EFFECTS OF CURCUMIN ON 3T3-L1 ADIPOCYTE DIFFERENTIATION

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To the Dean of the Graduate School:

I am submitting herewith a thesis written by Jegghna J Chheda entitled "Effects of Curcumin on 3T3-L1 Adipocyte Differentiation". I have examined 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.

Dr. Parakat Vijayagopal, Major Professor

We have read this thesis and recommend its acceptance:

(Department Chair)

Accepted:

Dean of the Graduate School

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ABSTRACT

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EFFECTS OF CURCUMIN ON 3T3-L1 ADIPOCYTE DIFFERENTIATION

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Curcumin, the principal curcuminoid found in the spice, turmeric, from rhizomes of *Curcuma Longa*, is a fat soluble polyphenolic compound. Several studies have shown its anticarcinogenic, antioxidant, anti-inflammatory, anti-septic and, antiangiogenic properties. In this study, we investigated the effect of curcumin on 3T3-L1 adipocyte differentiation in culture and gene expression involved in lipid metabolism. Curcumin inhibited 3T3-L1 adipocyte differentiation in dose-dependent manner by downregulating PPAR-γ expression. Curcumin was toxic to cells at a concentration of 30 μM. Curcumin also downregulated PPAR-α gene expression while not affecting Fabp4 gene expression indicating that fatty acid oxidation is not affected by curcumin in 3T3-L1 cells. Although adiponectin gene expression is known to be PPAR-γ dependent, in our study curcumin did not affect adiponectin gene expression despite downregulation of PPAR-γ gene suggesting that a change in PPAR-γ gene alone is not sufficient to alter adiponectin gene expression in these cells. Curcumin may benefit as a potential weight loss supplement.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF TABLES (MANUSCRIPT)

LIST OF FIGURES

LIST OF FIGURES (MANUSCRIPT)

CHAPTER I

INTRODUCTION

Excess energy intake that exceeds expenditure results in obesity (Mahan & Escott, 1996) and obesity is associated with many metabolic disorders. Collectively, the disorders such as non-insulin dependent diabetes mellitus (type II diabetes), coronary artery disease, stroke, high blood pressure, increased central or abdominal obesity, high blood levels of triglycerides and low levels of HDL are known as metabolic syndrome (Mahan & Escott, 1996; Totora & Grabowski, 1996; Gregoire, 2001).

The body reserve of energy is primarily stored as triglycerides in fat cells. Adipose tissue is made up of specialized fat cells (adipocytes), fibroblasts, blood vessels and other connective tissue cells. They are found in the subcutaneous layer of skin and around organs like heart and kidneys. Adult obesity results from either the increasing size of adipocytes (hypertrophy) and/or increasing number of adipocytes (hyperplasia) (Mahan & Escott, 1996; Totora & Grabowski, 1996; Gregoire, 2001). Agents that can inhibit adipogenesis can be very useful in the treatment of obesity.

Adipocytes are generated from fibroblasts. Fibroblasts are a kind of connective tissue cells which secrete reticular fibers and certain components of the ground substance of the extracellular matrix (Totora & Grabowski, 1996). Fibroblasts undergo differentiation to form mature adipocytes of different kinds (Gesta, Tseng, & Kahn,

2007). Apart from being the storage site for fat, adipose tissue plays a major role in energy metabolism by release of different hormones (Ntambi & Young-Cheul, 2000). The mouse embryonic fibroblasts 3T3-L1 and 3T3-F442A are considered preadipocyte cell lines. These cells have been used extensively to study adipocyte metabolism. 3T3-L1 preadipocytes differentiate in a synchronous manner in response to methylisobutylxanthine (MIX), dexamethasone (DEX) and/or insulin in fetal bovine serum. Within 5 days of differentiation, >90% of preadipocytes accumulate triacylglycerol. Transcription factors like CCAAT/enhancer binding proteins (C/EBP) and peroxisome proliferator-activated receptor (PPAR) work together and in a sequence to stimulate the genetic events that result in differentiation (Darlington, Ross & MacDougald, 1998; Cowherd, Lyle & McGehee, 1999). After the treatment with the differentiation inducers, an increase in transcription of C/EBPβ and C/EBPδ gene expression is seen which in turn induces the C/EBPα and PPARγ gene expression. After differentiation is induced, cells undergo two rounds of cell division. C/EBPα activates the gene encoding proteins involved in regulating the adipocyte phenotype (Djian, Phillips & Green, 1985; Darlington, Ross & MacDougald, 1998; Cowherd, Lyle & McGehee Jr, 1999; Ntambi & Young-Cheul, 2000).

Turmeric rhizome (Curcuma Longa) imparts yellow color to curry. It is grown in India and China and holds age-old medicinal properties. It is also used in the textile industry as a dye and as a food preservative. The rhizome portion of the turmeric plant consists of polyphenol rich compounds called curcuminoids. The three kinds of

curcuminoids are curcumin, demethoxycurcumin and bisdemethoxycurcumin. Of these, curcumin is the most abundant and active component. Extensive research has shown beneficial effects of curcumin such as antioxidant, anti-inflammatory, antimicrobial, anti-carcinogenic and cardio-protective properties (Katz, Trask & Lucchesi, 2009). Recent research has revealed that curcumin reduces obesity- and diabetes-induced cardiovascular complications such as hyperlipidemia, hyperhomocysteinemia, insulin resistance, cardiac hypertrophy and atherosclerosis (Katz, Trask & Lucchesi, 2009). A decrease in heart rate variability (HRV) by curcumin is associated with free fatty acid (FFA) -induced sympathovagal disturbances. Low levels of HRV is an independent risk factor for cardiovascular disease. Obese mice fed curcuminoids for 12 weeks showed improvement in hyperlipidemia, specifically free fatty acids and thus improvement in cardiac autonomic function (Katz, Trask & Lucchesi, 2009). To our knowledge, very little is known regarding the role of curcumin in adipocyte differentiation.

Purpose of the Study

The purposes of this study are:

(1) To determine the effect of curcumin on adipocyte differentiation.

(2) To study the effects of curcumin on genes that induce fat cell differentiation or lipolysis.

Hypotheses

The hypotheses for this study are:

(1) Curcumin will significantly inhibit the differentiation of 3T3-L1 fibroblasts into adipocytes

(2) Curcumin may inhibit the genes inducing lipogenesis and or inducing the genes involved in lipolysis.

CHAPTER II

REVIEW OF LITERATURE

Obesity

Obesity is the most visible health disorder observed around the globe. Nearly 1 billion people worldwide are overweight [body mass index (BMI) $\geq$25 kg/m^2] and at least 300 million people are thought to be obese (body mass index $\geq$30 kg/m^2) (Table 1) (Ford & Mokdad, 2008). Initially, obesity was observed more prominently in developed countries, principally United States, but is now observed in developing countries where previously malnutrition was the dominant concern. As developing countries have become wealthier, people have rapidly adopted Westernized lifestyles characterized by increased energy consumption and decreased energy expenditure along with migration from rural to urban areas. Increased prevalence of obesity and its association with many chronic diseases is imposing serious health concerns for future health of populations and costly health care expenditures. In 2005-2006, 33.3% of men and 35.3% of women were obese in United States (Ford & Mokdad, 2008). The cost of obesity in the United States was estimated to be $117 billion in 2000. From 2003-2006, the prevalence of obesity for ages 2-19yr was 16.3% (17.1% among males and 15.5% among females) and the prevalence among whites was 14.6% (males 15.6%, females 13.6%), 20.7% among African- Americans (males 17.4%, females 24.1%), and 20.9% among Mexican-Americans (males 23.2%, females 18.5%) (Ford & Mokdad, 2008).

Table 1:

Classification of Overweight and Obesity by BMI (Shils et al, 2006)

	BMI (kg/m^2)	Obesity Class
Underweight	<18.5	
Normal	18.5 – 24.9	
Overweight	25.0 – 29.9	
Obesity	30.0 – 34.9	I
	35.0 – 39.9	II
Extreme obesity	≥ 40	III

Obesity has resulted due to over nutrition and sedentary lifestyle. Metabolic processes such as action of insulin on glucose-lipid-free fatty acid metabolism and blood glucose control and blood pressure regulation get dysregulated due to persistent obesity. This then triggers a cluster of conditions: dysglycemia, dyslipidemia, hypertension, and procoagulant state, known as the metabolic syndrome. Both obesity and the metabolic syndrome are immediate precursors of type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD) (Misra & Khurana, 2008).

