EFFECTS OF CURCUMIN ON RETINOBLASTOMA PROTEIN AND P300 DURING ADIPOCYTE DIFFERENTIATION

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ABSTRACT

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The goal of the present study was to understand how curcumin suppresses PPARγ-mediated adipocyte differentiation in 3T3-F442 preadipocytes. We differentiated cultured 3T3-F442 cells with and without different doses of curcumin (0, 10μM, 15μM, and 20μM) and prepared cell lysates. We then performed Western Blot analysis to measure two upstream regulators of PPARγ, retinoblastoma protein (RB) and the p300 histone acetyltransferase in preadipocyte cells. Results were analyzed using one-Way ANOVA, and a Tuckey Post Hoc test. Curcumin caused a dose-dependent inhibition of adipocyte differentiation. Compared to control (no curcumin), dephosphorylated RB protein expression was higher in cultures exposed to 10 μM and 15 μM curcumin. However, at 20 μM the unphosphorylated RB protein decreased to the control level probably due to cell death. This suggests that curcumin inhibits 3T3-F442 preadipocyte differentiation by RB protein mediated inhibition of PPAR- γ. We did not detect the P300 protein in any cultures, including control by Western blot. This may be due to several reasons, including the cells not expressing P300, low sensitivity of detection technique, or denaturation of protein.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Over the last several decades, there has been a steady rise in obesity in the United States resulting in a U.S. public health expert declaration of obesity as an epidemic (Ross, Caballero, Cousins, Tucker, & Ziegler, 2014). Obesity is implicated in many health-related complications and diseases in humans including, type 2 diabetes (T2DM), hypertension, stroke, coronary heart disease, sleep apnea, gallbladder disease, osteoarthritis, and cancer (Nelms, Sucher, Lacey, & Roth, 2011). Although many programs have been developed to combat the obesity epidemic, the problem persists. This warrants a need to continue to explore and develop new ways of solving the obesity problem.

In a previous, unpublished study conducted in our laboratory, curcumin a turmeric polyphenolic compound, was found to be effective at preventing the pre-adipocyte cell 3T3-L1 from differentiating into mature adipocytes by reducing the gene expression of peroxisome proliferator-activated receptor γ (PPARγ) a key regulator of adipogenesis. The aim of the present study was to determine how curcumin suppresses PPARγ-mediated adipocyte differentiation. This was done by evaluating two separate upstream regulatory proteins that affect PPARγ expression, specifically retinoblastoma protein, a cell cycle regulator, and the histone acetyltransferase p300.

Hypothesis

(1) Curcumin prevents the phosphorylation of retinoblastoma protein during cell cycle re-entry preceding pre-adipocyte clonal expansion.

(2) Curcumin represses the p300 histone acetyltransferase during differentiation.

Specific Aims

(1) To determine the effect of curcumin on the phosphorylation of retinoblastoma protein during adipocyte differentiation.

(2) To determine whether curcumin represses p300 during adipocyte differentiation.

CHAPTER II

REVIEW OF LITERATURE

White Adipose Tissue

Adipose tissue functions primarily as an energy storage depot by storing and removing triglycerides (TGs) from their transporters in the blood (Tortora & Derrickson, 2011b). Adipocytes are considered endocrine cells as they can release hormones involved in appetite control, energy balance, body fat content, and reproduction (Nelms et al., 2011). There are two distinct types of adipose tissue: brown adipose tissue (BAT) and white adipose tissue (WAT). For this study, we focused only on WAT. White adipose tissue is found under the skin (subcutaneous) and inside the abdomen (visceral). Subcutaneous and visceral WAT have differences in their involvement in diseases with visceral fat associated with an altered metabolic profile (Ibrahim, 2010). Adipose tissue grows in two ways, hypertrophy (cell enlargement) and hyperplasia (increase in number). The balance between hyperplasia and hypertrophy is influenced by the interactions between genes, food, and lifestyle (Marti, Martinez-Gonzalez, & Martienez, 2008).

3T3-F442A Cells

Most research on adipocyte formation has been done with 3T3-L1 and 3T3-F442A preadipocyte cell lines from mice. These clonal-preadipocyte cells are morphologically indistinguishable from fibroblasts, but they have already been

programmed to becoming preadipocyte cells. These cells need only be treated with prodifferentiative agents (Insulin for 3T3-F442A cells) to undergo differentiation into mature adipose cells in four to six days after treatment (Green & Kehinde, 1974, 1975, 1976, 1979). The 3T3-F442A pre-adipocyte cells were used in this study.

