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I am submitting herewith a thesis written by Kimberly Causey entitled "Effects of 1,25-Dihydroxyvitamin $D_3$ on Adipocyte Differentiation." I have examined the final paper copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.

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# EFFECTS OF 1,25-DIHYDROXYVITAMIN $D_3$ ON ADIPOCYTE DIFFERENTIATION

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## DEDICATION

This thesis is dedicated to my parents, Mr. Thomas Causey and Mrs. Ruth Causey as well as my brother, Mr. Chris Causey. I would also like to dedicate this thesis to my grandmother, Mrs. Maxie Ray, and my aunt, Ms Sue Ray. Their loving support and encouragement enabled me to complete this thesis. I want to thank my parents for their guidance and the principles they have instilled in my life. They taught me that with determination, you can achieve whatever you may dream.

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## ABSTRACT

Several recent reports from this laboratory demonstrate a regulatory role for intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$) in modulating lipid metabolism in both human and murine adipocytes, with increased $[Ca^{2+}]_i$ coordinately stimulating lipogenesis and inhibiting lipolysis, thereby expanding lipid mass. Further, we have recently demonstrated that 1,25-dihydroxyvitamin-$D_3$ [1,25-$(OH)_2$-$D_3$] stimulates adipocyte membrane vitamin D receptor (mVDR)-mediated rapid $Ca^{2+}$ influx into adipocytes, resulting in the stimulation of lipogenesis and inhibition of lipolysis. However, increasing $[Ca^{2+}]_i$ in the early stages of differentiation inhibits human adipocyte differentiation, whereas increasing $[Ca^{2+}]_i$ in the late stage promotes human adipocyte differentiation Accordingly, we have investigated the role of 1,25-$(OH)_2$-$D_3$ in the differentiation of 3T3-L1 preadipocytes, using triglyceride (TG) accumulation and glycerol-3-phosphate dehydrogenase (GPDH) as markers. 3T3-L1 preadipocytes were placed in differentiation media upon confluence, and exposed to varying amounts (0, 1nM, and 10nM) of 1,25-$(OH)_2$-$D_3$ for either one-hour pulses or for sustained (24 hrs or 48 hrs) amounts of time throughout the differentiation process Exposure to one-hour pulses of 1nM 1,25-$(OH)_2$-$D_3$ throughout differentiation caused modest decreases (31%-38%) in TG accumulation ($p<0.0001$), with one-hour pulse exposure to10nM 1,25-$(OH)_2$-$D_3$ having little to no effect on TG accumulation. One-hour pulse exposure to both 1nM and 10nM 1,25-$(OH)_2$-$D_3$ suppressed GPDH activity early, but not late in differentiation. Sustained (24-hour) exposure to 1,25-$(OH)_2$-$D_3$ (1nM and 10nM) inhibited differentiation at 0-24 hrs, with decreases in both TG and GPDH of 41-81% ($p<0.0001$). Similarly, sustained exposure

of 1,25-$(OH)_2$-$D_3$ resulted in marked inhibition of GPDH activity and TG accumulation early in differentiation. In contrast, sustained exposure late in differentiation exerted no significant effects on either marker of differentiation PPAR-γ and pref-1 expression were also used as markers of differentiation. One-hour pulses of 10nM 1,25-$(OH)_2$-$D_3$ did not cause any changes in PPAR-γ expression compared to control. Sustained exposure to 1,25-$(OH)_2$-$D_3$ throughout differentiation decreased PPAR-γ expression, with a 92% decrease from 0-48h ($p<0.0001$). One-hour pulses of 1,25-$(OH)_2$-$D_3$ had no effect on Pref-1 expression, with the exception of an increase in expression at 47-48 hr ($p<0.0001$). Sustained exposure to 10nM 1,25-$(OH)_2$-$D_3$ at 0-48 hrs, 24-48 hrs and 47-48 hrs all caused significant increases (125%-146%) in the expression of Pref-1 ($p<0.001$). Thus, although 1,25-$(OH)_2$-$D_3$ stimulates lipogenesis, inhibits lipolysis and increases TG accumulation in mature human and murine adipocytes, it also modestly inhibits the differentiation of preadipocytes into mature adipocytes.

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# PART 1

# INTRODUCTION

# INTRODUCTION

Intracellular calcium ($[Ca^{2+}]_i$) plays a key role in the metabolic modifications associated with obesity (1). Studies involving the mechanisms of *agouti* gene-induced obesity found that $[Ca^{2+}]_i$ modulates *de novo* lipogenesis in both human and murine adipocytes. Previous studies show that an increase in $[Ca^{2+}]_i$ via receptor or voltage-mediated calcium channels stimulates the expression and activity of fatty acid synthase (FAS), a key enzyme in *de novo* lipogenesis, thereby causing an increase in lipogenesis and a decrease in lipolysis The resulting triglyceride accumulation leads to adipocyte hypertrophy (2).

$[Ca^{2+}]_i$ also appears to play a role in the regulation of adipogenesis, a contributing factor to both human and murine obesity (3). In order to investigate the specific role of calcium, both thapsigargin, a $Ca^{2+}$-ATPase inhibitor, and A23187, a calcium ionophore, were used as calcium agonists to stimulate an increase in $[Ca^{2+}]_i$ in human preadipocytes. Exposure early in differentiation suppressed triglyceride accumulation greatly. However, increasing $[Ca^{2+}]_i$ late in differentiation increased triglyceride accumulation. Similar results were also seen using KCl and agouti protein as $[Ca^{2+}]_i$ agonists (2, 3). Therefore, increasing $[Ca^{2+}]_i$ has a biphasic effect on human adipocyte differentiation, with an increase early inhibiting differentiation and an increase in the late stages stimulating adipocyte differentiation and lipid filling (3).

Recent studies have introduced a paradox by showing that a decrease in the expression of genes normally involved in adipogenesis is associated with obesity and diabetes mellitus. White adipose tissue from obese mice exhibited decreased expression

of genes important in adipocyte differentiation, as compared to the lean controls. Sterol responsive element binding protein (SREBP), responsible for positively regulating multiple genes coding for lipogenic enzymes, demonstrated a 2.7-fold decrease in expression. Several mRNAs that encode proteins involved in lipid metabolism were decreased, including glycerol 3-phosphate dehydrogenase and stearoyl CoA desaturase. A dedifferentiation was observed in 3T3-L1 adipocytes including a suppression of PPARγ and C/EBPα, as well as other lipogenic enzymes in response to tumor necrosis factor α and transforming growth factor β (4).

1,25-dihydroxyvitamin $D_3$ [1,25-$(OH)_2$-$D_3$] acts as a calcium agonist (5) Further, we have recently demonstrated that 1,25-$(OH)_2$-$D_3$ stimulates adipocyte membrane vitamin D receptor (mVDR)-mediated rapid $Ca^{2+}$ influx into adipocytes, resulting in the stimulation of lipogenesis and inhibition of lipolysis (5). This effect was mimicked by 1α,25-(OH)2-lumisterol$_3$, an agonist for the mVDR. These effects were not seen when the human adipocytes were pretreated with 1β,25-dihydroxyvitamin $D_3$, a specific antagonist for mVDR. 1,25-$(OH)_2$-$D_3$ was also shown to increase adipocyte fatty acid synthase (FAS), with 1α,25-$(OH)_2$-lumisterol$_3$ having a greater stimulatory effect on FAS activity. Pretreatment with 1β,25-dihydroxyvitamin $D_3$ also prevented the stimulation of FAS. 1,25-$(OH)_2$-$D_3$ and 1α,25-$(OH)_2$-lumisterol$_3$ also reduced adipocyte basal lipolysis, with 1β,25-dihydroxyvitamin $D_3$ preventing any inhibition of lipolysis (5) In addition, Norman et al. (6) demonstrated that 1α,25-dihydroxylumisterol$_3$ acts on the mVDR solely to generate non-genomic action in adipocytes, while 1,25-$(OH)_2$-$D_3$ may target both the mVDR and the nuclear vitamin D receptor (nVDR) to mediate genomic and non-genomic actions which may interact with each other in signal response and, thereby compromise

the modulation of lipid metabolism. Accordingly, we have investigated the role of 1,25-$(OH)_2$-$D_3$ in adipogenesis.

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# PART 2

# LITERATURE REVIEW

## I. Adipocyte Differentiation

### A. Models of adipocyte differentiation

While the developmental origins of adipocytes are poorly understood, it is known that the transformation of a fertilized egg to the determination and conversion of precursor cells into mature adipocytes occurs in a series of stages. Pluripotent fibroblasts (stem cells) differentiate into multipotential mesenchymal precursors which may become preadipocytes, chondroblasts, osteoblasts, or myoblasts (1, 2). In a human model, preadipocytes begin to differentiate into adipose tissue during late embryonic development, with the majority of the conversion taking place shortly after birth (3). The preadipocytes in rodent and murine models do not begin to differentiate into adipose tissue until after birth (4) Depending on energy needs, all species have the ability to differentiate preadipocytes throughout their lives.

#### 1. In vivo models of adipocyte differentiation

In vivo study of adipocyte differentiation is difficult due to the complexity of adipose tissue. Adipocytes account for only one third of fat tissue, with the remaining two thirds consisting of blood vessels, nerve tissue, fibroblasts, and preadipocytes in various stages of development (2). Comprehensive in vivo studies are confounded by the difficulty distinguishing preadipocytes from fibroblasts and the inability to coordinate preadipocytes at similar stages of development.

## 2. In vitro models of adipocyte differentiation

### a. Primary cultures of preadipocytes

Primary preadipocytes have been successfully obtained from multiple species including rats, mice, rabbits, pigs, and humans. Because the stage of differentiation and the lineage of preadipocyte cell lines have not been well established, primary cultures can be useful for validating results from preadipose cell lines. The use of primary culture has several advantages over preadipocyte cell lines. While cell lines are aneuploid, the diploid primary cells are a better representation of an in vivo situation. Another advantage is the ability of primary cultures to be derived from fat tissue obtained from various species at different postnatal stages of development and from multiple adipose depots Primary cultures can come from subcutaneous, epididymal, and perirenal fat stores (1). There are also several drawbacks of primary cultures. First, large amounts of adipose tissue are required due to the small amount of preadipocytes that make up the total fat tissue Second, it is difficult to isolate preadipocytes from other fibroblast-like cells that are found in adipose tissue. Finally, primary cultures have a limited life span in culture (2).

### b. Cell lines of preadipocytes

There are advantages and disadvantages to using a cell line to study preadipocyte differentiation. It can be advantageous to uses a cell line which is homogenous in cells that are all at the same stage of differentiation. This allows for detailed responses to treatments during differentiation. Another advantage is the ability of cell lines to provide a consistent source of preadipocytes because of their ability to be passaged indefinitely.

There is some variation in the differentiation requirements of each cell line depending on the developmental stage at which the cells were arrested and derived (2,5) A major disadvantage to using a cell line is the lack of multiple factors that are seen in whole tissue in vivo. Cell lines are unable to give a realistic view of true physiological conditions.

(1) Pluripotent fibroblasts

Pluripotent fibroblasts (10T1/2, Balb/c 3T3, 1246, RCJ3.1 and CHEF/18 fibroblasts) can be converted into several cell types. 10T1/2 fibroblasts, derived from C3H mouse embryos (6), may be converted to preadipose, premuscle, and precartilage tissue. Pluripotent fibroblasts are good models for understanding the events that lead to cellular determination.

(2) Unipotent preadipocytes

Unipotent preadipocytes (3T3-L1, 3T3-F422A, 1246, Ob17, TA1, and 30A5) are committed and can either remain as preadipocytes or differentiate into adipocytes. These cell lines are useful when studying the conversion of preadipocytes to adipocytes. The most widely used cell lines are 3T3-L1 and 3T3-F422A, which were isolated from Swiss 3T3 cells derived from disaggregated 17 to 19 day mouse embryos (7). Ob17 cells were derived from the epididymal fat pads of genetically obese (*ob/ob*) adult mice and are used to explore differentiation resulting from genetic obesity (8).

B. Process of differentiation of 3T3-L1 preadipocytes

The 3T3-L1 cell line is one the most well studied models of adipocyte differentiation. When injected into mice, 3T3-L1 preadipocytes form fat pads that are indiscernible from the normal fat tissue (9) The development of fat droplets mimics the actions of live adipose tissue (10).

3T3-L1 cells spontaneously differentiate into fat-cell clusters over a period of several weeks when cultured in fetal calf serum. This process can be accelerated when confluent 3T3-L1 preadipocytes are exposed to an adipogenic cocktail. This cocktail contains a glucocorticoid, a phosphodiesterase inhibitor, and fetal bovine serum Insulin has also been used in combination with the above and works via the insulin-like growth factor 1 (IGF-1) receptor. Dexamethasone (DEX) is a synthetic glucocoticoid agonist commonly used in a differentiation cocktail. Methylisobutylxanthine (MIX) is a phosphodiesterase inhibitor used to increase the intracellular cAMP levels in the adipogenic cocktail (1).

