2013

Histone Deacetylase Inhibitors Trichostatin A (tsa) And Sulforaphane (sfn) Modulate Vitamin D Responsive Cyp24 Gene Expression in 3t3-l1 Preadipocytes

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HISTONE DEACETYLASE INHIBITORS TRICHOSTATIN A (TSA) AND SULFORAPHANE (SFN) MODULATE VITAMIN D RESPONSIVE CYP24 GENE EXPRESSION IN 3T3-L1 PREADIPOCYTES

A Thesis Presented

by

EUNJEE AHN

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

September 2013

Department of Nutrition
HISTONE DEACETYLASE INHIBITORS TRICHOSTATIN A (TSA) AND SULFORAPHANE (SFN) MODULATE VITAMIN D RESPONSIVE CYP24 GENE EXPRESSION IN 3T3-L1 PREADIPOCYTES

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ACKNOWLEDGMENTS

First and foremost I offer my sincerest gratitude to my advisor, Dr. Richard Wood, who provided me with the opportunity to complete this project and guided me throughout my thesis with his patience and knowledge.

I would like to thank my committee member, Dr. Young-Cheul Kim, for his encouraging words and inspiring advice.

I would like to thank my committee member Dr. Yeonhwa Park, for her thoughtful comments and continuous support.

I would like to thank Jennifer Swick and Brianna Gray, with whom I worked together in the lab, for their support, help, and friendship.

I would like to give a special thanks to my family: my parents, brothers and sister for their unwavering love and support. Finally I would like to extend my deepest gratitude to my family: my husband, my son, Roy, and my daughter, Emily, for their love, patience, support and encouragement throughout my study. It was such a blessing to me to spend time together as a family over the last three years.
ABSTRACT

HISTONE DEACETYLASE INHIBITORS TRICHOSTATIN A (TSA) AND SULFORAPHANE (SFN) MODULATE VITAMIN D RESPONSIVE CYP24 GENE EXPRESSION IN 3T3-L1 PREADIPOCYTES

SEPTEMBER 2013

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Vitamin D plays an important role in preserving healthy bones, and has additional roles in the body, including modulation of cell growth, differentiation, neuromuscular and immune function, and anti-inflammatory function. The vitamin D receptor (VDR) is a member of the nuclear hormone receptor superfamily and regulates transcription of vitamin D-dependent target genes, such as those for key proteins involved in calcium and phosphorus absorption and bone development. Histone acetylation weakens the association of histones with DNA, and increases the accessibility of transcriptional regulatory proteins to chromatin templates, thereby increasing transcriptional activity of gene expression. Histone deacetylases remove the acetyl groups and condense chromatin structure, thereby preventing transcription. TSA is a potent histone deacetylase inhibitor and can significantly enhance gene expression. Bioactive food component, sulforaphane (SFN) is found in cruciferous vegetables and is known to be a histone deacetylase inhibitor, leading to transcriptional activation of gene expression. The objective of this study is to demonstrate that the bioactive food components modulate vitamin D action in adipocytes. To investigate the effects of TSA and SFN on vitamin D response, 3T3L1
mouse preadipocytes were treated with the combination of various concentrations of 1,25(OH)_2 vitamin D, TSA, and SFN. Upon harvesting cells, the amounts of 24-hydroxylase mRNA, marker of vitamin D response, were measured by semiquantitative reverse transcriptase-PCR analysis. The results showed that the cells treated with 1μM TSA increased 1,25(OH)_2 vitamin D-induced CYP24 mRNA level nearly 3.5-fold (p < 0.05) at 1nM 1,25(OH)_2 vitamin D and nearly 2.5-fold (p < 0.05) in 10 nM 1,25(OH)_2 vitamin D, and the cells treated with 5μM SFN increased 1,25(OH)_2 vitamin D-induced CYP24 mRNA level nearly 1.4-fold at 1nM 1,25(OH)_2 vitamin D and nearly 1.2-fold at 10 nM 1,25(OH)_2 vitamin D.
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CHAPTER 1
LITERATURE REVIEW

1.1 Introduction

Vitamin D is produced in the skin in response to UVB radiation and is found in a limited number of foods. Vitamin D status plays an important role in maintaining healthy bones by promoting calcium absorption in the gut and maintaining adequate serum calcium and phosphate concentrations. Serum 25-hydroxyvitamin D concentration reflects vitamin D exposure. Low serum 25-hydroxyvitamin D concentrations are associated with low bone mineral density and fractures, while sufficient serum 25-hydroxyvitamin D concentrations can prevent rickets in children, osteomalacia in adults, and osteoporosis in older adults. Vitamin D also has other roles in the body, including modulation of cell growth, differentiation, neuromuscular and immune function, and anti-inflammatory function.

The vitamin D receptor (VDR) is a member of the nuclear hormone receptor superfamily and regulates transcription of vitamin D-dependent target genes. Nuclear receptors act as ligand-inducible transcription factors and interact with DNA response elements of target genes (Aranda and Pascual, 2001). Upon activation by the active 1,25-dihydroxyvitamin D metabolite, the VDR binds to vitamin D response elements (VDREs) on the DNA of target genes, such as those for key proteins involved in calcium and phosphorus absorption and bone development.
Within the nucleus, chromatin represents DNA that is wrapped around histone protein complexes to form nucleosomes (Figure 1). Chromatin structure can be modified by altering the binding affinity, which is controlled by the opposing activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs), of DNA to histone proteins. Histone acetylation weakens the association of histones with DNA, thereby altering nucleosomal conformation and increasing the accessibility of transcriptional regulatory proteins to chromatin templates. (Norton et al., 1989). As a consequence, transcriptional activity of gene expression may increase. Histone deacetylases remove the acetyl groups, thereby increasing the positive charge of histone tails and encouraging high-affinity binding between the histones and DNA backbone. The increased DNA binding to histone protein condenses chromatin structure and prevents transcription (Struhl, 1998). Certain
chemicals have been found that can alter HDAC activity, such as trichostatin A (TSA). TSA is an organic antifungal antibiotic that is a potent inhibitor of the histone deacetylase (Yoshida et al., 1990) and can significantly enhance gene expression (Nan et al., 2004). For example, a study by Luo et al. demonstrated that inhibition of histone deacetylases by TSA increases the expression of vitamin D 24-hydroxylase (CYP24A1) in prostate cancer cells (Luo et al., 2010).

Certain plant foods contain bioactive food components that may be important for optimal health. Bioactive food components refer to nonessential biomolecules with biological activity that is predominantly found in plant foods such as whole grains, fruits, and vegetables. A diet, rich in nutrients and bioactive food components may play a vital role in improving health and reducing risk for chronic disease (Liu, 2003). In recent years, bioactive food components are being intensively studied to evaluate their effects on health. An underlying mechanism through which some bioactive food components may affect health is by influencing gene expression by altering epigenetic “marks” (methylation and acetylation) that control DNA access. For example, the bioactive food component sulforaphane (SFN) is found in cruciferous vegetables, such as broccoli and cauliflower, and is known to have histone deacetylase inhibitor activity (Myzak et al., 2004), which promotes hyperacetylation of chromatin and affects the expression of genes, leading to transcriptional activation of some genes (Struhl, 1998). Recent studies have found that epigenetic changes can lead to an increase in the expression of some genes and a decrease in the expression of others. Moreover, interestingly, only small subsets of genes are apparently affected by these epigenetic alterations, and these changes
appear also to be tissue specific. The identification of which tissues and genes are affected by various bioactive food components and how these changes affect health is a new and growing area of nutritional research.

1.2 Vitamin D

1.2.1 Production

Vitamin D is a fat-soluble vitamin that is naturally present in only a small number of foods, but is produced endogenously in the skin by UVB irradiation between 290-315 nm. The two major forms of vitamin D, which differ chemically in their side chains, are cholecalciferol and ergocalciferol. Cholecalciferol is known as vitamin D₃ and is produced in animals from cutaneous 7-dehydrocholesterol (7-DHC), while ergocalciferol is known as vitamin D₂ and is produced in plants from ergosterol, a plant sterol (Figure 2). Vitamin D₃ is translocated from the skin to bloodstream, bound in plasma to the vitamin D binding protein.

Figure 2 Production of vitamin D₂ and vitamin D₃: Photolysis of ergosterol in plants (a) and 7-dehydrocholesterol (b) in skin 7-DHC to previtamin D₂ (pre-D₂) and previtamin D₃ (pre-D₃) and their thermal isomerizations to vitamin D₂ (ergocalciferol) and vitamin D₃ (cholecalciferol), respectively. D₂ and D₃ differ only in the side chain in which D₂ has a double bond between C22–C23 and a methyl group at C24 (Bikle, 2010).
Vitamin D synthesized from sun exposure, or obtained from dietary sources is biologically inert and must undergo two hydroxylations to become the active hormone form (Figure 3).