Curcumin

Curcumin is a component of turmeric that comes from the rhizomes sometimes referred to as the root of plant *Curcuma longa*, a member of the ginger family. It is grown in the south and southeast tropical Asia. Turmeric has been used not only as a spice but is also used medicinally taken both orally and topically (Ammon & Wahl, 1991). Curcumin's bright golden color comes from its fat-soluble, polyphenolic pigments called curcuminoids. Of the curcuminoids, curcumin is known for being the most active polyphenol, and so is often the one of interest to researchers (Bar-Sela, Epelbaum, & Schaffer, 2010). Curcumin chemically is referred to as 1,7-bis (4hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, or diferuloylmethane. Curcumin has poor solubility in water and is quite unstable at basic pH, degrading in typically 30 minutes. Antioxidants such as ascorbic acid, or human blood, or 10% fetalbovine serum in culture media can prevent this degradation (Wang et al., 1997). Curcumin has been found to have antioxidant, anti-inflammatory, and antiseptic properties (Bengmark, 2006; Nishino et al., 2004; Maheshwari, Singh, Gaddipati, & Srimal, 2006). These properties allow curcumin to improve disease states that are rooted in oxidative and inflammatory conditions.

Historically turmeric has been a widely accepted treatment for a host of ailments including allergies, asthma, bronchial hyperactivity, coughs, sinusitis, liver disease,

digestive problems, blood sugar regulation, diabetic wounds, arthritis, sprains, and a large number of inflammatory conditions (Goel, Kunnumakkara, & Aggarwal, 2008). Inflammation is involved in a multitude of chronic neurodegenerative diseases (Begum et al., 2008; Kidd, 2008, 2009). Pathways promoting inflammation in the brain have been well studied in Alzheimer's disease. The known generators of inflammation are reactive oxygen species, reactive nitrogen species, lipid peroxides, and upregulated nuclear factor (NF)-κB and JNK gene. Curcumin is able to reduce these biological markers of inflammation in Alzheimer's disease (Huang et al., 1991; Begum et al., 2008; Pendurthi, Williams, & Rao, 1997). Curcumin is also highly effective at treating cancer in all stages: initiation, promotion, proliferation, and metastasis (Strimpakos, & Sharma, 2008; Jurenka 2009; Hatcher et al., 2008; Villegas et al., 2008). Curcumin has been shown to slow cancer, and block cancer promoting phorbol esters (Goel et al., 2008; Hatcher et al., 2008). In vitro models have shown curcumin induces apoptosis in cancer cells while at the same not inducing apoptosis in surrounding healthy cells (Hatcher et al., 2008; Villegas et al., 2008).

Curcumin has again shown positive effects on markers of oxidative stress for example, it was found to reduce serum lipid peroxides (Sahebkar, Serbanc, Ursoniuc, & Banach, 2015). Investigations using curcumin supplementation have found that curcumin is able to upregulate serum antioxidants such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSH; Banach et al., 2014; Menon & Sudheer, 2007; Panahi, Alishiri, Parvin, & Sahebkar, 2016). Curcumin's ability to sequester free radicals occurs through several mechanisms. First curcumin itself is a free radical scavenger, able to reduce reactive oxygen species and reactive nitrogen species (Menon & Sudheer, 2007).

Curcumin being lipophilic also makes it very effective at scavenging peroxyl radicals and as a result is considered a chain-breaking antioxidant (Priyadarsini et al., 2003). Second, it can increase the activity of antioxidant fighting proteins; catalase, GSH, and SOD enzymes (Lin et al., 2007; Marchiani, Rozzo, Fadda, Delogu, & Ruzza, 2014). Third, it can inhibit reactive oxygen generating enzymes such as lipoxygenase/cyclooxygenase and xanthine hydrogenase/oxidase (Lin et al., 2007).

Oxidative stress is implicated in many chronic diseases such as Parkinson, epilepsy, metabolic syndrome, diabetes, obesity, and many more (Panahi, 2016a). Oxidative stress can cause damage to proteins, lipids, and DNA leading to an increase in pro-inflammatory mediators

(Chatterjee, 2016). Tumor necrosis factor α (TNF-α) is one of those mediators and is responsible for inflammation found in most diseases. TNF-α is regulated by NF-κB and in reverse the presence of TNF-α increases activity of NF-κB. Both TNF-α and NF-κB are also stimulated by most inflammatory cytokines. Curcumin has shown have the ability to down-regulate NF-κB in the presence of different inflammatory stimuli and is thereby able to reduce inflammation (Panahi, 2016b). Curcumin also suppresses inflammation through other mechanisms, but these are beyond the scope of this study.