Twenty-four hours after the introduction of a differentiation cocktail, the preadipocytes experience a postconfluent mitosis and an ensuing growth arrest (11). The preadipocytes go through at least one round of DNA replication and cell division. The mitosis is believed to be needed to unwind DNA and allow the transcription factors access to transactivate adipocyte specific genes (5). Day 2 marks the end of the postconfluent mitosis and the beginning of a growth arrest called $G_D$ (12). After growth arrest, the preadipocytes are ready for differentiation and are committed to the formation of adipocytes. On day 3, growth-arrested cells begin to express late markers of differentiation such as multiple proteins that modulate adipocyte metabolism as well as

lipogenic and lipolytic enzymes. 3T3-L1 cells accumulate fat droplets and become spherical in appearance by day 5 to 7.

C. Changes during the differentiation of preadipocytes

While the 3T3-L1 cells have been heavily analyzed, the exact chronology of the earliest stages of adipocyte differentiation is still unknown. Growth arrest triggers the expression of lipoprotein lipase (LPL) mRNA in 3T3-L1 preadipocytes (2,5). While the presence of LPL has been thought to be an early sign of adipocyte differentiation, the expression of LPL occurs upon confluence independent of the addition of a differentiation cocktail. LPL is also found in other mesenchymal cell types and is therefore not adipocyte specific. These two properties leave LPL's role as an early marker of adipocyte differentiation ambiguous (1).

Within the first hour of differentiation, the expression of *c-fos, c-jun, junB*, and *c-myc* is seen (5). Fos and jun proteins have not been linked to any differentiation-specific events, but are believed to have mitogenic properties. *c-myc* has been shown to initiate mitogenesis in differentiating preadipocytes (5). The expression of the above proteins depletes 2-6 h after the initial exposure to the differentiation cocktail (2).

There are at least two families of transcription factors, CCAAT/enhancer binding proteins (C/EBP) and peroxisome proliferator-activated receptors (PPAR), which are induced early during adipocyte differentiation. C/EBP-β and C/EBP-δ are the first transcription factors induced in the differentiation process. The expression of these factors, controlled by exogenous differentiation promoters, induces and increases adipogenesis in response to hormones. C/EBP-β is responsive to DEX, while C/EBP-δ

responds to MIX (13). The activity of C/EBP-β and C/EBP-δ is thought to mediate the expression of PPAR-γ (14). PPAR-γ is largely adipocyte specific and is expressed at low, but detectable levels in preadipocytes. Its expression quickly increases once exposed to hormonal induction of differentiation It is transcriptionally induced on day 2 of differentiation with maximum levels of expression seen in mature adipocytes (1, 15). The decrease of C/EBP-β and C/EBP-δ expression in the early to mid stages of differentiation is followed by an increase in the expression of C/EBP-α which occurs directly before the expression of adipocyte-specific genes (15) (**Fig.1**).

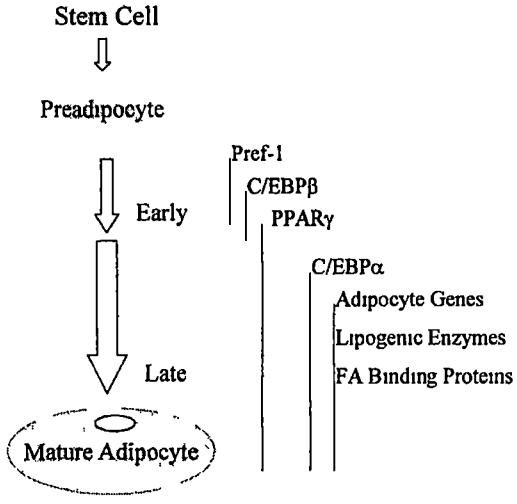


**Figure 1.** The process of differentiation from a multipotential stem cell to a mature, lipid filled adipocyte. Selected molecular events accompanying this process are indicated to the right, with their duration reflected by a solid line. Pref-1, preadipocyte factor-1; C/EBP, CCAT/enhancer –binding protein, PPAR-γ, peroxisome proliferator-activated receptor-γ; FA, fatty acid.

It appears PPAR-γ and C/EBP-α alone or in cooperation induce the transcription of many adipocyte genes encoding proteins and enzymes involved in creating the adipocyte phenotype. Once induced, PPAR-γ and C/EBP-α appear to cross-regulate each other to maintain their gene expression levels, although C/EBP-β and C/EBP-δ levels decrease after early transient expression (16). It is thought that an adipocyte-specific control of terminal differentiation might be found in PPAR-γ and C/EBP-α. While both are important for the late stages of differentiation in a synergistic manner, neither factor is expressed at high levels in preadipocytes, and therefore cannot be involved in early development. While coexpression promotes the differentiation process, ectopic expression of either factor does not promote differentiation to the same extent (15).

Sterol regulatory element binding protein-1/adipocyte determination and differentiation factor-1 (SREBP-1/ADD1) is another transcriptional factor that is induced early in adipocyte differentiation. This factor appears to up-regulate PPAR-γ expression (17).

Preadipocyte factor-1 (pref-1) is an inhibitor of adipocyte differentiation. It is thought to play a role in maintaining preadipocyte phenotype A decrease in pref-1 expression is seen during adipocyte differentiation. It is abundant in preadipocytes, but is undetectable in adipocytes. It is the only known gene whose expression is completely down-regulated during adipocyte differentiation (1).

As adipocytes enter the terminal phases of differentiation, they express an increase in lipogenesis and become sensitive to insulin. The activity, protein, and mRNA levels of enzymes found during triacylglycerol (TAG) metabolism increase 10 to 100 fold. These enzymes include ATP citrate lyase, malic enzyme, acetyl-CoA carboxylase,

stearoyl-CoA desaturase-1 (SCD-1), glycerol-3-phosphate acyltransferase, glycerol-3-phosphate dehydrogenase, fatty acid synthase, and glyceraldehyde-3-phosphate dehydrogenase (1)

1. Transcriptional control of adipocyte differentiation

a. Peroxisome proliferator-activated receptor (PPAR) family

The PPARs belong to a type II nuclear hormone receptor family and form heterodimers with the retinoid X receptor (RXR) (18). The PPARs regulate transcription through binding of PPAR-RXR heterodimers to a response element consisting of a direct repeat of the nuclear receptor hexameric DNA recognition motif spaced by one nucleotide (19). There are three known members of the PPAR family, including PPAR-α, PPAR-δ, and PPAR-γ. PPAR-α is weakly expressed in adipocytes. While PPAR-δ is not adipocyte specific, it is highly expressed in adipose tissue. Unfortunately, its role in adipose tissue development has not yet been demonstrated. PPAR-γ is the most adipose-specific of the PPARs, and it is induced before transcriptional activation of most adipocyte genes. Low but detectable expression of PPAR-γ is also seen in the liver and hematopoietic cells (20). The PPAR-γ gene gives rise to two isoforms, γ1 and γ2 PPAR- γ2 is highly enriched in adipose tissue and mediates gene expression regarding fatty acid metabolism (21). PPAR-γ plays a key role in the transcriptional control of adipocyte-specific gene expression, including aP2, PEPCK, LPL, GLUT-4, and leptin (1).

The binding of ligands to PPAR-γ enhances its transcriptional activity. Thiazolidinediones (TZDs) are synthetic ligands used to increase insulin sensitivity

clinically. These compounds bind to PPAR-γ with high affinity and increase its transcriptional activity (22). 15-deoxy-$_D$-$PGJ_2$ is an endogenous ligand for PPAR-γ (1)

PPAR-γ plays an important role in the transcriptional control of adipogenesis. PPAR-γ is expressed at low, but detectable levels in preadipocytes, with its expression increasing early in adipocyte differentiation (19). Retroviral expression of PPAR-γ in fibroblasts causes an adipocyte phenotype to appear, including morphological changes, lipid accumulation, and adipocyte-specific gene expression. However, a point mutation in the zinc finger of PPAR-γ required for DNA binding ablated its ability to induce differentition (23).

The role of PPAR-γ in adipogenesis has been demonstrated in PPAR-γ knockout mice. The homozygous null mutation is lethal in gestation, resulting from a placental dysfunction (24, 25). Chimeric embryos were created using a combination of wild-type tetraploid cells which contribute to the development of extraembryonic tissues. One PPAR-γ-null homozygous mutant developed to term, but died by postnatal day 5 from a variety of metabolic disorders. This animal lacked adipose tissue, suggesting that PPAR-γ is required for adipose tissue development in vivo (24). Chimeric mice were also created from both wild-type embryonic stem (ES) cells and PPAR-$\gamma^{-/-}$ ES cells (26) In these mice, PPAR-γ null ES cells do not contribute to the formation of adipose tissue, but do participate in the development of the other tissues This study shows that PPAR-γ is required for the in vitro differentiation of adipocytes from ES cells in addition to its adipogenic role in vivo.

b. CCAAT/enhancer binding protein (C/EBP) family

C/EBP proteins were the first transcription factors demonstrated to play an important role in adipocyte differentiation These transcription factors contain a basic transcriptional activation domain and a leucine zipper motif, which allows for homo- and heterodimerization. C/EBPs are not limited to adipose tissue, but rather are also found in other tissues, such as the liver, that metabolize lipids at high rates (1, 27).

The expression of C/EBPs during adipocyte differentiation exhibits a distinct pattern, with C/EBP-β and C/EBP-δ expressed early in response to hormone stimulation and C/EBP-α expressed immediately before the induction of most adipocyte-specific genes (15). In fact, several adipocyte-specific genes, such as SCD-1, GLUT-4, aP2, PEPCK, leptin, and the insulin receptor, contain C/EBP-α binding sites in their promoter regions (1, 28).

Ectopic expression of C/EBP-β is sufficient to induce differentiation of 3T3-L1 cells in the absence of hormone stimulation, while hormonal stimulation is required to induce differentiation in 3T3-L1 cells that over express C/EBP-δ, but adipogenesis in these cells is accelerated (29). Constitutive expression of C/EBP-α is sufficient to induce differentiation of 3T3-L1 cells in the absence of hormonal agents, and the expression of antisense C/EBP-α mRNA inhibits differentiation of cultured preadipocytes (21, 30). C/EBP-α has been shown to promote adipogenesis in a variety of mouse fibroblasts, including those that have little or no spontaneous capacity to develop into adipocytes (31). The autoactivation of the C/EBP-α gene seems to play a role in the maintenance of the adipocyte phenotype.

Mouse models lacking C/EBPs demonstrate the importance of these transcription factors in adipose tissue development. Mice lacking either C/EBP-β or C/EBP-δ usually survive without significant developmental of physiological defects. They develop normal white adipose tissue (WAT), but their brown adipose tissue (BAT) has a reduction in TG accumulation and UCP-1 expression. The double knockout mice have a very low survival rate (15%), but they exhibit a reduction in both WAT and BAT. The reduction in BAT is a result of decreased lipid accumulation, while decreased cell number causes the reduction in WAT (32). C/EBP-α knockout mice do not develop subcutaneous inguinal WAT (33) A high percentage of C/EBP-α deficient mice die from hypoglycemia that results from a failure to perform hepatic gluconeogenesis. The deficiency of C/EBP-α in the liver prevents it from regulating the genes involved in energy metabolism including glucose (33) All of these models illustrate the importance role the C/EBP family plays in adipogenesis.

c. Sterol regulatory element binding protein-1/adipocyte determination and differentiation factor-1 (SREBP1/ADD1)

SREBP1/ADD1 is a group of basic helix-loop-helix (bHLH) leucine zipper transcriptional factors (1). The SREBP family consists of three members, SREBP-1a, 1c, and 2. SREBP-1a and 1c are generated from the same gene in both human and murine models (34). ADD1, cloned from a rat cDNA library, is a homologue of human SREBP-1c (35).

SREBP1/ADD1 has been shown to regulate a number of genes involved cholesterol and fatty acid metabolism (1, 21). SREBP1/ADD1 is an inactive molecule

bound to the membrane of the endoplasmic reticulum. Once cells sense a decrease in cholesterol, SREBP1/ADD1 undergoes proteolytic cleavage and translocates to the nucleus to regulate the expression of target genes (36). While all of the SREBPs are capable of regulating similar gene expression, regulation of fatty acid biosynthesis is mediated primarily by SREBP-1a and SREBP-1c/ADD1. In vivo, adipose tissue expresses predominantly SREBP-1c/ADD1 over other forms of SREBP (21). Expression of a dominant negative form of SREBP-1c/ADD1 suppresses the expression of adipocyte marker genes and represses 3T3-L1 preadipocyte differentiation (37) Overexpression of SREBP-1c/ADD1 in preadipocytes increases the transcriptional activity of PPAR-γ by increasing the endogenous ligands (38).

2 Hormonal control of adipocyte differentiation

a. Insulin and insulin-like growth factor-1 (IGF-1)

Addition of insulin to differentiation media can promote adipogenesis and increase lipid accumulation in adipocytes (39). While preadipocytes express few insulin receptors, insulin may act by binding to the IGF-1 receptor (1). This may explain why pharmacological doses of insulin are required to achieve stimulatory effects on adipogenesis

IGF-1 is an essential factor for 3T3-L1 adipocyte differentiation. It is a critical component of fetal bovine serum for adipocyte differentiation, or it can be supplemented to enable differentiation to occur in a serum free environment. IGF-1 has a dose-dependent action on 3T3-L1 preadipocyte differentiation under both serum-containing and serum-free conditions (40).

