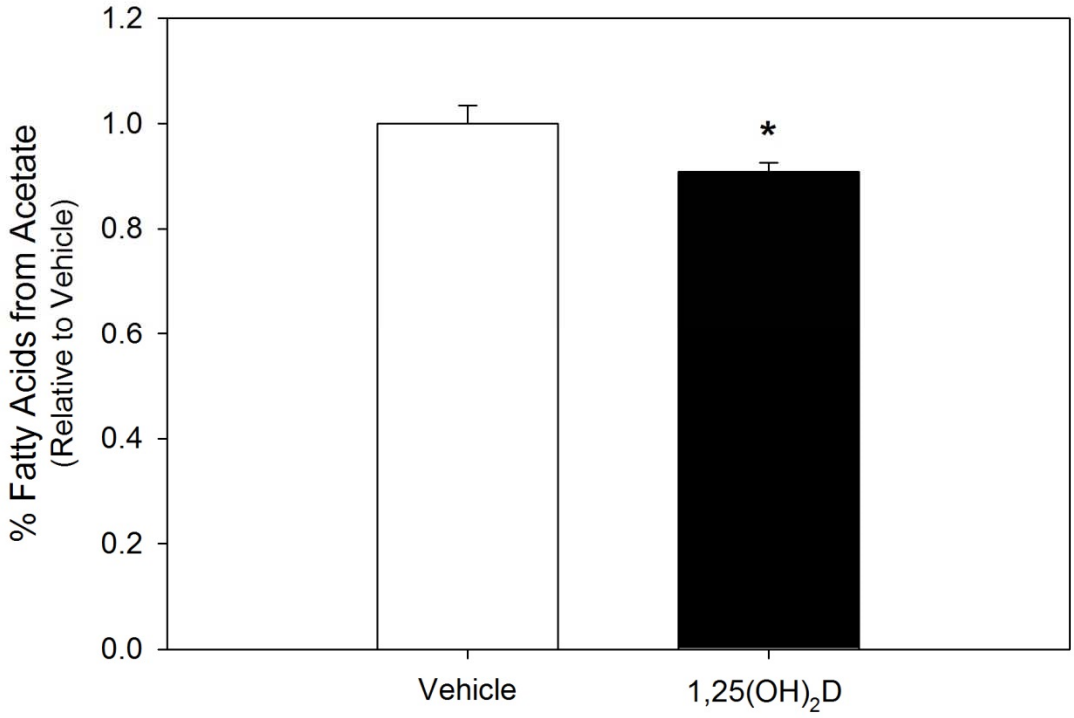


Figure 3.10. $1,25(OH)_2D$ reduces acetate incorporation into fatty acids. Differentiated 3T3-L1 adipocytes were stimulated with $1,25(OH)_2D$ (10 nM) or vehicle for 4 days. Cells were incubated with $[^{13}C_2]$acetate during the last 24 hours of treatment as described in Materials and Methods, and substrate incorporation into the cellular pool of palmitic, palmitoleic, stearic, and oleic acids was assessed using HPLC/ESI-MS/MS. Data are as the percent of labeled fatty acids containing ^{13}C, relative to vehicle. *P<0.05 compared to vehicle (n=6/group).

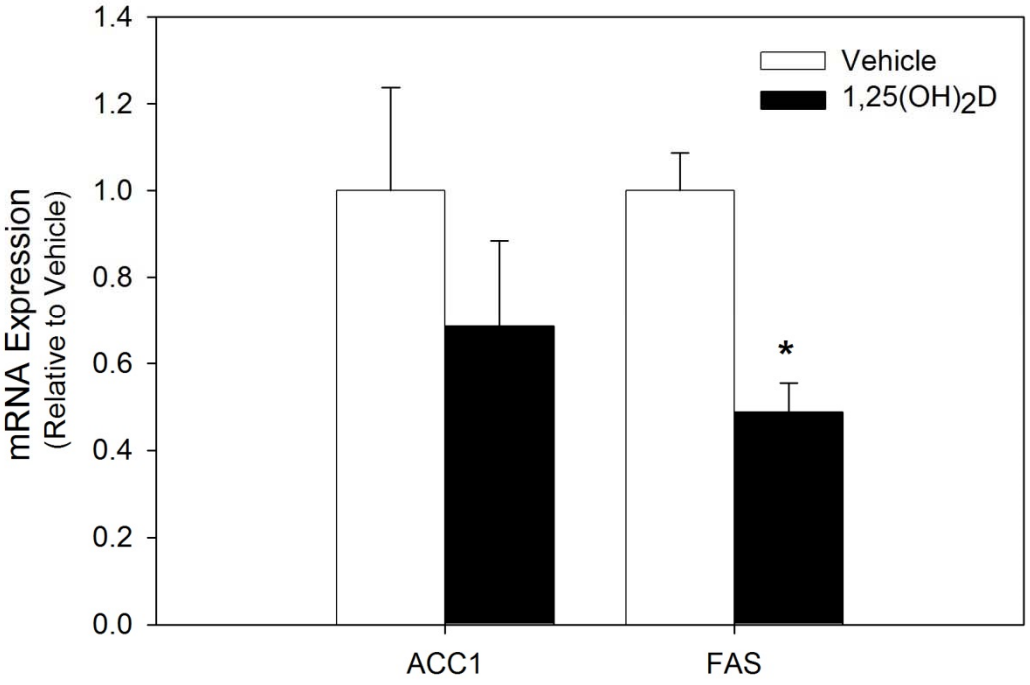


Figure 3.11. Effect of $1,25(OH)_2D$ on lipogenic gene expression. Differentiated 3T3-L1 adipocytes were treated with vehicle or $1,25(OH)_2D$ (10 nM) for 4 days. A) The mRNA expression of acetyl CoA carboxylase 1 (ACC1) is not significantly changed by $1,25(OH)_2D$. B) $1,25(OH)_2D$ significantly reduces fatty acid synthase (FAS) mRNA expression. *P<0.05 compared to vehicle (n=3/group).