The Cell Cycle

The cell cycle is a sequence of events in which a somatic cell (any cell of a living organism other than the reproductive cells) duplicates its contents and divides in two. This process is divided into four main phases G_1 phase, S (synthesis) phase, G_2 phase, and the M

(mitotic) phase. G_1 phase is located after the M phase and before the S phase in the cell cycle. During this phase no DNA replication occurs, instead, the cell is very metabolically active as it replicates many of its organelles and cytosolic components. Cells that remain in this G_1 phase for a long duration are likely never to divide again and are in G_0 phase. Following G_1 phase comes S phase during which DNA replication occurs. This is followed by the G_2 phase where cell growth continues, enzymes and other proteins are synthesized in preparation for cell division, and replication of centrosomes is completed. Finally, in the M phase, there is the formation of two identical cells following nuclear division and cytoplasmic division. M phase goes through a series of four seamless steps to achieve cytoplasmic division known as, prophase, metaphase, anaphase, and telophase (Tortora & Derrickson, 2011a).

Cell cycle regulators

The cell cycle transitions between phases through the control of regulatory proteins. Centrally important proteins during these transitions are cyclin-dependent kinases (CDKs), which are a family of serine/threonine protein kinases that are active at specific points of the cell cycle based on their required cyclins (Hunt, 1991; Pines & Hunter, 1991). Cyclin-dependent kinases are always present during the cell cycle with the rise and fall of cyclins influencing their activity (Evans, Rosenthal, Youngblom, Distel, & Hunt, 1983). In addition to this mechanism of control, a family of proteins called CDK inhibitors (CKIs) counteracts CDK activity. Cyclindependent kinases inhibitors are divided into two families. INK4 family includes p15, p16, p18, and p19, which act on CDK4 and CDK6 inside the G_1 phase, all of which bind directly to CDK, preventing

cyclin binding to the CDKs (Carnero & Hannon, 1998; Sherr & Roberts, 1995); The CIP/KIP family controls CDKs by inactivating CDK-cyclin complexes including p21, p27, and p57 which inhibit the CDK-cyclin complexes found in the G_1 phase (Koff, 2006; Harper et al., 1995; Hengst & Reed, 1998).

Control Mechanisms of the G1-Phase to S-Phase Transition

Positive regulators of the G_1 phase to S phase transition include CDK4, CDK6, CDK2, Cyclin D1, and Cyclin E activity. Negative regulators of this transition include p16, p21, p27, and most critically retinoblastoma tumor suppressor protein (RB). Retinoblastoma proteins consist of a small family called "Pocket Proteins" that includes Rb/p105, p107, and Rb2/p130. The name Pocket Protein comes from the pocket region used by pRb, p107, and Rb2/p130 to bind with oncoproteins and cellular proteins like E2F transcription factors. The pocket region structure is comprised of two functional domains (A and B) separated by a spacer region (S); each spacer region is unique for each Pocket Protein. The E2F family of transcription factors interact and binds with the A and B pocket domains (Gillet & Barnes, 2000).

RB is responsible for the regulation of the G1 checkpoint, capable of blocking S-phase entry and consequently cell growth, as well as promoting terminal differentiation by causing cell cycle exit and tissue-specific gene expression (Weinberg, 1995). RB family proteins are regulated post-translationally by phosphorylation. Phosphorylation inactivates RB proteins preventing binding with other proteins; the phosphorylation of RB family is a cell cycle-dependent event that is carried out by CDKs. In the early stages of G1 phase, D-cyclins couple with CDK4 and CDK6

and phosphorylate p105 and p130 (Dowdy et al., 1993). In the middle and later stages of G1, cyclins E and A form complexes with CDK2 that carry out the phosphorylation of p105, p130, and p107 (De Luca et al., 1997). The inactivation of RB family proteins is crucial for the release of E2F family proteins so that S phase of the cell cycle can begin.

The RB protein is central to the transition between the G_1 phase and S phase. Hyperphosphorylation of RB toward the end of the G_1 phase prevents RB from complexing with the E2F family proteins, allowing E2F proteins to begin a cascade of gene transcription necessary to begin the DNA replication to proceed with the S phase (Johnson, Schwarz, Cress, & Nevins, 1993). Initial phosphorylation of RB is carried out by Cyclin D-CDKs (can contain either CDK4/CDK6 or a CIP/KIP protein) beginning in the middle of G_1 phase. Later RB is phosphorylated for a second time by cyclin E-CDK2 to finish blocking RB activity. Conversely, CKIs can prevent CDKs from phosphorylating RB and halt the transition into S phase, leading to either growth arrest, repair, or apoptosis of the cell (Sherr & Roberts, 1994).