Insulin and IGF-1 may stimulate adipogenesis by activating downstream signal tranduction. Activation of ras by insulin or IGF-1 has been shown to mediate the stimulatory effects on adipogenesis. This was confirmed when it was shown that ectopic expression of ras promotes adipogenesis in the absence of hormonal stimulation (41). In addition, inactivation of ras inhibits adipogenesis. The timing of ras activation during differentiation is important in determining whether it will exhibit a stimulatory or inhibitory effect on differentiation. Ras is a small G protein that is also a mediator of the mitogen-activated protein kinase (MAPK) pathway, whose activation inhibits differentiation (42). IGF-1 and insulin also activate another down-stream signal, a serine/threonine kinase Akt (PKB) which is involved in adipocyte differentiation. Expression of constitutively active Akt/PKB in 3T3-L1 cells induces spontaneous differentiation (1).

b. Tumor necrosis factor-α (TNF-α)

TNF-α is a potent inhibitor of adipocyte differentiation. It also suppresses the expression of some adipocyte-specific gene expression and decreases lipid accumulation in newly differentiated adipocytes, serving to dedifferentiate these cells (43, 44) This inhibitory effect on adipogenesis is mediated by down-regulation of C/EBP-α and PPAR-γ. This may explain the down-regulation of those adipocyte-specific genes, such as aP2 and GLUT4, both of which contain binding sites for C/EBP-α and PPAR-γ in their promoters While these "dedifferentiated" cells may appear similar to preadipocytes, the expression of pref-1 is not restored in TNF-α treated adipocytes. Pref-1 expression is

therefore a fundamental difference between naïve preadipocytes and those that result from TNF-α treatment (43).

c. Nuclear hormone superfamily

The members of the nuclear hormone superfamily that influence adipocyte differentiation include glucocorticoids, 3,3',5-triiodothyronine ($T_3$), and retinoic acid (RA) It is believed that these hormones act on differentiation via activating nuclear hormone receptors, including PPAR-γ.

DEX, a synthetic glucocorticoid, is used to induce differentiation in 3T3-L1 cells The addition of DEX to a differentiation cocktail is either required for differentiation or is used to accelerate the differentiation procees, depending on the origin of the cells. DEX has been shown to induce C/EBP-δ expression in 3T3-L1 cells. This increase may contribute to the formation of C/EBP-δ/C/EBP-β heterodimers, which may lead to PPAR-γ expression (1). DEX has also been shown to promote adipogenesis by inhibiting pref-1 expression (45).

RA inhibits adipocyte differentiation when administered in pharmacological doses The inhibitory effect of RA on adipogenesis is mediated by the inhibition of the expression of C/EBP-β, C/EBP-α, and PPAR-γ. Physiological doses of RA increases adipogenesis The addition of RA either before or after treatment of inducing agents does not affect differentiation, suggesting that RA acts early in differentiation (1).

d. Prostaglandins

Both mature adipocytes and cultured preadipocytes produce large amounts of prostaglandins (PGs), including $PGF_{2\alpha}$, $PGE_2$, $PGD_2$, amd $PGI_2$. $PGE_2$ is a potent antilipolytic compound, suggesting that it may contribute to lipid accumulation in terminal adipogenesis. $PGD_2$ and its derivative, $PGJ_2$, are endogenous ligands for PPAR-γ and therefore act as adipogenic signals (46). Prostanoid FP receptor agonists have been shown to be potent inhibitors of the differentiation of 3T3-L1 cells. FP receptor stimulation causes a transient increase in intracellular $Ca^{2+}$, activation of calcium/calmodulin dependent protein kinase (CaM), and an increase in DNA synthesis that is associated with the inhibition of differentiation. The addition of a CaM kinase inhibitor in the presence of an FP receptor agonist reverses the inhibition of differentiation and suggests an important role for CaM kinase in adipocyte differentiation (47) $PGI_2$ is an agonist for PPAR-α, PPAR-δ, and PPAR-γ, suggesting that it may exert its adipogenic effect by the activation of the PPARs (48). The role of $PGE_2$ and $PGD_2$ in adipocyte differentiation is not clear at this time.

e. Intracellular secondary messengers

cAMP, G proteins, and intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$) are all important in adipocyte differentiation. The role of cAMP in adipogenesis has been demonstrated by the use of MIX in adipocyte differentiation. MIX has been shown to increase the expression of C/EBP-β. MIX causes intracellular cAMP accumulation by inhibiting phosphodiesterase and stimulating adenylyl cyclase activity by blocking protein $G_i$ (1). Studies have shown that synthetic cAMP analogs can replace MIX in differentiation cocktail, and increasing

cAMP through activation of adenylyl cyclase by forskolin can induce differentiation without MIX (49).

The G proteins, $G_{s\alpha}$ and $G_{i\alpha}$, have been shown to play an important role in the differentiation of 3T3-L1 cells independently of adenylyl cyclase. Either suppression of $G_{s\alpha}$ by $G_{s\alpha}$ antisense knockout or ectopic expression of constitutively active $G_{i\alpha}$ dramatically accelerated adipogenesis. These effects were exerted at ambient or elevated levels of cAMP, confirming that their effects on adipogenesis were independent of adenylyl cyclase (50). Further evidence confirmed this idea by demonstrating that the specific domains of $G_{s\alpha}$ and $G_{i\alpha}$ responsible for adipogenesis are distinct from those interacting with adenylyl cyclase (51).

$[Ca^{2+}]_i$ is also important in cellular signaling and adipogenesis. Increasing $[Ca^{2+}]_i$, by either inhibiting $Ca^{2+}$-ATPase or stimulating $Ca^{2+}$influx, inhibits the early stages of murine adipocyte differentiation. $[Ca^{2+}]_i$ exerted this inhibitory effect by blocking the postconfluent mitotic phase and mediating sustained levels of *c-myc* expression (52). As mentioned earlier, the stimulation of the FP receptor by $PGF_{2\alpha}$ causes an increase in $[Ca^{2+}]_i$, activating CaM and causing an inhibiton of adipocyte differentiation. This anti-adipogenic effect can be reversed by the addition of a CaM kinase inhibitor (47). Recent data confirm that increasing $[Ca^{2+}]_i$ exerts a biphasic regulatory role in human adipocyte differentiation, serving to inhibit the early stages of differentiation, while promoting the later stages of differentiation and lipid accumulation (53). The exact mechanism whereby $[Ca^{2+}]_i$ undergoes this transition is unknown. Increasing cAMP promotes adipocyte differentiation, while it inhibits the expression and activity of fatty acid synthase, a key enzyme in de novo lipogenesis, and stimulates lipolysis in mature

adipocytes (54). There is a significant interaction between calcium and cAMP signaling pathways. Studies have shown that adenylyl cyclases are associated with the site of $Ca^{2+}$ entry into the cell, and $Ca^{2+}$ influx causes an inhibition of type V and VI adenylyl cyclases. This results in a reduction of cAMP levels (55) Alternatively, other data has demonstrated that increasing adipocyte $[Ca^{2+}]_i$ stimulates phosphodiesterase 3B activity, resulting in a reduction of cAMP levels (56) Accordingly, increasing $[Ca^{2+}]_i$ in the early stages of differentiation may suppress preadipocyte cAMP levels and thereby inhibit differentiation In contrast, a $[Ca^{2+}]_i$-induced decrease in cAMP late in differentiation up-regulates lipogenesis and down-regulates lipolysis, promoting late adipocyte development and lipid accumulation.

3. Preadipocyte factor-1 (pref-1) control of adipocyte differentiation

Pref-1 is an inhibitor of adipogenesis. It is an EGF repeat-containing transmembrane protein that may link extracellular adipocyte differentiation signals to the interior cell (57). Expression of pref-1 in 3T3-L1 preadipocytes decreases to undetectable levels during differentiation. Down-regulation of pref-1 is required for adipogenic conversion, while constitutive expression of pref-1 inhibits differentiation This was confirmed by demonstrating that the exposure of antisense pref-1 to preadipocytes markedly increases adipogenesis (45). Wnt signaling maintains preadipocytes in an undifferentiated state through the inhibition of the expression of PPAR-$\gamma$ and C/EBP-$\alpha$. Correspondingly, disruption of Wnt signaling increases adipogenesis (58).

## II. Regulation of Metabolism in Adipocytes

Adipose tissue is a specialized connective tissue designed for synthesis, storage, and hydrolysis of triacylglycerols (TAGs). Triglycerides are stored as liquid droplets in the cytoplasm with an average half-life of only a few days. Thus, in a homeostatic situation, there is a continuous synthesis and breakdown of TAG in adipose tissue. The triglycerides found in adipocytes are derived from circulating lipoproteins or synthesized via *de novo* lipogenesis. *De novo* lipogenesis refers to the conversion of glucose to TAG. When energy is needed, TAG can also undergo lipolysis to release these fatty acids.

### A. De *novo* lipogenesis

There are several steps involved in *de novo* lipogenesis. First, glucose undergoes glycolysis to produce pyruvate, which can then be oxidized by glycolytic enzymes to form acetyl-CoA. Citrate synthase converts acetyl-CoA into citrate which can then be transported from the mitochondria to the cytosol. ATP-citrate lyase converts citrate back into acetyl-CoA which is converted into malonyl-CoA by acetyl-CoA carboxylase (ACC). The formation of malonyl-CoA is the rate limiting step of fatty acid synthesis. Fatty acid synthase (FAS) catalyzes the subsequent synthesis of palmitate from acetyl-CoA and malonyl-CoA using nicotinamide adenine dinucleotide phosphate (NADPH) as a reducing equivalent. NADPH is derived from malic enzyme converting malate to pyruvate, or by the pentose phosphate pathway where glucose-6-phosphate is converted by glucose-6-phosphate dehydrogenase and 6-phosphogluconate is converted by 6-phosphogluconate dehydrogenase. ACC and FAS are two key enzymes in *de novo*

lipogenesis. ACC catalyzes the rate-limiting reaction in fatty acid synthesis, while FAS catalyzes the subsequent synthesis of palmitate. The activity of ACC is regulated by short-term allosteric modification or covalent phosphorylation. FAS, however, is regulated at the transcriptional level, resulting in changes in FAS protein amount (59, 60).

Humans exhibit lower FAS activity in both liver and adipose tissue compared to rodents. This may be attributed to the lower metabolic rate in humans compared to rodents (61). Human adipocytes contain substantial levels of FAS expression and activity which is sensitive to both nutritional and hormonal modulation (62, 63). Studies in humans given a high carbohydrate diet demonstrate that adipose tissue plays an important role in fat synthesis under these diets (64, 65). Adipocyte FAS expression and activity are elevated in genetic obesity (66). Thus, up-regulation of adipocyte *de novo* lipogenesis may contribute to obesity

1. Fatty acid synthase (FAS)

The FAS found in *E coli* and higher plants consists of seven separate polypeptides that are tightly associated in a single, organized complex (67) However, FAS found in vertebrates is comprised of a single, large peptide. From its N-terminus to its C-terminus, the amino acids encoding β-ketoacyl synthatase, acetyl-CoA trnsacylase, molonyl-CoA transacylase, dehygratase, enoyl reductase, ketoacyl reductase, acyl carrier protein, and thioesterase are organized into discrete domains Active FAS is a homodimer with two identical 250 kDa subunits (68).

2. Nutritional, hormonal, and transcriptional regulation of fatty acid synthase

FAS is very sensitive to changes in nutritional status. In rats, the fasting state exhibits a decrease in the synthesis of FAS, while refeeding a high carbohydrate/low fat diet after fasting increases FAS synthesis by 20-25 fold (69). Using the same starvation and refeeding approach in mice produced an increase in FAS expression by up to 39 fold (70). This fasting/refeeding model alters the circulating glucose level, which induces the secretion of hormones mediating *de novo* lipogenesis. These hormones will be discussed later.

Carbohydrates play an important role in regulating FAS. Experiments in hepatocytes and adipocytes demonstrate that glucose stimulates the transcription of lipogenic genes including FAS (60). However, 3-O-methylglucose, a glucose analogue transported into the cell but not phosphorylated by hexokinases, does not induce FAS expression in adipose tissue. 2-deoxyglucose, a glucose analogue transported into the cell and phosphorylated to 2-deoxyglucose-6-phosphate, mimics the effect of glucose on FAS expression. This suggests that glucose-6-phosphate and its metabolites may be the signal that mediates the effect of glucose in its regulation of FAS (71).

Dietary polyunsaturated fatty acids (PUFAs) potently inhibit FAS activity and expression. Dietary supplement of 2% 18:2 (n-6) to a high carbohydrate/fat free diet results in a suppression of hepatic fatty acid synthesis and FAS activity There was no effect when saturated or monounsaturated fatty acids were used (71). This inhibitory effect has been extended to include all PUFAs of the n-3 and n-6 families (72).

Growth hormone (GH) and thyroid hormone ($T_3$) also mediate the regulation of FAS. GH appears to antagonize the effect of insulin on FAS stimulation It decreases

FAS expression in rats and in cultured pig adipose tissue (73, 74). Thyroid hormone stimulates FAS expression. Injection of $T_3$ into rats for seven days increased FAS activity in the liver (75). Moreover, both liver and WAT FAS expression is increased in hyperthyroid rats and decreased in hypothyroid rats (76).

Insulin and glucagon are both involved in the regulation of FAS. FAS expression is very low in streptozotocin-diabetic mice, but increases by 2 fold within 1 hr after the administration of insulin and 19 fold within 6 hrs after insulin administration (70). In contrast, administration of glucagon or dibutyryl cAMP during the refeeding of animals previously fasting blocked the elevation of FAS expression (70). These data suggest that glucagon, via the elevation of cAMP, antagonizes the stimulation of FAS expression by insulin.