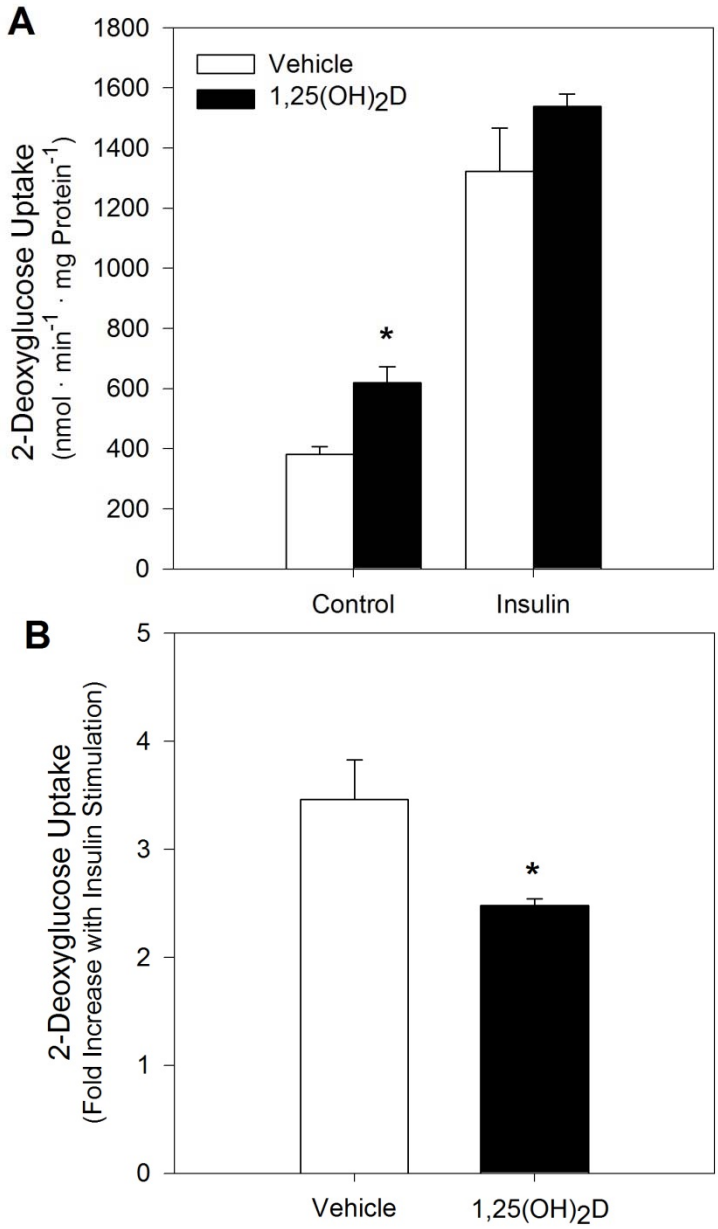


Figure 3.12. $1,25(OH)_2D$ stimulates 2-deoxyglucose uptake in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were treated with $1,25(OH)_2D$ (10 nM) or vehicle for 4 days. A) 2-deoxyglucose uptake was determined following a 20-minute incubation with 2-[1,2-^{3}H(N)]-deoxy-D-glucose (100 μM) as described in Materials and Methods. 2-deoxyglucose uptake is expressed as nmol•min^{-1}•mg protein^{-1}, and is normalized to total protein (n=4/group). B) $1,25(OH)_2D$ reduces insulin sensitivity in 3T3-L1 adipocytes. Data are expressed as fold increase in 2-deoxyglucose uptake with acute insulin stimulation, and are normalized to total protein (n=4/group). *P<0.05 compared to vehicle.

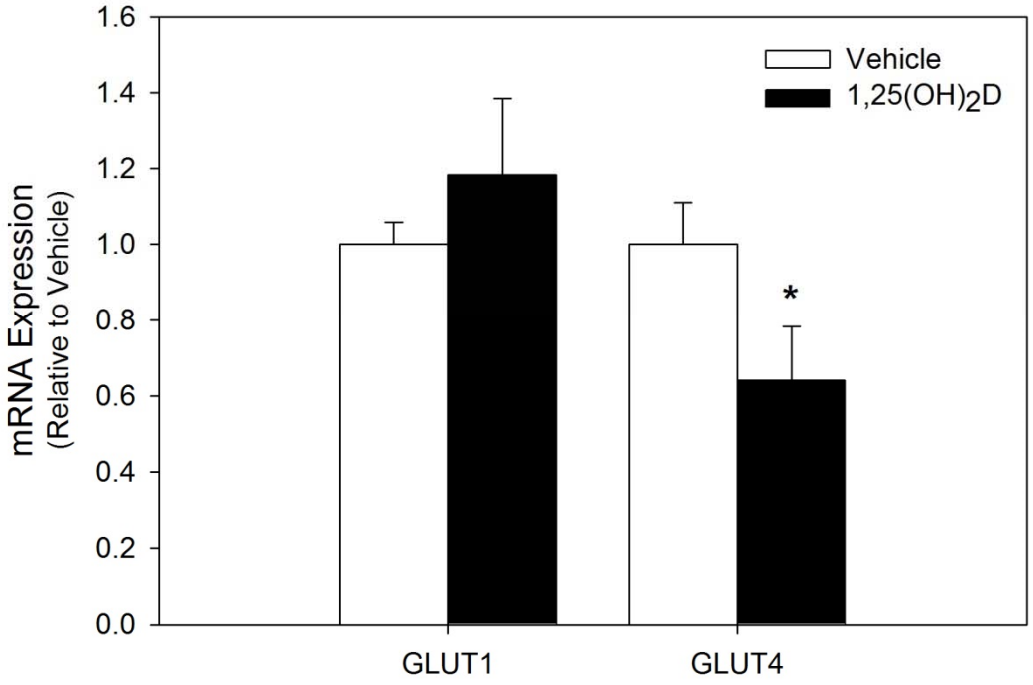


Figure 3.13. Effect of $1,25(OH)_2D$ on glucose transporter mRNA expression. Differentiated 3T3-L1 adipocytes were treated with vehicle or $1,25(OH)_2D$ (10 nM) for 4 days. GLUT1 and GLUT4 mRNA are expressed relative to vehicle. *P<0.05 compared to vehicle (n=3/group).

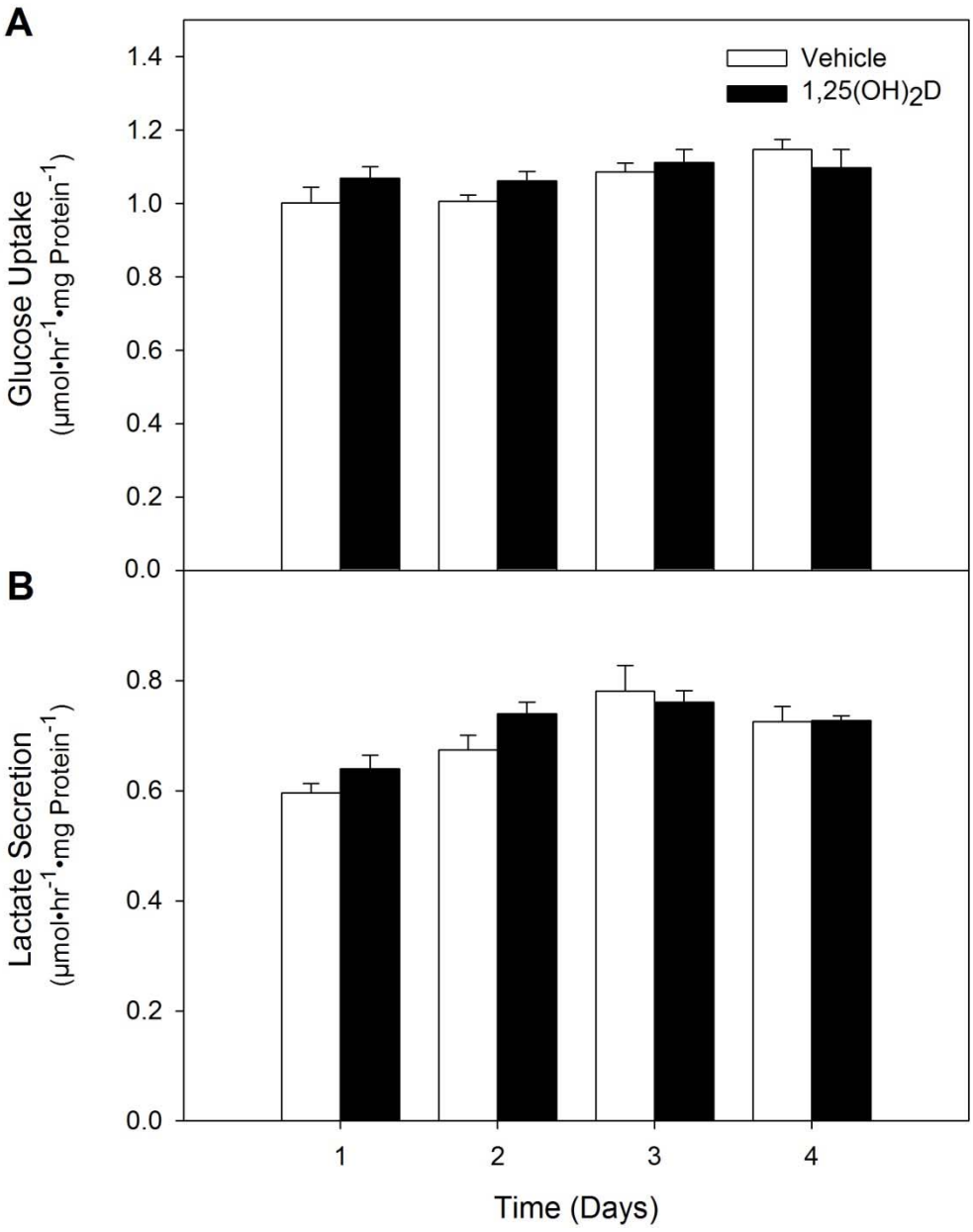


Figure 3.14. $1,25(OH)_2D$ does not affect glucose consumption or lactate secretion in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were treated with vehicle or $1,25(OH)_2D$ (10 nM) for 1-4 days as indicated. Glucose concentrations were assessed in spent media samples using a commercially available kit. Glucose consumption was calculated from the difference between glucose concentrations in spent and in fresh media samples. Data are expressed as $nmol \bullet min^{-1} \bullet mg\ protein^{-1}$, and are normalized to total protein. No effect of $1,25(OH)_2D$ is observed on glucose consumption at any of the time points tested (n=3/group).