Figure 3 Overview of vitamin D synthesis, intake, and activation (http://www.nap.edu/openbook.php?record_id=13050&page=76)
The first hydroxylation occurs in the liver due to the action of a P450 enzyme coded by the \textit{cyp2r1} gene and converts vitamin D to 25-hydroxyvitamin D (25(OH)D$_3$) known as calcidiol (Figure 3). The second hydroxylation occurs primarily in the kidney due to the action of the P450 enzyme (25-hydroxyvitamin D 1α-hydroxylase), coded by the \textit{cyp27b1} gene, and forms the physiologically active 1,25-dihydroxyvitamin D (1,25(OH)$_2$D$_3$) metabolite known as calcitriol. This vitamin D metabolite is the hormonal form of vitamin D that is responsible for most of its biological functions (Figure 3). The production of 1,25(OH)$_2$D$_3$ in the kidney is stimulated and tightly controlled by parathyroid hormone (PTH) and plasma phosphate concentration, which enhances the activity of renal 1α-hydroxylase, and is inhibited by raised plasma calcium and phosphate concentration. The 1α-hydroxylase is also inhibited by fibroblast growth factor 23 (FGF23), which is produced in bone cells. CYP24A1 gene encodes 24-hydroxylase that catalyzes the conversion of 25-hydroxyvitamin D$_3$ and 1,25-dihydroxyvitamin D$_3$ into 24-hydroxylated products, which constitute the degradation of the vitamin D molecule (Beckman et al., 1996, Holick, 2010).

\subsection*{1.2.2 Absorption, transport and metabolism}

Being fat-soluble, dietary vitamin D (both D$_2$ and D$_3$) is mixed with other fats and incorporated with the aid of bile salts into micelles in the small intestine from which it is absorbed and enters mucosal enterocytes by passive diffusion. Once absorbed, it is incorporated into chylomicrons, which are released by enterocytes and enter the lymphatic system. Lymphatic circulation transfers vitamin D in
chylomicrons into the blood stream. After vitamin D in chylomicron reaches the liver, 25-hydroxylation can occur and the 25-hydroxyvitamin D metabolite produced is attached to plasma vitamin D binding protein (DBP). Vitamin D binding protein and albumin are the primary transporters of vitamin D metabolites in blood (Wildman, 2011, SarDesai, 2012).

Since serum 25-hydroxyvitamin D (25(OH)D₃) is the predominant circulating form of vitamin D in the blood and increases in proportion to vitamin D intake and sunlight exposure, it is measured in the blood to determine the vitamin D status (Anderson et al., 2003). The unbound form of 25(OH)D₃ enters the kidney, where a second hydroxylation can occur by 1α-hydroxylase activity (25-hydroxyvitamin D-1α-hydroxylase, CYP27B1) forming the physiologically active 1,25-dihydroxyvitamin D (1,25(OH)₂D₃). Enzyme activity of CYP27B1 is predominant in the kidney, but is present in a number of non-renal tissues and cells, such as immune cells, pancreatic cells, muscle cells, adipocytes, etc. 1,25-dihydroxyvitamin D is the hormonal form of vitamin D responsible for most of its biological functions via its ability to activate the vitamin D receptor, a nuclear steroid hormone transcription factor. The production, which is controlled by parathyroid hormone, of 1,25(OH)₂D₃ from 25(OH)D₃ in the kidney acts to raise the serum calcium level to normal physiological range by stimulating intestinal calcium absorption via an increased expression of calcium transport proteins, such as TRPV6 and calbindin D. Low blood calcium level is associated with the increased release of parathyroid hormone from the parathyroid glands. In addition to its effect on 1α-hydroxylase to produce 1,25(OH)₂D₃, parathyroid hormone can stimulate the
release of calcium from bone and increase renal calcium reabsorption by increasing the activity of TRPV5 in the renal distal convoluted tubule.

The C-24 oxidation pathway plays a major role in the biodegradation of vitamin D metabolites in kidney (Figure 3) and other target tissues, and 24-hydroxylase (1,25(OH)_{2}D_3-24-hydroxylase, CYP24) functions in the catabolism of 1,25(OH)_{2}D_3 (Beckman et al., 1996, Holick, 2010). 24-hydroxylase converts 25(OH)D to 24,25 dihydroxyvitamin D (24,25(OH)_{2}D) or 1,25(OH)_{2}D to 1,24,25 trihydroxyvitamin D (1,24,25(OH)_{3}D). This hydroxylation is the first step of the C-24 oxidation pathway, which catabolizes vitamin D metabolites to the water-soluble calcitroic acid for its excretion by the kidney (Makin et al., 1989, Reddy and Tserng, 1989). Although CYP24 is highly expressed in the kidney tubule, it is also distributed in tissues including the intestine, osteoblasts, placenta, keratinocytes, and prostate. In general, CYP24 appears to be found wherever there is vitamin D receptor (VDR), which elicits the action of 1,25(OH)_{2}D_3. Overproduction of 1,25(OH)_{2}D_3 is regulated by a negative feedback mechanism, in that 1,25(OH)_{2}D_3 inhibits PTH synthesis and induces 24-hydroxylase (CYP24A1) activity, which catabolizes 1,25(OH)_{2}D_3 by hydroxylation on C24. Thus the biological activity of 1,25-dihydroxyvitamin D (1,25(OH)_{2}D_3) in a cell is determined mainly by the combination of the level of VDR expression and the local 24-hydroxylase activity (Anderson et al., 2003). However, other cellular factors may subsequently modulate vitamin D action by altering VDR genomic activity in the nucleus, such as epigenetic factors that influence chromatin structure.
1.2.3. Genomic actions

1.2.3.1. Calcemic functions

Production and accumulation of 1,25(OH)\(_2\)D\(_3\) is regulated either directly or indirectly by serum calcium concentration, and the major function of 1,25(OH)\(_2\)D\(_3\) is to increase the efficiency of intestinal calcium absorption, thereby increasing the availability of dietary calcium. As mentioned above, parathyroid hormone is the primary hormonal factor in determining synthesis of 1,25(OH)\(_2\)D\(_3\) in response to the concentration of free circulating calcium concentration (Garabedian et al., 1972). A drop in serum calcium concentration leads to induction of synthesis and secretion of parathyroid hormone, which then acts in the kidney to stimulate transcellular calcium reabsorption and in bone to stimulate osteoclast biogenesis (Thomas et al., 2001) and osteoclast bone-resorptive function. Calcium homeostasis in the blood is maintained by the coordinated regulation of intestinal calcium absorption, mobilization of calcium from bone, and renal reabsorption of calcium (DeLuca, 1981). Once these functions return serum calcium to normal, then 1,25(OH)\(_2\)D\(_3\) exerts a negative feedback signal on the parathyroid glands to suppress further synthesis and secretion of PTH (Cantley et al., 1985, Szabo et al., 1989). 1,25(OH)\(_2\)D\(_3\) also stimulates production of fibroblast growth factor (FGF23), produced primarily by bone by osteoblasts and osteocyte, and negatively control its own levels by reducing renal1α-hydroxylase (Kolek et al., 2005) (Figure 3).
1.2.3.2. Noncalcemic functions

The noncalcemic functions of 1,25-dihydroxyvitamin D include regulation of cell proliferation and differentiation, hormone secretion, and immunomodulation. A wide number of tissues and cells including the brain, gonads, pituitary gland, thymus, pancreas, stomach, breast, skin, adipocytes and lymphocytes possess vitamin D receptor (VDR) (Holick, 1995). The expression of VDRs in these tissues and cells suggests that they must serve functions there.

Proliferation and differentiation of normal and cancerous cells are affected by 1,25(OH)$_2$D$_3$ through directly or indirectly altering the transcription of cell regulatory genes, as the inhibition of the growth and differentiation of cancer by actions of 1,25(OH)$_2$D$_3$ was shown in leukemia and melanoma cells (Colston et al., 1981). Studies have shown that vitamin D inhibits adipogenesis by playing an important role in the cell growth and differentiation (Ishida et al., 1988, Kong and Li, 2006, Wood, 2008).

1,25(OH)$_2$D$_3$ has direct effects on the pancreatic islet cells and stimulates insulin secretion (Lee et al., 1994). VDR and calbindin-D28k, which was the first known target of vitamin D action and is a member of a family of high affinity calcium-binding proteins, are found in pancreatic beta cells (Morrissey et al., 1975). Calbindin-D28k can modulate depolarization-stimulated insulin release (Sooy et al., 1999) and protect beta cells against cytokine mediated destruction. A number of studies have suggested that vitamin D deficiency contributes to increased risk for type 2 diabetes mellitus (Bikle, 2009, Baz-Hecht and Goldfine, 2010).
1,25(OH)₂D₃ is a potent immune system modulator and can help in suppression of certain autoimmune diseases and cancers (DeLuca, 2004). It can induce phagocytic activity, cell surface antigen expression, and monocyte differentiation (Abe et al., 1981), and enhance lysosomal activity and interleukin 1 production. (Bar-Shavit et al., 1981, Holick, 1995). 1,25(OH)₂D₃ inhibits interleukin 2 production in activated T lymphocytes, and DNA synthesis and immunoglobulin (IgM and IgG) production in activated B lymphocytes (Provvedini et al., 1986).