Insulin's stimulatory effect on FAS activity is mediated by a cis-acting insulin response element (IRE). The IRE has been identified in the region from –67 to –52 on the FAS promoter (77). Additional studies demonstrate that the minimal region (-67/-52) that responds to insulin contains an E box (-65/-60) essential for transcriptional factor binding, suggesting that the transcriptional factors that are able to bind to the E box (-65/-60) may mediate insulin stimulation of FAS expression (78).

The cellular signaling pathway mediating insulin regulation of FAS is constantly being studied A phosphatidylinositol-3 kinase (PI-3 kinase) inhibitor, wortmannin, has been shown to block insulin stimulation of FAS (79). Ectopic expression of constitutive active p110 subunit of PI-3 kinase increased insulin stimulation of FAS, while overexpression of dominant negative p85 subunit suppressed insulin stimulation of FAS transcription. Moreover, overexpression of Akt/PKB, a downstream signal of PI-3

kinase, stimulated FAS transcription in the absence of insulin (79). These data suggest that PI-3 kinase and Akt/PKB are both mediators of insulin stimulation of FAS.

SREBP1 mediates insulin stimulation of FAS. FAS promoter contains two tandem SREBP1 binding sites split by the E box (-65/-60). These sites were first shown to mediate sterol modulation of FAS expression (80), but SREBP1 has also been shown to bind to this E box region and activate FAS transcription (81). In addition, SREBP1 levels decreased during fasting and increased with refeeding of a high carbohydrate diet. These observations suggest that SREBP1 may mediate insulin stimulation of FAS via the E box region.

Angiotensin II, which has been shown to be synthesized and secreted by adipocytes, stimulates FAS activity in both 3T3-L1 adipocytes and human adipose tissue (82). This effect is mediated by activation of SREBP1c and IRE (83).

The human homologue of agouti, which is expressed in human adipose tissue, has been shown to upregulate adipocyte FAS activity and expression in both 3T3-L1 and human adipocytes in a $Ca^{2+}$-dependent mechanism (84). Agouti stimulation of FAS is exerted at the transcriptional level. The effects of agouti and insulin on FAS expression are independent and additive. Studies have demonstrated an agouti/$Ca^{2+}$ response element mediating agouti stimulation of FAS transcription in FAS promoter (85).

B. Lipolysis

Lipolysis allows for fatty acids stored as TGs to be released and used for energy during the fasting state. Released free fatty acids (FFA) are transported in the bloodstream bound to albumin and taken up by other tissues. Fatty acids and their

derivatives also participate in many events involved in cell signaling and membrane biosynthesis.

Adipocyte lipolysis involves three reactions. The first reaction is the hydrolysis of a TAG to a diacylglycerol (DAG). Hormone-sensitive lipase (HSL) catalyzes this rate-limiting step. HSL also catalyzes the next step in lipolysis, the hydrolysis of a DAG to a monoacylglycerol (MAG). The last reaction of lipolysis, the conversion of a MAG into glycerol and FFA, is catalyzed by monoglyceride lipase (MGL). While there is no evidence of any regulation of MGL, HSL is recognized to be regulated by nutrition and hormones (86) Therefore, HSL is a key enzyme in adipocyte metabolism.

1. Hormone-sensitive lipase (HSL)

cDNA for HSL has been obtained from both rats and humans (87). The human HSL gene is composed of 9 exons, encoding a 775-amino acid protein. In addition to adipose tissue, HSL is also present in steroidogenic tissues such as the heart, muscle, adrenal cortex, pancreas, and ovary and testis (88, 89, 90, 91, 92). Regions upstream of exon 1 are expressed in a tissue-specific manner, which are either noncoding in adipose tissue or encode an N-terminal extension of the enzyme, as in the testis (92) and in the pancreas (91), leading to a relatively larger species in these tissues. HSL also has a significant activity against long chain esters of cholesterol (89).

HSL protein is composed of an N-terminal domain and a C-terminal catalytic domain. The catalytic domain includes a catalytic site, a regulatory module, and a putative lipid binding domain (93). The active catalytic triad of HSL consists of Ser-423, Asp-703, and His-733 (94). The N-terminal domain is thought to be involved in the

interaction of HSL with other cellular proteins, such as fatty acid binding protein (FABP) (95).

Adipocyte lipolysis is under acute hormonal control. Lipolytic hormones, such as catecholamines, act through β-adrenergic receptors, resulting in increases in cAMP The increases in cAMP then activate cAMP dependent protein kinase A (PKA). The activation of PKA phosphorylates and activates HSL. Insulin, on the other hand, phosphorylates and activates Akt/PKB via the insulin receptor and downstream PI-3 kinase. PKB phosphorylates and activates phosphodiesterase 3B, an isoform expressed in adipose tissue, which in turn catalyzes the degradation of cAMP. The decrease in cAMP and PKA activity leads to the inhibition of lipolysis (96, 97).

HSL is regulated by reversible phosphorylation. It is phosphorylated at two distinct serine residues, the regulatory and basal site. PKA phosphorylates HSL at the regulatory site (Ser-551 in humans), leading to the activation of HSL (98). The basal site (Ser-553 in humans) is phosphorylated by glycogen synthase kinase-4, $Ca^{2+}$/calmodulin dependent kinase II, and 5'-AMP-dependent protein kianse (AMPK) (99). Phosphorylation of the basal site prevents the phosphorylation of the regulatory site, thus exerting an anti-lipolytic effect (100). Two more serine sites, Ser-659 and Ser-660 in rats, have been identified to be phosphorylated by PKA. Site-directed mutagenesis has demonstrated that these two sites are critical in regulating HSL activity (101). The above suggests that HSL is subject to phosphorylation regulation at multiple serine sites.

The mechanism of HSL activation remains unclear. HSL is decreased 2 to 3 fold by PKA in vitro, while a more than 20 fold increase in lipolytic rate occurred in intact fat cells in response to hormonal stimulation (96) This may be explained by the

translocation of HSL from cytosol to lipid droplets upon isoproterenol stimulation (102, 103). In resting cells, HSL is localized in the cytosol Upon lipolytic stimulation, HSL translocates from the cytosol to the lipid droplets. This is seen in both 3T3-L1 and rat adipocytes (102, 103).

Perilipins are proteins found exclusively on the TAG-rich lipid droplets in adipocytes and the cholesterol ester-rich droplets in steroidogenic cells (104). They are phosphorylated by PKA upon lipolytic stimulation. In addition, it is believed that non-phosphorylated perilipins impose a barrier to HSL actions that is attenuated by PKA phosphorylation (104). When perilipin constructs were transfected to cells normally having small amounts of lipid droplets, perilipins localized to the lipid droplets and the intracellular TAG content increased as a result of decreased hydrolysis of lipids (104). This data suggests a protective role of perilipins against lipid hydrolysis. Moreover, TNF-α exerts a potent lipolytic effect via the inhibition of perilipin expression, leading to a reduction of perilipin immunostaining at the droplet surface (104). Overexpression of perilipin blocked TNF-α's effect (105), confirming the inhibitory role of non-phosphorylated perilipin on lipolysis Phosphorylation of perilipin by PKA results in the redistribution of perilipin on lipid droplets. This redistribution altered the structure of the lipid droplets surface, allowing HSL to interact with the core TAG in the lipid droplets (104). Another protein that is involved in HSL translocation and its interaction with lipid droplets is lipotransin. Lipotansin is an HSL-docking protein that may mediate the hormonally regulated redistribution of HSL (106).

Overexpression of HSL in 3T3-L1 adipocytes results in a diminished accumulation of TAG (107), confirming HSL's role as a key enzyme in lipolysis. HSL

knockout mice exhibit normal body weight, fat mass, and cold sensitivity (108). The male mice, however, are sterile because of oligospermia. They have normal gonadal and adrenal function, but the neutral cholesterol esterhydrolase activity is completely lost in the testis. WAT remained unchanged in these animals, and retained 40% of TAG lipase activity (108).

2. Nutritional and hormonal regulation of hormone-sensitive lipase

a. Nutritional regulation

Nutritional status regulates lipolysis Prolonged fasting for 3 to 5 days increases basal lipolysis rate, HSL activity, protein, and mRNA level in rat adipocytes (109). In addition, long-chain fatty acyl CoA and intermediary lipid metabolites, such as palmitoyl CoA and oleic acid exert an inhibitory effect on HSL activity (110), reflecting a feed-back inhibitory mechanism. Studies have shown that FABP may serve to limit the inhibitory effect of intermediary lipid metabolites on HSL activity (95). Ketone bodies produced during fasting, vigorous exercise, and uncontrolled diabetes act as potent inhibitors of lipolysis (111). Thus, ketone bodies, such as acetoacetate and β-hydroxybutyrate, may be important modulators of lipolysis under the above conditions.

b. Hormonal regulation

Catecholamines are powerful regulators of lipolysis that are mediated by four adrenergic receptors: β1, β2, β3, and α2 (112). Signals through β receptors result in increases in cAMP and stimulation of lipolysis, α2 receptors have the opposite effect on lipolysis. It is believed that α2 receptors predominate in the regulation of adipose tissue

lipolysis at rest, and β receptors assume a more important role during stress (113). Each receptor subtype may have a different signaling role.

Growth hormone (GH) stimulates lipolysis in vitro and at physiological concentrations in vivo (114, 115). Glucagon and the synthetic glucocorticoid dexamethasone also exert potent lipolytic effects in rat adipocytes (114). GH and glucagon exert lipolytic effects by stimulating acute HSL activity, while glucocorticoids increase lipolysis by stimulating both HSL activity and expression (114).

Tumor necrosis factor-α (TNF-α) is produced and secreted by adipose tissue, with its production increased in obesity (116). Studies have demonstrated TNF-α as a potent lipolytic factor. It stimulates lipolysis by inhibiting perilipin expression. This action was confirmed when overexpression of perilipin blocked TNF-α stimulation of lipolysis (117).

Insulin is by far the most potent anti-lipolytic hormone and has been extensively studied. Recent studies demonstrated that lipolysis in normal subjects is sensitive to insulin with a half-maximal effect ($ED_{50}$) occurring at a concentration of 12 pM (118). However, while insulin acutely inhibits hormone-stimulated lipolysis, chronic exposure of rat adipocytes to insulin resulted in stimulated lipolysis and activation of HSL (119). This long-term effect of insulin may also contribute the elevation of plasma FFAs in obesity and diabetes, which are frequently accompanied with hyperinsulinemia and insulin resistance.

Adenosine is also a potent anti-lipolytic factor. Intracellular cAMP is constantly exported from adipocytes to extracellular space where it is then converted to adenosine by phosphodiesterase and 5'-nucleotidase. Adenosine acts on the $A_1$ adenosine receptor,

which inhibits adenylyl cyclase via coupling $G_{i\alpha}$ This results in a reduction of cAMP levels (120). This may represent a transmembrane negative feedback mechanism which would limit excess cAMP production, and inhibit lipolysis.

The major prostaglandins, $PGI_2$ and $PGE_2$, also exert effects on lipolysis. $PGE_2$ is an anti-lipolytic agent, while $PGI_2$ stimulates lipolysis in rat adipocytes (121).

## III. The Role of Intracellular $Ca^{2+}$ in Obesity

### A. The role of intracellular $Ca^{2+}$ in syndrome X

Obesity is closely related to other metabolic disorders, including insulin resistance/hyperinsulinemia, hypertension, and dyslipidemia. These diseases have been integrated into a metabolic syndrome referred to as "Syndrome X" (122). Obesity, hypertriglyceridemia, hypertension, and insulin resistance/hyperinsulinemia has also been termed the "deadly quartet " There is much evidence to demonstrate that all of these disorders are characterized by an underlying impairment in intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$) levels Draznin et al. first reported that obese patients exhibited an elevation in basal adipocyte $[Ca^{2+}]_i$ (123). Sustained elevations of $[Ca^{2+}]_i$ have been observed in cardiomyocytes in patients with left ventricular hypertrophy (124), vascular smooth muscle cells, platelets, and erythrocytes in patients with hypertension (124-128), and skeletal muscle cells and adipocytes in patients with insulin resistance (123, 128, 129). To explain these tightly interacting abnormalities a unifying "ionic hypothesis" was proposed. This hypothesis suggests a common cell lesion underlying these disorders in different tissues characterized, in part, by elevations in steady-state intracellular $[Ca^{2+}]_i$ levels (124). Consistent with this hypothesis, antagonism of $Ca^{2+}$ influx results in clinical

improvements in blood pressure, insulin resistance, platelet aggregation, and left ventricular hypertrophy (124). Studies of the mechanisms of agouti-induced obesity also indicate that obesity may be partly a manifestation of a lesion in $[Ca^{2+}]_i$ regulation (130)

B. Calcium homeostasis and obesity

Obesity is associated with alterations in calcium homeostasis. Of the calcium found in plasma, 50% of the calcium is ionized, 35% is bound to albumin, and the remaining 15% is complexed with anions. Over half of the calcium found in serum is ionized calcium. Only the ionized calcium is biologically active and can be regulated by calcium-homeostatic hormones, including PTH, 1,25-$(OH)_2$-$D_3$, and calcitonin. Decreased serum ionized calcium, increased urinary calcium excretion, and increased calcitrophic hormones PTH and 1,25-$(OH)_2$-$D_3$ have been observed in obese humans (131-134). While serum calcium levels remain relatively stable, serum albumin, phosphate, and bicarbonate levels are decreased in obesity. In addition, serum citrate, lactate, fatty acids, and urate levels are increased (132). This causes the concentration of less readily resorbable anion in the renal ultrafiltrate, increasing urinary calcium excretion, and consequently decreasing serum ionized calcium (135). A decrease in the ionized calcium found in the serum stimulates the synthesis and release of calcitrophic hormones (132). An inverse correlation has been identified between serum ionized calcium levels and BMI (132). However, weight reduction does result in the normalization of serum phosphate, bicarbonate, lactate, and fatty acid levels, as well as an increase in serum ionized calcium and a decrease in calcitrophic hormone levels (136).

