Ind J Clin Biochem (Oct-Dec 2018) 33(4):414–421
https://doi.org/10.1007/s12291-017-0692-z

ORIGINAL RESEARCH ARTICLE

Citral, a Monoterpene Inhibits Adipogenesis Through Modulation of Adipogenic Transcription Factors in 3T3-L1 Cells

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Received: 13 June 2017 / Accepted: 31 August 2017 / Published online: 12 September 2017

Abstract Obesity is considered as a major global human health problem which significantly increases the risk for development of type 2 diabetes. Citral, a bioactive compound widely found in a variety of foods that are consumed daily. In this study, we investigated the inhibitory effect of citral against adipogenic genes in 3T3-L1 cells. The mouse fibroblast 3T3-L1 pre-adipocytes were differentiated into adipocytes using adipogenic cocktail (5 g/ml insulin, 0.5 mM isobutylmethylxanthine and 10 M dexamethasone). Differentiation of adipocytes was evaluated by assessing triglyceride accumulation assay and cell viability by MTT assay. The PI3K/AKT signaling, adipogenic specific transcription factors (PPARγ, SREBP-1c, FAS and CPD) and inflammatory genes (TNF-α, IL-6 and MCP-1) were analyzed by western blotting and reverse transcriptase PCR in differentiated 3T3-L1 cell lines. In this study, triglyceride accumulation was increased in adipogenic cocktail induced 3T3-L1 cells, whereas treatment of citral significantly decreased levels of triglyceride accumulation in concentration dependent manner. Further, MTT assay shows that there was no reduction of cell viability during the differentiation of 3T3-L1 cells. The differentiated 3T3-L1 cell significantly increases the expression of PI3K/AKT, adipogenic transcription factors (PPARγ, SREBP-1c, FAS and CPD) and inflammatory biomarkers (TNF-α, IL-6 and MCP-1). Conversely, cells were treated with citral significantly suppress the expression of PI3K/AKT, PPARγ, SREBP-1c, FAS, CPD, TNF-α, IL-6 and MCP-1 in dose dependent manner. Thus, citral exhibits beneficial effects to inhibit adipogenesis in 3T3-L1 adipocytes through the modulation of adipogenic transcription factors and inflammatory markers.

Keywords Adipogenesis · Citral · Inflammation · Obesity

Introduction

Obesity is a serious health problem that implicated in several diseases which including type 2 diabetes, hypertension, coronary heart diseases, and possible for cancer [1]. Obesity is a result of the imbalance between energy intake and energy expenditure, which leads to the adipogenesis [2]. Diet is closely associated with the development of obesity and it is caused from excess intake of sugars and lipid contents. Moreover, genetic factors, including gene mutations also cause obesity [1, 3].

Adipose tissue, a major organ of energy reservoir that accumulates triglycerol (TG), it releases free fatty acid (FFA) and nutritional excess when energy is required [4]. It has been recognized as an endocrine organ that synthesizes and secretes biologically active molecules called adipokines, which influence various homeostatic systems [5]. Adipogenesis is a process in which pre-adipocytes differentiate into lipid-laden and insulin-responsive, mature adipocytes under the regulation of several transcriptional factors, such as sterol regulatory element binding protein (SREBP), CCAAT/enhancer binding proteins (C/EBPs) and peroxisome proliferator-activated receptor γ (PPAR-γ) [6]. There are several studies have postulated that obesity has been considered as chronic and systematic inflammatory disease that stimulates insulin resistance and the production of inflammatory cytokines [7]. Adipocytes release

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adipokines and bioactive peptides, including leptin, adiponectin, tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), macrophage chemoattractant protein-1 (MCP-1) and it is circulated via the vascular system to insulin target tissues such as liver, muscle and islet cells inducing insulin resistance [2, 8].

The phosphatidylinositol 3 kinase (PI3K/AKT) signaling pathway involves the differentiation, inflammation, proliferation and regulation of glucose transporters [9]. AKT plays a pivotal role in the insulin signaling pathway; the activation of AKT signaling has been confirmed as the pathogenesis of obesity and diabetes. Therefore, inhibition of PI3K/AKT signaling has considered being an important target for prevention of obesity [10].