A study by Srivastava, Chen, Siddiqui, Sarva, and Shankar (2007) examined the molecular mechanisms by which curcumin induced cell cycle arrest in LNCaP and PC-3 prostate cancer cells. Srivastava et al. showed curcumin induced the expression of CKI p16, p21, and p27 in both prostate cancer cell lines. Srivastava et al. further showed that p21 can bind CDK2 and cyclin E preventing the phosphorylation of RB, which leads to growth arrest. p21 was able to block cyclin D preventing CDK4 from phosphorylating RB leading to higher levels of unphosphorylated RB protein and thus cell cycle arrest. The suppression of positive cell cycle modulators by curcumin prevented cell cycle

progression past the G_1/S phase juncture and induced apoptosis. The magnitude of curcumin's effect on cell cycle regulators increased in a dose-dependent manner (0, 5, 10, and 20 μM). E2F1 and RB are critical proteins in the control of adipogenesis where they serve as the gatekeepers of cell cycle re-entry as preadipocytes must enter and exit the cell cycle frequently to go through periods of clonal expansion. E2F1 positively regulates PPARγ (Fajas et al., 2002b). PPARγ is the quintessential protein needed to drive fat cell differentiation. PPARγ is required to bind fat-selective enhancers for the ap2 and PEPCK (Phosphoenolpyruvate Carboxykinase) genes (Tontonoz, Hu, Graves, Budavari, & Spiegelman, 1995). This means that unphosphorylated RB can reduce PPARγ. Unphosphorylated RB is also able to bind PPARγ directly and inhibit its activity (Fajas et al., 2002a).

We hypothesized that curcumin induces hypophosphorylation of RB and thus prevents cell cycle progression in 3T3-F442A preadipocyte cells.

Regulation of Gene Expression

Essential to epigenetic regulation of gene expression are histone deacetylases (HDACs) that are responsible for removing acetyl groups attached to the chromatin's histone tails (as well as other proteins), opposite of the HDACs are histone acetyltransferases (HATs) that play the role of adding acetyl groups to chromatin (and other proteins). Acetylation takes place on the lysine residues on the histone tails, this action removes the positive charge on the histone and causes the chromatin structure to relax and change shape increasing accessibility of transcription factors for their targets (Shahbazian & Grunstein, 2007). Actions of both deacetylation and acetylation have each

been shown to be responsible for the regulatory modification of gene transcription (Kouzarides, 2000). The state of acetylation is a key regulatory element that controls gene expression, developmental processes, and disease states. Acetylation is controlled through the antagonistic action of the two enzyme family's the HATs and HDACs (Haberland, Montgomery, & Olson, 2009).

P300 and CBP Protein Complex

P300 and CBP (CREBBP or CREB binding protein) proteins (EP300 or E1A binding protein p300) constitute the histone acetyltransferase KAT3 family1. P300 and CBP form a complex together and both interact directly and indirectly with several hundred different proteins (Bedford, Kasper, Fukuyama, & Brindle, 2010). The ability to bind with so many proteins comes from its many protein binding domains. The many domain regions are unique to p300/CBP protein and enable it to be the core structure of many multicomponent transcription complexes and thus a central nexus to many protein-protein interactions in the mammalian interactome
(Bedford, et al., 2010). The p300/CBP transcriptional coactivator is a HAT that is involved in multiple processes, including proliferation, differentiation, and apoptosis (Chan & Thangue, 2001).

Curcumin and Iron Chelation

Curcumin has been found to modulate proteins of iron metabolism. Curcumin is believed to exert its effects through iron chelation by increasing the activity of iron regulatory proteins (IRPs) and transferrin receptor-1 in liver cells (Jiao et al., 2006). A study using Saccharomyces cerevisiae to model eukaryotic cells also found that curcumin

entered the yeast cells and concentrated in the endoplasmic reticulum (ER). Curcumin reduced intracellular iron and stalled the cell cycle in G1 phase for a period. This G1 stalling was quickly reversed when supplemental iron was added (Minear et al., 2011).