C. Intracellular $Ca^{2+}$ and adipocyte metabolism

$[Ca^{2+}]_i$ plays an important role in the regulation of adipocyte metabolism. Obese patients exhibit an elevation in basal adipocyte $[Ca^{2+}]_i$ (123) This elevation in $[Ca^{2+}]_i$ has some physiological consequences that affect lipogenesis and lipolysis. Rat adipocytes were treated with A23187, a calcium ionophore, which caused an increase in both basal and insulin-stimulated lipogenesis. This action was completely blocked by the calcium channel antagonist verapamil and by the calmodulin inhibitor calmidazolium (137). $[Ca^{2+}]_i$ has also had an inhibitory effect on adipocyte lipolysis. $[Ca^{2+}]_i$ modulation plays a role in the regulation of both lipolytic and anti-lipolytic actions of hormones in adipocytes (138-140). The anitlipolytic effect of epidermal growth factor (EGF) is mediated by an increase in $[Ca^{2+}]_i$. The interaction between EGF and $[Ca^{2+}]_i$ increases the interaction between $G_i$ and adenylyl cyclase, thereby reducing cAMP and inhibiting lipolysis (139, 140). Thyroid hormone modulates lipolysis by increasing $[Ca^{2+}]_i$ via alterations in phosphodiesterase activity (141). In addition, increasing $[Ca^{2+}]_i$ by the calcium ionophore A23187 in hamster adipocytes stimulates phosphodiesterase activity and decreases cAMP levels, resulting in an inhibition of lipolysis (142).

D. Regulation of adipocyte intracellular $Ca^{2+}$

There are many nutritional, hormonal, and pharmacological factors that regulate $[Ca^{2+}]_i$. Some of these factors include agouti, calcitrophic hormones PTH and 1,25-$(OH)_2$-$D_3$, sulfonylureas, angiotensin II, as well as many others.

1. Agouti regulation of intracellular $Ca^{2+}$

*Agouti* was the first obesity gene cloned (143). $[Ca^{2+}]_i$ mediated increases in lipogenesis and decreases in lipolysis have also been studied in agouti. $A^{vy}$ mice exhibit increases in both steady-state $[Ca^{2+}]_i$ and $Ca^{2+}$ influx in several tissues (144, 145). The increase in $[Ca^{2+}]_i$ is correlated with the degree of ectopic agouti expression and body weight (144). $A^{vy}$ mice also exhibit elevated adipocyte fatty acid synthase expression, suggesting that adipocyte *de novo* lipogenesis is increased in this model (146). In addition, in vitro studies demonstrate that recombinant agouti protein stimulates dose-responsive increases in $Ca^{2+}$ influx and steady-state $[Ca^{2+}]_i$ in a variety of cell types, including both murine and human adipocytes (144, 145) The physiological consequence of this increased $[Ca^{2+}]_i$ was explored by studying adipocyte lipogenic genes and their activity. Agouti stimulates FAS expression and activity in a $Ca^{2+}$-dependent manner. This action can be mimicked by $Ca^{2+}$ channel activation and reversed by $Ca^{2+}$ channel antagonism (146). These actions are mediated by an agouti/$Ca^{2+}$ response element on the FAS promoter region (85). Moreover, agouti expression is highly correlated with in vivo FAS expression and activity, suggesting that agouti protein, which is normally expressed in human adipose tissue, may play a role in human obesity (147).

In addition to the effect of agouti on lipogenesis, agouti protein inhibits basal and agonist-stimulated lipolysis in human adipocytes via a $Ca^{2+}$-dependent mechanism (130). The mechanism of the anti-lipolytic effect of $[Ca^{2+}]_i$ was recently shown to be increased $[Ca^{2+}]_i$ activation of phosphodiesterase 3B, reducing cAMP levels, and thereby inhibiting HSL activity (148). Moreover, agouti regulation of adipocyte $[Ca^{2+}]_i$ appears to promote

triglyceride accumulation in adipocytes by stimulating lipogenesis and inhibiting lipolysis.

Agouti acts upon $Ca^{2+}$ channels through a homology between the carboxyl terminus of agouti and ω-conotoxins and plectoxins (149). The antagonism of $[Ca^{2+}]_i$ by either blocking $Ca^{2+}$ channels or inhibiting $Ca^{2+}$ agonists would seem to be a possible therapeutic treatment for obesity. Calcium channel blockade has been shown to reduce body weight and fat pad mass effectively in several animal models. Treating transgenic mice overexpressing *agouti* with the $Ca^{2+}$ channel antagonist nifedipine resulted in decreases in adipocyte lipogenesis and reduction in fat pad mass (150).

2. Sulfonylurea receptor regulation of intracellular $Ca^{2+}$

Sulfonylureas, such as glibenclamide, are insulin secretagogues used to stimulate insulin secretion for the treatment of non-insulin-dependent diabetes mellitus. They bind to the sulfonylurea receptor (SUR) of pancreatic β-cells and then block the conductance of an ATP-dependent potassium channel ($K_{ATP}$ channel) (151). The attenuation of potassium current by blocking this channel depolarizes the β-cells and induces $Ca^{2+}$ entry via L-type calcium channels, causing increased insulin secretion (152, 153).

Patients treated with glibenclamide frequently experience weight gain. Conversely, diazoxide, which inhibits SUR by activating $K_{ATP}$ channels, exerts an antiobesity effect in obese Zucker rats and hyperinsulinemic, obese humans (154-156). The effects of glibenclamide and diazoxide on body weight have been attributed to their effect on circulating insulin rather than any direct effect on adipocytes (154-156).

However, it has been demonstrated that human adipocytes express a SUR that mediates adipocyte $[Ca^{2+}]_i$ signaling (157).

Adipocytes exhibit a corresponding glibenclamide dose-responsive increase in $[Ca^{2+}]_i$. Glibenclamide exerts lipogenic and antilipolytic effects in human adipocytes, with significant increases in FAS and GPDH activity and a decrease in glycerol release (157). Diazoxide blunted each of the above effects (157). These data suggest that the SUR found in human adipocytes regulates $[Ca^{2+}]_i$ and thereby mediates lipogenesis and lipolysis. Consistent with these findings, glibenclamide increased $[Ca^{2+}]_i$ in isolated rat adipocytes in a dose-dependent manner by stimulating $Ca^{2+}$ influx through voltage-dependent $Ca^{2+}$ channels, with this effect blocked by nitrendipine (123). While more studies are needed, these data suggest a potential role for the human SUR in modulating energy storage, and therefore obesity.

3. 1,25-dihydroxyvitamin $D_3$ regulation of intracellular $Ca^{2+}$

The active form of vitamin D, 1,25-$(OH)_2$-$D_3$, generates biological responses via genomic and non-genomic pathways. 1,25-$(OH)_2$-$D_3$ was originally believed to solely function via a nuclear receptor in a manner similar to other members of the steroid hormone superfamily (158). 1,25-$(OH)_2$-$D_3$ binds to and activates a specific nuclear hormone receptor, nVDR. The activated nVDR interacts with another nuclear receptor, RXR, and forms a heterodimer complex This complex functions as a transcriptional factor to act on the direct repeat response element named DR-3 in the promoter region of certain genes. It either stimulates or suppresses the transcription of these genes (158). nVDR knockout mice exhibit impaired bone formation and growth retardation (159,

160). A high calcium, phosphorous and lactose diet fed at 16 days of age in nVDR knockout mice allows normal mineral ion homeostasis, reversing the vitamin D deficient phenotype, and suggesting that an alternate pathway may act to preserve intestinal calcium absorption and subsequent bone modeling in the absence of a functional nVDR (161). It has been shown that 1,25-$(OH)_2$-$D_3$ stimulates intestinal transcaltachia, the rapid stimulation of $Ca^{2+}$ transport into intestinal epithelium, which may contribute to the intestinal calcium absorption in these nVDR ablated mice, although might not be sufficient to prevent the phenotype of vitamin D deficiency in these mice fed normal calcium diets(162)

1,25-$(OH)_2$-$D_3$ also generates rapid, non-genomic signal transduction via a membrane vitamin D receptor (mVDR) in a variety of cells (163). It is responsible for the modulation of calcium channels, activation of phospholipase and subsequent production of DAG and inositol triphosphate, activation of protein kinase C, and stimulation of MAP kinase. 1α,25-dihydroxylumisterol$_3$, a 6-s-cis-locked analog of 1,25-$(OH)_2$-$D_3$, exerts a non-genomic action, including stimulation of $Ca^{2+}$ influx, via the mVDR (164, 165) An A-ring diasteromer analog of 1,25-$(OH)_2$-$D_3$, 1β,25-dihydroxyvitamin $D_3$, antagonizes the before mentioned action, but has no effects on the nVDR (166)

1,25-$(OH)_2$-$D_3$ elicits both genomic and non-genomic actions in adipocytes, resulting in modulation of lipid and energy metabolism (167-170). It was recently demonstrated that 1,25-$(OH)_2$-$D_3$ stimulates adipocyte $[Ca^{2+}]_i$, promotes lipogenesis, and inhibits lipolysis in human adipocytes via a rapid non-genomic action (168, 169). These

actions were mimicked by 1α,25-dihydroxylumisterol$_3$, a specific mVDR agonist, and can be prevented by 1β,25-dihydroxyvitamin $D_3$, a specific mVDR antagonist.

In contrast, 1,25-$(OH)_2$-$D_3$, exerts an inhibitory effect on adipocyte uncoupling protein-2 (UCP2) expression in human adipocytes independently of the mVDR via genomic action (170). mVDR agonists and antagonists failed to affect 1,25-$(OH)_2$-$D_3$'s inhibitory effect on adipocyte UCP2 expression, while nVDR knockout by antisense oligodeoxynucleotide (ODN) prevents the inhibitory effect of 1,25-$(OH)_2$-$D_3$ on adipocyte UCP2 expression, demonstrating that the nVDR mediates 1,25-$(OH)_2$-$D_3$ inhibition of human adipocyte UCP2 expression (170).

The role of 1,25-$(OH)_2$-$D_3$ in human obesity is still being explored. nVDR gene polymorphism is associated with the susceptibility to obesity in humans with type 2 diabetes (171). Circulating levels of 1,25-$(OH)_2$-$D_3$ are elevated in obese humans (132). 1,25-$(OH)_2$-$D_3$ has been shown to stimulate $Ca^{2+}$ and insulin release in pancreatic β-cells, indicating that 1,25-$(OH)_2$-$D_3$, elevated with the disruption of calcium homeostasis commonly seen in obese humans, may contribute to the development of hyperinsulinemia via non-genomic action (172-174). Antagonism of 1,25-$(OH)_2$-$D_3$ may offer new interventions for obesity. Suppression of 1,25-$(OH)_2$-$D_3$ levels by increasing dietary calcium decreased adipocyte intracellular $Ca^{2+}$, stimulated lipolysis, inhibited lipogenesis, increased adipocyte UCP2 expression and core temperatures in aP2-agouti transgenic mice (167-169). The dietary calcium not only attenuated diet-induced obesity, but also accelerated weight loss and fat mass reduction secondary to caloric restriction (167-169).

Recent data have demonstrated that this mechanism is relevant to humans. Population-based observation of the NHANES III survey revealed that the odds ratio of being in the highest body fat quartile was significantly reduced to 0.16 if they were in the highest calcium intake quartile (167) In an earlier clinical trial involving the antihypertensive effects of dairy products in African-Americans, it was noted that adding calcium-rich dairy foods (yogurt) to the diet resulted in a loss of body fat, decreased circulating insulin, decreased intracellular calcium, and antihypertensive effects (168). More recently, a 6-month clinical trial in obese adults showed that those who consumed a calcium-rich (either from dairy or supplement), hypocaloric diet had greater loss in weight and fat mass as compared to those who consumed a low calcium, hypocaloric diet (168). Interestingly, increasing dietary calcium caused more fat loss from the abdominal region of the body (50% of total fat loss with supplement and 66% loss with dairy) as compared to the low calcium diet (19% of total fat loss) (168). Davies et al. (175) reported that a significant negative association between calcium intake and body weight was observed in two cross-sectional and two longitudinal studies. The odds ratio of being overweight was 2.25 for women in the lower half of calcium intakes (175) They also found a significant weight loss in subjects maintained on a calcium supplement in a double-blinded, placebo-controlled, randomized clinical trial (175). Another study exploring the dietary intakes of 53 children showed that the higher the dietary intake of calcium, the lower the body fat mass (176). All of these studies support the relationship between increased calcium intake and reductions in body weight specific to fat mass in both genders, over a wide range of ages, and in many races.