There are several phytochemicals, which includes retinoic acid, genistein, naringenin and conjugated linoleic acid are reduced the risk of obesity [11–13]. Citral (3,7-dimethyl-2,6-octadienal) otherwise called as lemonal, a mixture of terpenoids and it is a major constituent of *Cymbopogon citratus* [14]. Citral has been used in perfumery, cosmetic, microbiological and pharmaceutical industries for eliminating pathogens [15]. Citral exhibits several pharmacological properties such as antioxidants, antifungal, antiviral, antiinflammation etc. [15]. Previously, citral has been potentially exhibits renoprotective agent for focal segmental glomerulo sclerosis (FSGS) by activating the Nrf2 pathway [16]. Moreover, citral has been increased energy dissipation and also reduced lipid accumulation in diet-induced obesity in animal model [17]. The antiadipogenic effect of citral and molecular mechanism were still not yet studied. Therefore, we aimed to investigate the inhibitory role of citral against adipogenesis through modulation of adipogenic transcriptional factors, PI3K/AKT signaling and inflammatory signaling in 3T3-L1 pre-adipocytes cell line.

Materials and Methods

Chemicals

Citral, insulin, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, propidium iodide, ribonuclease (Rnase), trizol and protease inhibitor were purchased from Sigma, St. Louis, MO, USA. Dulbecco's modified Eagle's medium (DMEM), Phosphate buffered saline (PBS), fetal bovine serum (FBS), trypsin-ethylenediamine tetra acetic acid (EDTA) were purchased from Himedia, Mumbai, India. Primary antibodies such as PI3K, p-AKT, TNF-α, IL-6 and MCP-1 and secondary antibodies for anti-mouse and anti-goat IgG-HRP were purchased from Sigma chemicals Co., St. Louis, USA. RNA isolation kit, reverse transcription kit and primers were purchased from Qiagen, USA. All other chemicals and solvents of analytical grade were obtained from SD Fine chemicals, Mumbai and Fisher Inorganic and Aromatic Limited, Chennai.

3T3-L1 Cell Differentiation

3T3-L1 mouse pre-adipocytes cell lines were obtained from National Centre for Cell Sciences (NCCS) Pune, and it was maintained with DMEM medium containing 10% fetal bovine serum, 100 mg/ml of streptomycin, and 100 U of penicillin. For adipogenesis, 3T3-L1 pre-adipocytes were grown into confluence in a 25 mm tissue culture flask or six-well plate, then were differentiated into adipocytes using a standard protocol. The 3T3-L1 cells were incubated in the adipogenic cocktail (5 g/ml insulin, 0.5 mM isobutylmethylxanthine, and 10 M dexamethasone) for 2 days. This was followed by incubation in insulin-supplemented medium for additional 4 days. The normal medium was used on day 7 to maintain the adipocytes.

Cell Viability Assay

To assess the cell viability of citral was analyzed by MTT assay [18]. The 3T3-L1 pre-adipocytes were seeded in 24 well plates at a density of 2×10^4 cells/well. 10–100 µM concentrations of citral were added to the confluent 3T3-L1 pre-adipocytes during the differentiation period. At the end of the treatment period, the medium was removed and replaced with 100 µl of 3-(4,5-dimethylthiazol-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma Aldrich) solution. Then, cells were kept incubation 3 h at 37 °C in CO_2 and dissolved with 500 µl dimethyl sulfoxide (DMSO). The optical density (OD) of each sample was measured at 570 nm by using multimode reader.

Triglyceride Assay

The total triglyceride contents were determined in pre-adipocytes and matured adipocytes using the triglyceride determination kit (Sigma) according to the manufacturer's instruction [19]. Briefly, after treatment, cells were collected and sonicated in lysate buffer. Then, the samples were mixed with 40 µl of triglyceride reagent in the 96 well plates, incubated for 15 min at 37 °C, and then measured for the light absorbance at 540 nm a microplate reader. For all measurements, at least four biological replicates were used in this experiment.