Adipose Tissue

In higher eukaryotes, the major energy reserve is in the form of white adipose tissue (WAT). Its main purpose is to store triacylglycerol during energy excess period and breakdown during the energy deprivation period. An excess of WAT has resulted in

increased obesity incidences (Gregoire, Smas & Sul, 1998; Gesta, Tseng & Kahn, 2007). Accumulation of fatty acids needs to be tightly controlled as they can act as detergents that can rapidly dissolve plasma membrane and cause cell lysis if allowed to accumulate. Therefore fatty acids need to be converted efficiently into triglycerides and stored in the adipocytes (Öst, Örtegren, Gustavsson, Nystrom, & Strålfors, 2005). Development of WAT is known to begin before birth and increased expansion is seen soon after birth (Gregoire, 1998 ; Ntambi & Young-Cheul, 2000). Expansion results from increase in fat cell size and increase in fat cell number. Potential new fat cells also can be generated in adulthood. In severely obese people, increase in fat cell number has been observed. Precursors of fat cells have been isolated from adult WAT in vitro and differentiated into mature adipocytes. For the past thirty years, adipocyte differentiation has been widely studied in in-vitro systems. This has led to the knowledge of molecular and cellular events involved in bringing the transition from undifferentiated fibroblast like pre-adipocytes into a mature round fat cells or adipocytes (Gregoire, 1998). Every species throughout its life span is known to have the ability to differentiate preadipocytes in response to the body's fat storage demands. Study on preadipocyte differentiation in vivo is difficult. Fat tissue of animals consists of approximately one third adipocytes and two-thirds of small blood vessels, nerve tissue, fibroblasts and preadipocytes in different stages of development. This makes a difficult distinction between preadipocytes and fibroblasts and the inability to have preadipocytes in similar developmental stages (Ntambi & Young-Cheul, 2000).The most frequently used cell lines to study adipocyte

metabolism are 3T3-F442A and 3T3-L1 which are clonally isolated from Swiss 3T3 cells derived from disaggregated 17-to 19-day mouse embryos (Green & Kehinde, 1976; Gregoire,1998). 3T3-L1 and 3T3-F442A cell lines can be passaged indefinitely providing a consistent source of preadipocytes for further studies. 3T3-L1 and 3T3-F442A exhibit the same morphological characteristics of the fibroblast preadipose cells present in the stroma of adipose tissue (Cowherd, et al., 1999; Ntambi & Young-Cheul, 2000) A cloned cell line is a homogenous cell population as it is at the same stage of differentiation. This helps in definitive response to treatments. Development of fat droplets and appearance in vitro is similar to the live adipose tissue (Ntambi & Young-Cheul, 2000). Like other fibroblasts cell lines, 3T3 cells make fibrous collagen and proteoglycans and appear fusiform in sparse culture indicating that these 3T3 cells are fibroblasts and were classified as endothelial cells earlier because of their mosaic appearance (Green & Kehinde, 1976). Earlier studies from Green & Kehinde, 1976 have revealed that Swiss 3T3-L1 fibroblast cells have the ability to convert into adipocytes in culture. This transition is called differentiation which begins when the growth of cells becomes reduced or arrested and during which there is a sharp increase in the triglyceride synthesis from precursors. Earlier stages of differentiation are less frequently seen in presence of insulin because insulin accelerates the accumulation of triglyceride. The 3T3 fibroblasts then differentiate into adipose cells. As triglyceride starts accumulating, the highly extended and flattened processes of cells appear similar to those of the nonfatty cells in the same cultures. However as the differentiation proceeds, the processes thicken and

retract and the cells acquire round shape of mature adipose cells. The fat droplet forms around the nucleus and at a distance from it giving a necklace effect before it forms a large central fat droplet which is characteristics of a mature adipocyte (Green & Kehinde, 1976).

3T3-L1 Preadipocyte Differentiation

The 3T3-L1 cell line is one of the most well defined and reliable models to study the transition of preadipocytes into adipocytes. Development of a multicellular organism occurs from fertilization of a single egg cell. This conversion of preadipose tissue from a fertilized egg cell is unknown. The pluripotent fibroblasts or stem cells are known to have aroused from mesodermal cells and can undergo differentiation to form preadipocytes, cartilage, bone and muscle tissue (Figure 1) (Ntambi & Young-Cheul, 2000; Gesta, Tseng & Kahn, 2007) .

Differentiation in the confluent 3T3-L1 preadipocytes is initiated by adding adipogenic cocktail. The adipogenic cocktail is comprised of methylisobutylxanthine (MIX) – a cAMP-phosphodiesterase inhibitor, dexamethasone (Dex)– a synthetic glucocorticoid agonist and insulin which is abbreviated here as MDI. The MDI is known to stimulate cAMP –dependent protein kinase pathway, the glucocorticoid receptor pathway and the insulin-like growth factor 1 (IGF1) receptor (Ntambi & Young-Cheul, 2000).

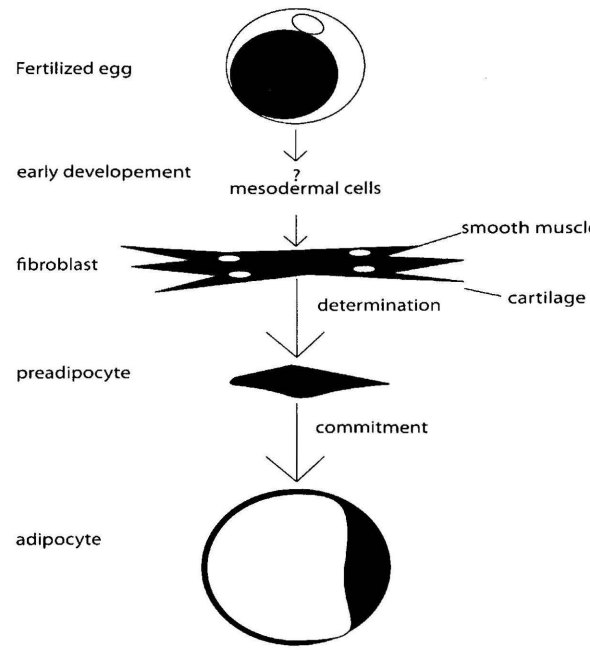


Figure 1: The developmental stages of mature adipocytes from a fertilized egg cell (Ntambi & Young-Cheul, 2000).

The process by which adipocytes differentiate from pre-adipocytes to mature adipocytes involves four stages: growth arrest, clonal expansion, early differentiation and terminal differentiation (Gesta, et al., 2007). After 24 hours of inducing differentiation with MDI, preadipocytes undergo postconfluent mitosis with at least one round of DNA replication, cell division and subsequent growth arrest. By day 2 of differentiation, mitosis is completed and cells enter into an unusual growth arrest (G_D). During mitosis, DNA unwinds and allows the transcription factors access to regulatory response elements present in genes which are involved in modulating the mature adipocyte phenotype. After the growth arrest, the preadipocyte cells are committed to become adipocytes. At day 3 of differentiation, growth arrested cells express late

markers of differentiation. These late markers consist of lipogenic and lipolytic enzymes, proteins which modulate the mature adipocyte phenotype. By day 5-7 the cells accumulate fat droplets and round up and become terminally differentiated adipocytes (Green & Kehinde, 1976; Djian, Phillips & Green, 1985; Darlington, Ross & MacDougald, 1998; Cowherd, Lyle & McGehee Jr, 1999; Ntambi & Young-Cheul, 2000; Gregoire, 2001)

Gene Expression During 3T3-L1 Preadipocyte Differentiation

As 3T3-L1 pre-adipocytes reach confluence, early markers of adipocyte differentiation are expressed. The cell-cell contact induces lipoprotein lipase and type VI collagen gene expression. Within 1.0 hr of MDI [methyl isobutyl xanthine (MIX), dexamethasone (DEX) and insulin] addition for 3T3-L1 conversion to adipocytes, the expression of *c-fos, c-jun, jun-B, c-myc* and CCAAT/enhancer binding proteins (C/EBP) β and δ is seen. Twenty-four hours post-MDI, the differentiating pre-adipocytes undergo a final mitosis and subsequent growth arrest (G_D) (Darlington, Ross & MacDougald, 1998; Cowherd, Lyle & McGehee Jr, 1999; Ntambi & Young-Cheul, 2000).

Increased *c-myc* expression is the branch point in directing the growth arrested cells to commit to terminal differentiation. After exposure to MDI, C/EBP β and C/EBP δ are expressed to induce and accelerate adipogenesis under the influence of these hormonal inducers. C/EBP β is known to respond to DEX and C/EBP δ responds to increased cAMP induced by MIX. The activity of C/EBP β and C/EBP δ brings

about the expression of peroxisome proliferator-activated receptor γ (PPARγ) and C/EBPα which in turn cross regulate with each other and induce the transcription of many adipocyte genes responsible for encoding proteins and enzymes necessary for creating and maintaining the adipocyte phenotype. C/EBPα has anti-mitotic activity that prevents post-confluent cell division in pre-adipocytes as well as preventing the intermediate differentiation. In response to MDI, an endogenous ligand for PPARγ is produced, thus suggesting that 3T3-L1 pre-adipocytes have no endogenous PPARγ ligand before treatment. The up regulation of sterol responsive element binding protein (ADD1/SREBP-*1c*) gene expression with preadipocyte differentiation causes the production of endogenous PPARγ ligands. These endogenous ligands are known to be fatty acid like molecules (Darlington, Ross & MacDougald, 1998; Cowherd, Lyle & McGehee Jr, 1999; Ntambi & Young-Cheul, 2000).

Role of Polyphenols

The side-effects of drugs have led researchers to use non-drug options which can prevent adipocyte formation and or reduce fat in mature adipocytes. A natural food component as a non-drug option seems to be the safe alternative to drugs to prevent obesity. Many phytochemicals, polyphenols and flavonoid rich foods are being tested as chemopreventive and anti-obesity agents. A series of phytochemicals known as polyphenols are synthesized by plants (Table 2; Meskin, 2004).