At the molecular level, it has been shown that 1,25(OH)₂D₃ can inhibit adipogenesis through a vitamin D receptor (VDR)-dependent inhibition of CCAAT enhancer binding protein-alpha (C/EBPα) and peroxisome proliferator-activated receptor-gamma (PPARγ) expression in a dose-dependent and time-sensitive manner, a decrease in PPARγ transactivating activity, and a stabilization of the inhibitory VDR protein in the pre-adipocyte. Treatment of the 3T3-L1 mouse preadipocytes cell line with 1,25(OH)₂D₃ stabilizes the inhibitory VDR protein levels, which are normally increased during the early phase of adipogenesis, peaking at 4 – 8 hours and subsiding afterward throughout the rest of the differentiation (Kong and Li, 2006).

1.2.4. Molecular mechanisms of genomic actions of vitamin D

Nuclear receptors are a class of proteins found within cells and can act as ligand-inducible transcription factors that specifically regulate the expression of target genes involved in metabolism, development, and reproduction. Their primary function is to mediate the transcriptional response in target cells to steroid
hormones or other lipid soluble compounds. More than 100 nuclear receptors are known to exist and these proteins comprise the nuclear receptor superfamily (McKenna et al., 1999). All of the nuclear receptors have common structural features and are composed of several independent, but interacting, functional modules. These are an amino-terminal regulatory domain (A/B domain containing the activation function 1 (AF-1)), a DNA-binding domain (DBD; region C), a hinge region (region D), a ligand-binding domain (LBD; region E containing the activation function 2 (AF-2)), and a carboxyl-terminal domain (region F) (Giguère, 1999, Olefsky, 2001).

The vitamin D receptor is a member of the nuclear receptor superfamily that regulates the expression of vitamin D responsive gene and mediates the biological activity of its ligand 1,25(OH)2D3. The liganded VDR can form a VDR-RXR heterodimer that is able to enter the deep groove of DNA and recognize vitamin D responsive elements (VDREs) in promoter region of vitamin D target genes. Binding of 1,25(OH)2D3 to the VDR appears to cause a conformational change at the COOH terminus of the VDR, permitting the AF-2 domain (ligand-dependent transcriptional activation domain) to interact with other transcription factors including coactivator proteins that lead to activation of vitamin D target gene transcription (Masuyama et al., 1997). Some of these coactivator proteins are known to possess intrinsic histone acetylase activity and are capable of chromatin modification by histone acetylation (Jones et al., 1998, Haussler et al., 2008). Therefore, a large multiprotein coactivator complex, which assembles on the heterodimeric VDR-RXR complex, functions to remodel condensed chromatin and can interact with RNA polymerase II located at
the transcriptional start site (Näär et al., 2001). These interactions are necessary to alter the rate of gene expression (Figure 4).

Figure 4 Genomic actions of 1,25(OH)₂D₃. 1,25(OH)₂D₃ molecules pass through the plasma membrane with the help of vitamin D binding protein (DBP). Ligand binding to the VDR induces a conformational change in the receptor and subsequent heterodimerization with RXR on vitamin D responsive element (VDRE). Corepressor (CoR) proteins are released from the surface of the VDR, allowing interaction with coactivator (CoA) proteins. These molecules modulate chromatin structure and allow the interaction of the receptor with the RNA polymerase II transcriptional complex (POL II), thus activating transcription of the target gene (Seshadri et al., 2011).

The VDR interacts with other transcription factors, such as transcription factor IIB (TFIIB), and coactivator proteins, such as SRC-1 and TIF-1. Nuclear receptor coactivator protein (ACTR) has been shown to enhance transcription from a vitamin D target gene (Chen et al., 1997, McKenna and O'Malley, 2002). Several corepressor proteins, NCoR, SMRT, and Alien, have been shown to interact with the
VDR and block VDR-mediated transcriptional activities (Polly et al., 2000). The hairless gene product HR is another protein that binds directly to VDR and VDR-mediated transactivation is strongly inhibited by HR in a manner relieved by 1,25(OH)_{2}D_{3} (Xie et al., 2006, Bikle, 2009, Malloy et al., 2009). These corepressors typically bind VDR in the absence of 1,25(OH)_{2}D_{3} and are displaced when 1,25(OH)_{2}D_{3} binding recruits the coactivators to the VDR. Corepressors recruit histone deacetylases that repress transcription by removing acetyl groups from histone proteins in the nucleosome and stabilizing chromatin (Marks et al., 2001).

Various genes have been shown to be responsive to vitamin D and most of these genes play a direct role in calcium endocrinology or bone formation including osteocalcin, osteopontin, PTH, the hydroxylases cyp24 and cyp27b1, and the calbindin genes. Expression of the bone protein osteocalcin, which is secreted by osteoblasts and play a significant role in maintaining bone integrity, is upregulated by 1,25(OH)_{2}D_{3}, whereas several genes have been shown to be downregulated by 1,25(OH)_{2}D_{3}, including the gene for PTH, which plays a critical role in controlling the levels of 1,25(OH)_{2}D_{3} in serum. Calbindins are another set of genes that have been shown to be regulated by vitamin D. Calbindin D9K, which plays a role in absorption of calcium from the mammalian gut, is expressed primarily in upper small intestine, whereas the calbindin D28K, which may be involved in calcium reabsorption from glomerular filtrate, is expressed in mammalian kidney and other tissues (Jones et al., 1998). 1,25(OH)_{2}D_{3} stimulates the differentiation of many cell types by through induction of arrays of genes and stimulation of signal transduction pathways (Marks et al., 2000, Samuel and Sitrin, 2008). Phospholipase C (PLC)
activity is upregulated by 1,25(OH)$_2$D$_3$ at the transcriptional level in normal human keratinocytes via a DR6 type vitamin D responsive element (VDRE) in the PLC-1 promoter (Xie and Bikle, 1997).

The CYP24 hydroxylase, an immediate target gene of VDR, is one of the most highly regulated genes that respond to vitamin D. In the presence of ligand, it is a sensitive marker for VDR function, because its promoter region contains multiple VDREs and its transcription is very responsive to 1,25(OH)$_2$D$_3$ treatment (Chen and DeLuca, 1995, Alimirah et al., 2010). 1,25(OH)$_2$D$_3$ is thought to be the sole physiological inducer of 24-hydroxylase activity (Shinki et al., 1992). In the proposed studies, I plan to use cyp24 gene expression as a measure of VDR transcriptional activity to assess to what extent histone deacetylase inhibitors (trichostatin A and sulforaphane) alter cellular vitamin D action.

1.2.5. Molecular mechanisms of non-genomic actions of vitamin D

1,25(OH)$_2$D$_3$ also exerts biologic effects that do not appear to require gene regulation and may work through a membrane receptor rather than its nuclear hormone receptor. 1,25(OH)$_2$D$_3$ has been shown to be a biological regulator of calcium channels (Yukihiro et al., 1994) by acting as a potent modulator of calcium channel function in osteosarcoma cells and affecting the intracellular Ca$^{2+}$-dependent signaling processes (Caffrey and Farach-Carson, 1989). It regulates chloride channel activity by promoting the rapid enhancement of outwardly rectifying Cl$^-$ currents in a concentration-dependent manner in osteoblasts (Zanello and Norman, 1997), and plays a role in protein kinase C activation and distribution.
by increasing the breakdown of membrane phosphoinositides, raising intracellular calcium concentration, and translocating protein kinase C (PKC) from the cytosolic to the particulate fraction of Caco-2 cells (Bissonnette et al., 1994). These rapid nongenomic effects occurring in minutes could be elicited by the classical nuclear VDR acting at the cytoplasmic membrane or by a separate membrane VDR, and have been most extensively studied in the intestine (Bikle, 2009). Transcaltachia, which describes the rapid hormonal stimulation of intestinal calcium transport, occurs via nongenomic mechanisms, which involve a plasma membrane receptor (Nemere et al., 1984). The physiological role of Transcaltachia is unknown.