Western Blot Analysis

Proteins were subjected to 10% SDS-PAGE and transferred to PVDF membranes for the analysis of PI3K, p-AKT,

TNF-α, IL-6 and MCP-1 [20]. Membranes were washed with tris-buffered saline (TBS) containing 0.05% Tween 20 (TBST), and were then blocked for 1 h in TBS containing 3% BSA. The membranes were then washed with TBST and then incubated overnight at 4 °C with the primary antibodies with a dilution of 1:1000. Membranes were washed with TBST and treated with HRP-conjugated secondary antibodies for 60 min at room temperature. The PVDF membranes were then washed with TBST thrice with 10 min interval and the developed bands were detected using a chemiluminescence substrate. The images were acquired by Image Studio J software.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Cells were seeded onto a culture plate at a density of 1×10^5 cells/ml. After treatment of differentiation and/or citral, the total RNA was isolated from cells using the Trizol reagent (Sigma, Mumbai). cDNA was synthesized using 5 μg total RNA by superscript III reverse transcriptase enzyme (Invitrogen). cDNA was amplified in 20 μl total volume containing Taq polymerase enzyme and oligonucleotides, sequences of the appropriate primers were used for specific amplifications. For cDNA synthesis, 25 °C for 10 min, 42 °C for 50 min and 75 °C for 15 min. For DNA amplification, 2 min initial denaturation at 95 °C followed by 35 cycles with 15 s, denaturation at 94 °C, 30 s primer annealing at 58 °C and 30 s of extension at 72 °C. For quantitation, differences between treatments were analyzed by comparing mRNA levels to the control after normalization to GAPDH mRNA levels.

Statistical Analysis

All the values were expressed as means ± Standard Deviation (SD) of six (n = 6) determinations. The data were statistically analyzed using one-way analysis of variance (ANOVA) on SPSS (statistical package for social sciences) and the group means were compared by Duncan's Multiple Range Test (DMRT). The results were considered statistically significant if the p value was less than 0.05.

Results

Effect of Citral on Triglyceride Accumulation in Normal Pre-adipocyte and Differentiated Adipocytes 3T3-L1 Cells

The triglyceride accumulation of normal pre-adipocyte and differentiated adipocytes 3T3-L1 cells were analyzed by triglyceride accumulation assay. In this study, we found that normal 3T3-L1 pre-adipocytes showed there is no triglyceride accumulations were observed (Fig. 1). Conversely, adipogenesis cocktail induced 3T3-L1 cells showed increased intracellular triglyceride accumulations were observed and it is indicated as differentiation of pre-adipocytes into adipocytes. However, 3T3-L1 cells treated with citral significantly inhibits the formation of intracellular lipid accumulation in a concentration depended manner. Furthermore, 30, 40, and 50 μM concentration of citral exhibits significant inhibition of triglyceride accumulation than 10 and 20 μM concentration of citral. Hence, we have chosen 30, 40, and 50 μM concentration of citral for further experiments.

Effect of Citral on the Proliferation of 3T3-L1 Pre-adipocytes

The cells were incubated with various concentrations of citral (10–100 μM); the proliferative activity was assessed by using MTT assay. The findings revealed that citral did not caused significant reduction on the cell proliferation up to 100 μM concentrations in 3T3-L1 cells (Fig. 2).

Effect of Citral on Adipogenic Transcription Factors Gene Expression in 3T3-L1 Cells

Adipocyte differentiation is triggered by a set of interacting transcription factors (C/EBPα, PPARγ, SREBP-1c, and FAS) contributes obesity and diabetic complications. PCR analysis was adopted to analyze the effect of citral on adipogenic transcription factors expressions in 3T3-L1 cells (Fig. 3). We found that differentiated control 3T3-L1 cells showed increased mRNA expression of adipogenic transcription factors such as C/EBPα, PPARγ, SREBP-1c, and FAS; whereas, citral treatment significantly downregulates mRNA expression of C/EBPα, PPARγ, SREBP-1c, and FAS in 3T3-L1 cells.

Effect of Citral on PI3K/AKT Signaling 3T3-L1 Cells

The overexpression of PI3K/AKT signaling has been considered as cell survival, differentiation and inflammation. Therefore, effect of citral on PI3K/AKT protein expressions were assessed by western blotting (Fig. 4). In this study, there was over-expression of PI3K and phosphorylated AKT were observed in differentiated control 3T3-L1 cells. However, treatment with citral significantly decreased the expression of PI3K and phosphorylated AKT in 3T3-L1 cells in a concentration dependent manner.

