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Curcumin inhibits 3T3-L1 preadipocyte proliferation by mechanisms involving post-transcriptional p27 regulation

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ABSTRACT

Previous reports from our lab have shown that Skp2 is necessary for p27 degradation and cell cycle progression during adipocyte differentiation. Data presented here demonstrate that the anti-inflammatory, anti-obesity phytochemical curcumin blocked Skp2 protein accumulation during early adipocyte hyperplasia. In addition, curcumin dose-dependently induced p27 protein accumulation and G1 arrest of synchronously replicating 3T3-L1 preadipocytes. Of note, p27 protein accumulation occurred in the presence of decreased p27 mRNA suggesting a role for post-transcriptional regulation. In support of this hypothesis, curcumin markedly increased p27 protein half-life as well as attenuated ubiquitin proteasome activity suggesting that inhibition of targeted p27 proteolysis occurred through curcumin-mediated attenuation of Skp2 and 26S proteasome activity. While we observed no cytotoxic effects for curcumin at doses less than 20 μM, it is important to note an increase in apoptotic signaling at concentrations greater than 30 μM. Finally, data presented here demonstrate that the anti-proliferative effect of curcumin was critical for the suppression of adipocyte differentiation and the development of the mature adipocyte. Collectively, our data demonstrate that curcumin-mediated post-transcriptional accumulation of p27 accounts in part for the anti-proliferative effect observed in 3T3-L1 preadipocytes.

1. Introduction

Obesity-associated co-morbidities such as diabetes and cardiovascular disease contribute to the rising incidence of morbidity and mortality as well as add to the tremendous burden plaguing our health care system [19,43]. Obesity is defined as excess white adipose tissue accumulation that can occur due to increased adipocyte cell volume (hypertrophy) and cell number (hyperplasia). Though preadipocyte (PA) replication slows during adulthood, periods of positive energy balance at any age can trigger proliferation and subsequent differentiation of dormant PAs known to exist throughout the lifespan [5,15,18,32]. Obesity resulting from adipocyte hyperplasia is associated with the poorest prognosis of treatment [11,46]. Thus, understanding of molecular and cellular events involved in adipogenesis is critical for elucidating targets in the treatment and prevention of obesity and its co-morbidities.

The hormonal and nutritional mechanisms underlying adipocyte hyperplasia have been extensively studied using the murine 3T3-L1 preadipocyte cell line [24]. In response to a standard adipogenic cocktail, growth-arrested PAs synchronously re-enter the cell cycle for 1–2 rounds of cell division known as mitotic clonal expansion (MCE), a required and necessary step for adipogenesis [26,40]. MCE is driven by the convergence of mitogen-dependent signaling pathways on sequential expression of regulatory cyclin proteins needed for cyclin-dependent kinase (Cdk) activation. Timely activation of Cdks is critical for orderly cell cycle progression. Transition through the G1/S phase restriction point represents a pivotal and decisive point of cellular autonomy where cell cycle control becomes mitogen-independent [4,37,38]. Cell cycle progression through this restriction point is potently suppressed by the cyclin-dependent kinase inhibitor (CKI) p27 [7]. In contrast to the mitogenic activation of cyclins, p27 accumulates in response to anti-mitotic stimuli (e.g. nutrient deprivation, density-arrest, differentiation) to ensure cell cycle arrest and extended quiescence [14,28,30,31]. Indeed, p27 gene ablation has been shown to markedly increase fat mass in mice due to adipocyte hyperplasia [27]. Thus, molecular mechanisms that maintain an optimal balance in p27 during PA replication appear critical in the etiology of obesity.

Curcumin is a bioactive phytochemical found in the rhizome of the perennial herb *Curcuma longa* Linn (turmeric). Widely used as a culinary agent, curcumin is known to have anti-oxidant, anti-inflammatory, and anti-carcinogenic properties [36,44]. Of note, high doses of curcumin (12 g/day) have shown no significant cytotoxic effects in humans demonstrating that curcumin is a safe

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bioactive food component [36,44]. Recent experimental evidence demonstrated an anti-inflammatory, anti-diabetic, and anti-obesity role for curcumin in mouse models of obesity and diabetes [10,35,45]. While these studies postulated a preventive role of curcumin in diet-induced obesity, findings from Ejaz et al. [10] demonstrated an inhibitory role for curcumin during adipocyte differentiation. More recently, it was demonstrated that curcumin blocks adipogenesis during early adipocyte differentiation by inhibiting post-confluent PAs during MCE [17]. Despite these findings that suggest that curcumin is effective in regulating new adipocyte generation, the mechanistic actions of curcumin during adipogenesis remain unclear.