1.3 Epigenetic modulation of gene expression

Conrad Waddington originally defined epigenetics as “the study of the causal interactions between genes and their products, which bring the phenotype into being”, referring to all molecular pathways modulating the expression of a genotype into a particular phenotype. Today, epigenetics has been generally accepted as “the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence, including histone variants, posttranslational modifications of amino acids on the amino-terminal tail of histones, and covalent modifications of DNA bases.” (Dupont et al., 2009). Epigenetic modifications altered by external or internal environmental factors can change gene expression and maintain the acquired phenotype permanently. Thus, epigenetics can provide a new model for the search for etiological factors of many diseases, in particular environment-associated diseases known to be affected by many
environmental factors (Choi and Friso, 2010). Epigenetic modification includes DNA methylation, histone modifications, and chromatin remodeling.

1.3.1 Histone acetylation

Histone proteins and their posttranslational modifications have an important role in chromatin structure, nucleosome assembly, and regulation of gene transcription. Histone acetylation is one of the most extensively studied histone modifications associated with transcriptional activation (Figure 5) (Dupont et al., 2009).

![Diagram of histone acetylation and deacetylation](http://www.biotek.com/resources/articles/cellular-lanthascreen-histone.html)

**Figure 5** Regulations of chromatin structure and transcription by activities of HATs and HDACs. The amino termini of histones, which are called histone tails, can be post-translationally modified. HAT acetylates lysine residues (K) of the histones and HDAC removes the acetyl group (http://www.biotek.com/resources/articles/cellular-lanthascreen-histone.html).

A nucleosome is a building block of chromatin and consists of double-stranded DNA and an octamer of four different (H2A, H2B, H3, H4) histones.
Chromatin can regulate transcriptional processes through modifications of DNA and the histone. The location of the reversible histone modifications is at the N-terminal histone tails, where the lysine residues are on the surface of the nucleosome. (Choi and Friso, 2010). Acetylation of the lysine residues neutralizes the positive charges on the lysine amino groups and decreases their affinity for DNA, thus weakening the association between histones and DNA and altering the nucleosomal conformation (Figure 6. (Hong et al., 1993). This chromatin conformational change can increase the accessibility of transcriptional regulatory proteins to chromatin templates, leading to increased transcriptional activity (Struhl, 1998). Histone acetylation status is modulated by histone acetyltransferase (HAT) and histone deacetylase (HDAC) activity (Figure 5, (Zhang et al., 2002)).

Figure 6 Acetylation and deacetylation of the lysine residue. A HAT catalyzes the transfer of an acetyl group from acetyl CoA to the amino group of the lysine residue. HAT, histone acetyltransferase; HDAC, histone deacetylase (Yang and Seto, 2007).
1.3.2 Histone deacetylase inhibitors (HDACIs)

Histone deacetylases (HDACs) catalyze the removal of acetyl groups on the amino-terminal lysine residues of core nucleosomal histones. This activity is associated generally with transcriptional repression (Figure 5). Histone deacetylase inhibitors (HDACIs) inhibit the activity of HDAC and induce hyperacetylation of core histones, thereby modulating the chromatin structure and affecting the gene expression (de Ruijter et al., 2003)

*Inhibitors of HDAC activity can induce upregulation and downregulation of a small subset of cellular genes, suggesting that HDACIs act selectively on specific genes.*

In fact, HDACIs alter the transcription of only approximately 2% of expressed genes with a variable transcriptional outcome (Van Lint et al., 1996, Marks et al., 2001, Moreira et al., 2003). Furthermore, chromatins of normal and transformed cells are differentially affected by histone deacetylase inhibitors. Normal cells are up to ten times more resistant to histone deacetylase inhibitor (HDACI)-induced cell death compared with transformed cells due to the difference in chromatin conformation and accessibility between normal and transformed cells (Kim et al., 2003). The transformed cells have different nuclear sizes and cellular shapes compared with the normal cells (Zink et al., 2004). In transformed cells, acetylation of K16 on histone H4 (H4-K16Ac) is markedly reduced (Fraga et al., 2005), and levels of histone acetylation can directly impact chromatin accessibility at specific loci (Ehrenhofer-Murray, 2004). Altered chromatin structure may increase the accessibility to the histone tails, indicating that the structural characteristic of transformed cells predispose them to HDACI sensitivity. However, the mechanisms
that affect chromatin structure and the molecular processes underlying this selectivity for transformed cells are still not well understood (Nalabothula and Carrier, 2011).

Sodium butyrate, phenylbutyrate, trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA), trapoxin (TPX), MS-27-275, apicidin, oxamflatin, and FR901228 are the known compounds that inhibit HDAC activity and cause a variety of effects, including cell growth inhibition, cell differentiation and apoptotic cell death (Moreira et al., 2003).

1.3.2.1 Trichostatin A (TSA)

![Trichostatin A (TSA)](image)

*Figure 7* Trichostatin A (TSA, 7-[4-(dimethylamino)phenyl]-N-hydroxy-4,6-dimethyl-7-oxohepta-2,4-dienamide)

There have been many histone deacetylase inhibitors discovered, but the most potent is trichostatin A (TSA, 7-[4-(dimethylamino)phenyl]-N-hydroxy-4,6R-dimethyl-7-oxo-2E,4E-heptadienamide, Figure 7). TSA is a fermentation product of Streptomyces and belongs to the hydroxamic acid family. Originally it was used as an anti-fungal agent, but later it was found to have potent proliferation-inhibitory
properties with cancer cells. TSA is effective at nanomolar concentrations in vitro (Yoshida et al., 1990, de Ruijter et al., 2003). It interacts reversibly with the HDAC catalytic site to prevent binding of the substrate and leads to histone acetylation (Finnin et al., 1999). It has been known to induce cell cycle arrest, differentiation, and apoptosis. (Finnin et al., 1999, Marks et al., 2000, Tóth et al., 2004). Table 1 provides information on the experimental parameters tested in published TSA studies. These studies show that TSA modified histone acetylation status and gene expression levels of some genes at nano- or micromolar concentrations. TSA at concentration of 1 μM was used in 3T3L1 cells in my study.
### Table 1: TSA study experimental parameters

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>TSA Dosage</th>
<th>Exposure Time</th>
<th>Outcome Measure</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human breast carcinoma cells MDA 468 and 435, human bladder carcinoma cells T24</td>
<td>0.1, 0.3, 1 (μM)</td>
<td>24 h</td>
<td>Histone hyperacetylation, p21, Hep27, α-Fucosidase, Histone H2B, TRPM-2, α-Tubulin, Glutaredoxin, Metallothionein 1L, Thymidylate synthetase, TRP, Importin β, APRIL, CTP synthase expression, acetylation of α-tubulin in T24 cells and expression of a core set of genes</td>
<td>p21, Hep27, α-Fucosidase, Histone H2B, TRPM-2, α-Tubulin, Glutaredoxin, Metallothionein 1L were up-regulated by 2.8 - 48.3 folds, Thymidylate synthetase, TRP, Importin β, APRIL, CTP synthase were down-regulated by 3.7 - 7.4 folds.</td>
<td>(Glaser et al., 2003)</td>
</tr>
<tr>
<td>HeLa-H2A-YFP cell line</td>
<td>12.5, 25, 50, 100, 200 (ng/ml)</td>
<td>2, 6, 12, 24 h</td>
<td>Histone acetylation on the interphase chromatin conformation</td>
<td>Cell density was reduced to about 50%</td>
<td>(Tóth et al., 2004)</td>
</tr>
<tr>
<td>Human prostate cancer cell line LNCaP, human breast cancer cell lines T-47D, ZR-75-1</td>
<td>100, 200 (ng/ml)</td>
<td>24 h</td>
<td>Expression of housekeeping genes including 18S rRNA, β-actin, GAPDH and ribosomal highly-basic 23-kDa protein (rb 23-kDa, RPL13A)</td>
<td>Either up-regulated (LNCaP and ZR-75-1 at 100 ng/ml TSA) or down-regulated (prostate explants, T-47D, LNCaP and ZR-75-1 at 200 ng/ml TSA)</td>
<td>(Mogal and Abdulkadir, 2006)</td>
</tr>
<tr>
<td>Human prostate BPH-1 cells, prostate cancer PC-3 and LNCaP cells</td>
<td>100 ng/ml</td>
<td>48 h</td>
<td>P21Cip1/Waf1 and Bax protein expression, HDAC activity level, acetylated histone levels, p21 expression, multi-caspase activity</td>
<td>Lowered HDAC activity by 30%</td>
<td>(Myzak et al., 2006)</td>
</tr>
<tr>
<td>Human prostate cancer cell lines; DU145, LNCaP, PC3</td>
<td>50, 100, 200, 300, 400 (nM)</td>
<td>8 h</td>
<td>Expression of CYP24A1 mRNA</td>
<td>CYP24A1 enzyme activity was increased by 1.6 - 4 fold</td>
<td>(Luo et al., 2010)</td>
</tr>
<tr>
<td>Mouse 3T3-L1 cells</td>
<td>100 nM</td>
<td>1, 2, 4, 6, 8 days</td>
<td>Expression of adipogenic marker genes: LIPE, AdipoQ, and adip, expression of PPARγ and Fabp4, and SREBP-1c or C/EBPβ</td>
<td>HDACs block adipogenesis.</td>
<td>(Haberland et al., 2010)</td>
</tr>
<tr>
<td>Human lung fibroblasts 2BS, Human embryonic kidney HEK293T</td>
<td>1, 2 (μM)</td>
<td>12, 24 h</td>
<td>Changes of HBP1 acetylation, p16INK4A expression</td>
<td>Transcriptional activity of p16INK4A promoter was increased to about 10-fold</td>
<td>(Wang et al., 2012)</td>
</tr>
</tbody>
</table>
Bioactive food components are defined as a constituent in foods or dietary supplements, other than those needed to meet basic nutritional needs that may be responsible for changes in health status. Sulforaphane (1-isothiocyanato-4-(methylsulfinyl)butane or SFN, Figure 8) is a bioactive food component found in cruciferous vegetables, such as broccoli, cauliflower, brussels sprouts, cabbages or kale. It is a naturally occurring sulfur-containing isothiocyanate derivative produced during the hydrolysis of glucosinolates. When cruciferous vegetables are chopped or chewed, myrosinase interacts with glucosinolates and releases isothiocyanates from their precursors (Figure 9. (Zhang, 2004)).
Sulforaphane has multiple biological effects, including an ability to affect xenobiotic metabolism by inhibiting and inducing various biotransformation enzymes, and to alter gene expression by inducing epigenetic modifications (Mahéo et al., 1997, Basten et al., 2002, Myzak et al., 2004). It also exhibits many other biological activities such as preservation of normal cell cycle regulation (Chiao et al., 2002), inhibition of proliferation and induction of apoptosis (Singh et al., 2009), anti-inflammatory activity (Heiss et al., 2001), antibacterial activity (Fahey et al., 2002), and antidiabetic activity (Song et al., 2009). Furthermore, sulforaphane is known to be a histone deacetylase inhibitor, which induces acetylation of chromatin protein and affects the expression of genes by modulating transcriptional activation of the genes. Myzak et al. reported that SFN acted as HDACIs with evidence of altered histone acetylation status and increased p21\(^{Gp1/Waf1}\) expression in human embryonic kidney 293 cells and HCT116 colon cancer cells (Myzak et al., 2004). Table 2 provides information on the experimental parameters tested in published SFN studies. These studies show that SFN modified histone deacetylase activity level or histone acetylation status, and gene expression levels of some genes at various micromolar concentrations. SFN at concentration of 5 μM was used in 3T3L1 cells in my study.
### Table 2 SFN study experimental parameters