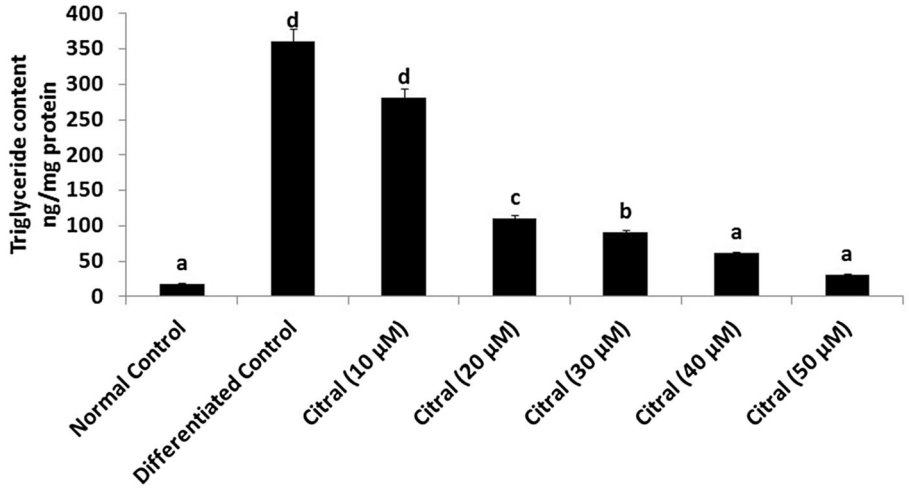

Fig. 1 Effect of citral on the differentiation and adipogenesis of 3T3-L1 cells. 3T3-L1 pre-adipocytes were incubated with adipogenesis cocktail (DMEM with 3-isobutyl-1-methylxanthine, dexamethasone, and insulin) for 2 days and then replaced with DMEM containing insulin with or without citral (0, 10, 20, 30, 40 and 50 µM) for 8 days. Intracellular triglyceride concentrations are presented as the mean ± SD from three independent experiments. Values not sharing a common marking (a, b, c and d) differ significantly at $P < 0.05$ (Duncan's multiple-range test)

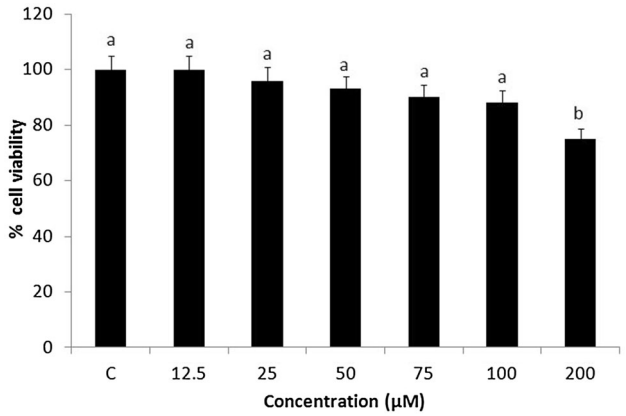

Fig. 2 Effect of citral treatment on cell viability in 3T3-L1 cells. 3T3-L1 pre-adipocytes were incubated with adipogenic cocktail (DMEM with 3-isobutyl-1-methylxanthine, dexamethasone, and insulin) for 2 days and then replaced with DMEM containing insulin with or without citral (0, 12.5, 25, 50, 100 and 200 µM) for 8 days. Cell viability was determined by MTT assay. Data are presented as the mean ± SD from three independent experiments. Values not sharing a common marking (a) differ significantly at $P < 0.05$ (Duncan's multiple-range test)

Effect of Citral on Inflammatory Signaling in 3T3-L1 Cells

The effect of citral on inflammatory protein expression was assessed by western blotting analysis (Fig. 5). Inflammatory protein such as TNF-α, IL-6, and MCP-1 were over expressed in differentiated control in 3T3-L1 cells (Fig. 5). Whereas, treatment of citral significantly decreases the expression of inflammatory genes such as TNF-α, IL-6, and MCP-1 in 3T3-L1 cells in a concentration dependent manner.

Discussion

The incidence of obesity is increasing at an alarming rate throughout the world [21]. Sedentary lifestyles, environmental factors, and change in dietary habits are the most important contributors to this sudden surge [17]. Prevention of obesity through education and changes to the obesogenic environment are long-term goals [22]. Moreover, psychological therapies such as cognitive behavioral therapy cannot easily be delivered on a mass scale and long-term results are disappointing [23]. Plant derived phytochemicals are effectively preventing the formation of obesity. For these purposes, citral, a terpenoids from lemon grass inhibits adipogenesis in 3T3-L1 pre-adipocytes by modulation of adipogenic transcriptional factors, inflammatory responses and PI3K/AKT cell survival signaling.