The most abundant antioxidants in our foods are polyphenols which are widely distributed in fruits, vegetables, cereals, olives, dry legumes, spices, chocolate and

beverages such as tea, coffee and wine. The delay in polyphenol research is due to its wide variety and complexity of chemical structures. Antioxidant properties protect cells from oxidative damage and thus prevent various degenerative diseases associated with oxidative stress. Experimental studies have supported role of polyphenols in prevention of cardiovascular disease, cancer, osteoporosis, diabetes mellitus and neurodegenerative disease (D'Archivio, Filesi, Di Benedetto, Gargiulo, Giovannini & Masella, 2007). However, pro-oxidant effects of polyphenols have opposite effects to antioxidant properties. Antioxidants protect cells from damage, but as pro-oxidants, induce apoptosis and block cell proliferation. Polyphenols are also known to inhibit or reduce different enzymes such as telomerase, cyclooxygenase and lipoxygenase and the interaction with signal transduction pathways and cell receptors, affect caspase-dependent pathways, cell cycle regulation and platelet functions. These protective effects have gained polyphenols more attention as potential therapeutic agents against several chronic degenerative diseases (D'Archivio, Filesi, Di Benedetto, Gargiulo, Giovannini & Masella, 2007).

Table 2:

Classification of Polyphenols and Polyphenol-Containing Foods. (D'Archivio, Filesi, Di Benedetto, Gargiulo, Giovannini & Masella, 2007):

Polyphenols	**Sources**
Anthocyanins	
Cyanidine 3-glucoside	Orange juice, Red fruit extract
Malvidin 3-glucoside	Red wine, Red grape juice

Table 2. Continued

Polyphenols	Sources
Flavanols	
Epigallocatechin gallate	Green tea extract and infusion
Catechin	Red wine, Black tea
Epicatechin	Chocolate
Procyanidin B1	Grapeseed extract
Flavanones	
Hesperidin	Orange juice
Hesperetin	Pure compound
Naringenin	Orange juice, Grapefruit juice
Flavanones	
Hesperidin	Orange juice
Hesperetin	Pure compound
Naringenin	Orange juice, Grapefruit juice
Flavanones	
Hesperidin	Orange juice
Hesperetin	Pure compound
Naringenin	Orange juice, Grapefruit juice
Isoflavones	
Daidzein	Soy milk, extract and nuts
Genistein	Soy milk, extract and nuts
Glycitein	Soy milk, Pure compound
Hydroxybenzoic acids	
Gallic acid	Assam black tea, Red wine
Hydroxycinnamic acids	
Chlorogenic acid	Coffee
Caffeic acid	Red wine
Hydrocinnamic acids	Apple cider

Curcumin

Curcumin is the principal curcuminoid (polyphenolic compound) found in the spice, turmeric. Turmeric is a dried spice derived from the rhizomes (underground stem) of *Curcuma Longa,* a member of the ginger family. Turmeric contains 2-9 % curcuminoids, and of this, curcumin is 75% of total curcuminoids, with 10-20% being demethoxycurcumin and <5% is bisdemethoxycurcumin (Figure 2; Jurenka, 2009). It is widely used in Indian, Southeast Asian and Middle Eastern cuisines. Commonly, turmeric is used as a spice and a yellow coloring and flavoring agent and the coloring properties are imparted by curcumin. As well, curcumin is available as a dietary supplement (Meskin, 2004; Micronutrient research for optimum health- Curcumin, 2009; Kang & Chen, 2009). It is widely used in Indian medicines and several studies have shown its anticarcinogenic, antioxidant, anti-inflammation, anti-septic and antiangiogenic properties. Curcumin is also known to modulate multidrug-resisitance gene and protein function (Duvoix et al., 2005; Supachai, Wittaya, Spiridione, & Pornngarm, 2009).

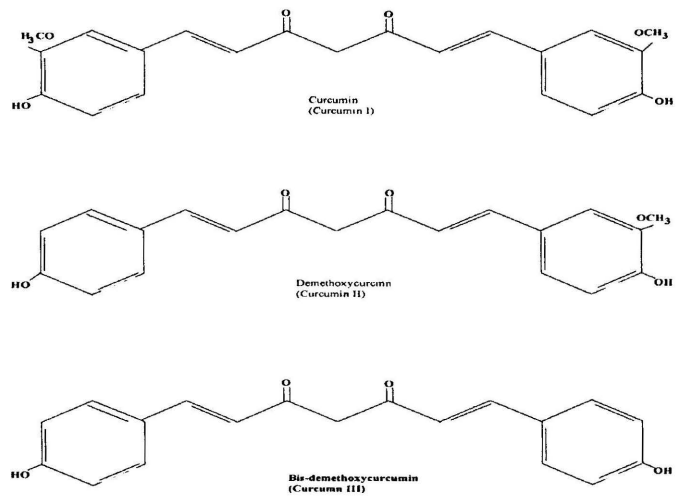


Figure 2: The types of curcuminoids

Absorption of Curcumin

The glycosidic polyphenols consists of polar groups which minimize intestinal uptake by passive mechanisms. Polyphenols, with 1-2 hydroxyl groups, are absorbed easily, whereas those with 5-hydroxyl groups are more hydrophobic. Once absorbed in the enterocyte, some polyphenols can be transported back into the intestinal lumen by the multi-drug resistance transporter (MDR-2). The intestinal uptake of polyphenols consists of polar conjugates that make up > 95% of total polyphenols which appear in circulation (Meskin, 2004).

Jurenka reported the poor absorption of conventional curcumin preparations (Jurenka, 2009). This has led researchers to investigate the benefits of complexing curcumin with other substances to increase systemic bioavailability. In vivo studies

have shown that curcumin is rapidly metabolized, conjugated in the liver, and excreted in the feces, therefore having limited systemic bioavailability (Jurenka, 2009).

In phase I clinical trials, 25 cancer patients with various precancerous lesions were given oral doses of 4, 6, and 8 g curcumin daily for three months and yielded serum curcumin concentrations of only 0.51 ± 0.11, 0.63 ± 0.06, and 1.77 ± 1.87 μM, respectively, indicating curcumin is poorly absorbed and may have limited systemic bioavailability. Serum levels peaked between one and two hours post-dose and declined rapidly. Curcumin metabolites and urinary excretion of curcumin were found undetectable in this study (Jurenka, 2009).

Curcumin's rapid plasma clearance and conjugation has shown limited therapeutic usefulness leading researchers to investigate the benefits of complexing curcumin with other substances to increase systemic bioavaility. Currently curcumin is being complexed with a phospholipid, known as a phytosome. The phosphatidylcholine-curcumin complex is more readily incorporated into lipophilic cell membranes, making it significantly more bioavailable than unbound curcumin. Most of the published clinical trials have shown that curcumin appears to be extremely safe, even at doses up to 8 g daily (Jurenka, 2009). There is little evidence about the toxicity and side effects of curcumin (Meskin, 2004).

Role of Curcumin in Health

For centuries, turmeric has been widely used as a treatment for allergy, asthma, bronchial hyperactivity, runny nose, cough, sinusitis, liver disease, digestion, dental

problems, blood sugar control, diabetic wounds, arthritis, sprains, and a host of inflammatory problems. Curcumin protects DNA against oxidative damage and hence lowers the risk for mutations and other genetic damage. The curcuminoids can become pro-oxidative in the presence of free iron or copper. This property may be relevant to their pro-apoptotic effects on cancer cells, especially when applied at high concentrations (Kidd, 2009).

In cell culture, curcumin showed an increased anti-inflammatory activity by indirectly inhibiting the activity of inflammatory enzymes and/or by enhancing the synthesis of glutathione (Micronutrient research for optimum health- Curcumin, 2009). In addition, curcumin induced apoptosis by inhibiting cell-signaling pathways in various cancer cell lines grown in culture (Micronutrient research for optimum health-Curcumin, 2009). In cultured vascular endothelial cells, curcumin prevented tumor invasion and inhibited angiogenesis. Orally administered curcumin inhibited development of intestinal adenomas in Apc min/+ mice that have a mutation in Apc (adenomatous polyposis coli) gene similar to that found in humans. In phase I clinical trials, curcumin, inhibited gastrointestinal cancers (Micronutrient research for optimum health- Curcumin, 2009).

Ejaz et al observed significant suppression of differentiation of preadipocyte 3T3-L1 fibroblasts into mature adipocytes with curcumin treatment (20μmol/L). Curcumin inhibited angiogenesis in rat adipose tissue and reduced accumulation of lipids in adipocytes and liver. However, curcumin did not suppress mRNA expression

for PPARγ and C/EBP α, key transcriptional factors involved in adipogenesis and lipogenesis (Ejaz, Wu, Kwan, & Meydani, 2009)

The curcuminoids influence many genes involved with initiation and regulation of inflammation: (a) they can down-regulate NF-kB, the nuclear transcription factor and critical upstream regulator of genes that control acute and chronic inflammation cascades; (b) With NF-kB down regulation, curcuminoids downregulate other pro-inflammatory enzymes such as lipoxygenase and inducible nitric oxide synthase (iNOS); (c) curcuminoids inhibit other transcription factor products such as signal transducer and activator of transcription (STAT), peroxisome proliferator-activated receptor-g (PPAR-g), and β-catenin; (d) cyclooxygenase-2 (COX-2) is the inducible form of COX that predominates at inflammatory sites and also likely plays a critical role in tumor promotion. Curcumin inhibits COX-2 activation by pro-inflammatory agents (Supachai, Wittaya, Spiridione & Pornngarm, (2009).