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# PART 3

# EFFECTS OF 1,25-DIHYDROXYVITAMIN $D_3$ ON ADIPOCYTE DIFFERENTIATION

## I. Abstract

Several recent reports from this laboratory demonstrate a regulatory role for intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$) in modulating lipid metabolism in both human and murine adipocytes, with increased $[Ca^{2+}]_i$ coordinately stimulating lipogenesis and inhibiting lipolysis, thereby expanding lipid mass. Further, we have recently demonstrated that 1,25-dihydroxyvitamin-$D_3$ [1,25-$(OH)_2$-$D_3$] stimulates adipocyte membrane vitamin D receptor (mVDR)-mediated rapid $Ca^{2+}$ influx into adipocytes, resulting in the stimulation of lipogenesis and inhibition of lipolysis. However, increasing $[Ca^{2+}]_i$ in the early stages of differentiation inhibits human adipocyte differentiation, whereas increasing $[Ca^{2+}]_i$ in the late stage promotes human adipocyte differentiation. Accordingly, we have investigated the role of 1,25-$(OH)_2$-$D_3$ in the differentiation of 3T3-L1 preadipocytes, using triglyceride (TG) accumulation and glycerol-3-phosphate dehydrogenase (GPDH) as markers 3T3-L1 preadipocytes were placed in differentiation media upon confluence, and exposed to varying amounts (0, 1nM, and 10nM) of 1,25-$(OH)_2$-$D_3$ for either one-hour pulses or for sustained (24 hrs or 48 hrs) amounts of time throughout the differentiation process. Exposure to one-hour pulses of 1nM 1,25-$(OH)_2$-$D_3$ throughout differentiation caused modest decreases (31%-38%) in TG accumulation ($p<0.0001$), with one-hour pulse exposure to10nM 1,25-$(OH)_2$-$D_3$ having little to no effect on TG accumulation. One-hour pulse exposure to both 1nM and 10nM 1,25-$(OH)_2$-$D_3$ suppressed GPDH activity early, but not late in differentiation. Sustained (24-hour) exposure to 1,25-$(OH)_2$-$D_3$ (1nM and 10nM) inhibited differentiation at 0-24 hrs, with decreases in both TG and GPDH of 41-81% ($p<0.0001$). Similarly, sustained exposure of 1,25-$(OH)_2$-$D_3$ resulted in marked inhibition of GPDH activity and TG accumulation

early in differentiation. In contrast, sustained exposure late in differentiation exerted no significant effects on either marker of differentiation PPAR-γ and pref-1 expression were also used as markers of differentiation. One-hour pulses of 10nM 1,25-$(OH)_2$-$D_3$ did not cause any changes in PPAR-γ expression compared to control. Sustained exposure to 1,25-$(OH)_2$-$D_3$ throughout differentiation decreased PPAR-γ expression, with a 92% decrease from 0-48h ($p<0.0001$). One-hour pulses of 1,25-$(OH)_2$-$D_3$ had no effect on Pref-1 expression, with the exception of an increase in expression at 47-48 hr ($p<0.0001$). Sustained exposure to 10nM 1,25-$(OH)_2$-$D_3$ at 0-48 hrs, 24-48 hrs and 47-48 hrs all caused significant increases (125%-146%) in the expression of Pref-1 ($p<0.001$). Thus, although 1,25-$(OH)_2$-$D_3$ stimulates lipogenesis, inhibits lipolysis and increases TG accumulation in mature human and murine adipocytes, it also modestly inhibits the differentiation of preadipocytes into mature adipocytes.

## II. Introduction

Intracellular calcium ($[Ca^{2+}]_i$) plays a key role in the metabolic modifications associated with obesity (1). Studies involving the mechanisms of *agouti* gene-induced obesity found that $[Ca^{2+}]_i$ modulates *de novo* lipogenesis in both human and murine adipocytes. Previous studies show that an increase in $[Ca^{2+}]_i$ via receptor or voltage-mediated calcium channels stimulates the expression and activity of fatty acid synthase (FAS), a key enzyme in *de novo* lipogenesis, thereby causing an increase in lipogenesis and a decrease in lipolysis The resulting triglyceride accumulation leads to adipocyte hypertrophy (2).

$[Ca^{2+}]_i$ also appears to play a role in the regulation of adipogenesis, a contributing factor to both human and murine obesity (3). In order to investigate the specific role of calcium, both thapsigargin, a $Ca^{2+}$-ATPase inhibitor, and A23187, a calcium ionophore, were used as calcium agonists to stimulate an increase in $[Ca^{2+}]_i$ in human preadipocytes. Exposure early in differentiation suppressed triglyceride accumulation greatly. However, increasing $[Ca^{2+}]_i$ late in differentiation increased triglyceride accumulation. Similar results were also seen using KCl and agouti protein as $[Ca^{2+}]_i$ agonists (2, 3) Therefore, increasing $[Ca^{2+}]_i$ has a biphasic effect on human adipocyte differentiation, with an increase early inhibiting differentiation and an increase in the late stages stimulating adipocyte differentiation and lipid filling (3).

Recent studies have introduced a paradox by showing that a decrease in the expression of genes normally involved in adipogenesis is associated with obesity and diabetes mellitus. White adipose tissue from obese mice exhibited decreased expression of genes important in adipocyte differentiation, as compared to the lean controls. Sterol responsive element binding protein (SREBP), responsible for positively regulating multiple genes coding for lipogenic enzymes, demonstrated a 2 7-fold decrease in expression. Several mRNAs that encode proteins involved in lipid metabolism were decreased, including glycerol 3-phosphate dehydrogenase and stearoyl CoA desaturase A dedifferentiation was observed in 3T3-L1 adipocytes including a suppression of PPARγ and C/EBPα, as well as other lipogenic enzymes in response to tumor necrosis factor α and transforming growth factor β (4).

1,25-dihydroxyvitamin $D_3$ [1,25-$(OH)_2$-$D_3$] acts as a calcium agonist (5). Further, we have recently demonstrated that 1,25-$(OH)_2$-$D_3$ stimulates adipocyte membrane

vitamin D receptor (mVDR)-mediated rapid $Ca^{2+}$ influx into adipocytes, resulting in the stimulation of lipogenesis and inhibition of lipolysis (5). This effect was mimicked by 1α,25-(OH)2-lumisterol$_3$, an agonist for the mVDR These effects were not seen when the human adipocytes were pretreated with 1β,25-dihydroxyvitamin $D_3$, a specific antagonist for mVDR. 1,25-$(OH)_2$-$D_3$ was also shown to increase adipocyte fatty acid synthase (FAS), with 1α,25-$(OH)_2$-lumisterol$_3$ having a greater stimulatory effect on FAS activity. Pretreatment with 1β,25-dihydroxyvitamin $D_3$ also prevented the stimulation of FAS. 1,25-$(OH)_2$-$D_3$ and 1α,25-$(OH)_2$-lumisterol$_3$ also reduced adipocyte basal lipolysis, with 1β,25-dihydroxyvitamin $D_3$ preventing any inhibition of lipolysis (5). In addition, Norman et al. (6) demonstrated that 1α,25-dihydroxylumisterol$_3$ acts on the mVDR solely to generate non-genomic action in adipocytes, while 1,25-$(OH)_2$-$D_3$ may target both the mVDR and the nuclear vitamin D receptor (nVDR) to mediate genomic and non-genomic actions which may interact with each other in signal response and, thereby compromise the modulation of lipid metabolism Accordingly, we have investigated the role of 1,25-$(OH)_2$-$D_3$ in adipogenesis.

## III. Materials and Methods

A. Culture and differentiation of murine preadipocytes

3T3-L1 preadipocytes obtained from ATCC were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Confluent cells were differentiated in a standard differentiation medium consisting of DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, isobutylmethylxanthine (IBMX), dexamethasone, and varying amounts of 1,25-$(OH)_2$-$D_3$

(0, 1nM, 10nM) in ethanol. Eight of the treatment groups were subjected to one hour pulse exposures to 1,25-$(OH)_2$-$D_3$ (1nM or 10nM). These exposures occurred at 0-1hour (h), 23-24 h, 47-48 h, and 71-72 h. Six of the treatment groups were subjected to sustained (24 hour) exposures to 1,25-$(OH)_2$-$D_3$ (1nM or 10nM) at 0-24 h, 24-48 h, and 48-72 h. Cells used for gene expression analysis were exposed to 0 or 10nM 1,25-$(OH)_2$-$D_3$ at the intervals stated above, with the addition of an exposure from 0 to 48 h of differentiation. The cells were allowed to differentiate for 72 hours. The differentiation cocktail was changed everyday for every sample to control for pH The cells incubated for 24 hours in DMEM containing 10% FBS and 1% penicillin/streptomycin before they were collected.

B. Triglyceride content

The medium was removed from the 3T3-L1 cells. 250uL of homogenization buffer were added to each well. The cells were scraped and collected for subsequent sonication. Triglyceride accumulation was measured spectrophotometrically at 500nm using a triglyceride assay kit (Sigma procedure 336, St.Louis MO).

C. Glycerol-3-phosphate dehydrogenase (GPDH) activity

GPDH activity was measured spectrophotometrically. The GPDH buffer solution consisted of 1M triethanolamine, 1mM β-mercaptoethanol, 25mM ethylenediaminetetraacticacid (EDTA), and water. A combination of the GPDH buffer, NADH, and the sample/homogenization buffer was allowed to incubate at 37°C for 5

minutes. DHAP was added, and the oxidation rate of NADH was read spectrophotometrically at 340nm for 5 minutes

D. Protein content

Total protein content was measured for correction purposes by a modified Bradford method using Coomassie Blue dye (Pierce, Rockford, IL).

E. RNA isolation

Total RNA from 3T3-L1 adipocytes was extracted using a Totally RNA™ total RNA isolation kit (Ambion, Austin, TX). Purity and quantity was measured spectrophotometrically at 800nm and 200nm and RNA was stored in formamide.

F. Gene expression

Gene expression was measured using real time reverse transcription-polymerase chain reaction (RT-PCR). Reverse transcriptase occured at 48° C for 30 minutes. Initial denaturation occured at 95° C for 10 minutes. It was followed by 40 cycles of denaturation at 95° C for 15 seconds and re-annealing and elongation at 60° C for 1 minute. The PPAR-γ forward primer, reverse primer, and probe had the following sequences. forward primer-(5'-GCCTATGAGCACTTCACAAGAAATT-3'), reverse primer-(5'-TGCGAGTGGTCTTCCATCAC-3'), and probe-(5' CAL Red-TCTGGCCCACCAACTTCGGAATCAG-BHQ-2 3'). The sequences for the Pref-1 forward primer, reverse primer, and probe were as follows: forward primer-(5'-TTCGGCCACAGCACCTATG-3'), reverse primer-(5'-

ACATTGTCAGCCTCGCAGAA-3'), and probe (5'CAL Red-CCACCCTGCGACCCCCAGTATG-BHQ-2 3'). The 18s forward primer, reverse primer, and probe had the following sequences: forward primer-(5'-AGTCCCTGCCCTTTGTACACA-3'), reverse primer-(5'-GATCCGAGGGCCTCACTAAAC-3'), and probe-(5' 6-FAM-CGCCCGTCGCTACTACCGATTGG-BHQ-1 3')(Biosearch Technologies, Inc., Novato, CA).

G. DNA content

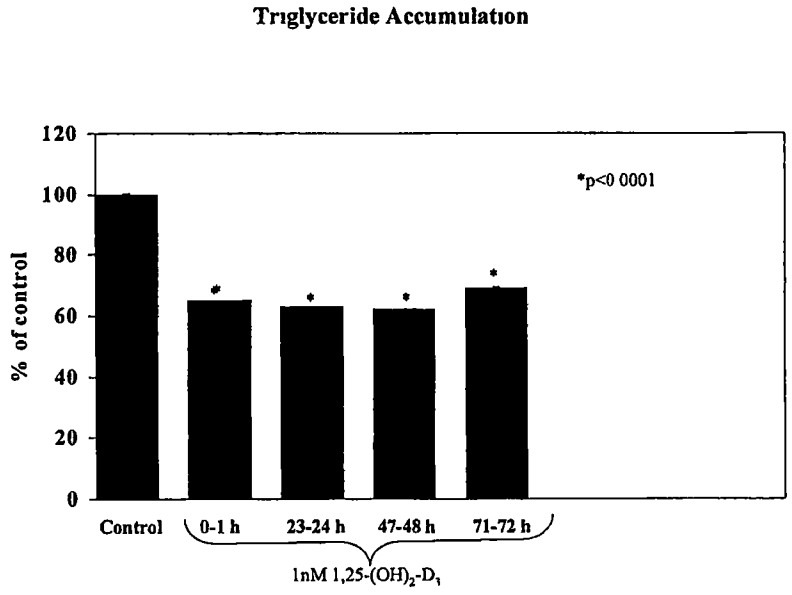
Cell density in culture was determined by measuring DNA using CyQuant® cell proliferation assay kit (Molecular Probes, Eugene,OR).

H. Statistical analysis

SPSS was used for statistical analysis. After normality of distribution was verified, analysis of variance (ANOVA) was used to evaluate statistical significance, with significantly different group means separated using the least significant different (LSD) or Tukey test. All data are expressed as mean ± SE.