Depletion of cellular iron by curcumin leads to an alteration in histone acetylation and degradation of Sml1p in Saccharomyces cerevisiae. Once more, supplemental iron was able to reverse the effects (Azad, Singh, Golla, & Tomar, 2013). Curcumin's effect on histone acetylation is attributed to inhibition of HATs. However, no effects on histone deacetylases HDACs were found (Kang, Chen, Shi, Jia, & Zhang, 2005). Another study found that curcumin is able to repress the p300/CBP protein and inhibit p300-mediated acetylation of p53 *in vivo* (Balasubramanyam et al., 2004).

Critical to preadipocyte differentiation is the CCAAT/enhancer-binding protein β (C/EBPβ). This protein has a role in mitotic clonal expansion and terminal differentiation (Tang, Otto, & Lane, 2003; Tang et al., 2005). C/EBPβ is required for the activation of PPAR-γ and C/EBPβ (Tang, Jiang, & Lane, 1999). C/EBPβs action is upregulated by acetylation from p300/CBP (Zhao et al., 2013).

Suppression of the p300/CBP coactivator by curcumin-mediated iron chelation is a possible mechanism for inhibition of PPAR-γ and prevention of adipocyte differentiation.

CHAPTER III

MATERIALS AND METHODS

The study examined the effect of the polyphenol curcumin on the state of RB phosphorylation and P300 histone acetyltransferase in pre-adipocyte 3T3-F442A cells. The experiment used one control group (no curcumin) and three test groups consisting of different curcumin dosages: 10µM, 15µM, and 20µM. Each group contained 12 cell cultures. RB's state of phosphorylation and p300 were measured by Western Blot analysis.

3T3-F442A Cell Culture

3T3-F442A preadipocytes were cultured in 6-well culture plates from our stock culture in the laboratory. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum at 37 C in a 10% CO_2 incubator. When cells reached 70-80% confluency, the culture medium was changed to induction medium (DMEM + 10% fetal bovine serum, FBS, + 167 nM insulin) with and without different doses of curcumin (0, 10µM, 15µM, and 20µM). After 48 h, the cells were switched to induction medium without insulin ± different doses of curcumin. Analyses were performed after seven days of differentiation.

Oil Red-O staining

3T3-F442A cells were differentiated in 6-well plates in the presence and absence

of curcumin. On day 8, cells were fixed in formalin and stained with Oil Red-O.

Cell Lysis

3T3-F442A cells were differentiated in the presence and absence of different doses of curcumin. On day 8, cells were harvested. To begin this process, the medium was removed, and cells were washed with HBSS. Next cells were lysed with 0.3ml per well of 1X RIPA buffer containing protease inhibitors. The plates were then incubated on ice for five minutes, the cells were scraped off and cell lysate transferred to individual tubes. The lysates were sonicated three times for 10 seconds each to prevent overheating the sample. Following sonication, tubes were centrifuged for 10 minutes and the supernatant stored at -80º C for later use.

BCA Protein Assay

Protein content of the cell lysates was determined by using the Pierce BCA Protein Assay Kit.

Western Blotting

We used Western blot analysis to determine the state of phosphorylation on the RB protein and the status of p300 protein. Proteins in cell lysates were resolved in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and proteins were transferred onto nitrocellulose membranes. Membranes were blocked in 1% bovine serum albumin dissolved in TBS-0.1% Tween-20 (TBST) for one-hour. The blots were then washed and incubated with mouse RB protein monoclonal antibody, P-300 histone acetyltransferase monoclonal antibody, and rabbit β-actin polyclonal antibody for 1 h.

The membranes were then washed and incubated with secondary antibodies for 1 h. and the protein bands were visualized using chemifluorescence imager (Li-cor Odyssey).

Statistical Analysis

The data were analyzed using One-Way ANOVA and Tuckey Post Hoc were used to compare differences in RB, between groups. All results are expressed as mean ± SEM. $P < 0.05$ is considered significant.

CHAPTER IV

EFFECTS OF CURCUMIN ON RETINOBLASTOMA PROTEIN AND P300 DURING

ADIPOCYTE DIFFERENTIATION

A PAPER TO BE SUBMITTED TO THE JOURNAL OF MEDICINAL FOOD (J Med Food)

Jonathan Rea, Victorine Imrhan, Chandan Prasad and Parakat Vijayagopal

Abstract

The goal of the present study was to understand how curcumin suppresses PPARγmediated adipocyte differentiation in 3T3-F442 preadipocytes. We differentiated cultured 3T3F442 cells with and without different doses of curcumin (0, 10µM, 15µM, and 20µM) and prepared cell lysates. We then performed Western Blot analysis to measure two upstream regulators of PPARγ, retinoblastoma protein and the p300 histone acetyltransferase in preadipocyte cells. Results were analyzed using One-Way ANOVA, and a Tuckey Post Hoc test. Curcumin caused a dose-dependent inhibition of adipocyte differentiation. Compared to control (no curcumin), dephosphorylated RB protein expression was higher in cultures exposed to 10 µM and 15 µM curcumin. However, at 20 µM the unphosphorylated RB protein decreased to the control level probably due to cell death. This suggests that curcumin inhibits 3T3-F442 preadipocyte differentiation by

RB protein mediated inhibition of PPAR- γ. We were unable to detect the P300 protein in any culture, including control by Western blot. This may be attributed to several reasons, including the cells not expressing P300, low sensitivity of detection technique, and denaturation of protein.