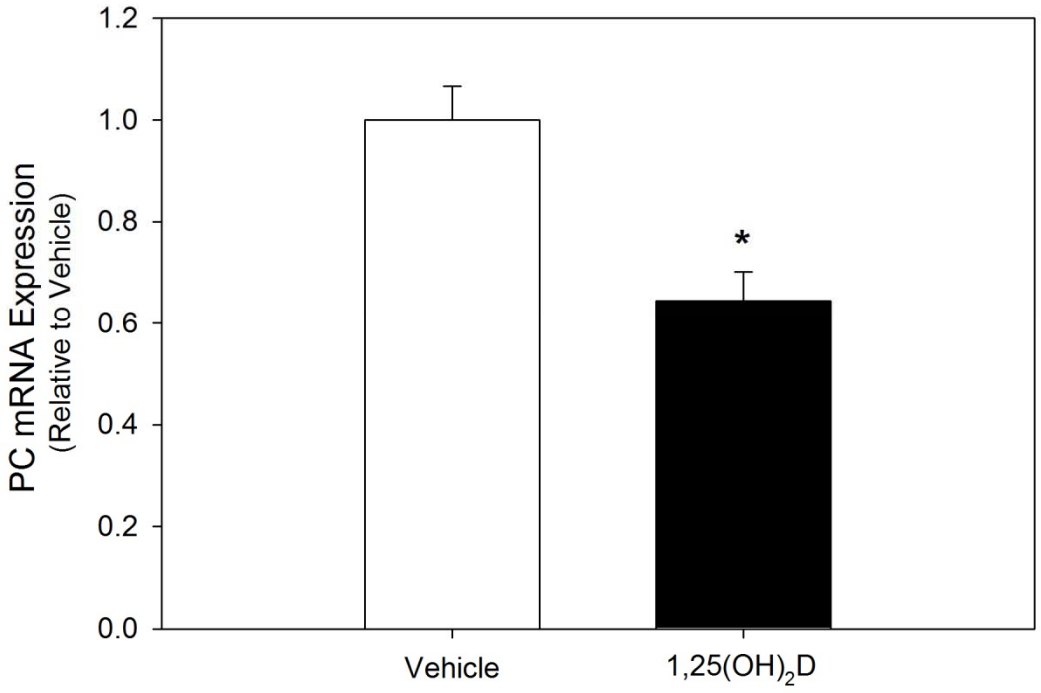


Figure 3.15. $1,25(OH)_2D$ reduces pyruvate carboxylase mRNA expression in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were treated with vehicle or $1,25(OH)_2D$ (10 nM) for 2 days. The mRNA expression of pyruvate carboxylase is expressed relative to vehicle. *P<0.05 compared to vehicle (n=3/group).

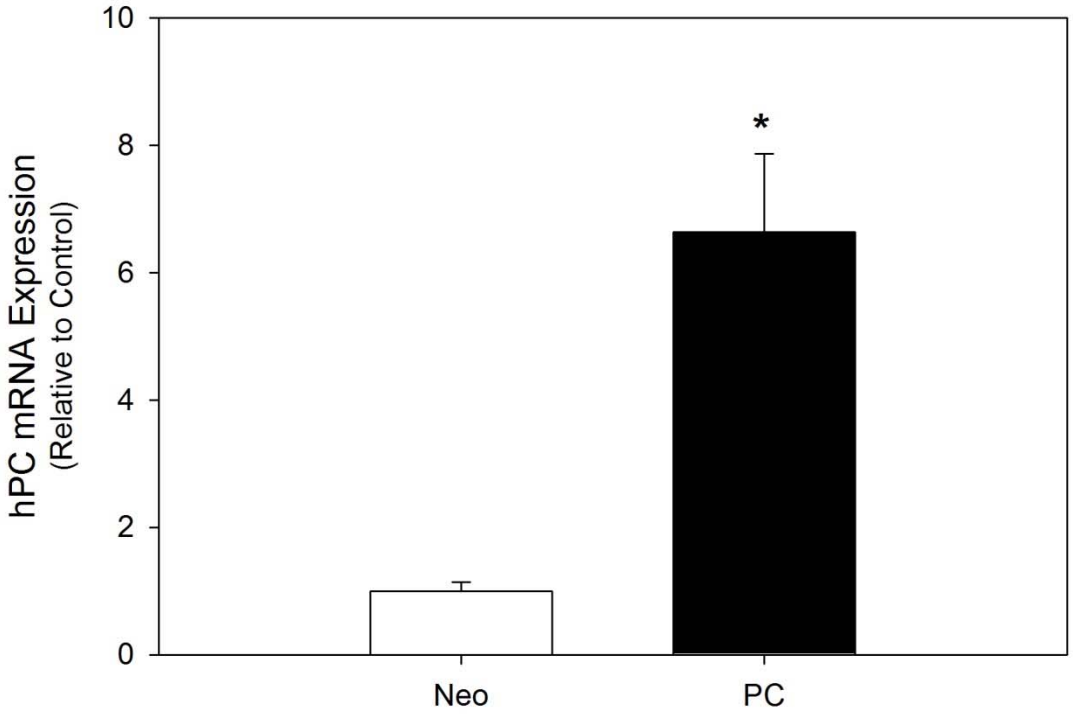


Figure 3.16. Human PC mRNA abundance with PC overexpression. Differentiated 3T3-L1 adipocytes were transfected with a plasmid containing the human PC gene as described in Materials and Methods. The mRNA expression of hPC is expressed relative to control cells. *P<0.05 compared to control (n=3/group).