In Alzheimer's disease (AD), known molecular mediators are reactive oxygen species (ROS), reactive nitric oxide species generated by iNOS, lipid peroxidation products, and the genes NF-kB and phosphorylated JNK. The curcuminoids have been shown to block these mediators at effective concentrations between 1 and 2 μM and are attainable in humans (Kidd, 2009).

The curcuminoids are effective against all stages of cancer – initiation, promotion, proliferation, and metastasis. In vivo, they have chemopreventive effects against cancers of colon, duodenum, esophagus, stomach, and mouth. They have also

blocked cancer promotion by phorbol esters and other experimental agents in animals, and can interfere with angiogenesis and metastasis. In vitro, they have induced apoptosis in cancer cells while leaving normal cells unaffected (Kidd, 2009).

In vivo models, curcumin ameliorates diet-induced hypercholesterolemia and CCl4-induced hepatic fibrosis. In vitro studies have shown induction of PPARγ gene in activated hepatic stellate cells (HSC) with curcumin leading to inhibition of HSC activation (Kang & Chen, 2009). Curcumin suppresses expression of LDLR gene in activated HSCs, in vitro, by activating PPARγ and suppressing SREBP-2 gene expression leading to the reduction in level of cellular cholesterol and to attenuation of stimulatory effects of LDL on HSC activation. PPARγ activation mediated the inhibitory effect of curcumin on regulating SREBP-2 expression (Kang & Chen, 2009).

Very little information is available regarding the effect of curcumin on obesity. Therefore, in the present study we investigated the effect of curcumin in 3T3-L1 preadipocyte differentiation in culture.

CHAPTER III

MATERIAL AND METHODS

3T3-L1 Fibroblasts Cell Culture and Differentiation

The mouse embryonic fibroblasts 3T3-L1 cells (Dr.Howard Green's Lab-Harvard Medical School) were cultured in Dulbecco's Modified Eagle's Medium (DMEM- Invitrogen) containing 10% calf serum (bovine) (BCS- Hyclone laboratories) and 1% Penicillin streptomycin (Invitrogen) at 37 ^{o}C in a humidified CO_2 (10%) incubator.

Differentiation of 3T3-L1 Cells

Cells were grown to 80% + confluence and then placed in a differentiating medium consisting of DMEM, 10% fetal bovine serum (FBS-Hyclone laboratories), 1% of penicillin/streptomycin in the presence and absence of different concentrations of curcumin (0, 10, 20, 30 μM) and MDI [0.5 mM 3-Isobutyl-1-methylxanthine (MIX - Sigma Aldrich), 1.0 μM dexamethasone (Sigma Aldrich), 1.0 μg/ml insulin (Sigma-Aldrich)]. After 48hrs, the medium was changed to differentiating medium and 1.0 μg/ml insulin ± curcumin. Thereafter, the medium was changed at two day intervals. Experiment was terminated on day 10. The cells were then analyzed for the accumulation of fat and specific gene expression. Insulin was dissolved in 0.02 M HCl,

MIX in 100% dimethylsulfoxide (DMSO) and dexamethasone in 100% ethanol. Curcumin (VWR) was dissolved in dimethylsulfoxide (DMSO). The flow diagram of differentiation is given in figure 3 and the protocol for cell culture experiments is given in Table 3.

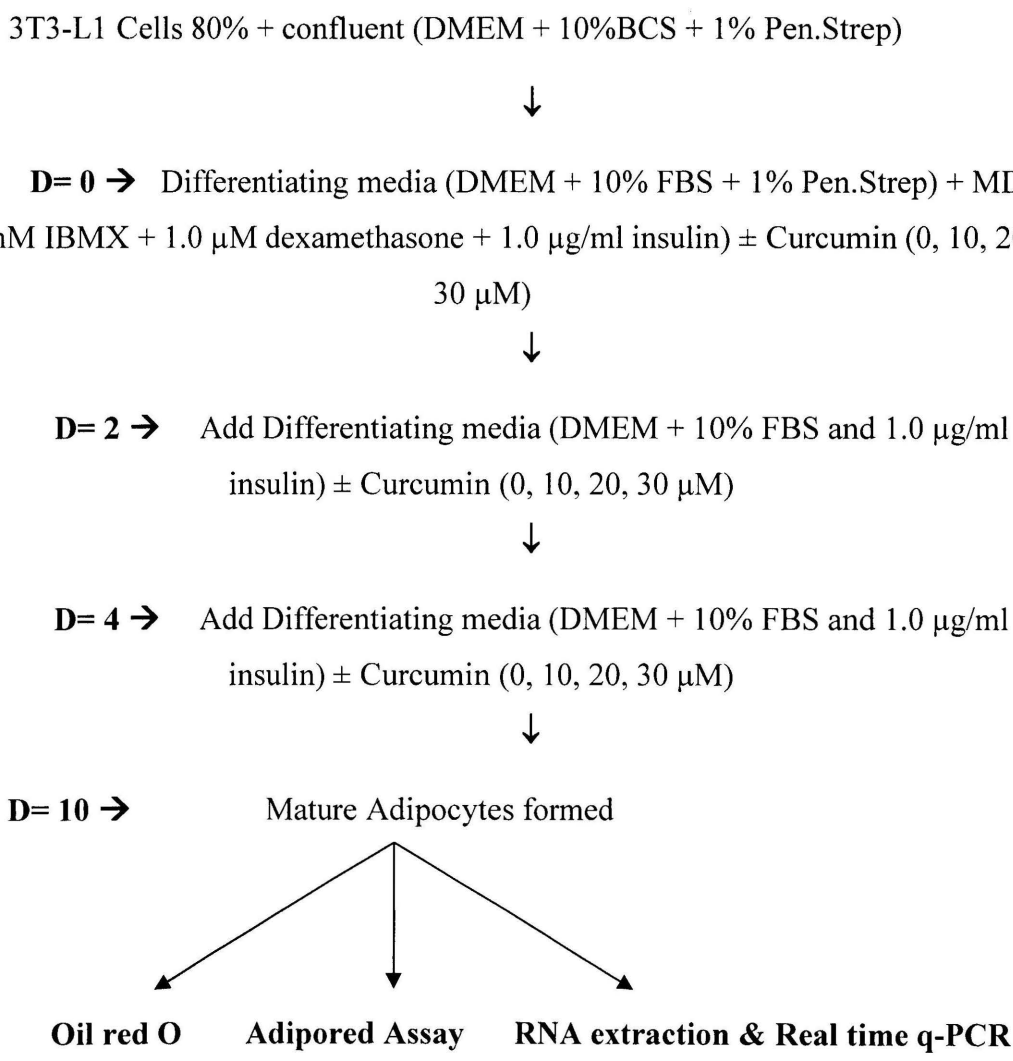


Figure 3: Flow Diagram: 3T3-L1 fibroblasts cell culture and differentiation

Table 3:

Protocol for Cell Culture Experiments

Undifferentiated 3T3-L1 cells	Differentiated 3T3-L1 cells	Differentiated 3T3-L1 cells	Differentiated 3T3-L1 cells
Bovine calf serum	Fetal Bovine serum MDI	Fetal bovine serum MDI DMSO	Fetal bovine serum MDI Curcumin treatment (0,10,20,30μM) in DMSO

Oil Red-O Staining

Cells were grown at 1.4 X 10^5 density in 12 well plate to 80% confluence. Coverslips were added to the well-plates prior to culturing. Experiments were performed in triplicate. As discussed earlier, on d=10, cells had accumulated fat. To assay fat, cells were rinsed with Hank's balanced salt solution (HBSS) twice and fixed in Davidson's fixative for an hour. The cells were washed once with HBSS and stained with 36% oil red-O (ORO) (Fisher-Scientific) and tri-ethyl phosphate (TEP) working solution for 30 min. Stained cells were rinsed with HBSS twice and fixed on coverslip with crystal mount and left overnight. The coverslips were then fixed with permount

and left for drying for 3-4 days. The slides were viewed under the microscope. ORO/TEP stock was prepared by dissolving 500mg oil red-O in 60ml tri-ethyl phosphate and 40ml DI water. The working solution of ORO/TEP was prepared by dissolving 12ml of stock solution in 8ml of DI water (Kinkel et al., 2004).

Adipored Assay

Cells were grown at 5.6 X 10^4 density in 12 well plates to 80% confluence and differentiated as discussed earlier. Three independent experiments were performed. On d= 10, each well was rinsed with HBSS. To each well, solution consisting of 2ml HBSS and 60ul of AdipoRed was added. The plate was incubated for 15min at room temperature and the fluorescence was measured in a spectrophotometer with excitation at 485nm and emission at 572nm (Lonza, 2008, pg 1-3).