In this study, first we evaluated the anti-adipogenic role of citral using 3T3-L1 cells. This 3T3-L1 preadipocyte cell line is one of the well-characterized and reliable experimental models for studying adipogenesis and high fat induced obesity [24]. Adipogenesis, a process during which fibroblast-like pre-adipocytes developed into mature lipid-loaded, insulin-responsive adipocytes [25, 26]. The adipogenic cocktail and/or citral treatment induced pre-adipocytes into mature adipocytes were determined by triglyceride accumulation assay. In this study, we found that an adipogenic cocktail (5 g/ml insulin, 0.5 mM isobutylmethylxanthine, and 10 M dexamethasone)

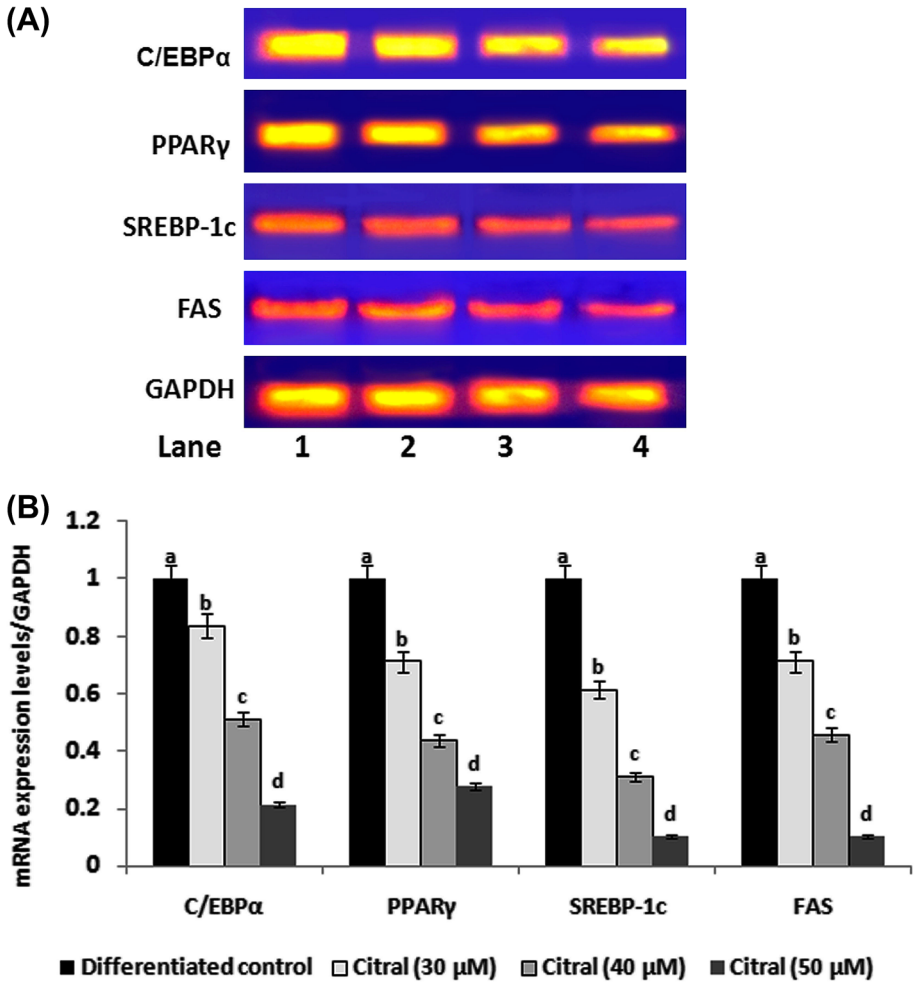

Fig. 3 Effect of citral on the mRNA expression of adipogenic transcriptional factor in 3T3-L1 cells. The mRNA levels of adipogenic genes were examined by reverse transcritase-PCR. Lane 1: differentiated control, Lane 2: citral 30 μM, Lane 3: citral 40 μM, Lane 4: citral 50 μM. Data are presented as the mean ± SD from three independent experiments. Values not sharing a common marking (a, b, c and d) differ significantly at $P < 0.05$ (Duncan's multiple-range test)

induced 3T3-L1 pre-adipocytes showed increased triglyceride accumulation when compared to normal undifferentiated control 3T3-L1 cells (Fig. 1). The adipogenic cocktail (insulin, isobutylmethylxanthine and dexamethasone) are excellent factors for involving differentiating the pre-adipocytes into adipocytes [27]. Several studies have been used these agents for inducing differentiated cells [26, 27]. In this study, we tested 10–50 μM concentration of citral against triglyceride accumulation in 3T3-L1 cells. Results shows that citral at the dose ranges from 30 to 50 μM inhibits triglyceride accumulation and 50 μM of citral effectively inhibits intracellular triglyceride accumulation in 3T3-L1 cells.