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>SFN Dosage</th>
<th>Exposure Time</th>
<th>Outcome Measure</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human embryonic kidney 293 (HEK293) cells, Human HCT116 colorectal cancer cells</td>
<td>3, 9, 15 (µM)</td>
<td>47 h</td>
<td>HDAC activity; activity of a β-catenin-responsive reporter (TOPflash) p21&lt;sup&gt;Cip1/Waf1&lt;/sup&gt; expression</td>
<td>SFN alone increased reporter activity by 3.9-fold and &gt;8-fold in combination with TSA (100 ng/ml). About 4-fold increase in p21&lt;sup&gt;Cip1/Waf1&lt;/sup&gt; expression</td>
<td>(Myzak et al., 2004)</td>
</tr>
<tr>
<td>Human prostate BPH-1 cells, prostate cancer PC-3 and LNCaP cells</td>
<td>3, 9, 15 (µM)</td>
<td>48 h</td>
<td>P21&lt;sup&gt;Cip1/Waf1&lt;/sup&gt; and Bax protein expression, HDAC activity level, acetylated histone levels, p21 expression, multi-caspase activity</td>
<td>SFN (15 µM, for 48 h) inhibited HDAC activity by 40, 30 and 40%, p21 protein expression was increased by 50–100%, and pro-apoptotic protein Bax was increased by 50%</td>
<td>(Myzak et al., 2006)</td>
</tr>
<tr>
<td>Human colorectal cancer Caco-2, HT-29 and SW480 cells</td>
<td>1, 10, 20 (µM)</td>
<td>24 and 48 h</td>
<td>Expression of HBD-2, VDR, PPARγ, ERK1/2, phospho-ERK1/2, p38 MAPK and phospho-p38 MAPK</td>
<td>SFN (20 µm) increased β-defensin-2 mRNA expression up to 5.5-fold (HT-29) and HBD-2 protein expression up to 104% (Caco-2)</td>
<td>(Schwab et al., 2008)</td>
</tr>
<tr>
<td>Human prostate cancer LNCaP and C4-2 cells</td>
<td>10, 20 or 40 (µM)</td>
<td>24 h</td>
<td>Levels of androgen receptor (AR) protein, Ser210/213-phosphorylated AR, prostate specific antigen (PSA) protein and AR mRNA</td>
<td>40–80% decrease in AR promoter activity. Phosphorylated AR, PSA protein, and AR mRNA levels in both cell lines were decreased</td>
<td>(Kim and Singh, 2009)</td>
</tr>
</tbody>
</table>
Sulforaphane can readily permeate the enterocytes in the perfused human jejunal segment. At a physiologically relevant concentration $11.0 \pm 2.7 \ \mu$M, the percentage of absorption ($\pm$ S.D.) is $74 \pm 29\%$ (Petri et al., 2003). Chewing of raw cruciferous vegetables may increase glucosinolate contact with plant myrosinase and increases the amount of isothiocyanates absorbed, and the myrosinase activity of human intestinal bacteria also allows for some formation and absorption of isothiocyanates (Shapiro et al., 1998). It has been estimated that the amount of SFN in 1 g of dry broccoli florets ranges from 507 to 684 $\mu$g (Campas-Baypoli et al., 2010) and approximately 20 $\mu$mol/l SFN can be derived from consumption of 50 g of broccoli (Chuang L.T. et al., 2013). The bioavailability study showed that higher amounts of SFN were found in the blood and urine when 200 g broccoli was eaten raw (bioavailability of 37%) versus cooked (3.4%). Absorption of SFN was delayed when cooked broccoli was consumed (peak plasma time of 6 h) compared to raw broccoli (1.6 h). Half-lives of excretion were comparable, 2.6 hours and 2.4 hours on average, for raw and cooked broccoli, respectively (Vermeulen et al., 2008).

Glucosinolates are hydrolyzed by myrosinase, an enzyme found in plants and bowel microflora and completely inactivated by heat, to form isothiocyanates. In vivo, isothiocyanates are conjugated with glutathione and then sequentially metabolized to mercapturic acids, which are found in the urine (Shapiro et al., 2001). These isothiocyanate metabolites are highly correlated with dietary intake of cruciferous vegetables (Seow et al., 1998).
1.3.3 Epigenetic modulation of vitamin D-mediated gene expression by HDACI

Various studies (Gommersall et al., 2004b, Banwell et al., 2006, Wang et al., 2008, Luo et al., 2010, Schwab et al., 2008) have revealed that the function of 1α,25(OH)₂D₃ can be enhanced in combination with HDACIs, thereby modulating the expressions of vitamin D-mediated genes. Table 3 shows the studies that demonstrated the modulated effects of vitamin D-mediated gene expression by HDACIs.

TSA activity on CYP24A1 expression was tested in prostate cancer cells and the result showed that the treatment with trichostatin A (TSA) enhanced the expression of CYP24A1 in LNCaP and PC3 cells and induced its expression in a dose-dependent and 1,25(OH)₂D₃-dependent manner (Luo et al., 2010). Furthermore, the epigenetic effect of HDACI activity on the binding of the VDR to the CYP24A1 promoter region was also tested in PC3 cells treated with TSA and a significant increase in binding of VDR to the CYP24A1 promoter with TSA treatment was observed (Luo et al., 2010).
Table 3 Modulation of vitamin D-mediated gene expression by HDACIs