RNA Extraction

Cells were grown in 35 mm culture dishes at 0.5 X 10^6 density for nucleic acid extraction. The differentiated cells were grown along with curcumin in duplicate cultures and the experiments were repeated three times. For RNA extraction, cells were washed with Hank's balanced salt solution (HBBS) twice and then 1.0 ml of TRIzol (Invitrogen) Reagent was added. The dissolved cells were scraped from the culture plates with rubber policemen and transferred to polypropylene tubes. The Mixture was incubated at room temperature (30 ^{o}C) for 10 minutes followed by addition of 0.25 ml chloroform and then the samples were shaken vigorously for 30 sec. The samples appeared rose-pink in color. Samples were placed on ice for 5 min followed by

centrifugation at 7500 rpm for 20 min at 4 °C to separate the RNA containing upper aqueous phase from the protein containing organic phase with DNA forming the interphase. The upper aqueous phase was collected using transfer pipets and placed in separate tubes. A 1:1 ratio of 100% isopropanol was added, mixed and allowed to stand for 20 min to precipitate the RNA. The precipitate was collected by centrifugation at 10,000 rpm for 20 min at 4 °C. Isopropanol was discarded and 75% ethanol (volume same as isopropanol added earlier) was added to the RNA pellet and mixed to wash the RNA pellet. This helped to get rid of any traces of phenol. The washed RNA was again collected by centrifugation at 10,000 rpm for 20 min at 4 °C .The tube was drained of ethanol and the remaining pellet was air-dried for 15 min followed by solution of 100µl of Tris-EDTA (Tris-Cl, 1mM and EDTA at 0.1mM, pH 7.2-7.5). The RNA was stored at -20 °C. The purity of the total RNA preparation was established with an A_{260nm}/A_{280nm} ratio and the yield of total RNA was established in mg/ml as $A_{260nm}/25$ $A_{260nm/mg\ RNA}$/ml x vol. in ml of sample. Each sample was then diluted to 0.5mg/ml using TE buffer (Tris Cl, 1.0 mM and EDTA 0.1 mM, pH 7.2). Three micrograms of each sample was separated by size on a 1.5% agarose gel using TAE buffer (40 mM Tris-acetate, 1.0 mM EDTA, pH 8.0 to check the integrity of the total RNA.

Reverse Transcription and Polymerase Chain Reaction (RT-PCR)

Each sample of total RNA was reverse transcribed (RT) to produce complementary DNA (cDNA) using Invitrogen's SuperScript III First-Strand Synthesis kit for reverse transcription. Two micrograms of total RNA for each sample, 1.0 µl of

Oligo-dT_{20}, 1.0 µl of dNTP, was brought to 10 µl with dI-H_2O. After mixing, samples were incubated at 65 °C for 5min. in the thermal minicycler to denature RNA. The enzyme mixture consisting of 2µl of 10X RT-buffer, 1.3µl of $MgCl_2$, 1µl DTT, 1µl RNaseOUT, 1µl Superscript III-RT were brought to 10µl with dI-H_2O and then after mixing was added to each sample. The samples were vortexed and incubated at 48 °C for 50min in the thermal minicycler to synthesize cDNA. The reaction was stopped by heating to 85 °C for 5min and then placed on ice for 10 min. One microliter of RNAse H was added to all the respective tubes to remove the RNA from the cDNA. The samples were mixed, spun and incubated in the thermal minicycler at 37 °C for 40min. Ten µl of cDNA samples was diluted to 250 µl of water containing acetylated bovine serum albumin (25µg/µl BSA) carrier. These diluted cDNAs were then stored at -20 °C until PCR was performed.

Real-Time Quantitative PCR

To perform qPCR, sample cDNA was added to primer sets designed for the specific gene and the Platinum Taq, SYBR Green I qPCR Super Mix-UDG kit (Invitrogen) was used for dsDNA synthesis. The primers were designed using Vector NTI, v11 software from Invitrogen, Inc (Table 4). All sequences had a melt point of about 65 °C. All primers were synthesized at Biosynthesis, Inc. Lewisville, TX.

Table 4:

The Primer Sequence Used for Real-time PCR

Gene name	Reference ID	Primer
mus PabpN1	09-201	up+449
		GCAATGCTGGCCCAGTGATCATG
	09-202	dn-683
		AAGGCCAGGGACGTCCTCACTGAC
mus FABP4	09-161	up+68
		GTGTGATGCCTTTGTGGGAACCTGG
	09-162	dn-293
		TGCGGTGATTTCATCGAATTCCACG
mus Adiponectin	09-149	up+555
		CGGCAGCACTGGCAAGTTCTACTGC
	09-150	dn-788
		TTGTGGTCCCCATCCCCATACACCT
mus PPARα	09-185	up+6846
		TGTCCTGGCCACATTTCTCCTTGGC
	09-186	dn-7061
		CACAGGGGTGAAGGGCTGCAAATC
mus PPARγ	09-189	up+1243
		AGAGGGCCAAGGATTCATGACCAGG
	09-190	dn-1488
		TTCAGCTTGAGCTGCAGTTCCAGGG

Statistical Analysis

All experiments were performed in duplicate and repeated at least three times. Results are expressed as mean ± SE. SPSS (version 15.0 for windows) was used for determining the treatment effects by one-way ANOVA. An alpha level of $p<0.05$ was considered significant.

CHAPTER IV

EFFECTS OF CURCUMIN IN 3T3-L1 ADIPOCYTE DIFFERENTIATION

Jegghna J. Chheda, Nathaniel Mills, Parakat Vijayagopal

A Paper Submitted to Biochemical and Biophysical Research Communication

Curcumin, the principal curcuminoid found in the spice, turmeric, from rhizomes of *Curcuma Longa*, is a fat soluble polyphenolic compound. Several studies have shown its anticarcinogenic, antioxidant, anti-inflammatory, anti-septic and, antiangiogenic properties. In this study, we investigated the effect of curcumin on 3T3-L1 adipocyte differentiation in culture and gene expression involved in lipid metabolism. Curcumin inhibited 3T3-L1 adipocyte differentiation in dose dependent manner by downregulating PPAR-γ expression was observed in this study. Curcumin appears to be toxic to cells at a concentration of 30 μM. Curcumin also downregulated PPAR-α gene expression while not affecting Fabp4 gene expression indicating that fatty acid oxidation is not affected by curcumin in 3T3-L1 cells. Although adiponectin gene expression is known to be PPAR-γ dependent, in our study curcumin did not affect adiponectin gene expression despite downregulation of PPAR-γ gene suggesting that a change in PPAR-γ gene alone is not sufficient to alter adiponectin expression in these cells. Curcumin may be used as a potential weight loss supplement.

Key Words: Cucumin; 3T3-L1; Adipocyte differentiation; PPAR-α; PPAR-γ; Adiponectin; Fabp4

Introduction

Over the last few years there have been major advances in regulating obesity through molecular mechanisms (1). Preadipocyte differentiation has been studied using in vitro models of adipogenesis (2). The murine cell lines such as 3T3-L1, F442A and ob1771 preadipocytes are similar morphologically to fibroblastic preadipose cells found in stroma of the adipose tissue. Once these cells undergo differentiation they exhibit almost all the characteristics of the adipocytes present within the adipose tissue. Activation of adipogenesis is achieved over a period of 4-6 days by treating with a hormonal cocktail. The cocktail consists of insulin, isobutylmethylxanthine and dexamethasone in fetal bovine serum. The differentiation process of 3T3-L1 preadipocytes is divided into four stages: (1) preconfluent proliferation; (2) confluence/ growth arrest; (3, 10, 11) hormonal induction/ clonal expansion; and finally (4) permanent growth arrest/ terminal differentiation (1-5). Specific genes and proteins are expressed during each of these stages. The proteins involved in lipid metabolism and regulation of normal adipocyte function include lipoprotein lipase (LPL), stearyl-CoA-desaturase (SCD), fatty acid synthetase (FAS) and glucose transporter 4 (GLUT 4). Some secretory proteins involved are angiotensinogen, tumor necrosis factor α (TNFα) and leptin. The adipocyte-specific genes are gene encoding aP2 which is lipid-binding protein. The genes encoding these proteins are expressed later during differentiation and

do not play a critical role in differentiation but occur as a result of adipocyte differentiation (1).

Curcumin is the principal curcuminoid (polyphenolic compound) found in the spice – turmeric (6). Turmeric is a dried spice derived from the rhizomes of *Curcuma Longa,* a member of the ginger family. It is widely used in Indian, Southeast Asian and Middle Eastern cuisines. In cell culture, curcumin had an increased anti-inflammatory activity by indirectly inhibiting the activity of inflammatory enzymes and/or by enhancing the synthesis of glutathione (6). In addition, curcumin induced apoptosis by inhibiting cell-signaling pathways in various cancer cell lines grown in culture (6). In cultured vascular endothelial cells, it prevented tumor invasion and inhibited angiogenesis (6). In this study, we investigated the effect of curcumin in adipogenesis.

Material and Methods

3T3-L1 fibroblasts cell culture and differentiation. The mouse embryonic fibroblasts 3T3-L1 cells (Dr.Howard Green's Lab- Harvard Medical School) were cultured in Dulbecco's Modified Eagle's Medium (DMEM- Invitrogen) containing 10% bovine calf serum (Hyclone laboratories) and 1X Penicillin streptomycin (Invitrogen) at 37 ^{o}C in a humidified CO_2 (10%) incubator.

When cells reached 80% confluence they were placed in differentiating medium consisting of DMEM, 10% fetal bovine serum (FBS-Hyclone laboratories), penicillin/streptomycin in the presence and absence of different concentrations of curcumin and MDI [0.5 mM 3-Isobutyl-1-methylxanthine (MIX - Sigma Aldrich), 1.0

μM dexamethasone (Sigma Aldrich), 1.0 μg/ml insulin (Sigma-Aldrich)]. After 48 hrs the medium was changed to DMEM, 10% FBS and 1.0 μg/ml insulin ± Curcumin. Thereafter, the media was replaced with identical media every 2 days until the experiment was terminated on day 10. Cells usually differentiate by day 7 to 10 following first placement in the differentiating medium.