CCAAT element-binding proteins (C/EBP) and sterol response element-binding protein 1 (ADD1/SREBP1) are active during the early stages of the differentiation process and induce the expression and/or activity of the PPARγ, a pivotal coordinator of adipocyte differentiation [28, 29]. Activated PPARγ induces cell cycle, and in cooperation with C/EBPα, stimulates the expression of many metabolic genes [28]. The differentiation of 3T3-L1 pre-adipocytes is a complex process that needs activating ability of several transcription factors, which mainly includes PPAR-γ, C/EBP, ADD1/SREBP1 [30]. Numerous studies have been postulated that modulation and/or activation of PPARγ, C/EBPα, SREBP-1c, and FAS resulted in adipogenesis [28, 31]. In this present study, citral inhibits the differentiation and adipogenesis through blocking adipogenic transcriptional factors 3T3-L1 cells (Fig. 3). Similarly, blueberry peel extracts inhibit adipogenesis through blocking PPARγ, C/EBPα, SREBP-1c and FAS in 3T3-L1 cells and reduce high-fat diet-induced obesity [32].

PI3K/AKT is serione therionine kinase, which regulates cell survival, differentiation and inflammatory responses for the development of adipogenesis [33]. The PI3K pathway that is activated by the phosphorylation of AKT is an important component of adipogenesis. The cascade reaction of the PI3K/AKT pathway plays an important role in the differentiation of fat and activates PPARγ and C/EBPα during the differentiation of 3T3-L1 pre-adipocytes [34]. In this present study adipogenic cocktail induced 3T3-L1 cells showed that there was over expression of PI3K and phosphorylated AKT, which indicates the differentiation of 3T3-L1 cells. Previously, 3T3-L1 pre-

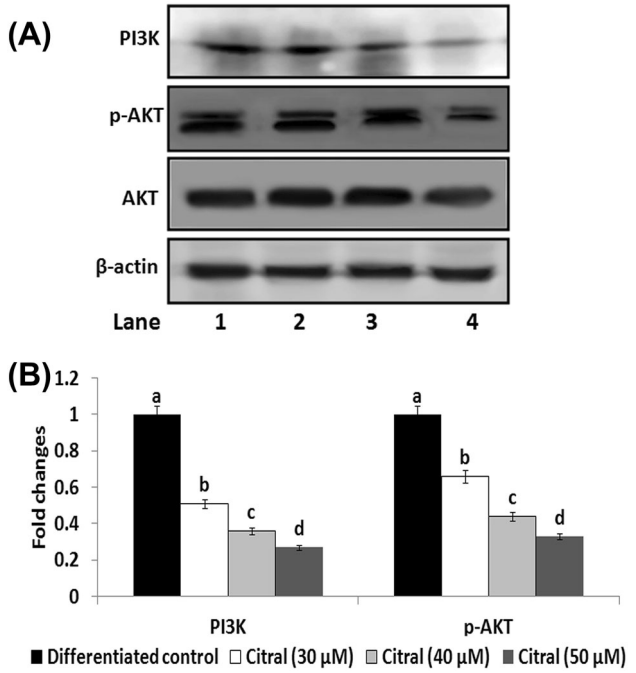

Fig. 4 Effect of citral on the protein levels of inflammatory signalling in 3T3-L1 cells. The protein levels of TNF-α, IL-6, MCP-1 in 3T3-L1 cells were determined by western blot analyses. **a** Representative images for western blot. Lane 1: differentiated control, Lane 2: citral 30 μM, Lane 3: citral 40 μM, Lane 4: citral 50 μM. **b** Densitometric analyses. Data are presented as the mean ± SD from three independent experiments. Values not sharing a common marking (a, b, c and d) differ significantly at $P < 0.05$ (Duncan's multiple-range test)