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Dosage of HDACI and 1α,25(OH)₂D₃</th>
<th>Exposure Time</th>
<th>Gene/Protein</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostate cancer cell lines; PC-3</td>
<td>15 nM TSA 100 nM 1α,25-(OH)₂D₃</td>
<td>7 h</td>
<td>GADD45α</td>
<td>Induction of gene expression was increased by 4.3-fold</td>
<td>(Gommersall et al., 2004a)</td>
</tr>
<tr>
<td>PZ-HPV-7 cell line</td>
<td>10 μM SAHA 50 nM 25-(OH)D₃</td>
<td>24 h</td>
<td>1α-OHase</td>
<td>2-fold increase in 1α-OHase promoter activity and more than a 30-fold stimulation in 1α-OHase mRNA expression</td>
<td>(Wang et al., 2008)</td>
</tr>
<tr>
<td>Human prostate cancer cell lines; DU14, LNCaP, PC3</td>
<td>50, 100, 200, 300, and 400 nM TSA, 100 nM 1α,25(OH)₂D₃</td>
<td>8 h for TSA followed by 24h for 1α,25-(OH)₂D₃</td>
<td>CYP24A1</td>
<td>CYP24A1 enzyme activity was increased by about 1.6–4 fold and CYP24A1 mRNA expression was increased by about 4–10 fold</td>
<td>(Luo et al., 2010)</td>
</tr>
<tr>
<td>Human colorectal cancer Caco-2, HT-29 and SW480 cells</td>
<td>10 - 20 μM SFN</td>
<td>24 and 48 h</td>
<td>VDR</td>
<td>Increased the production</td>
<td>(Schwab et al., 2008)</td>
</tr>
<tr>
<td>SCC4, MDA-MB231</td>
<td>100 nM Triciferol</td>
<td>8 h</td>
<td>1,25D₁-dependent gene (cdkn1c/kip2, alox12, and pex) VDR</td>
<td>Gene regulatory profile is distinct from that of 1,25D</td>
<td>(Tavera-Mendoza et al., 2008)</td>
</tr>
</tbody>
</table>

| | | | Cyp24 | Induced strong expression within a factor of ≈10 as potent as 1,25D | |
| | | | p57kip2 (cdkn1c/kip2) | Markedly up-regulated | |
| | | | Col13a1 | Induced VDR binding to the VDRE | |
| | | | P160 coactivator AIB1 | Induced its recruitment to VDR-bound target genes | |

TSA can upregulate the expressions of target genes of VDR and combined use of 1α,25(OH)₂D₃ and TSA may be an effective therapeutic for some cancers (Gommersall et al., 2004b). Combinations of 1α,25(OH)₂D₃ compounds
(1α,25(OH)₂D₃ or its analogue 1α,25-dihydroxy-16,23Z-diene-26,27-hexafluoro-19-nor 1α,25(OH)₂D₃ (RO-26-2198), 100 nmol/L) with TSA (15 nmol/L) restored VDR antiproliferative signaling in breast cancer cell lines T-47D, ZR-75-1, MCF-7, and MDA-MB-23. Cotreatment with RO-26-2198 and TSA showed significant enhancement of mRNA accumulation of target genes GADD45α, VDUP-1 and CYP24 in MDA-MB-231 cells notably at 12 hours (Banwell et al., 2006).

Triciferol, a hybrid molecule in which the 1α,25(OH)₂D₃ side chain is replaced with TSA, binds directly to the VDR ligand binding domain and functions as an agonist with 1α,25(OH)₂D₃-like potency on several 1α,25(OH)₂D₃ target genes. Triciferol (100 nM) induced strong cyp24 expression and exhibited more efficacious antiproliferative and cytotoxic activities than 1α,25(OH)₂D₃ (100 nM) alone in human squamous carcinoma cells SCC4 and SCC25, and human breast cancer cells MCF-7 and MDA-MB231 (Tavera-Mendoza et al., 2008).

Suberoylanilide hydroxamic acid (SAHA) increases the expression of genes that modulate cell cycle progression, tumor suppression, differentiation and apoptosis in PZ-HPV-7 prostate cells. SAHA upregulates the expression of the 1α-hydroxylase gene (CYP27B1) that controls the synthesis of 1α,25(OH)₂D₃ regulating prostate growth and differentiation in an autocrine/paracrine fashion (Wang et al., 2008).

Schwab et al. demonstrated that sulforaphane (SFN, 10–20 μmol/L), a dietary HDACI, increased the production of VDR protein in HT-29 and Caco-2 cells (Schwab et al., 2008) after 24-hour treatment.
1.4 Vitamin D and adipogenesis in 3T3L1 Cells

Adipocyte differentiation, adipogenesis, is the process of development of fat cells from preadipocytes, and has been one of the most intensely studied models of cellular differentiation. To investigate the regulatory mechanism of adipocyte differentiation, many studies have utilized the well-established mouse 3T3-L1 preadipocyte experimental systems as a cell culture model (Ishida et al., 1988, Lagace and Nachtigal, 2004, Kong and Li, 2006). The 3T3-L1 cells are fibroblastic cells already determined to the adipocyte lineage and originally derived from mouse embryos. Fully differentiated 3T3-L1 adipocytes possess most of the morphological, biochemical, and hormonal response characteristics of adipocyte (Gregoire, 2001).

Peroxisome proliferator-activated receptor-gamma (PPARγ) and CCAAT enhancer binding protein-alpha (C/EBPα) are essential in adipocyte differentiation. PPAR family and the CCAAT/enhancer-binding proteins are important transcriptional factors involved in the regulation of differentiation. PPARs are members of the nuclear-receptor superfamily and nuclear transcription factors, and form heterodimers with RXRs prior to binding of DNA in the promoter of PPAR-responsive genes (Chu et al., 1995). Permanent exit from the cell cycle with the coexpression of PPARγ and C/EBPα establishes the irreversible commitment to adipocyte differentiation (Shao and Lazar, 1997). C/EBPα terminates the mitotic clonal expansion preceding entry into the terminally differentiated state (Umek et al., 1991) and activates several adipogenic genes that create and maintain the adipocyte phenotype by autoactivating transcription of its own gene (Lin et al., 1993).
1α,25(OH)$_2$D$_3$ inhibits differentiation of preadipocyte to adipocyte (Ishida et al., 1988) and reduces triacylglycerol accumulation by 50% of that of fully differentiated control cells through a vitamin D receptor (VDR)-dependent inhibition of C/EBPα and PPARγ expression (Kong and Li, 2006, Wood, 2008). Kong et al. demonstrated the molecular mechanism whereby 1α,25(OH)$_2$D$_3$ inhibited 3T3-L1 cell adipogenesis by inhibiting only the early events of adipogenesis in which 1α,25(OH)$_2$D$_3$ could effectively inhibit 3T3-L1 cell adipogenesis within the first 48 hours after the differentiation program was initiated in a dose-dependent manner (from $10^{-11}$ to $10^{-7}$ M). The blockade of 3T3-L1 cell differentiation by 1α,25(OH)$_2$D$_3$ involves direct suppression of C/EBPα and PPARγ upregulation, antagonization of PPARγ activity, and stabilization of the inhibitory VDR protein (Kong and Li, 2006).

Vitamin D receptor (VDR) mediates 1α,25(OH)$_2$D$_3$-mediated inhibition of adipogenesis in 3T3-L1 cells. The study by Kong et al. demonstrated that the level of VDR protein was increased during this early phase of adipogenesis, peaked at 4-8 hours after the cells were switched to differentiation media (hormonal cocktail containing insulin, dexamethasone, and isobutyl methylxanthine) containing $10^{-8}$ M 1α,25(OH)$_2$D$_3$, and subsided afterward along the progression of the differentiation so that the VDR protein became barely detectable in mature adipocytes at day 8. The study also showed that overexpression of VDR in 3T3-L1 cells blocked adipogenesis. These results indicate that 1α,25(OH)$_2$D$_3$ treatment increased and stabilized the VDR levels and confirms that VDR is inhibitory on 3T3-L1 cell adipogenesis (Kong and Li, 2006). Given that VDR and PPARγ share the same heterodimeric partner RXR, Kong et al. suggested that VDR directly suppress the transacting activity of
PPARγ by sequestering the limited amount of RXR in 3T3-L1 cells. (Kong and Li, 2006).
CHAPTER 2

PURPOSE OF THE STUDY

Although some studies have examined the effects of TSA on 1,25-dihydroxyvitamin D mediated gene expression in various cell types including human prostate cancer cells (Luo et al., 2010), human lung fibroblasts, human embryonic kidney cells (Wang et al., 2008), and human colorectal cancer cells (Schwab et al., 2008), to my knowledge, no studies have investigated the role of TSA in murine 3T3-L1 in preadipocytes or other fat cell lines. In addition, the ability of the dietary histone deacetylase inhibitor SFN to alter VD/VDR–mediated gene expression has not been explored in any cell types.