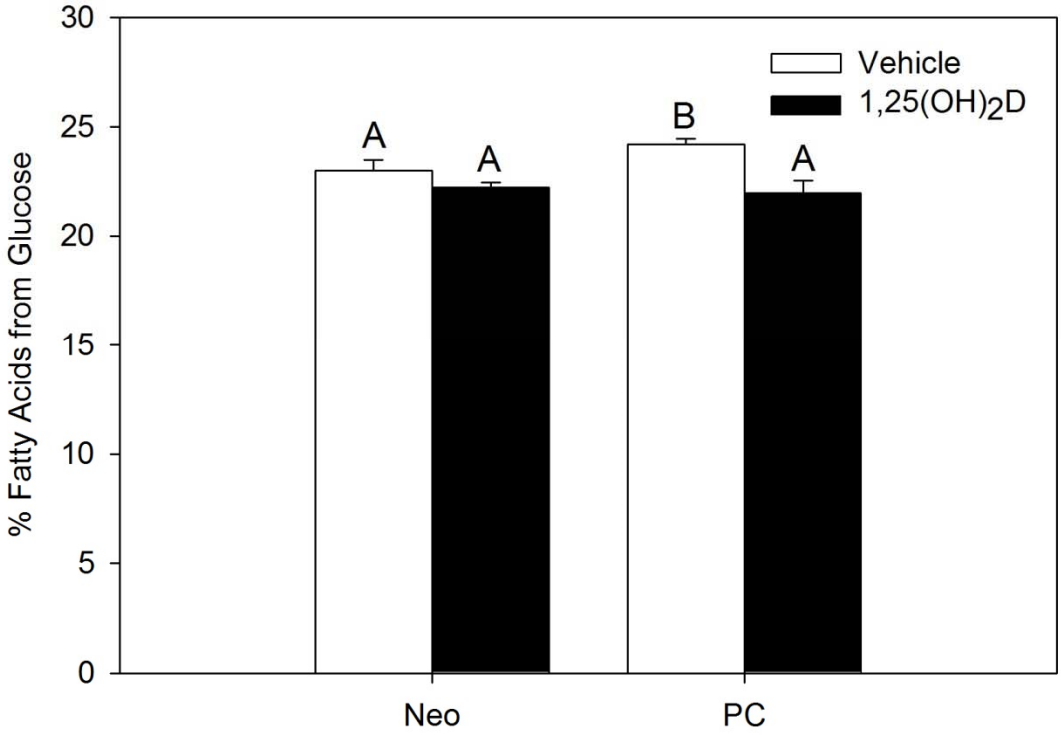


Figure 3.17. Overexpression of pyruvate carboxylase increases glucose incorporation into fatty acids. Differentiated 3T3-L1 adipocytes were transfected as described in Materials and Methods. 24 hours following transfection with the PC or control plasmid, cells were stimulated with 1,25$(OH)_2$D (10 nM) or vehicle for 4 days. Cells were incubated with D-[U-^{13}C]glucose during the last 48 hours of treatment as described in Materials and Methods, and substrate incorporation into the cellular pool of palmitic, palmitoleic, stearic, and oleic acids was assessed using HPLC/ESI-MS/MS. Data are as the percent of labeled fatty acids containing ^{13}C, relative to vehicle. *P<0.05 compared to vehicle (n=3/group).

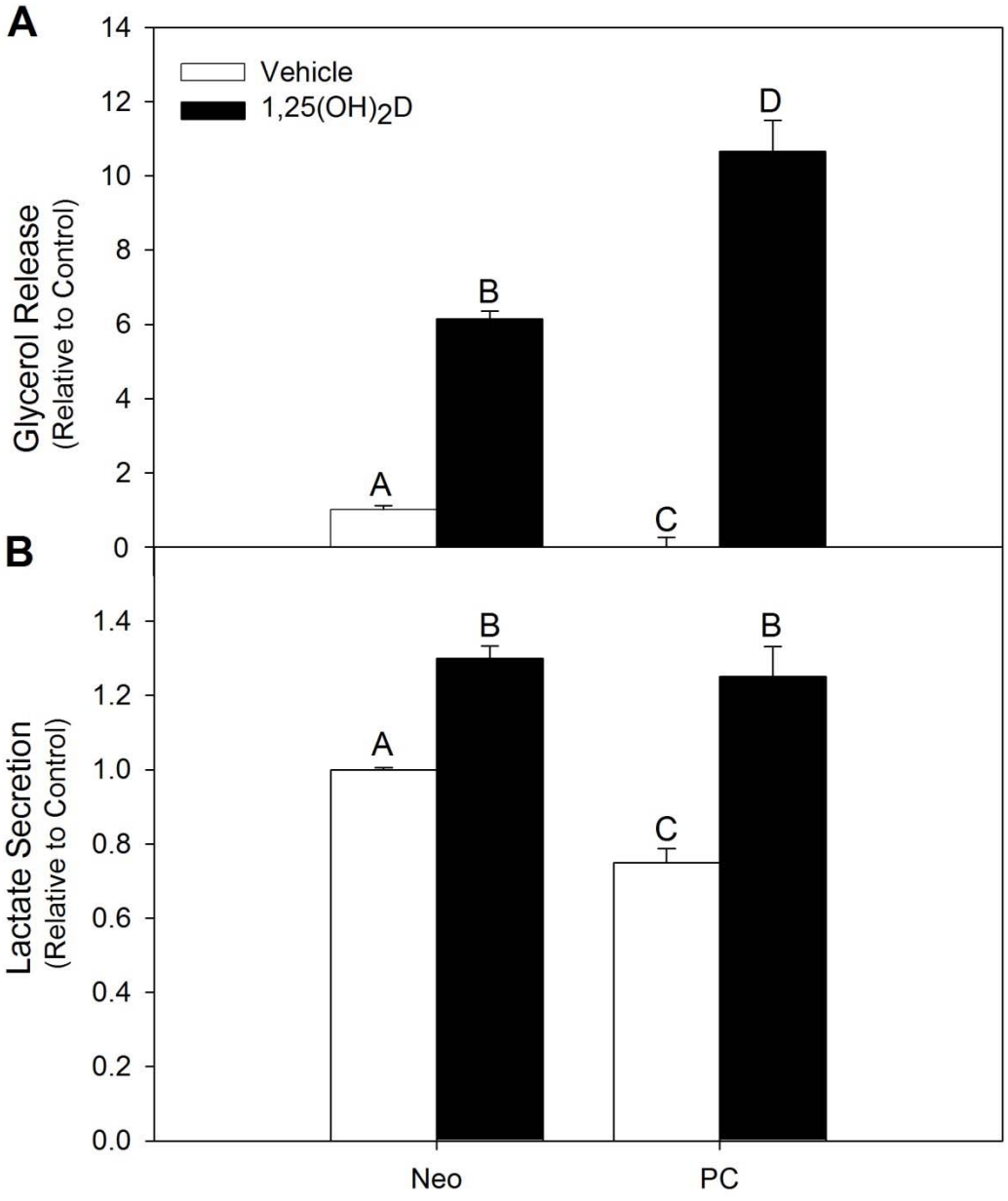


Figure 3.18. Overexpression of pyruvate carboxylase reduces glycerol and lactate secretion. Differentiated 3T3-L1 adipocytes were transfected as described in Materials and Methods. 24 hours following transfection with the PC or control plasmid, cells were stimulated with 1,25$(OH)_2$D (10 nM) or vehicle for 2 days. Glycerol (A) and lactate (B) release were assessed in spent media samples as described in Materials and Methods. Bars with different letters are significantly different (P<0.05) (n=3/group).

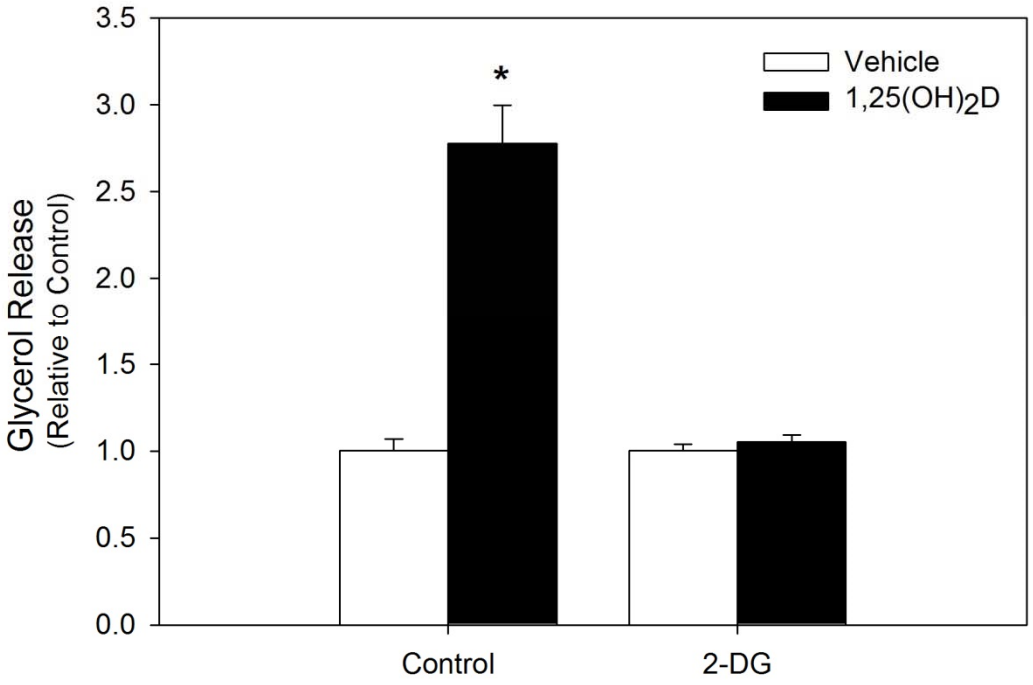


Figure 3.19. Inhibition of glycolysis blunts $1,25(OH)_2D$-stimulation of glycerol release in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were co-treated with $1,25(OH)_2D$ (10 nM) and 2-deoxyglucose (20 mM) for 2 days. Glycerol release was assessed in spent media samples by quantitative enzymatic determination of free glycerol using a commercially available kit. *P<0.05 compared to vehicle at the same time point (n=3/group).