Previous reports from our lab have demonstrated that S-phase kinase-associated protein-2 (Skp2) regulates p27 degradation during S and G2 phase progression of replicating 3T3-L1 PAs during early adipocyte differentiation [2,3]. Furthermore, we and others have shown that Skp2 promotes cell cycle progression by targeting specific proteins for ubiquitylation and degradation by the 26S proteasome [2,6,39]. In this study, we demonstrated that the inhibitory actions of curcumin on adipocyte differentiation occur early during PA proliferation. Moreover, we demonstrated that curcumin arrests proliferating PAs in late-G1 phase and promotes p27 protein accumulation during density arrest by post-transcriptional mechanisms involving Skp2 and 26S proteasome degradation. Together, these data demonstrate an effect of curcumin on p27 protein accumulation and provide a link between cell cycle progression and adipocyte hyperplasia.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle's Medium (DMEM), calf bovine serum (CS), and trypsin-EDTA were purchased from Invitrogen. Fetal bovine serum (FBS) was obtained from HyClone. Propidium Iodide was purchased from Sigma. The following antibodies used for western blot analysis: p27 (Transduction Laboratories); Cleaved caspase-3, cleaved PARP, and ubiquitin (Cell Signaling); and cyclin A, PPARγ, C/EBPα, adipsin, and Skp2 (Santa Cruz Biotechnology). Curcumin was purchased from Alexis Biochemical.

2.2. Cell Culture

Murine 3T3-L1 PAs, purchased from Howard Green, Harvard Medical School, were cultured and differentiated as previously described [2]. Briefly, cells were propagated in DMEM supplemented with 10% CS until density-arrest at 2 days post-confluence then stimulated a hormonal cocktail containing 10% FBS, 0.5 mM 1-methyl-3-isobutylxanthine, 1 μM dexamethasone, and 1.7 μM insulin (MDI). Time post-MDI refers to the time since the administration of MDI to culture medium.

2.3. Immunoblotting

Relative protein abundance was assessed by immunoblotting as previously described [2].

2.4. Oil Red O (ORO)

Cells were fixed in formalin and stained with ORO, washed in deionized water, dried and scanned for analysis.

2.5. Flow cytometry

Cell cycle progression was assessed by DNA histograms generated by propidium iodide staining as previously described [2].

2.6. Real-Time RT-PCR

Relative mRNA abundance was assessed by RT-PCR using the $2^{-\Delta\Delta C_T}$ method as previously described [12].

2.7. Proteasome assay

Proteasome activity was assessed using the 20S Proteasome Assay Kit per manufacturer's instructions (BostonBiochem, Cambridge, MA).

3. Results

3.1. Curcumin inhibits 3T3-L1 adipocyte differentiation during early stages of adipogenesis

To examine the effects of curcumin on adipocyte differentiation, density-arrested PAs were dose-dependently treated with curcumin and stimulated to differentiate with MDI. On day 8 (d8), cells were fixed and stained with Oil Red O (ORO) to assess lipid accumulation. As shown by ORO, curcumin dose-dependently inhibited adipocyte differentiation (Fig. 1A). Inhibition of differentiation resulted from suppression of PPARγ and C/EBPα, the dominant transcriptional regulators of adipogenesis (Fig. 1B).

Mitotic clonal expansion (MCE) is an 'obligatory' step of adipocyte differentiation. Inhibition of MCE has been shown necessary for the activation of PPARγ and C/EBPα and acquisition of the mature adipocyte phenotype [24–26,33]. To elucidate a role for curcumin during MCE, we treated density-arrested PAs in the absence or presence of 20 μM curcumin at 0 h, 24 h, 48 h, or 72 h post-MDI stimulation. For each treatment, protein expression was assessed on day 8 (d8) for C/EBPα, PPARγ, and adipsin, an adipocyte-specific serine protease. Compared to untreated PAs (d0), MDI-stimulation led to marked increases in protein expression of all three proteins (Fig. 1C). However, treatment with curcumin at the onset of differentiation (0 h) suppressed protein expression of C/EBPα, PPARγ, and adipsin, with only modest suppression observed for curcumin treatment 24 h post-MDI and no effect seen with treatment 48 h or 72 h post-MDI (Fig. 1C).

The findings demonstrated that curcumin blocked early stages (<24 h) of adipocyte differentiation. To determine if curcumin suppressed MCE, density-arrested PAs were stimulated with MDI for 20 h in the absence or presence of increasing doses of curcumin. Cells were fixed and DNA stained with propidium iodide for flow cytometric analysis of cell cycle progression. As shown in Fig. 1D, two DNA peaks in the 2n and 4n range, representing G0/G1 and G2/M cell populations, were present in all histograms. Stimulation with MDI resulted in a marked shift from G0/G1 to S and G2/M phase populations demonstrating synchronous cell cycle progression in the absence of curcumin (Fig. 1D). In contrast, curcumin dose-dependently inhibited MDI-induced cell cycle progression prior to G1/S phase transition (Fig. 1D).

Curcumin has been shown to induce apoptosis in various cancer cell lines [21,29], To assess cytotoxicity, density-arrested PAs were stimulated with MDI for 20 h in the absence or presence of increasing concentrations of curcumin or 1000 J/m^2 UV, a known activator of apoptosis. Protein expression was examined for caspase 3 and PARP cleavage, mid- and late-stage markers of apoptosis.