The long-term objective of this line of research is to determine to what extent bioactive food components can be used to promote health. The objective of this study is to determine whether epigenetic modification can increase vitamin D-induced gene expression in adipocytes. This hypothesis will be tested by successfully completing the following Specific Aim:

Specific Aim: Enhancement of vitamin D cellular action by epigenetic modification

Changes in gene expression can drive changes in cellular function. Recently, there has been increased appreciation of the role of epigenetic modification in the control of gene expression. Moreover, some bioactive food components, such as SFN, have been shown to cause epigenetic changes in nuclear chromatin due to histone
deacetylase inhibitor activity. The \textit{working hypothesis} tested in this specific aim is that treatment of 3T3-L1 mouse preadipocytes with the histone deacetylase inhibitors (tricostatin A or sulforaphane) will increase 1,25(OH)$_2$ vitamin D-induced gene expression, as evidenced by an increase in CYP24 mRNA levels following 1,25(OH)$_2$ vitamin D and HDACI treatment compared to 1,25(OH)$_2$ vitamin D alone. This hypothesis is based on strong support in the published literature showing that the expression of certain genes are increased following treatment with histone deacetylase inhibitors, and a specific report that vitamin D-induced CYP24 gene expression in human squamous carcinoma cell lines and human breast cancer cell lines are increased by treatment with triciferol, a hybrid 1,25(OH)$_2$ vitamin D molecule with a modified side chain containing tricostatin A (Tavera-Mendoza et al., 2008). To our knowledge, modulation of vitamin D-induced gene expression by sulforaphane, a natural histone deacetylase compound found in cruciferous vegetables such as broccoli and cabbage, has not been investigated, nor has the effects of any HDACIs in preadipocytes.

The rationale for performing this research is that since 1,25(OH)$_2$ vitamin D has been shown to influence gene expression and adipogenesis in 3T3-L1 preadipocytes and other adipocyte cell lines, the findings of this research will be significant in advancing our understanding of how altered intake of bioactive food components that cause specific epigenetic changes in cells may modulate the action of the vitamin D hormone in adipose tissue. These findings could have future implications in the design of vitamin D intervention studies to influence adipose
metabolism and help combat obesity, or other diseases in which vitamin D may be involved.
3.1. Experimental design

My experiment investigated the effect of two histone deacetylase inhibitors, TSA and SFN, on 24-hydroxylase CYP24 gene expression levels. The 3T3-L1 preadipocytes cell culture system was used for the study due to their well-documented response to vitamin D (Sato and Hiragun, 1988, Kamei et al., 1993, Kong and Li, 2006). I measured CYP24 expression levels by PCR-based quantitation of CYP24 mRNA following incubation of cells with 1,25(OH)$_2$ vitamin D alone and 1,25(OH)$_2$ vitamin D in combination with either 1µM TSA or 5µM SFN. CYP 24 induction is a well-known measure of 1,25(OH)$_2$ vitamin D responsiveness in cells. In order to confirm the cells’ dose-dependent responses to 1,25(OH)$_2$ vitamin D, three different 1,25(OH)$_2$ vitamin D concentrations of 0, 1, and 10 nmol/L were used. The incubation time of 24 hours was kept as a constant. Concentrations of TSA and SFN and incubation period were set in accordance with literature values (Schwab et al., 2008, Wang et al., 2012).

3.2. Cell culture and treatments

Mouse 3T3-L1 preadipocytes cells were grown in 6-well culture plates to 100% confluence in growth media (89% DMEM, 10% calf serum, 1% pen-strep). The media was changed with fresh growth media every 48 hours. At confluence, cells were treated with 3 different concentrations of hormonal form of vitamin D (1,25-dihydroxycholecalciferol 0 nM, 1 nM, or 10 nM) alone the 3 different
concentrations of 1,25(OH)$_2$ vitamin D in combination with either 1μM TSA (Trichostatin A, Sigma-Aldrich) or 5μM SFN (Sulforaphane, Sigma-Aldrich) for 24 hours. An experiment consisted of 6 different treatment combinations that were performed in three 6-well plates, and so there were 2 replicate wells for each treatment combination including vehicles (no vitamin D treatment (control); 2 different concentrations of 1,25(OH)$_2$ vitamin D; 1 and 10 nmol/L with either 1μM TSA or 5μM SFN). The experiment was repeated 7 times (n = 7). In additional experiments, 1,25(OH)$_2$ vitamin D dose-response experiment at 0, 1, 2, 5 and 10 nM were also conducted in the presence and absence of 5μM SFN. These experiments were repeated 3 times (n = 3).

3.3. Gene Expression Analysis

3.3.1. RNA isolation

After 24 hours treatment incubation, the media was discarded and cells were harvested to assess 24-hydroxylase mRNA expression. Cells were scraped in 1 mL Trizol reagent (Invitrogen) and RNA was isolated from the treated cells according to the manufacturer’s protocol. In brief, after collecting Trizol-treated cells in the tube, 200μL chloroform was used for phase-separation. To get the total RNA pellet, RNA obtained from the upper aqueous phase layer was precipitated in 100% isopropanol (1:1), centrifuged to obtain an RNA pellet, and then washed with 75% ethanol followed by centrifugation. After discarding the supernatant, the washed RNA pellet was resuspended in DEPC-treated water (20μl) and then heated to 55°C for 10 min.
3.3.2 RNA Quantification

2μl of the RNA sample in DEPC-treated water was diluted (1:500) in 998μl DEPC-treated water and read at OD of 260nm on a spectrophotometer to determine RNA concentration.

3.3.3 Reverse transcriptase PCR (RT-PCR)

First-strand cDNA was synthesized from isolated RNA by reverse transcriptase with SuperScript III First-Strand Synthesis System for RT-PCR kit (Invitrogen) and then followed by PCR amplification to obtain the specific mRNA of interest. cDNAs were synthesized from RNA in a final volume of 20μl using 2 μg of total RNA as input. PCR reactions were performed with 1μl of cDNA per reaction according to the protocol provided by manufacturer. Semiquantitative RT-PCR method was used to assess relative 24-hydroxylase (CYP24) and GAPDH gene expression. The sequences for 24-hydroxylase and GAPDH primers used for PCR are as follows (Shao et al., 2001).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
</tr>
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<tbody>
<tr>
<td>Cyp24</td>
<td>Forward 5’-CTCATGCTAAATACCCAGGTG-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’-TCGCTGGCAAAACGCGATGGG-3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward 5’-CCATGGAGAAGGCTGGGG-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’-CAAAGTTGTCAATGGATGACC-3’</td>
</tr>
</tbody>
</table>
PCR was performed for 30 cycles, which falls within the linear range of amplification for both 24-hydroxylase and GAPDH. The annealing temperature that was used for PCR for both genes is 55°C.

3.3.4 Agarose Gel Electrophoresis

5 μl of loading dye was added to each 25μl of PCR product. Then, 6 μl of the PCR sample of CYP24 mRNA, a gene of interest, and GAPDH mRNA, a housekeeping gene, were loaded into wells on a 2.5% agarose gel made of Tris acetate-EDTA (TAE) buffer and containing ethidium bromide. The bands obtained after electrophoresis were visualized under UV light and a digital picture of the gel with the PCR product bands was obtained. DNA band intensity was quantified using Image J software (NIH) and relative band intensity of 24-hydroxylase was calculated against the respective samples GAPDH housekeeping gene. The fold-change in expression of the cyp24 mRNA compared to control (no treatment with either 1,25(OH)₂ vitamin D or HDACI) was calculated.

3.4 Statistical Analysis

Relative mRNA expression was analyzed in Prism version 5.0 (GraphPad Software) and, when appropriate, the difference between treatment groups for 24-hydroxylase mRNA expression was assessed by post-hoc comparison of treatment means. Main effects of treatments on 24-hydroxylase mRNA expressions was analyzed using two-way ANOVA to test the effects of 1,25(OH)₂ vitamin D and HDACI (TSA or SFN), and interaction at P < 0.05.
CHAPTER 4

RESULTS

CYP24 mRNA Expression is Dependent on 1,25(OH)₂ Vitamin D Dose in 3T3-L1 Mouse Preadipocytes

In the absence of 1,25(OH)₂ vitamin D there was no evidence of CYP24 mRNA by PCR for 30 cycles (representative PCR bands shown in Figure 10); however, there was an evident induction of CYP24 mRNA expression at 1 nM and 10 nM 1,25(OH)₂ vitamin D. The response of CYP24 mRNA level to 1,25(OH)₂ vitamin D treatment was dependent on the dose (0, 1, 2, 5 and 10 nM for 24h) of 1,25(OH)₂ vitamin D administered (Figure 11), which increase by 6.3-fold at 10 nM 1,25(OH)₂ vitamin D, the highest vitamin D dose investigated compared to 1 nM 1,25(OH)₂ vitamin D, the lowest dose to evoke an apparent increase in CYP24 expression (Figure 10). However, the greatest relative response of CYP24 mRNA to vitamin D was apparent at the lower doses between 1 and 2 nM 1,25(OH)₂ vitamin D, suggesting an approaching saturation of the CYP24 response.