Key Words: 3T3-F442A Cells; Adipocyte differentiation; p300 histone acetyltransferase; Retinoblastoma protein.

Introduction

Over the last several decades, there has been a steady rise in obesity rates resulting in a US public health expert declaration of obesity as an epidemic[1]. Obesity is implicated in many health-related complications and diseases in humans including, T2DM, hypertension, stroke, coronary heart disease, sleep apnea, gallbladder disease, osteoarthritis, and cancer[2]. Although many methods have been developed to combat the obesity epidemic, the problem persists. This warrants a need to continue to explore and develop new ways of solving the obesity problem. We believe curcumin could be one of these new ways.

In a previous study conducted in our laboratory, the turmeric polyphenolic compound curcumin was found to be effective at preventing the pre-adipocyte cell 3T3-L1 from differentiating into mature adipocytes by reducing the gene expression of peroxisome proliferator-activated receptor γ (PPARγ), a key regulator of adipogenesis. The aim of the present study was to determine how curcumin suppresses PPARγ-mediated adipocyte differentiation. This was done by evaluating two separate upstream

regulatory proteins that affect PPARγ expression, specifically Retinoblastoma protein, a cell cycle regulator, and the histone acetylase p300.

Materials and Methods

The study examined the effect of the polyphenol curcumin on the state of RB phosphorylation and P300 histone acetyltransferase in pre -adipocyte 3T3 -F442A cells. The experiment used one control group (no curcumin) and three test groups consisting of different curcumin dosages; 10µM, 15µM, and 20µM. Each group contained 12 cell cultures. RB's state of phosphorylation and p300 was measured using the Western Blot method.

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Statistical Analysis

The data were analyzed using One-Way ANOVA, and a Tuckey Post Hoc test was used to compare differences in RB, between groups. All results are expressed as mean ± SEM. $P< 0.05$ is considered significant.

Results

Effect of curcumin on adipocyte differentiation

3T3-F442 preadipocytes were incubated in the presence of these different doses of curcumin and curcumin's ability to inhibit adipocyte differentiation was measured by oil red O staining. Figure 1 shows that curcumin dose-dependently inhibited lipid accumulation over eight days posttreatment. The effect of curcumin was strongest at 15µM and 20µM treatment.

Microscope images of the effects of curcumin on adipocyte differentiation:

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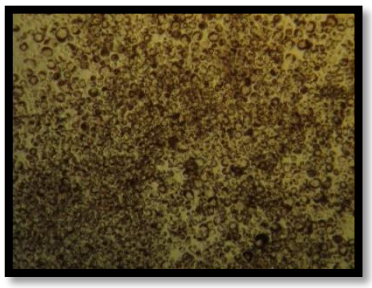
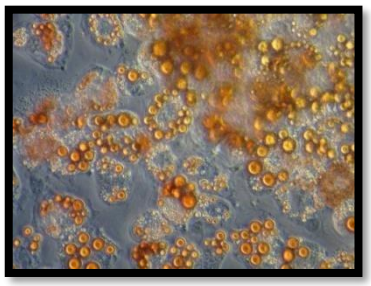
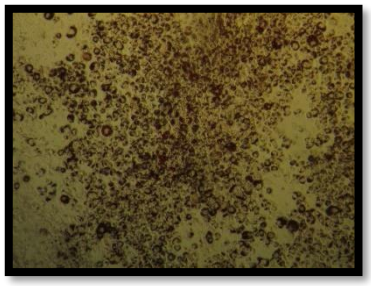
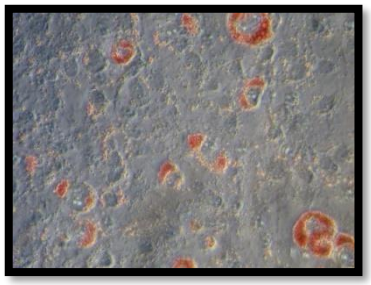
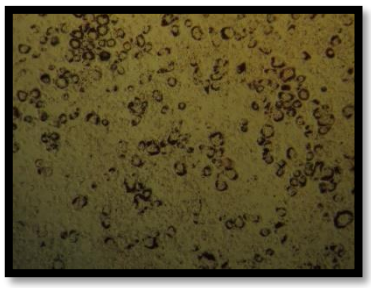
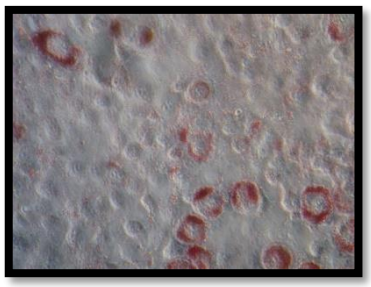
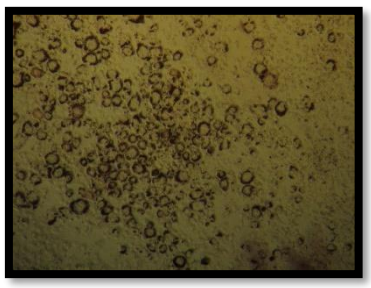
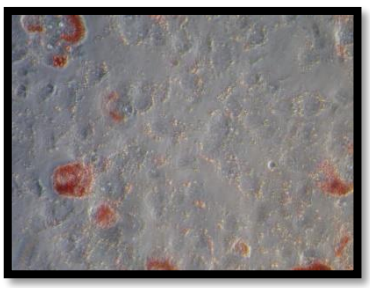
Control Group (No Curcumin Treatment):		
Group 1 (10µM):		
Group 2 (15µM):		
Group 3 (20µM):		

Figure 1. Results from the Oil Red-O staining test shows the effects of curcumin treatments on 323-F442A cells.

Retinoblastoma Protein

We quantified RB protein in cultures treated with and without different doses of curcumin by Western blot analysis. Table 1 shows the results from one representative gel. Figure 2 shows the histogram of unphosphorylated RB protein. Compared to control (no curcumin), RB protein expression was higher in cultures exposed to 10 µM and 15 µM curcumin. However, at 20 µM the unphosphorylated RB protein decreased to the control level.

Table 1

Data from The Western Blot Done On Retinoblastoma Protein

10/23/2017 Gel 1	Actin	Normalized Actin	RB	Normalized RB/Actin	Normalized RBActin/RB
C1	2020	1	1650	1650	1
C2	1730	0.85643564	1610	1879.88439	1.139323875
C3	1380	0.68316832	1460	2137.10145	1.295213
G10:1	1230	0.60891089	1980	3251.70732	1.970731707
G10:2	1050	0.51980198	1790	3443.61905	2.087041847
G15:1	1210	0.5990099	1900	3171.90083	1.922364137
G15:2	1120	0.55445545	1740	3138.21429	1.901948052
G20:1	1490	0.73762376	1200	1626.84564	0.985967053
G20:2	1380	0.68316832	1640	2400.57971	1.454896794

Note. Data from a representative Western Blot done on retinoblastoma protein. C: Control group. G10: 10µM curcumin treatment. G15: 15µM curcumin treatment. G20: 20µM curcumin treatment.

Visual of Gel-1:

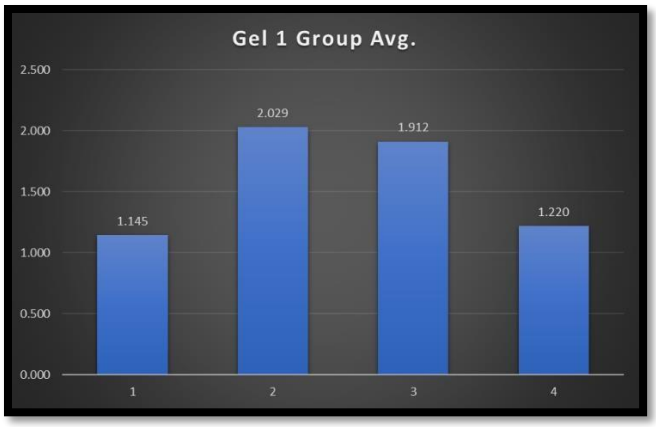


Figure 2. Group results for unphosphorylated retinoblastoma protein from a representative Western blot gel. 1: Control group. 2: 10µM curcumin treatment. 3: 15µM curcumin treatment. 4: 20µM curcumin treatment.