## IV. Results

Triglyceride (TG) accumulation and glycerol-3-phosphate dehydrogenase (GPDH) activity were used as markers of differentiation. The data showing exposure to one-hour pulses of 1nM and 10nM 1,25-$(OH)_2$-$D_3$ throughout differentiation was inconclusive (**Fig. 2, Fig. 3**). Sustained (24-hour) exposure to 1,25-$(OH)_2$-$D_3$ (1nM and



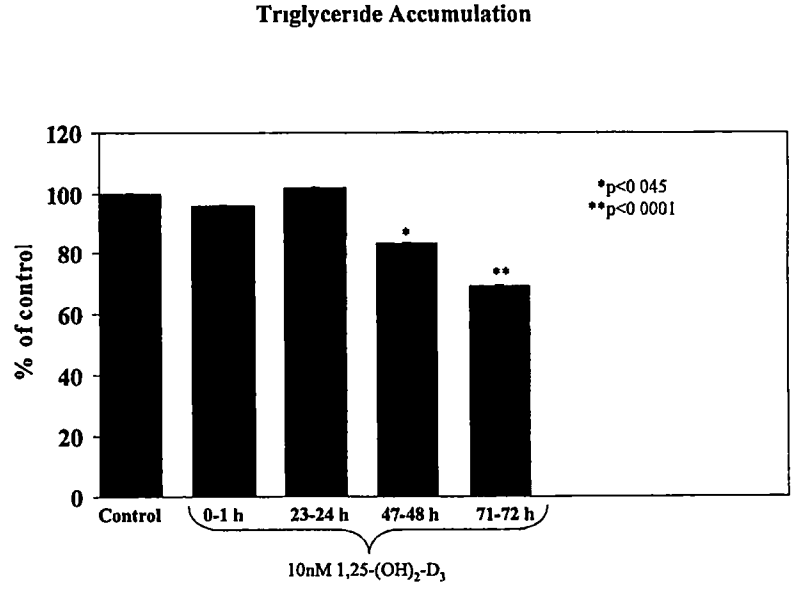




**Figure 2.** Effect of treatment with 1nM and 10nM 1,25-$(OH)_2$-$D_3$ for one-hour pulses on triglyceride accumulation throughout 3T3-L1 preadipocyte differentiation Data are expressed as % of control ± SE.

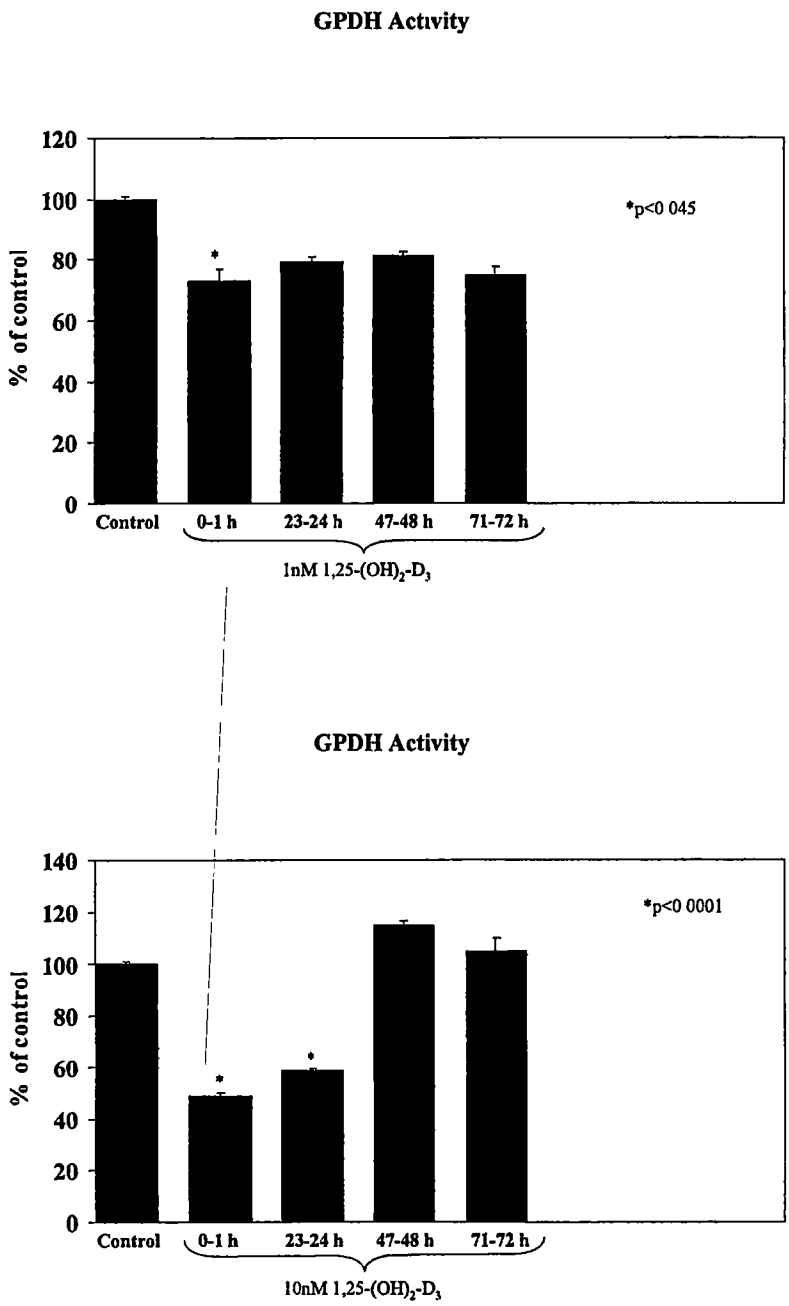



**Figure 3.** Effect of treatment with 1nM and 10nM 1,25-$(OH)_2$-$D_3$ for one-hour pulses on GPDH activity throughout 3T3-L1 preadipocyte differentiation. Data are expressed as % of control ± SE.

10nM) inhibited differentiation at 0-24 hrs, with decreases in both TG and GPDH of 41-81% ($p<0.0001$) (**Fig. 4 and Fig. 5**). Similarly, sustained exposure of 1,25-$(OH)_2$-$D_3$ resulted in marked inhibition of GPDH activity and TG accumulation early, but not late, in differentiation (**Fig. 4 and Fig. 5**). In contrast, sustained exposure late in differentiation exerted no significant effects on either marker of differentiation (**Fig. 4 and Fig. 5**).

PPAR-γ and pref-1 expression were also used as markers of differentiation. One-hour pulses of 10nM 1,25-$(OH)_2$-$D_3$ did not cause any changes in PPAR-γ expression compared to control (**Fig. 6**). Sustained exposure to 1,25-$(OH)_2$-$D_3$ throughout differentiation decreased PPAR-γ expression, with a 92% decrease from 0-48h ($p<0.0001$, **Fig. 6**). One-hour pulses of 1,25-$(OH)_2$-$D_3$ had no effect on pref-1 expression, with the exception of an increase in expression at 47-48 hr ($p<0.0001$, **Fig. 7**). Sustained exposure to 10nM 1,25-$(OH)_2$-$D_3$ at 0-48 hrs, 24-48 hrs and 48-72 hrs all caused significant increases (125%-146%) in the expression of Pref-1 ($p<0.001$, **Fig. 7**).

The DNA measurement data demonstrate that there were similar concentrations of cells in both types of plates used for the experiments While there are some differences in the amount of DNA found in the 24-well plates (**Table 1**), none of the amounts are significantly different from control, and there are no significant differences among the treatments in the 6-well plates (**Table 2**).

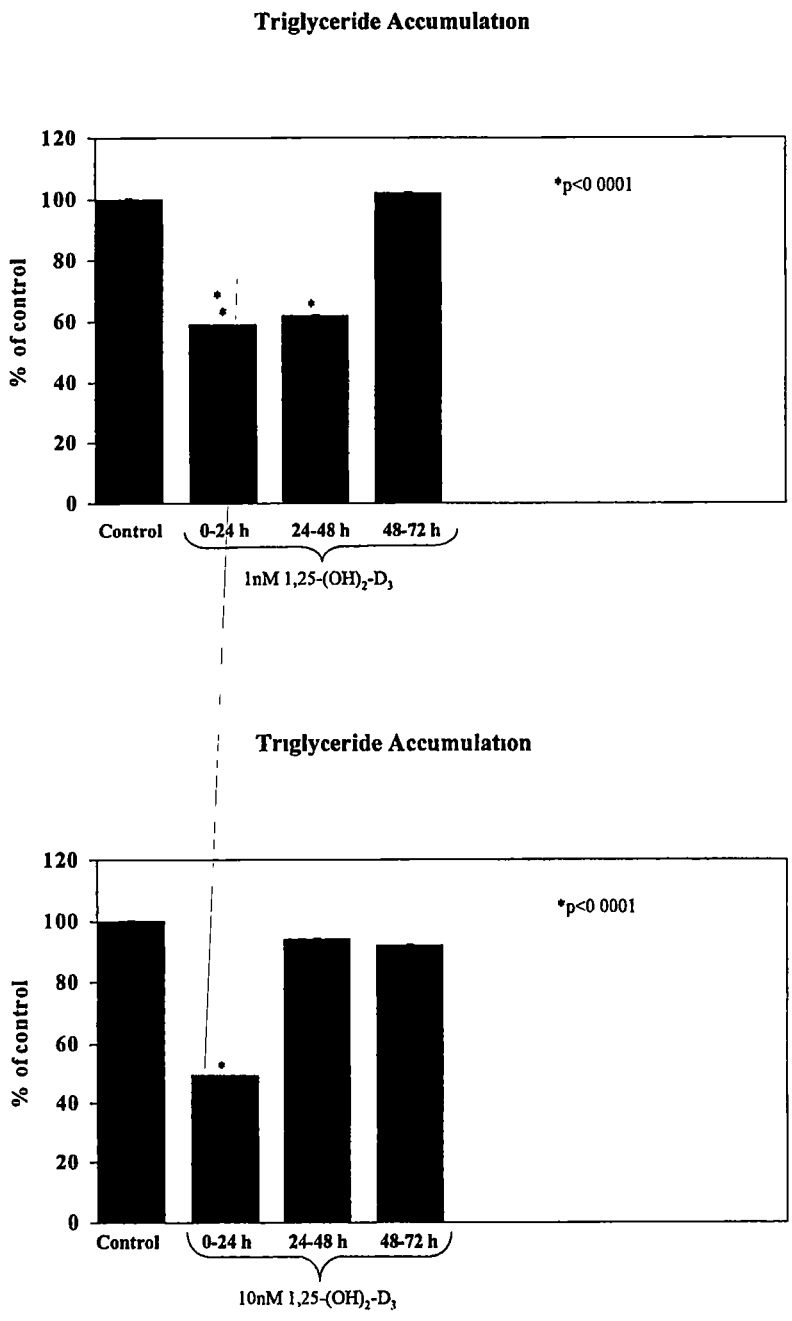
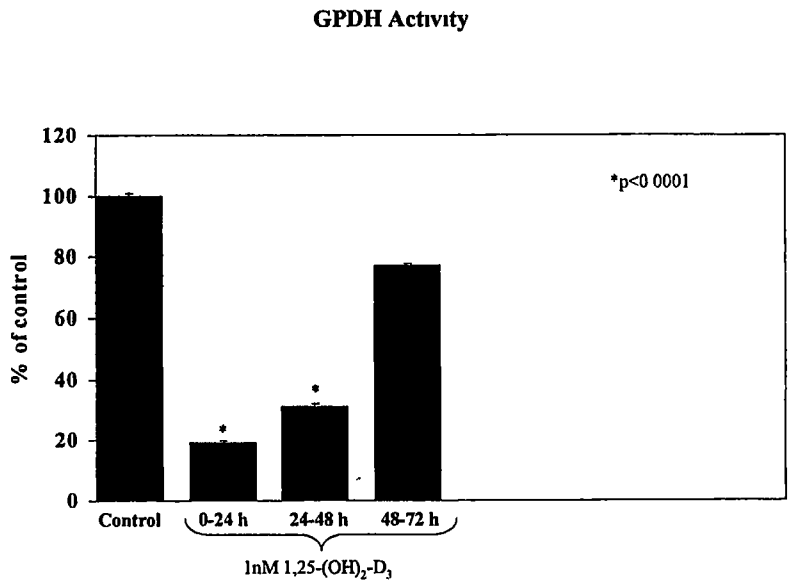



**Figure 4.** Effect of treatment with 1nM and 10nM 1,25-$(OH)_2$-$D_3$ for a sustained exposure time on triglyceride accumulation throughout 3T3-L1 preadipocyte differentiation. Data are expressed as % of control ± SE.