CHAPTER 4. DISCUSSION

As the prevalence of obesity across age, ethnic, and socioeconomic groups increase, so does the need to identify safe and effective measures by which we can prevent and reverse the disease. Both hypertrophy (increase in cell size) and hyperplasia (increase in cell number) have been targeted as pathways that might be modulated in an attempt to ameliorate the progression of obesity and associated metabolic disorders. While both of these pathways contribute to the maintenance of white adipose tissue, a study by Spalding et al. demonstrate that changes in fat mass, even in cases of significant weight gain or loss, are due to changes in adipocyte size rather than alterations in adipose cell number (105). In this study, Spalding et al. observed an annual adipocyte turnover rate of 10%, independent of age and BMI, yielding an average adipocyte lifespan of approximately 10 years. These data highlight the significance that regulation of lipid accumulation in existing adipocytes plays in the modulation of adipose mass.

Observation of the inverse relationship between vitamin D status and measures of adiposity in humans has triggered a high level of interest in the relationship between vitamin D and obesity. Many studies have been conducted to determine the impact of vitamin D on adipogenesis, or differentiation of

adipocytes; however, there is little knowledge of the impact of vitamin D on lipid storage and metabolism in differentiated adipocytes. In the present studies, $1,25(OH)_2D$ stimulated a significant reduction in TAG accumulation in differentiated 3T3-L1 adipocytes. We demonstrate that mitochondrial β-oxidation is stimulated by $1,25(OH)_2D$ in these adipocytes, and the data suggest that $1,25(OH)_2D$ stimulates a reduction in the utilization of glucose for fatty acid synthesis, while increasing glucose utilization for the synthesis and secretion of glycerol. To our knowledge, these results are the first to demonstrate directly that $1,25(OH)_2D$ alters fatty acid synthesis and oxidation in differentiated adipocytes.

It is well-established that vitamin D is stored in adipose tissue, and as a result, many studies have been performed to determine the impact of vitamin D on adipogenesis, or hyperplasia. Studies utilizing the 3T3-L1 preadipocyte cell line found that $1,25(OH)_2D$ inhibits differentiation of these cells, and subsequent lipid accumulation, by downregulating differentiation “master regulator” PPARγ and C/EBPα (205-208). Downregulation of these genes inhibits the subsequent expression of genes necessary for lipid and glucose metabolism, and consequently, preservation of the preadipocyte phenotype. However, the impact of vitamin D on adipocyte differentiation remains controversial. While $1,25(OH)_2D$ inhibits the differentiation of murine 3T3-L1 adipocytes, $1,25(OH)_2D$ stimulates maturation and lipid accumulation during the adipogenic differentiation of porcine mesenchymal stem cells (212) and human subcutaneous preadipocytes (213). The discrepancy between these different cell lines, while not fully understood, may be attributed to the different roles that adipose tissue plays with regard to

energy balance in these species, or due to the stage of differentiation that isolated human subcutaneous adipocytes are in compared to the 3T3-L1 preadipocyte cell line.

Reports of vitamin D regulation of lipid metabolism and accumulation in differentiated adipocytes are limited. One study performed by Zoico et al. (248) examined the impact $1,25(OH)_2D$ on the lipopolysaccharide (LPS)- induced inflammatory response in 3T3-L1 adipocytes that had been differentiated for 5 days. In this study, stimulation with LPS (1.0 μg/mL) for 24 hours triggered a significant increase in the secretion of pro-inflammatory adipokines IL-6 and TNFα, with a concomitant increase in lipid accumulation, measured by Oil Red-O staining. However, when differentiated 3T3-L1 adipocytes were pre-treated with $1,25(OH)_2D$ (100 nM) for 24 hours, the LPS-induced increases in cytokine secretion and lipid accumulation were attenuated. These data suggest a role for vitamin D in the regulation of inflammation-mediated lipid metabolism; however, no impact of $1,25(OH)_2D$ was observed on lipid accumulation in the absence of LPS-induced inflammation.

In contrast to the null results described by Zoico et al. with regard to the impact of $1,25(OH)_2D$ on lipid accumulation in a non-inflammatory state, a recent study published by Chang and Kim documented a reduction in lipid accumulation in 3T3-L1 adipocytes with $1,25(OH)_2D$ stimulation (249). In these studies, 3T3-L1 adipocytes that had been differentiated for 6 or 7 days were stimulated for 24 hours with 1-100 nM $1,25(OH)_2D$ (249). An approximate 15% reduction in lipid accumulation, measured by Oil Red O staining, was observed following 24 hours

of 1 nM 1,25$(OH)_2$D stimulation, with similar reductions in lipid accumulation occurring with 10 and 100 nM 1,25$(OH)_2$D treatment. Consistent with the studies described in this dissertation, Chang and Kim observed a significant increase in basal glycerol release with 24 hours of 1,25$(OH)_2$D treatment, and the authors report 1,25$(OH)_2$D enhancement of isoproterenol-induced glycerol release. Further, the mRNA expression of lipogenic genes (C/EBPα, FAS, PPARγ, SCD-1) were significantly reduced by 1,25$(OH)_2$D, while lipid oxidizing genes (CPT-1, PGC-1α, PPARα, UCP-1) were significantly increased following 1,25$(OH)_2$D stimulation. These 1,25$(OH)_2$D-induced changes in lipid accumulation and lipid metabolizing gene expression occurred with a concomitant increase in sirtuin-1 (SIRT1) mRNA expression and activity. SIRT1 promotes fatty acid mobilization from adipose tissue (250), and these data suggest that 1,25$(OH)_2$D may reduce lipid accumulation in 3T3-L1 adipocytes by activating this metabolic pathway. However, 1,25$(OH)_2$D regulation of adipocyte lipid accumulation remains far from fully understood.

While the studies described by Chang and Kim provide some insight into the actions of 1,25$(OH)_2$D in adipocytes, the role of 1,25$(OH)_2$D in regulating adipose function and pathophysiology remain incompletely defined. The authors report an increase in NAD/NADH ratio and SIRT1 activity with 1,25$(OH)_2$D treatment (249). SIRT1 acts as a metabolic sensor, acting in coordination with AMPK to increase oxidative capacity in tissues such as skeletal muscle (251), and promote lipid mobilization from adipose tissue to support fatty acid oxidation in peripheral tissues (250). As AMPK is known to activate SIRT1 by increasing

the NAD/NADH ratio (252), it would have been interesting to know if 1,25$(OH)_2$D stimulates activation of AMPK in these cells as well. Further, given the extensive history of discrepancies between 1,25$(OH)_2$D action *in vitro* and the impact of dietary vitamin D on adipose tissue *in vivo* (215), it would have been valuable if functional outcomes such as fatty acid oxidation and fatty acid synthesis had been measured in these studies directly, rather than relying on the mRNA expression of genes related to FA metabolism alone to determine the impact of 1,25$(OH)_2$D on adipose tissue physiology.