Oil Red-O staining. Cells were plated on coverslips in 12 well culture plates at a density of 1.4×10^4. When the cells reached 80% confluence, differentiation was initiated in the presence and absence of curcumin. Oil red O (ORO) staining was performed on day 10. The cells were rinsed with Hank's balanced salt solution (HBSS) twice and fixed in Davidson's fixative for an hour. The cells were then washed once with HBSS and stained with 36% ORO (Fisher-Scientific) in tri-ethyl phosphate (TEP) for 30 min. Stained cells were rinsed with HBSS twice and fixed with crystal mount and left overnight. The coverslips were then fixed with permount and left for drying for 3-4 days. The slides were viewed under a microscope and photographed. ORO/TEP stock was prepared by dissolving 500mg oil red-O in 60ml tri-ethyl phosphate and 40ml DI water. The working solution of ORO/TEP was prepared by dissolving 12ml of stock solution in 8ml of DI water (7).

Adipored Assay. 3T3-L1 cells were plated in 12 well culture plates to 80% confluence and differentiated in the presence and absence of curcumin as discussed earlier. On d= 10, each well was rinsed with HBSS. To each well, solution consisting of 2ml HBSS and 60ul of AdipoRed (8) was added. The plate was then incubated for

15min at room temperature and fluorescence was measured with excitation at 485nm and emission at 572nm (8).

RNA extraction. Cells were grown in a 35 mm culture dishes at 0.5 X 10^6 density for nucleic acid extraction. The differentiated cells were grown along with curcumin treated cells. On day 10 for extraction, cells were washed with Hank's balanced salt solution (HBBS) twice and then 1.0 ml of TRIzol (Invitrogen) Reagent was added. The dissolved cells were scraped from the culture plates with a rubber policeman and transferred to polypropylene tubes. The Mixture was incubated at room temperature for 10 minutes, 250µl chloroform was added and the samples were shaken vigorously for 30 sec. The samples were then placed on ice for 5 min followed by centrifugation at 7500 rpm for 20 min at 4 °C to separate the RNA containing upper aqueous phase from the protein containing organic phase with DNA forming the interphase. The upper aqueous phase was collected using transfer pipets and placed in separate tubes. A 1:1 ratio of 100% isopropanol was added, mixed and allowed to stand for 20 min to precipitate the RNA. The precipitate was collected by centrifugation at 10,000 rpm for 20 min at 4 °C. Isopropanol was discarded and 75% ethanol (volume same as isopropanol added earlier) was added to the RNA pellet and mixed to wash the RNA pellet. This helped to get rid of any traces of phenol. The washed RNA was again collected by centrifugation at 10,000 rpm for 20 min. at 4 °C .The tube was drained of ethanol and the remaining pellet was air-dried for 15 min followed by addition of 100µl of Tris-EDTA (Tris-Cl, 1mM and EDTA at 0.1mM, pH 7.2-7.5). The RNA was stored

at 20°C. The purity of the total RNA preparation was established with an A_{260nm}/A_{280nm} ratio and the yield of total RNA was established in mg/ml as $A_{260nm}/25$ $A_{260nm/mg\ RNA}$/ml x vol. in ml of sample. Each sample was then diluted to 0.5mg/ml using TE buffer (Tris Cl, 1.0 mM and EDTA 0.1 mM, pH 7.2). Three micrograms of each sample was separated by size on a 1.5% agarose gel using TAE buffer (40 mM Tris-acetate, 1.0 mM EDTA, pH 8.0to check the integrity of the total RNA.

Reverse Transcription and Polymerase Chain Reaction (RT-PCR). Each sample of total RNA was reverse transcribed (RT) to produce complementary DNA (cDNA) using Invitrogen's SuperScript III First-Strand Synthesis. Two micrograms of total RNA for each sample, 1.0 µl of Oligo-dT_{20}, 1.0 µl of dNTP, was brought to 10 µl with dI-H_2O. After mixing, samples were incubated at 65 °C for 5min in the thermal minicycler to denature RNA. The enzyme mixture consisting of 2µl of 10X RT-buffer, 1.3µl of $MgCl_2$, 1µl DTT, 1µl RNaseOUT, 1µl Superscript III-RT were brought to 10µl with dI-H_2O and then after mixing was added to each sample. The samples were vortexed and incubated at 48 °C for 50min in a thermal minicycler to synthesize cDNA. The reaction is stopped by heating to 85 °C for 5min and then placed on ice for 10min. One microliter of RNase H was added to all respective tubes to remove the RNA from the cDNA. The samples were mixed, spun and incubated in the thermal minicycler at 37 °C for 40min. Ten µl of cDNAs was diluted to 250µl of water containing acetylated bovine serum albumin (25µg/µl BSA). These diluted cDNAs were then stored at -20°C until PCR is performed.

Real-Time Quantitative PCR. To perform qPCR, sample cDNA was added to primer sets designed for the specific gene and the Platinum Taq, SYBR Green I qPCR Super Mix-UDG kit (Invitrogen) was used for dsDNA synthesis. The primers were designed using Vector NTI, v 11 software from Invitrogen, Inc (Table 1). All sequences had a melt point of about 65°C. All primers were synthesized at Biosynthesis, Inc. Lewisville, Tx.

Table 1:

The Primer sequence used for real-time PCR

Gene name	Reference ID	Primer
mus PabpN1	09-201	up+449 GCAATGCTGGCCCAGTGATCATG
	09-202	dn-683 AAGGCCAGGGACGTCCTCACTGAC
mus FABP4	09-161	up+68 GTGTGATGCCTTTGTGGGAACCTGG
	09-162	dn-293 TGCGGTGATTTCATCGAATTCCACG
mus Adiponectin	09-149	up+555 CGGCAGCACTGGCAAGTTCTACTGC
	09-150	dn-788 TTGTGGTCCCCATCCCCATACACCT
mus PPARα	09-185	up+6846 TGTCCTGGCCACATTTCTCCTTGGC
	09-186	dn-7061 CACAGGGGTGAAGGGCTGCAAATC

mus PPARγ	09-189	up+1243 AGAGGGCCAAGGATTCATGACCAGG
	09-190	dn-1488 TTCAGCTTGAGCTGCAGTTCCAGGG

Statistical Analysis. All experiments were performed in triplicate. The values were determined as mean ± SE (standard error). SPSS (version 15.0 for windows) was used for determining the treatment effects by one-way ANOVA. An alpha level of $p<0.05$ was considered significant.

Results

(1) Effect of curcumin on 3T3-L1 adipocyte differentiation. Oil red O is a fat soluble dye which stains intracellular triglycerides. Curcumin inhibited adipocyte differentiation in 3T3-L1 adipocytes in a dose dependent manner (Figure 1). At 30uM concentration, curcumin was toxic to the cell as indicated by extensive cell detachment from the plate. DMSO treatment alone did not affect adipocyte differentiation.

(2) Effect of curcumin on total triglyceride accumulation in differentiated 3T3-L1 adipocytes. Adipored Assay quantifies the accumulation of intracellular triglycerides. Curcumin significantly decreased intracellular triglycerides at 20μM concentration as compared to the DMSO control (Figure: 2).

(3) Effect of curcumin on PPARα, PPARγ, and Fabp4 and Adiponectin gene expression in 3T3-L1 adipocytes.

During the initial stages of our study with undifferentiated and differentiated 3T3-L1 adipocytes, expressions of the following 25 genes were studied: PabpN1(Poly A Binding Protein Nuclear 1), Adiponectin, Fabp4 (Fatty acid binding protein 4), Peroxisome Proliferator-Activated Receptor- PPAR-α, PPAR-γ, PPAR-δ, Leptin, Seipin, Lipin1, Lipin 2, Lipin 3, HmgCoA Reductase, Akt3 [v-akt murine thymoma viral oncogene homolog 3 (protein kinase B, gamma)], AGPAT6 [1-acylglycerol-3-phosphate O-acyltransferase 6 (lysophosphatidic acid acyltransferase, zeta)], Ghrelin, mTOR [Mechanistic target of rapamycin (serine/threonine kinase)], RPL22 (Ribosomal protein L22), Testin, Camkk2 (calcium/calmodulin-dependent protein kinase kinase 2, beta), Kcjn11 (potassium inwardly-rectifying channel, subfamily J, member 11), Tcf7l2 [Transcription factor 7-like 2 (T-cell specific, HMG-box)], Jmjd2a (jumonji domain containing 2A), EsR1 (Estrogen receptor 1), EsR2 (Estrogen receptor 2), CathepsinL.

Out of these 25 genes, 4 genes, PPAR-γ, PPAR-α, Adiponectin, and Fabp4 showed the highest fold changes between the undifferentiated and differentiated cells. Therefore, we chose these four genes to further investigate the effect of curcumin on their expression.

Curcumin showed a significant reduction in PPAR-γ gene expression at 20μM conc. as compared to control and also as compared to 10μM conc (Figure: 3A). Curcumin also significantly reduced PPAR-α gene expression at 20 μM conc (Figure: 3B). Curcumin did not affect adiponectin and Fabp4 gene expression (Figure: 3C and 3D).

Figure 1: Effect of curcumin on 3T3-L1 adipocyte differentiation. The cells were stained with Oil Red O at day 10 and pictures were taken under density filter with 20X magnification. The experiment was performed in duplicate and repeated three times.