Table 2

Summary Statistics

Groups	*Count*	*Sum*	*Average*	*Variance*	*Std. Dev.*
C	3	3.434537	1.144846	0.021811	0.147684
G10	2	4.057774	2.028887	0.006764	0.082244
G15	2	3.824312	1.912156	0.000208	0.014436
G20	2	2.440864	1.220432	0.109948	0.331583

Note. G10: 10µM curcumin treatment. G15: 15µM curcumin treatment. G20: 20µM curcumin treatment.

ANOVA test revealed significant differences between some of the groups; *P*-value 0.006 (see Table 2). The Post Hoc analysis by the Tuckey test revealed significant differences between groups C & G10 ($P = 0.011$), C & G15 ($P = 0.02$), G10 & G20 ($P =$

0.023), and G15 & G20 (P = 0.042). No significant differences were found between the control group and the 20µM treatment as well as the 10µM treatment and 15µM treatment.

P300 Protein

We did not detect P300 protein in any of the cultures.

Discussion

These results show that curcumin inhibits 3T3-F442A preadipocyte differentiation in a dose-dependent manner. This is indicated by a progressive reduction in Oil red O staining in cultures incubated with increasing doses of curcumin. Earlier studies in our laboratory (unpublished), and by Ferguson et al. and Ejaz et al., have shown that curcumin inhibited adipocyte differentiation in 3T3-L1 preadipocytes as well[3,4]. To our knowledge, this is the first study to show this effect of curcumin in 3T3-F442 cells.

Retinoblastoma Protein

PPAR-γ is the master regulatory gene for adipocyte differentiation. Differentiation is initiated following the upregulation of PPAR-γ. This, in turn, activates several genes involved in lipogenesis. We (unpublished data) and Ejaz et al. have observed that curcumin inhibits PPAR-γ expression and prevents differentiation in 3T3-L1 cells[3]. Fajas et al. have shown that unphosphorylated RB protein can bind PPAR-γ directly and inhibit its activity[5]. This will prevent adipocyte differentiation. In our present study, curcumin increased unphosphorylated RB protein at 10 µM and 15 µM concentrations. This raises the possibility that curcumin inhibits 3T3-F442 preadipocyte differentiation by RB

protein-mediated inhibition of PPAR-γ. The increase of unphosphorylated RB protein following curcumin treatment was also seen in a study by
Srivastava et al. found that curcumin at 10 µM and 20 µM concentrations increased unphosphorylated of RB protein levels in LNCap and PC-3 prostate cancer cells[6]. Another study in 3T3-L1 preadipocyte cells tested a more bioavailable version of curcumin called Curcumin3,4-Dichloro Phenyl Pyrazole (CDPP). Gupta et al. (2017) examined upstream regulators of RB and not RB itself, and found downregulation of Cyclin D1, Cyclin D3, CDK2, CDK4, CDK6, and an increase in p27 at dosages of CDPP between 5–20 uM. The decrease in RB's upstream phosphorylation proteins by the CDPP compound further supports our findings[7].

Our results also show that in cultures treated with 20 µM curcumin, the unphosphorylated RB protein expression was not significantly different from control. This observation contrasts with the results of Oil red O staining where the 20 µM curcumin caused the maximum inhibition of adipocyte differentiation. One explanation for this inconsistency is that the 20µM dose of curcumin may be toxic to the cells and the reduced Oil red O staining may partly be due to increased cell death. It is well documented that curcumin induces apoptosis in many cancer cell lines including acute T-cell leukemias, prostate PC-3 cells, prostate LNCaP cells, leukemia HL60 cells, colon HCT-116 and colon SW480 cells.[8,9,10] Ferguson et al. also found curcumin treatment to be lethal but starting at concentrations of 20 µM or higher was highly lethal to 3T3L1 cells[4]. Therefore, cell death is a likely reason for the reduced expression of unphosphorylated RB protein in our study. However, we cannot confirm this without measuring cell viability.

P300 and Western Blot Problems

We were unable to detect the P300 protein in any cultures, including control by Western blot. There may be several reasons for this**.** One possibility is that these cells do not express P300 protein. Another likely reason may be that the concentration of P300 protein inside the cells is below the level of detection by Western blot. A third possibility for the failure to detect the P300 could be the instability of the large protein due to repeated freezing and thawing.

Summary

Curcumin inhibited preadipocyte differentiation in a dose-dependent manner. Curcumin at low doses also increased unphosphorylated RB protein suggesting that curcumin inhibits 3T3F442 preadipocyte differentiation by RB protein mediated inhibition of PPAR- γ. We did not detect P300 proteins in all of our cultures.

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