As the available evidence regarding the impact of vitamin D on differentiated adipocytes remains limited, the collective aim of the present studies was to determine the impact of 1,25$(OH)_2$D on lipid metabolism and storage in 3T3-L1 adipocytes. In these studies, we observed a 21% reduction in TAG accumulation after 4 days of stimulation with 10 nM 1,25$(OH)_2$D (Figure 3.1). This reduction in TAG accumulation persisted for up to 7 days, and was achieved without negatively impacting cell viability (Figure 3.2). In addition to the reduction in TAG accumulation, we found that 1,25$(OH)_2$D stimulates glycerol release after 1-4 days, with a maximal increase in glycerol release following 48 hours of treatment (Figure 3.3). These data are consistent with the recently published study by Chang and Kim, who demonstrate a significant increase in glycerol release and reduction in lipid accumulation following 24 hours of 10 nM 1,25$(OH)_2$D treatment (249).

Typically, glycerol release is indicative of lipolysis. However, while several changes in the lipolysis signaling pathway were observed in response to

1,25$(OH)_2$D, collectively, the data obtained from the current studies do not suggest that 1,25$(OH)_2$D stimulates TAG hydrolysis. Consistent with TAG hydrolysis, 1,25$(OH)_2$D-induced glycerol release is abolished upon chemical inhibition of PKA (Figure 3.5A). Further, 1,25$(OH)_2$D stimulates increased phosphorylation of HSL at PKA phosphorylation site Ser660 (Figure 3.5B), and interestingly, an increase and decrease in ATGL and CGI-58 protein expression, respectively (Figure 3.5C). Previous studies have demonstrated that retroviral-mediated overexpression of ATGL increases basal glycerol and NEFA release in 3T3-L1 adipocytes, while siRNA-mediated knockdown of ATGL has the opposite effect (253). Given the increase in basal glycerol release by 1,25$(OH)_2$D, downregulation of ATGL protein expression is contrary to what might be expected. However, ATGL requires activation by CGI-58 to hydrolyze TAG, and it has been demonstrated that the molar ratio of CGI-58:ATGL is positively associated with ATGL hydrolase activity (85). Therefore, the increase in CGI-58 protein expression in concert with downregulation of ATGL protein expression results in an elevated CGI-58:ATGL molar ratio, and consequently, is consistent with elevated glycerol release.

While these changes in the lipolysis signaling pathway suggest that 1,25$(OH)_2$D may stimulate glycerol release by hydrolyzing TAG, not all data are consistent with this hypothesis. First, the reduction in TAG accumulation that is achieved following 4 days of 1,25$(OH)_2$D treatment (-0.514 µmols, based on an average TAG mw of 846.09 g/mol) does not quantitatively account for the increase in the number of moles of glycerol that are secreted following 1-4 days

of 1,25$(OH)_2$D stimulation (+29.68 µmols). Further, while glycerol release is typically indicative of lipolysis, as complete TAG hydrolysis yields NEFA and glycerol moieties in a 3:1 molar ratio, no increase in NEFA release was observed with 1,25$(OH)_2$D (data not shown). Finally, intracellular cAMP levels, which become elevated in response to lipolytic stimuli in order to activate PKA, were not changed following 1,25$(OH)_2$D treatment (Figure 3.6). These data suggest that 1,25$(OH)_2$D may stimulate stimulates glycerol synthesis from glycolytic or glyceroneogenic precursors, rather than hydrolysis of TAG. Previous studies have demonstrated that adipocytes dispose of glucose as glycerol, particularly in response to high glucose concentrations (95) such as the standard culture medium used in these studies.

To test the hypothesis that 1,25$(OH)_2$D stimulates synthesis of glycerol from glycolytic/glyceroneogenic precursors, adipocytes were co-treated with 1,25$(OH)_2$D and 2-deoxyglucose, a known inhibitor of glycolysis. Inhibition of glycolysis completely prevented 1,25$(OH)_2$D stimulation of glycerol release in 3T3-L1 adipocytes (Figure 3.19). Since lactate, the primary substrate for glyceroneogenesis in adipocytes, is produced from pyruvate obtained through glycolysis, this assay is not capable of distinguishing between glycerol produced from non-glucose precursors (glyceroneogenesis) or directly from glucose (glycolysis). However, these data do suggest that 1,25$(OH)_2$D stimulates synthesis of glycerol via one of these pathways, rather than hydrolysis of TAG. These data also highlight the possibility of altered glucose metabolism in

response to 1,25$(OH)_2$D, and the potential contribution of *de novo* lipogenesis to the 1,25$(OH)_2$D-induced reduction in TAG accumulation.

In the current studies, 1,25$(OH)_2$D reduced glucose incorporation into fatty acids by 30% compared to vehicle-treated cells (Figure 3.9), without altering the rate of glucose consumption (Figure 3.14A). To determine whether the reduction in glucose incorporation into fatty acids was a consequence of an impaired ability to synthesize fatty acids, in separate experiments, acetate incorporation into fatty acids was also measured. Acetate incorporation into fatty acids, a measure of fatty acid synthesis, was reduced by only 10% in response to 1,25$(OH)_2$D (Figure 3.10). These data suggest that while fatty acid synthesis is slightly inhibited in response to 1,25$(OH)_2$D, possibly via reduced enzymatic activity of fatty acid synthase and/or acetyl-CoA carboxylase, 1,25$(OH)_2$D stimulates a reduction in the utilization of glucose specifically for the synthesis of fatty acids. As glucose consumption (Figure 3.14A) and lactate secretion (Figure 3.14B) were not impacted following 1-4 days of treatment, the reduction of glucose incorporation into fatty acids is consistent with adipocytes disposing of excess glucose as glycerol. The possibility that 1,25$(OH)_2$D stimulates glucose disposal as glycerol as a consequence of reduced glucose flux into the TCA cycle was explored.

As 1,25$(OH)_2$D reduced the utilization of glucose for fatty acid synthesis, it is possible that 1,25$(OH)_2$D reduces flux of glucose into the TCA cycle. By entering into the TCA cycle, glucose-derived pyruvate serves as a source of ATP. Additionally, by replenishing TCA cycle intermediates, pyruvate supplies carbon substrate in the form of acetyl-CoA for fatty acid synthesis. Previous

studies by our laboratory demonstrate that 1,25$(OH)_2$D reduces pyruvate carboxylase expression and enzymatic activity in human breast epithelial cells, leading to reduced glycolysis and glucose flux into the TCA cycle (unpublished data). Consistent with these studies, results from the current studies demonstrate that 1,25$(OH)_2$D reduces PC mRNA expression in 3T3-L1 adipocytes (Figure 3.15). To determine whether 1,25$(OH)_2$D reduces glucose utilization as a substrate for fatty acid synthesis, and consequently TAG accumulation, by reducing pyruvate carboxylase activity, human PC (hPC) was overexpressed in differentiated 3T3-L1 adipocytes. Despite a 6.5-fold increase in PC mRNA expression, PC overexpression failed to inhibit 1,25$(OH)_2$D-induced changes in glycerol release (Figure 3.18A) and glucose incorporation into fatty acids (Figure 3.17), suggesting that PC does not mediate the 1,25$(OH)_2$D-induced changes in fatty acid synthesis and accumulation. Whether 1,25$(OH)_2$D stimulates reduced glucose flux into the TCA cycle remains to be determined. Collectively, the available data do suggest that 1,25$(OH)_2$D does stimulate glucose disposal as glycerol.

The impact of elevated glycerol release may have implications in hepatic gluconeogenesis and systemic glucose homeostasis. Glycerol and lactate are the major substrates for hepatic gluconeogenesis, and under fasted conditions, glycerol release from adipocytes by lipolysis is a major contributor to hepatic glucose synthesis (254). Previous studies have demonstrated that glycerol delivery to the liver can stimulate hepatic glucose production by a substrate-dependent push mechanism, independent of insulin signaling (255-257). Since

hepatic gluconeogenesis is a major contributor to the hyperglycemia that is observed in type 2 diabetes (258), this raises the concern about the impact of 1,25$(OH)_2$D-stimulated glycerol release in adipocytes *in vivo*. However, many cross-sectional studies have reported a positive relationship between vitamin D status and insulin sensitivity (259), as well as a positive impact of vitamin D on hepatic glucose and lipid metabolism (260). While overall the existing evidence does not support that vitamin D supplementation improves insulin sensitivity in an insulin-resistance state (187), the available evidence does suggest that it unlikely that vitamin D action in adipocytes disrupts global glucose homeostasis in humans.