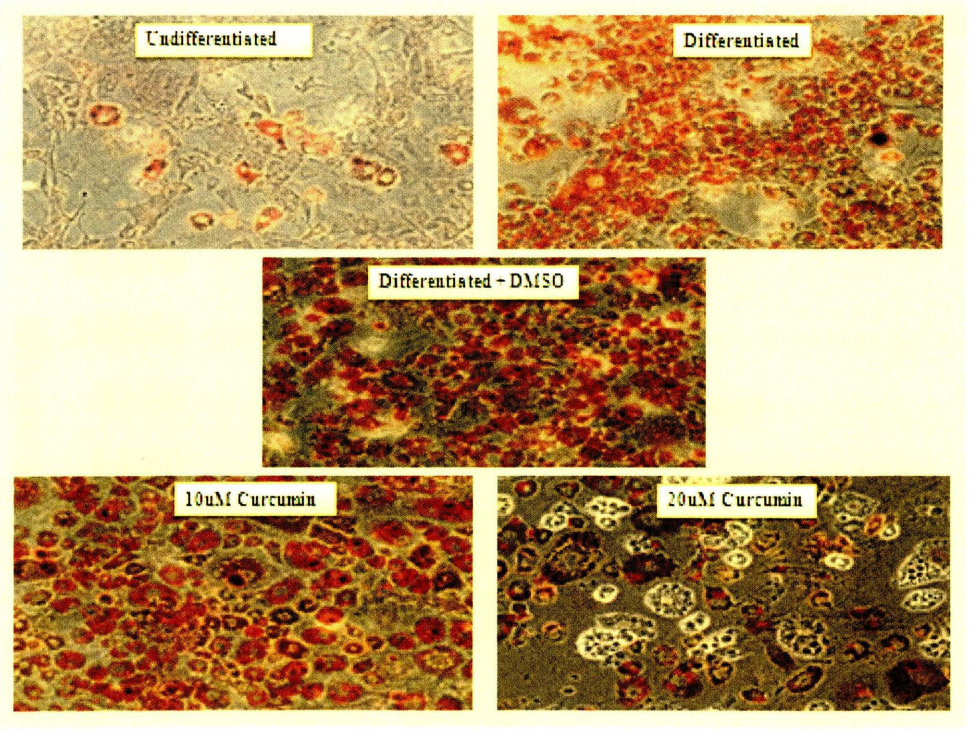


Figure 2: Effect of curcumin on total triglyceride accumulation in differentiated 3T3-L1 adipocytes. At day 10, cells were treated with solution consisting 2ml HBSS + 60µl adipored reagent and incubated for 15min. Cells were measured for fluorescence at excitation at 485nm and emission at 572nm. The values are from three independent experiments. The fluorescence values were measured and expressed as percentage of DMSO control and represented as mean ± SE. * $p<0.05$.

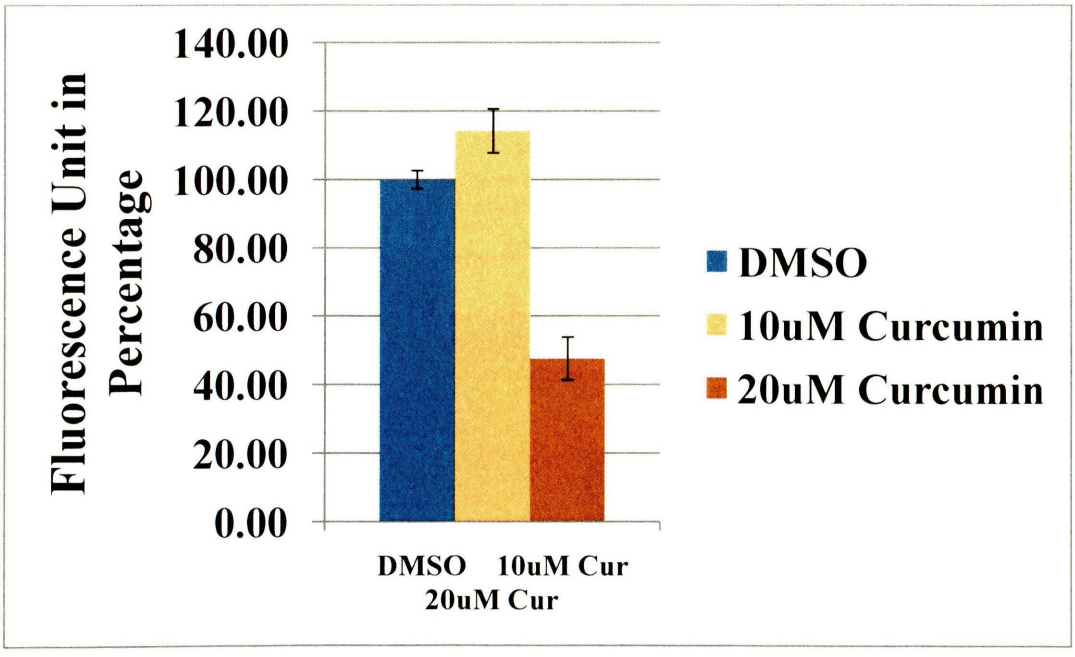
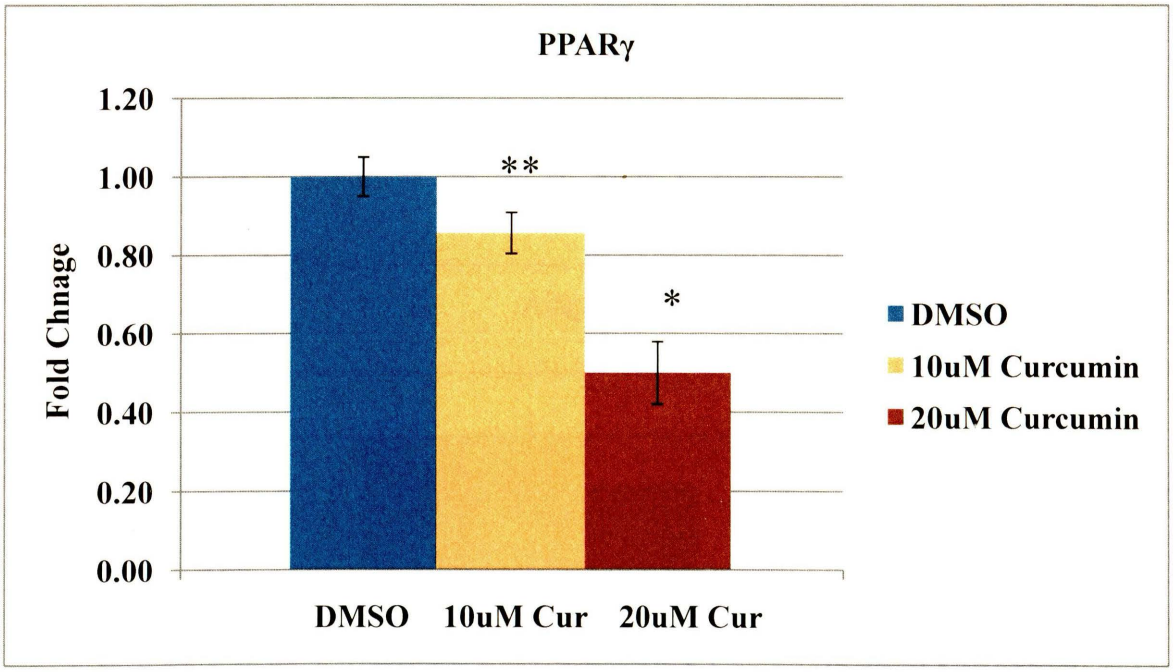
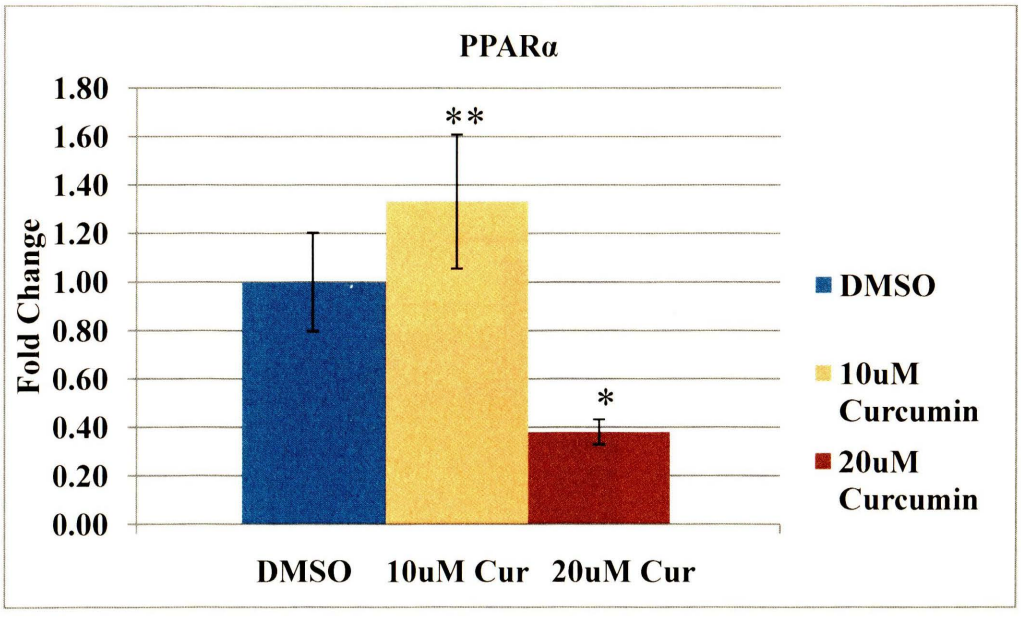


Figure 3: Effect of curcumin on PPARα, PPARγ, and Fabp4 and Adiponectin gene expression in 3T3-L1 adipocytes. Figure 3A and 3B- Real time PCR showed that curcumin suppressed PPARγ, PPARαgene expression in 20μM curcumin treated cells and Figure 3C and 3D showed no significant difference in Adiponectin and Fabp4gene expression. The values are from three independent experiments and PabpN1 was the housekeeping gene for all the experiments. Data are presented as mean ± SE. * $p<0.05$.