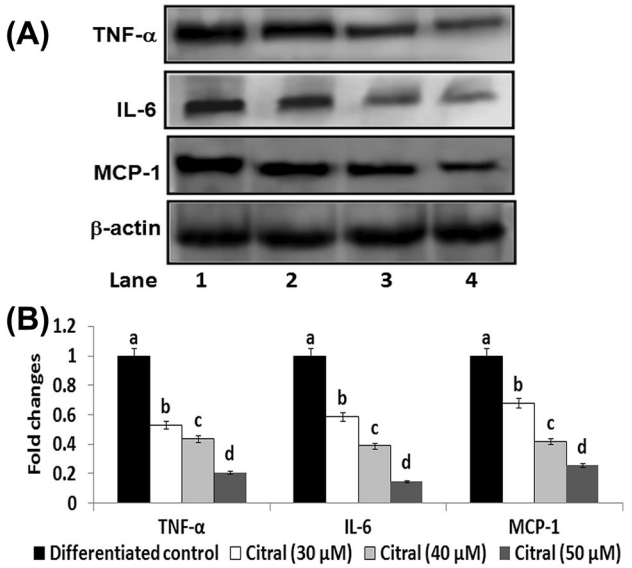

Fig. 5 Effect of citral on the protein levels of cell survival signalling in 3T3-L1 cells. The protein levels of PI3K/AKT in 3T3-L1 cells were determined by western blot analyses. **a** Representative images for western blot. Lane 1: differentiated control, Lane 2: citral 30 μM, Lane 3: citral 40 μM, Lane 4: citral 50 μM. **b** Densitometric analyses. Data are presented as the mean ± SD from three independent experiments. Values not sharing a common marking (a, b, c and d) differ significantly at $P < 0.05$ (Duncan's multiple-range test)

adipocytes treated with PI3K inhibitor LY294002 could inhibit differentiation by suppressing the C/EBPa and PPARγ expressions [35]. Our results also demonstrated that the over expression of PI3K and phosphorylated AKT were suppressed by citral treatment with 3T3-L1 cells in a concentration dependent manner (Fig. 4). Similarly, *Citrus aurantium* flavonoids inhibits adipogenesis through the modulating effect of Akt signaling pathway in 3T3-L1 cells [36].

Activation of inflammatory signaling pathways links obesity with metabolic disorders. The increased expression of PI3K/AKT signaling directly associated with inflammatory responses which induces adipogenesis [37]. The PI3K/AKT mediated over-expression of inflammatory proteins such as TNF-α, IL-6, and MCP-1 have been well documented [8]. Our results suggest that adipogenic cocktail induced over-expression of TNF-α, IL-6, and MCP-1 were suppressed by citral treatment in 3T3-L1 cells in a concentration dependent manner (Fig. 5). Recently, lunasin attenuates obesity-related inflammation responses specifically inhibits MCP-1, IL-6, TNF-α, and IL-1β in RAW264.7 cells [38]. These results imply that there citral inhibits PI3K/AKT mediated signaling pathway, transcription factors, PPARγ, C/EBPα, SREBP-1c and FAS and inflammatory responses in 3T3-L1 adipocyte differentiation induction.

Conclusion

In the present study, we examined the anti-obesity effects of citral on adipocyte differentiation and the associated molecular mechanisms in 3T3-L1 cells. Citral treatment significantly attenuated intracellular triglyceride accumulation and adipocyte differentiation of 3T3-L1 cells in a dose-dependent manner. The treatment of citral modulates the adipogenic transcriptional factor and subsequently down-regulated the activation of the key transcriptional gene PPARγ, C/EBPα, SREBP-1c and FAS in 3T3-L1 adipocytes. Moreover, treatment with citral suppressed PI3K/AKT signaling and inflammatory signaling in which associated with adipocyte differentiation in 3T3-L1 cells. These results suggest that the anti-obesity effects of citral result of a decrease in adipogenesis through modulating adipogenic transcriptional factors, PI3K/AKT signaling and inflammatory signaling and that citral has a beneficial effect by reducing the body weight gain in an obesity.

Acknowledgements The author S. Sri Devi acknowledges the financial assistance awarded by University Grants Commission

(UGC) under Basic Science Research (BSR) Scheme, New Delhi, India in the form of Senior Research Fellow.

Compliance with Ethical Standards

Conflicts of interest All the authors are declared that no conflicts of interest.

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