In the current studies, FA oxidation was increased approximately 2.5-fold by 1,25$(OH)_2$D in 3T3-L1 adipocytes (Figure 3.8A). This is, to our knowledge, the first report of increased fatty acid oxidation in response to 1,25$(OH)_2$D in white adipocytes. While no reports of vitamin D stimulation of fatty acid oxidation in white adipocytes have been made, a few studies have documented evidence that vitamin D may play a role in fatty acid oxidation in other tissues, and contribute positively to the maintenance of energy homeostasis. A study by Marcotorchino *et al.* examined the effect of dietary vitamin D on high fat diet-induced weight gain and glucose homeostasis in C57BL/6 mice (261). In this study, despite no difference in energy intake between mice given a high fat diet with standard vitamin D (1,500 IU/kg food, HF) and those given a high fat diet with high vitamin D (15,000 IU/kg food, HFVD), HFVD mice gained significantly less body and adipose mass than HF mice after 10 weeks. The authors found that HFVD mice

had a significantly lower respiratory quotient, and increased energy expenditure and lipid oxidation compared to HF mice, reflecting the preferential use of lipids vs. carbohydrates as an energy source. Gene expression of enzymes related to fatty acid oxidation were elevated in BAT, liver, and skeletal muscle of HFVD compared to HF mice. While fatty acid oxidation was not examined in white adipose tissue specifically, these data do demonstrate the ability of vitamin D to increase global energy expenditure, and consequently, protect against excessive accumulation of adipose tissue. The data obtained by Marcotorchino *et al.* and those described from the current studies both provide mechanistic support for the inverse relationship between vitamin D and obesity.

In addition to the global impact of dietary vitamin D on fatty acid oxidation described above, vitamin D has been reported to increase FAO and reduce FA synthesis specifically in hepatocytes (262). In this study, male Sprague-Dawley rats were fed a control diet, or a high fat diet plus intraperitoneal injection of 1, 2.5, or 5 μg/kg $1,25(OH)_2D$. Administration of $1,25(OH)_2D$ ameliorated HF diet-induced body and liver weight gain, increased the hepatic mRNA expression of FAO-related genes (PPARα and CPT-1), and reduced the hepatic mRNA expression of genes involved in lipogenesis (SREBP-1c, ACC, FAS). These data suggest that $1,25(OH)_2D$ protects against hepatic steatosis by stimulating FAO, while inhibiting lipogenesis in the liver. Again, these data highlight the role of vitamin D in the regulation of energy expenditure and lipid homeostasis, and provide mechanistic support of the inverse relationship between vitamin D and obesity. However, it must be noted that the studies described by Marcotorchino

et al. (261) and Yin *et al.* (262) examined only the impact of vitamin D (dietary or intraperitoneal injection of 1,25$(OH)_2$D, respectively) on high fat diet-induced weight gain and metabolism, without including a control group to examine the impact of vitamin D on energy and lipid metabolism in the non-obese state. Such studies are essential in order to fully understand the impact of vitamin D on lipid oxidation and metabolism.

The studies described above demonstrate that vitamin D may impact lipid oxidation in oxidative tissues such as the liver, skeletal muscle, and BAT. However, very limited data exist regarding the impact of fatty acid oxidation in WAT. While β-oxidation is most well-studied in these oxidative tissues, oxidative capacity in adipocytes is reduced during obesity and in the diabetic state (263, 264), and targeting oxidative metabolism in WAT is an effective treatment measure in type 2 diabetes (265, 266). Consistent with our observation of increased β-oxidation with 1,25$(OH)_2$D stimulation, the recent report by Chang and Kim demonstrates that 1,25$(OH)_2$D stimulates the mRNA expression of several genes related to fatty acid oxidation in 3T3-L1 adipocytes, including CPT-1α, PGC1α, UCP-1, and PPARα, providing additional support that vitamin D may increases FAO in these cells (249).

This increase in fatty acid oxidation that was observed in the present studies is interesting, given the reduction in CPT-1 mRNA expression, and increase in ACC1 mRNA expression, in response to 1,25$(OH)_2$D (Figure 3.8B). Flux of the β-oxidation pathway is determined primarily by CPT-1 (267, 268) and by substrate supply (267, 269). When cellular energy stores are low, AMPK

phosphorylates ACC, thereby inactivating it (270). This phosphorylation event inhibits ACC-mediated production of malonyl-CoA, a potent CPT-1 inhibitor, allowing ATP-yielding processes such as β-oxidation to occur, while limiting ATP-consuming processes such as *de novo* lipogenesis. Conversely, during times of adequate cellular energy states, ACC1 remains in its active, unphosphorylated state, producing malonyl-CoA for the synthesis of fatty acids, and inhibiting the ATP-producing process of β-oxidation. As ACC1 is regulated primarily by phosphorylation rather than transcriptionally, its mRNA levels in the cell may not necessarily reflect its enzymatic activity. These findings are significant, given that many of the previously described studies of $1,25(OH)_2D$ action on adipocytes report only changes in the mRNA expression of lipogenic and oxidative enzymes, without examining enzyme functionality or activity. For example, the impact of $1,25(OH)_2D$ on CPT-1 expression described herein is in contrast to the findings described by Chang and Kim, who report a significant increase in CPT-1 mRNA expression in 3T3-L1 adipocytes in response to $1,25(OH)_2D$ (249). Nonetheless, the data from both the current studies and those described by Chang and Kim are consistent with $1,25(OH)_2D$ stimulation of fatty acid oxidation in differentiated 3T3-L1 adipocytes.

To determine if $1,25(OH)_2D$ reduces TAG accumulation in 3T3-L1 adipocytes by inhibiting the uptake of exogenous fatty acids, the impact of $1,25(OH)_2D$ on uptake of the fluorescent C_{16} BODIPY fatty acid was observed. Despite the observed reduction in TAG accumulation, uptake of the C_{16} BODIPY fatty acid was elevated in response to $1,25(OH)_2D$ (Figure 3.7). These data

suggest that 1,25$(OH)_2$D enhances the adipocyte's ability to take up exogenous fatty acids. However, under the experimental conditions used for these studies, the contribution of exogenous fatty acids to the intracellular TAG pool remains limited. In these experiments, exogenous fatty acids were not directly supplied during or after differentiation. Rather, the contribution of exogenous lipids to the intracellular TAG pool was limited to the amount of fatty acids that were present in the cell culture sera, supplied in the culturing media in a 10% (vol:vol) ratio. In an environment where exogenous fatty acids are more freely or frequently available, such as those found in circulation *in vivo* postprandially, the contribution of exogenous lipids to intracellular TAG pool may be significantly larger, and may impact the overall effect of 1,25$(OH)_2$D on TAG accumulation *in vivo*.

It is established that long-chain fatty acids (LCFA) stimulate FAO by acting as PPAR ligands, stimulating the transcription of lipid-oxidizing genes (271). As 1,25$(OH)_2$D stimulates uptake of BODIPY FL_{16} in 3T3-L1 adipocytes, the possibility that FAO is increased indirectly by 1,25$(OH)_2$D, as a result of increased uptake of 1-^{14}C-palmitate, was raised. To address this question, uptake of 1-^{14}C-palmitate was determined under conditions used for the FAO assay. Under these conditions, uptake of 1-^{14}C-palmitate is not different between vehicle- and 1,25$(OH)_2$D-treated cells (Figure 3.8C). The discrepancy between uptake of BODIPY fatty acid and 1-^{14}C-palmitate may be due to the difference in substrate concentration used in each of these assays. As fatty acid uptake occurs via both diffusion and protein-mediated uptake (71), it is likely that at low

substrate concentrations, such as those used for the BODIPY FA uptake assay, differences in protein mediated uptake may be observed. Conversely, under conditions of high substrate availability, protein-mediated and passive diffusion of fatty acids across the cell membrane are maximized, and differences in protein-mediated uptake may not be detected. Since no difference in 1-^{14}C-palmitate uptake was observed between vehicle- and 1,25$(OH)_2$D-treated cells, it is unlikely that indirect stimulation of FAO by increased FA uptake is the mechanism by which 1,25$(OH)_2$D stimulates FAO in these cells.