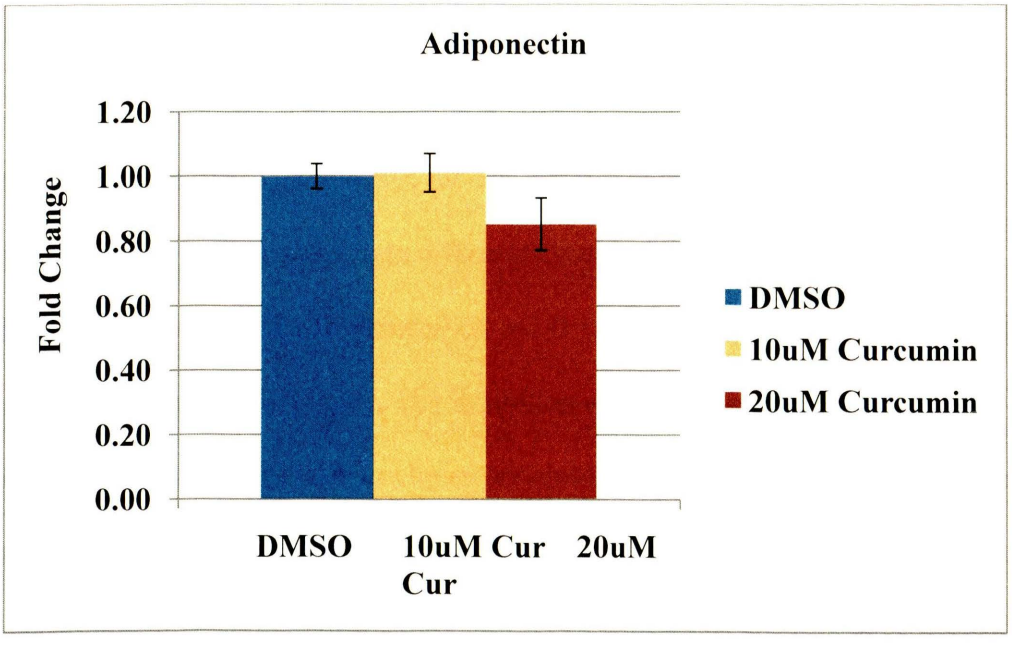
(A) * DMSO and 20uM Curcumin p=0.000

** 10uM and 20uM Curcumin p=0.003

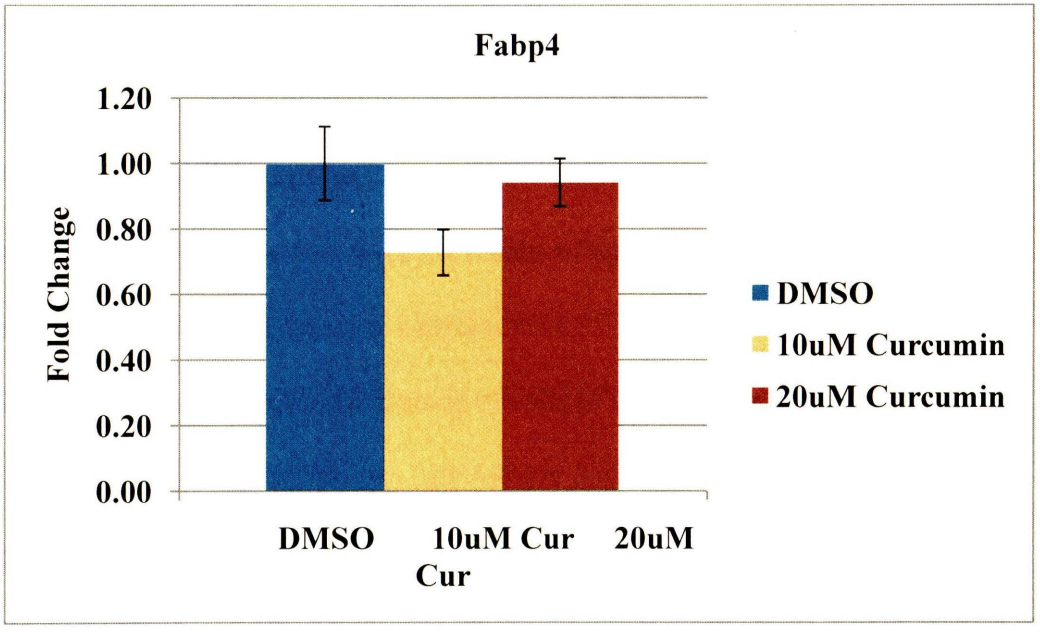


(B) *DMSO and 20uM Curcumin p=0.072

** 10uM and 20uM Curcumin p=0.046



(C)



(D)

Discussion

The aim of the study was to determine effect of curcumin on adipocyte differentiation and on genes involved in fat cell differentiation/lipolysis. Our result shows that curcumin inhibits adipocyte differentiation in a dose-dependent manner. This is indicated by progressively reduced oil red O staining in cultures incubated with 10 μM and 20 μM curcumin (Fig. 1). The inhibition of adipocyte differentiation by curcumin is further substantiated by reduced accumulation of triglycerides in cells (Fig 2). These results are consistent with the recent report by Ejaz et al showing inhibition of 3T3-L1 preadipocyte differentiation by curcumin (9). Curcumin appears to be toxic to cells at a concentration of 30 μM. This may be due to induction of apoptosis by

curcumin. Although we did not determine the effect of curcumin in apoptosis, polyphenols derived from green tea has been shown to increase apoptosis in 3T3-L1 adipocytes (12).

Curcumin significantly downregulates the expression of PPAR-γ at 20 uM conc (Fig.3A). This is not in agreement with the results of Ejaz et al. who found that curcumin did not affect PPAR-γ gene expression in 3T3-L1 adipocytes (9). This could be due to the difference in time of exposure of cells to curcumin in our study vs that of Ejaz et al; 10 days vs 24 h respectively. PPAR-γ is the master regulatory gene that regulates adipocyte differentiation (12). PPAR-γ is upregulated prior to initiation of adipocyte differentaiton. This in turn stimulates lipogenesis. Therefore, one mechanism by which curcumin inhibits adipogenesis in 3T3-L1 cells is through inhibition of PPAR-γ. Curcumin also has been reported to inhibit mitogen-activated protein (MAP) kinase phosphorylation required for adipocyte differentiation (13). Thus curcumin may interfere with multiple steps in adipocyte differentiation.

Interestingly, curcumin also significantly decreased PPAR-α gene expression which is involved in the stimulation of β-oxidation of fatty acids (Fig.3B). Although we did not determine fatty acid oxidation in our study, the downregulation of PPAR-α gene expression by curcumin suggests that there is less lipid degradation in cells exposed to curcumin. Therefore, it appears that curcumin works predominantly by inhibiting adipogenesis without affecting lipolysis.

Adipose tissue produces a variety of factors, including adipocyte fatty acid binding protein 4 (FABP4). FABP4 is implicated in hyperlipidemia, insulin resistance, type 2 diabetes, and atherogenesis (14). It is proposed that FABP4 promotes hypertriglyceridemia by inhibiting lipoprotein lipase. Elevated plasma level of FABP4 is a risk factor for obesity (15). FABP4 transports fatty acids into cell for β-oxidation. We did not find any significant change in FABP4 gene expression in cells treated with curcumin. This observation combined with the downregulation of PPAR-α by curcumin further confirms that fatty acid oxidation is not affected by curcumin in 3T3-L1 cells.

Adiponectin is secreted by adipocytes, skeletal muscle cells, cardiac myocytes and endothelial cells (16). It is involved in glucose and lipid homeostasis. There is a negative correlation between obesity and circulating adiponectin (17). Marked decrease in serum adiponectin levels has been seen in individuals with visceral obesity and states of insulin resistance such as non-alcoholic liver disease, atherosclerosis and type 2 diabetes mellitus(18,19). In 3T3-L1 cells, induction of adiponectin gene expression appears to be PPAR-γ dependent (20).This means that downregulation of PPAR-γ gene should decrease adiponectin gene expression. However, in our study, curcumin did not affect adiponectin gene expression despite the downregulation of PPAR-γ gene. This suggests that a change in PPAR-γ gene alone is not sufficient to alter adiponectin expression in these cells.

In summary, our study shows that curcumin is a potent inhibitor of adipocyte differentiation and the inhibitory action of curcumin is mediated through the downregulation of PPAR-γ gene expression.

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