<table>
<thead>
<tr>
<th>1,25 Vitamin D (nM)</th>
<th>0</th>
<th>1</th>
<th>10</th>
<th>0</th>
<th>1</th>
<th>10</th>
<th>0</th>
<th>1</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFN 5μM</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TSA 1μM</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

24-hydroxylase

Figure 10. Relative CYP 24 mRNA amounts measured by semiquantitative reverse transcription–PCR in 3T3L1 preadipocytes. CYP 24 mRNA expressions at 3 different 1,25(OH)₂ vitamin D (nM) concentrations in combination with either 1μM TSA (Trichostatin A) or 5μM SFN (Sulforaphane) in 3T3-L1 preadipocytes.
Figure 11. Dose-dependent effect of 1,25(OH)₂ vitamin D on CYP 24 mRNA expression in 3T3-L1 preadipocytes. With increasing doses of 1,25(OH)₂ vitamin, CYP24 mRNA expression increased (p < 0.05). The sharpest increase in CYP24 mRNA appeared to occur at the lower doses with tapering of response at the higher vitamin D treatment levels. Results indicate mean ± SEM, n = 3 independent experiments.

TSA Histone Deacetylase Inhibitor Treatment Increases Vitamin D-induced CYP24 mRNA Expression

To examine the effects of the potent fungus-derived histone deacetylase inhibitor TSA on vitamin D-induced CYP24 gene expression in mouse preadipocytes, preadipocytes were treated with 3 different concentrations of 1,25(OH)₂ vitamin D (0, 1 and 10 nM for 24h) in combination with a high dose (1μM) of TSA. The denser PCR bands evident in Figure 10 following 1,25(OH)₂ vitamin D and TSA treatment compared to 1,25(OH)₂ vitamin D alone (far right set of bands compared to far left set of bands in the figure) indicate an apparent TSA-induced enhancement of CYP24
Figure 12. CYP 24 mRNA expressions at 3 different 1,25(OH)_2 vitamin D concentrations in combination with either 1μM TSA (Trichostatin A) or 5μM SFN (Sulforaphane) in 3T3-L1 preadipocytes. Relative CYP 24 mRNA amounts measured by semiquantitative reverse transcription–PCR in 3T3L1 preadipocytes. TSA treatment had a significant effect on CYP24 mRNA expression. SFN treatment also increased CYP24 mRNA expression, but to a lesser extent. Data are shown as means ± SEM of n = 7 separate experiments.
mRNA expression in the presence of 1,25(OH)₂vitamin D. Note also that TSA alone had no effect on CYP24 mRNA.

Cells treated with TSA in 7 independent experiments (Figure 12) had higher mean vitamin D-induced CYP24 mRNA expression, by 3.8-fold (p < 0.05) at a low (1nM) 1,25(OH)₂ vitamin D dose and by 2.4-fold (p < 0.05) at a high (10 nM) 1,25(OH)₂ vitamin D dose.

**SFN Increases Vitamin D-induced CYP24 mRNA Expression**

To test whether the bioactive food component SFN, which has been shown to have HDACI activity, could also influence vitamin D-induced CYP24 gene expression in 3T3-L1 preadipocytes, cells were treated with 3 different concentrations of 1,25(OH)₂ vitamin D (0, 1 and 10 nM for 24h) with or without 5μM SFN, followed by measurement of vitamin D-induced CYP24 mRNA expression. The apparently denser PCR bands, shown in the middle set of bands in Figure 10, following SFN and 1,25(OH)₂ vitamin D treatment compared to 1,25(OH)₂ vitamin D treatment alone (far left set of bands in the figure) suggest an apparent up regulation of CYP24 mRNA due to SFN treatment. This notion would be supported by the data, shown in Figure 13, in which a vitamin D-induced dose-response of CYP24 expression was compared with and without concomitant SFN treatment. Over 3 independent experiments, cells treated with 5μM SFN had increased mean vitamin D-induced CYP24 mRNA, by 3.6-fold at 1nM 1,25(OH)₂ vitamin D, 6% at 2 nM, 74% at 5 nM, and 45% at 10 nM 1,25(OH)₂ vitamin D. However, the main effect of SFN on CYP24 mRNA was not statistically significant by ANOVA (P = 0.0876). The main effect
difference in CYP24 mRNA between treatments without and with SFN was $2842 \pm 2908$. The reason for the apparent lack of response to SFN at 2 nM $1,25(OH)_2$ vitamin D in these experiments is unknown, but may reflect a possible experimental anomaly given the apparent effect of SFN at the both lower and higher vitamin D doses.

**Figure 13.** Dose-dependent effect of $1,25(OH)_2$ vitamin D in combination with SFN on CYP 24 mRNA expression in 3T3-L1 preadipocytes. The main effect of SFN on CYP24 mRNA was not statistically significant by ANOVA ($P = 0.0876$). Results indicate mean ± SEM, $n = 3$ independent experiments.

Overall, our findings suggest that CYP24 mRNA expression is a highly dependent upon the presence of $1,25(OH)_2$ vitamin D in 3T3-L1 mouse preadipocytes. In addition, treatment with a HDACI can increase the responsiveness of 3T3-L1 cells to the vitamin D hormone, at least as judged by increased CYP24
mRNA expression. Based on our findings, it appears that the ability of SFN to increase CYP24 induction by $1,25(\text{OH})_2\text{vitamin D}$ is not as effective as TSA.
CHAPTER 5
DISCUSSION

1,25(OH)_2 vitamin D has been known for a long time to inhibit the differentiation of preadipocytes to adipocytes (Ishida et al., 1988) in a vitamin D receptor (VDR)-dependent manner. In this study, we used the mouse 3T3-L1 preadipocyte experimental system, which is a well-established cell culture model to study the mechanism of adipocyte differentiation, to investigate the effects of HDACI on vitamin D-induced gene expression. To study how histone deacetylase inhibitor influences VD/VDR-mediated gene expression, and to what extent histone deacetylase inhibitor can modulate the VD/VDR-mediated gene expression in preadipocytes, we examined the individual effect of two HDACIs, TSA and SFN, on CYP24 mRNA expression. Our central hypothesis was that treatment of 3T3-L1 mouse preadipocytes with either histone deacetylase inhibitor (tricostatin A or sulforaphane) would increase 1,25(OH)_2 vitamin D-induced CYP24 mRNA levels.

Our observations are consistent with the idea that both TSA and SFN can increase 1,25(OH)_2 vitamin D-induced CYP24 mRNA expression in 3T3-L1 preadipocytes (Figure 12, 13). This finding of up regulation of CYP24 gene expression by TSA in preadipocytes is consistent with previous studies, which tested TSA in different cell lines, such as various prostate cancer cell lines (Gommersall et al., 2004b, Luo et al., 2010). To our knowledge, our observations concerning the effect of TSA in 3T3-L1 mouse preadipocytes are novel and support the view that concomitant vitamin D and HDACI treatment could influence the
effects of the vitamin D hormone on adipocyte biology. The dietary histone deacetylase inhibitor (HDACi) sulforaphane (SFN) is one of the biologically active compounds in the human diet and is present at high levels in some cruciferous vegetables, such as broccoli. SFN has received a great deal of attention because of its ability to inhibit histone deacetylase enzymes, which was identified in human embryonic kidney 293 (HEK293), HCT116 human colon cancer cells (Myzak et al., 2004), and prostate epithelial cells (BPH-1, LnCaP and PC-3) (Myzak et al., 2006), and its potential use in cancer chemoprevention or treatment.

Our novel observation that SFN increased vitamin D-induced CYP24 mRNA level in our 3T3-L1 preadipocytes model system supports the idea that dietary consumption of foods containing bioactive food components with HDACi activity, such as certain cruciferous vegetables, may be able to affect adipogenesis by altering vitamin D-induced gene expression. Adipogenesis in human primary adipocytes studied in culture has been recently reported to be increased by 1,25(OH)₂ vitamin D [add reference from the BU group here]. It is thus possible that HDACi could further increase the adipogenic response in humans to vitamin D compounds. On the other hand, there are studies that showed the inhibitory effect of TSA and SFN on adipogenesis in murine 3T3-L1 preadipocytes (Lagace and Nachtigal, 2004, Haberland et al., 2010, Choi et al., 2012).

Additional studies are needed to investigate the influence of HDACIs on cell function because of the potential complex effects of these compounds. For example, an increase in CYP24 expression induced by HDACIs could reduce the intracellular bioavailability of vitamin D metabolites because its main biochemical function is to
initiate vitamin D catabolism and thereby reduce VDR signaling. The extent to which HDACIs, including SFN, affect other vitamin D-dependent genes that may be involved with regulating adipocyte biology needs further exploration, as well as the net effect of HDACI-induced effects on complex physiological processes, such as adipogenesis.
CHAPTER 6

FUTURE DIRECTIONS

Although SFN treatment increased CYP24 mRNA expression 1.4-fold at 1nM 1,25(OH)_2 vitamin D and nearly 1.2-fold at 10 nM 1,25(OH)_2 vitamin D in 3T3-L1 mouse preadipocytes, the main effect of SFN on CYP24 mRNA was not statistically significant. Testing different concentrations of SFN would be helpful to understand the effect of SFN on CYP24 mRNA expression.
REFERENCES


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