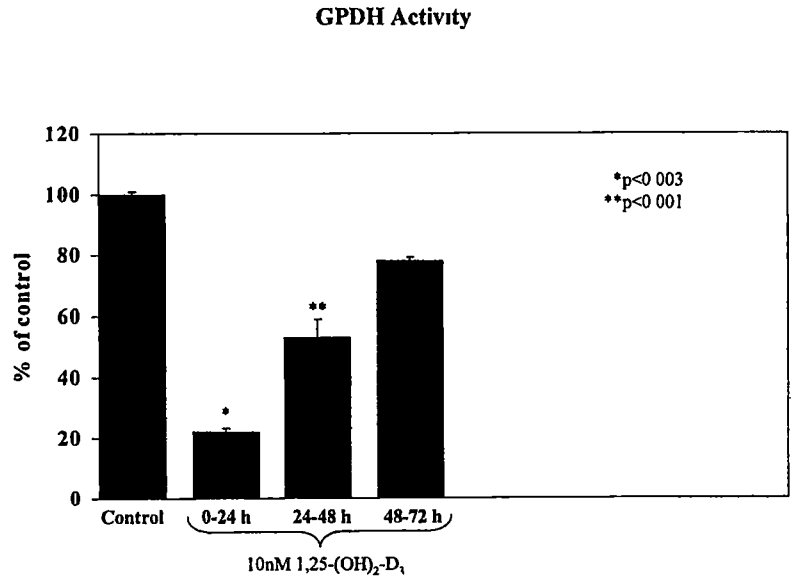




**Figure 5.** Effect of treatment with 1nM and 10nM 1,25-$(OH)_2$-$D_3$ for one-hour pulses on GPDH activity throughout 3T3-L1 preadipocyte differentiation. Data are expressed as % of control ± SE.

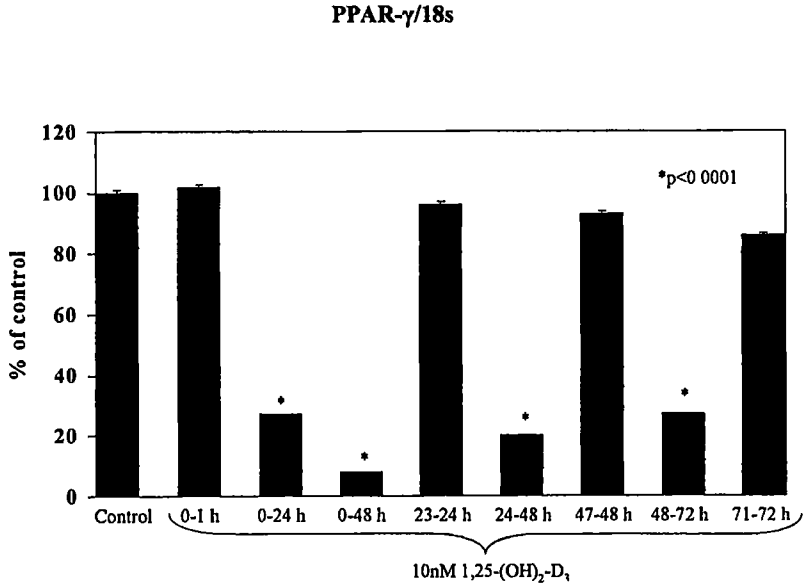



**Figure 6.** Effect of treatment with 10nM 1,25-$(OH)_2$-$D_3$ on PPAR-γ expression throughout 3T3-L1 preadipocyte differentiation. Data are expressed as % of control ± SE.

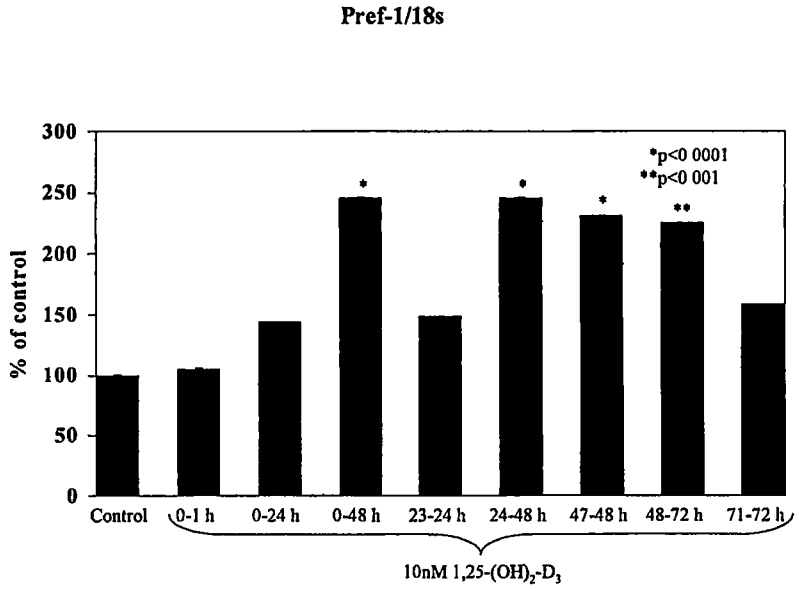



**Figure 7.** Effect of treatment with 10nM 1,25-$(OH)_2$-$D_3$ on Pref-1 expression throughout 3T3-L1 preadipocyte differentiation. Data are expressed as % of control ± SE.

**Table 1. DNA Measurement Data for 24-well Plates**

| Concentration of 1,25-$(OH)_2$-$D_3$ and Time of Treatment | 24-well plates (TG and GPDH) DNA ug/sample |
|---|---|
| Control | $.090 \pm .003^{a,b,c}$ |
| 1nM 0-1 h | $099 \pm .003^{b,c}$ |
| 10nM 0-1 h | $098 \pm .003^{a,b,c}$ |
| 1nM 0-24 h | $.093 \pm .005^{a,b,c}$ |
| 10nM 0-24 h | $.104 \pm .004^{c}$ |
| 1nM 23-24 h | $.094 \pm .003^{a,b,c}$ |
| 10nM 23-24 h | $.101 \pm .002^{c}$ |
| 1nM 24-48 h | $.102 \pm .002^{c}$ |
| 10nM 24-48 h | $.088 \pm .003^{a,b,c}$ |
| 1nM 47-48 h | $.088 \pm .002^{a,b,c}$ |
| 10nM 47-48 h | $089 \pm .003^{a,b,c}$ |
| 1nM 48-72 h | $.092 \pm .005^{a,b,c}$ |
| 10nM 48-72 h | $.084 \pm .002^{a,b}$ |
| 1nM 71-72 h | $.082 \pm .004^{a}$ |
| 10nM 71-72 h | $.081 \pm .004^{a}$ |

a,b,c Non-similar superscripts within columns indicate significant differences at $p<0.05$.
Values are Mean ± SE.

**Table 2. DNA Measurement Data for 6-well Plates**

| Concentration of $D_3$ and Time of Treatment | 6-well plates (Gene Expression) DNA ug/sample* |
|---|---|
| Control | .173 ± 008 |
| 10nM 0-1 h | 191 ± .021 |
| 10nM 0-24 h | .228 ± .013 |
| 10nM 0-48 h | .223 ± .012 |
| 10nM 23-24 h | .211 ± .011 |
| 10nM 24-48 h | 217 ± .008 |
| 10nM 47-48 h | 217 ± .015 |
| 10nM 48-72 h | .216 ± .003 |
| 10nM 71-72 h | .182 ± .007 |

*No significant differences among groups at $p<0.05$.
Values are Mean ± SE.

## V. Discussion

We have recently demonstrated that increasing $[Ca^{2+}]_i$ has a biphasic effect on adipogenesis, inhibiting the early stages and accelerating late differentiation and lipid filling (3). We have also reported that 1,25-$(OH)_2$-$D_3$ stimulates rapid adipocyte $[Ca^{2+}]_i$ influx via a membrane vitamin D receptor (mVDR) (5) Accordingly, it may be anticipated that 1,25-$(OH)_2$-$D_3$ will have similar effects on markers of differentiation as other $Ca^{2+}$ agonists. However, although our data does demonstrate an inhibitory effect of early exposure to 1,25-$(OH)_2$-$D_3$, late exposure to 1,25-$(OH)_2$-$D_3$ did not stimulate differentiation or lipid filling. Accordingly, it is likely that in addition to mVDR-mediated $[Ca^{2+}]_i$ influx affecting adipogenesis, nuclear vitamin D receptor (nVDR) signaling may modify these effects.

1,25-$(OH)_2$-$D_3$ was originally believed to solely function via a nuclear receptor in a manner similar to the other members of the steroid hormone superfamily (8). However, 1,25-$(OH)_2$-$D_3$ may target both mVDRs and nVDRs to mediate genomic and non-genomic actions which may interact with each other in signal response and, thereby affect the modulation of lipid metabolism. For example, 1,25-$(OH)_2$-$D_3$ generates rapid, non-genomic signal transduction, including modulation of calcium channels, via a mVDR (5, 9). 1,25-$(OH)_2$-$D_3$ stimulates adipocyte $[Ca^{2+}]_i$, promotes lipogenesis, and inhibits lipolysis via this non-genomic action (5). However, 1,25-$(OH)_2$-$D_3$ generates genomic actions via binding to a specific nVDR (10). 1,25-$(OH)_2$-$D_3$ exerts an inhibitory effect on adipocyte uncoupling protein 2 (UCP2) expression via nVDR-mediated genomic action (10).

1,25-$(OH)_2$-$D_3$ binds to and activates a specific nuclear hormone receptor, nVDR. The activated nVDR then interacts with another nuclear receptor, retinoid X receptor (RXR), and forms a heterodimer complex This complex functions as a transcriptional factor to act on the direct repeat response element, DR-3, in the promoter region of target genes, thereby stimulating or suppressing transcription of those genes encoding for proteins that carry out a variety of functions (5) The ablation of the (RXR) gene in adipocytes of transgenic mice caused a delay in the formation of fat depots and a resistance to dietary and chemically induced obesity (11). In addition, multiple studies (12, 13) suggest that retinoic acid inhibits adipogenesis by blocking the induction of/ inhibiting the expression of PPAR-γ. Sato et al. (14) also demonstrated an inhibitory effect of 1,25-$(OH)_2$-$D_3$ on the differentiation of 3T3-L1 cells by demonstrating an inhibition of triacylglycerol accumulation. Accordingly, our data suggest that early exposure to lower doses of 1,25-$(OH)_2$-$D_3$ may result in nVDR-mediated inhibition of differentiation, while higher doses and/or later exposure may result in nVDR-mediated events.

Alternatively, the discrepancy between the findings may be due to the source of the cells. Ntambi et al. (15) also did not find a stimulatory effect of increasing $[Ca^{2+}]_i$ in the late stages of differentiation in 3T3-L1 cells. 3T3-L1 cells are derived from mouse embryos (16), while the human preadipocytes used by Shi et al. (3) originated from human stromal-vascular cells in the subcutaneous fat depot. Cell models derived from different species and of different developmental stages may exhibit distinct differentiation properties

The normal physiological level of 1,25-$(OH)_2$-$D_3$ is $\leq$0.1nM, but past studies demonstrated that differentiation of 3T3-L1 preadipocytes is not affected at this concentration of 1,25-$(OH)_2$-$D_3$ (14). The reason why preadipocytes require a high dose of 1,25-$(OH)_2$-$D_3$ to exert its inhibitory effect on differentiation is unclear. However, in addition to the differences between cell lines and adipose tissue, one possible reason could be the presence of serum in the culture media causing a reduction in the affinity of the receptor for 1,25-$(OH)_2$-$D_3$.

In summary, the TG and GPDH data suggest that 1,25-$(OH)_2$-$D_3$ may modestly inhibit adipocyte differentiation when exposed early in differentiation, but has no effect late in differentiation. Sustained exposure to 1,25-$(OH)_2$-$D_3$ throughout differentiation decreased PPAR-$\gamma$ expression, and increased pref-1 expression However, one-hour pulses had no effect on the expression of PPAR-$\gamma$ or pref-1.

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# PART 4

# SUMMARY AND CONCLUSIONS

## SUMMARY AND CONCLUSIONS

Regulation of adipocyte differentiation and metabolism via 1,25-$(OH)_2$-$D_3$ signaling pathways may play an important role in the development of obesity in vivo. Several lines of evidence demonstrate that the circulating level of 1,25-$(OH)_2$-$D_3$ is elevated in obese humans. Suppression of 1,25-$(OH)_2$-$D_3$ by increasing dietary calcium decreases adipocyte intracellular $Ca^{2+}$, stimulates lipolysis, inhibits lipogenesis, and increases UCP2 expression in transgenic mice, thereby reducing body weight and fat mass in these animals Similar effects have also been observed in recent clinical trials. Accordingly, it is important to understand the role of 1,25-$(OH)_2$-$D_3$ both in adipocyte metabolism as well as the differentiation of preadipocytes to adipocytes.

Previous data demonstrate that agents which increase adipocyte cytosolic $Ca^{2+}$ stimulate lipogenesis, inhibit lipolysis, and increase triglyceride accumulation. Our data demonstrate that 1,25-$(OH)_2$-$D_3$ has a similar effect as other $Ca^{2+}$ agonists when used in low doses early in differentiation, while higher doses and late exposure do not mimic these effects. Accordingly, we propose that the effects of low doses early in differentiation are mediated via the membrane vitamin D receptor (mVDR) and increases in $[Ca^{2+}]_i$. However, higher doses of 1,25-$(OH)_2$-$D_3$ and/or late exposure during differentiation may exert a counter-regulatory effect which is mediated via the nuclear vitamin D receptor (nVDR). More research is needed to fully understand the role of 1,25-$(OH)_2$-$D_3$ and its various receptors on adipocyte differentiation and metabolism.

## VITA

Kimberly Causey was born in Charlottesville, Virginia. She studied at Auburn University for four years, and earned her Bachelor of Science degree in Nutrition and Food Science in 1999 She entered the University of Tennessee, Knoxville as a graduate student and became a Registered Dietitian in 2001, as well as earned her Master of Science degree in Nutrition Science in 2003.



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