The data from these studies demonstrate that 1,25$(OH)_2$D acts in differentiated adipocytes to regulate lipid and glucose metabolism, leading to reduced TAG accumulation. While the impact of vitamin D supplementation on weight loss remains controversial (272), these data provide mechanistic support for the inverse relationship between vitamin D status and measures of adiposity. However, studies to determine the clinical significance of these findings are needed. Many *in vitro* studies have been performed to determine the impact of vitamin D on adipose tissue physiology, specifically, on adipocyte differentiation, but the impact of 1,25$(OH)_2$D on adipogenesis in humans is still unclear. 1,25$(OH)_2$D inhibits adipogenesis in murine 3T3-L1 preadipocytes, while promoting differentiation in human preadipocytes and primary mouse adipocytes at an advanced stage of differentiation (272). These data suggest that the impact of 1,25$(OH)_2$D on adipogenesis is dependent on stage of differentiation. On the other hand, while limited in quantity and restricted to the 3T3-L1 cell line, the available evidence regarding 1,25$(OH)_2$D regulation of already differentiated

adipocytes consistently demonstrate that 1,25$(OH)_2$D reduces lipid accumulation in these cells, at least in part by regulating metabolic pathways that are independent of the signaling pathways that control early adipogenesis. Whether 1,25$(OH)_2$D has the same impact on differentiated human adipocytes remains to be determined.

That 1,25$(OH)_2$D modulates adipocyte metabolism and lipid storage independently from its effects on the adipogenic program highlights the potential for vitamin D to impact adipose tissue physiology through its effects on existing adipocytes, in addition to its potential effects on differentiation of preadipocytes and mesenchymal stem cells. This may have therapeutic potential, as significant changes in adipose mass are mainly due to alterations in cell size rather than cell number (105). It has been demonstrated that adipocyte size is positively correlated with serum insulin and leptin concentrations, insulin resistance, and risk of developing type 2 diabetes (273-281). Obese individuals with few, large adipocytes are more glucose intolerant and hyperinsulinemic than those having the same degree of adiposity but in many small adipocytes (276-278, 282). Further, large adipocyte size may impair adipose function by promoting local inflammation and altered adipokine secretion (283-287). These data suggest that for a given state of adiposity, a higher number of smaller adipocytes is more metabolically favorable than fewer, larger adipocytes.

Given that it is more metabolically favorable to distribute adipose mass over a larger number of smaller adipocytes, agents such as 1,25$(OH)_2$D that reduce adipocyte size by reducing accumulation in existing (differentiated)

adipocytes, or possibly by distributing the existing fat mass over a larger number of adipocytes through stimulation of adipogenesis, may be beneficial in preserving metabolic function. In support of this, a study by Caron-John et al. demonstrated that in women undergoing abdominal gynecological surgery, intake of vitamin D was inversely correlated with omental adipocyte size, independent of calcium intake, BMI, total body fat mass, season, and physical activity.(288). In this study, Serum 25(OH)D was also negatively correlated with omental adipocyte size, and with total fat mass. Dietary intakes of vitamin D, calcium, and dairy products were assessed using a food frequency questionnaire, and mean adipocyte size was calculated by measuring the diameter of 250 adipocytes per patient. These data provide support for vitamin D regulation of adipose tissue physiology in humans, and highlight a potential mechanism by which dietary vitamin D may protect against obesity-associated metabolic disorders.

CHAPTER 5. CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Conclusions

In conclusion, $1,25(OH)_2D$ impacts several processes related to fatty acid and glucose metabolism in differentiated 3T3-L1 adipocytes, leading to reduced TAG accumulation. $1,25(OH)_2D$ stimulates glycerol release, likely due to stimulation of glycerol synthesis from glucose-derived substrates (e.g. during glycolysis, or from lactate or pyruvate). Fatty acid uptake and oxidation are stimulated by $1,25(OH)_2D$, while glucose utilization as a substrate for fatty acid synthesis is reduced. Collectively, these changes in adipocyte metabolism may contribute to the reduction in TAG accumulation that is stimulated by $1,25(OH)_2D$. The results of this study may aid in the identification of metabolic pathways targeted by $1,25(OH)_2D$, and in the development of *in vivo* studies designed to examine the impact of dietary vitamin D on obesity and related metabolic disorders.

5.2 Future Directions

While the studies described herein improve our understanding of vitamin D regulation of adipose tissue metabolism, several questions remain regarding vitamin D impact on adipose tissue metabolism and function.

First, the studies described in this dissertation utilized the murine 3T3-L1 preadipocyte cell line to examine the impact of $1,25(OH)_2D$ on glucose and lipid metabolism and TAG accumulation in adipocytes. Given the discrepancies in $1,25(OH)_2D$ action on preadipocyte differentiation between mouse and human preadipocytes, it will be essential to examine the impact of $1,25(OH)_2D$ on energy substrate metabolism and TAG accumulation in differentiated human adipocytes. The discrepancy in $1,25(OH)_2D$ action between mouse and human preadipocytes may be due to differing actions of $1,25(OH)_2D$ on the adipogenic transcriptional cascade, and may be dependent on the stage of differentiation that the cell is in. Therefore, the impact of $1,25(OH)_2D$ on human adipocyte metabolism after the differentiation program is complete will need to be studied directly. This knowledge is key in understanding the vitamin D regulation of adipose tissue in humans.

Second, it will be useful to determine the minimum concentration of $1,25(OH)_2D$ necessary to elicit changes in glucose and lipid metabolism, and in TAG accumulation in adipocytes. The concentration of $1,25(OH)_2D$ used in the current studies (10 nM) was chosen in order to ensure the ability to capture the cellular response to $1,25(OH)_2D$ stimulation. While adipose tissue concentrations of $1,25(OH)_2D$ and rate of local adipose production of $1,25(OH)_2D$ are not known, serum $1,25(OH)_2D$ concentrations are generally in the picomolar range. Chang and Lee demonstrated that $1,25(OH)_2D$ reduces neutral lipid accumulation in concentrations as low as 1 nM (249), suggesting that the $1,25(OH)_2D$-induced

changes in glucose and lipid metabolism may also occur at lower 1,25$(OH)_2$D concentrations.

Finally, the impact of dietary vitamin D on adipocyte metabolism and substrate handling must be examined *in vivo*. A study designed to examine: 1) whether dietary vitamin D may elicit changes in adipose tissue metabolism and total fat mass that are consistent with the current *in vitro* studies (e.g. elevated glycerol release and fatty acid oxidation in adipose tissue); and 2) the impact of altered adipose metabolism on systemic energy balance, lipid profile, and glucose homeostasis. It is possible that chronically elevated glycerol supplied to the liver by adipose tissue would stimulate gluconeogenesis leading to elevated fasting blood glucose. However, this scenario is unlikely, given the positive association between vitamin D status and insulin sensitivity (259), and the favorable impact of high dietary vitamin D on liver glucose and lipid metabolism (260). While several questions remain to be answered, the studies described in this dissertation provide information that is critical to improve our understanding of the actions of 1,25$(OH)_2$D on adipose tissue metabolism and function, and that provides mechanistic support for the inverse relationship between vitamin D status and obesity.

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VITA

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PUBLICATIONS

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Camarillo, I., Clah, L., Zheng, W., Zhou, X., **Larrick, B**., Blaize, N., Breslin, E., Patel, N., Johnson, D., Teegarden, D., Donkin, SS., Gavin, T., Newcomer, S. Maternal exercise during pregnancy reduces risk of mammary tumorigenesis in rat offspring. European Journal of Cancer Prevention. 2014; 23: 502-505.

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