10-Gingerol, a novel ginger compound, exhibits antiadipogenic effects without compromising cell viability in 3T3-L1 cells

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Abstract. Obesity is defined as excessive fat accumulation that can be detrimental to health and currently affects a large part of the global population. Obesity arises from excessive energy intake along with a sedentary lifestyle and leads to adipocytes with aggravated hypertrophy. Strategies have been designed to prevent and treat obesity. Nutrigenomics may serve a role in prevention of obesity using bioactive compounds present in certain foods with anti-obesogenic effects. Ginger (*Zingiber officinale* Roscoe) contains gingerols, key bioactive compounds that inhibit hypertrophy and hyperplasia of adipocytes. The present study aimed to evaluate the antiadipogenic activity of 10-gingerol (10-G) in the 3T3-L1 cell line. Three study groups were formed: Negative (3T3-L1 preadipocytes) and positive control (mature 3T3-L1 adipocytes) and 10-G (3T3-L1 preadipocytes stimulated with 10-G during adipogenic differentiation). Cell viability and lipid content were evaluated by MTT assay and Oil Red O staining, respectively. mRNA expression of CCAAT enhancer-binding protein α (*C/ebpα),* peroxisome proliferator-activated receptor γ (*Pparγ),* mechanistic target of rapamycin complex *(Mtor),* sterol regulatory element binding transcription factor 1 (*Srebf1)*, acetyl-coenzyme A carboxylase (*Acaca)*, fatty acid binding protein 4 (*Fabp4),* and 18S rRNA (*Rn18s*), was quantified by quantitative PCR. The protein expression of C/EPBα was analyzed by western blot. In the 10-G group, lipid content was decreased by 28.83% (P<0.0001) compared with the positive control; notably, cell viability was not affected (P=0.336). The mRNA expression in the 10-G group was higher for *C/ebpα* (P<0.001) and lower for *Acaca* (P<0.001), *Fabp4* (P<0.001), *Mtor* (P<0.0001) and *Srebf1* (P<0.0001) compared with the positive control group, while gene expression of *Pparγ* did not present significant changes. The presence of 10-G notably decreased C/EBPα protein levels in 3T3-L1 adipocytes. In summary, the antiadipogenic effect of 10-G during the differentiation of 3T3-L1 cells into adipocytes may be explained by mRNA downregulation of adipogenic transcriptional factors and lipid metabolism-associated genes.

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Introduction

Obesity is defined as an abnormal or excessive fat accumulation that typically leads to health problems and currently affects >2 billion people of the world population (1-3). Its primary etiology is excessive energy intake combined with physical inactivity, which leads to adipocytes with exacerbated hypertrophy (1,4). Obesity is an important risk factor for development of metabolic disorders, type 2 diabetes, cancer, depression, dyslipidemia and cardiovascular diseases (2,4). Therefore, it is important to seek strategies focused on its prevention (3).

Strategies used for the prevention and treatment of obesity include promoting regular physical activity at a moderate intensity and following a diet with both adequate caloric content and a correct distribution of macronutrients (1,2,4). However, nutrigenomics may play an important role in the prevention of obesity via the use of the bioactive compounds present in certain foods with anti-obesogenic effects (5).

Ginger is a widely consumed plant around the world with several beneficial health effects, such as improving blood circulation, lowering blood lipids and glucose, and providing anti-inflammatory, antioxidant and anti-obesogenic effects (6-8). The anti-obesogenic effect is associated with gingerols, the major pungent compounds present in the rhizomes of ginger (9). Studies have confirmed the antiadipogenic and/or lipolytic capacity of both 6-gingerol (10-12) and 6-shogaol (13), the most abundant phenols in fresh and dried ginger root, respectively (6). 6-gingerol inhibits adipogenesis, decreases the accumulation of lipid droplets (10) and promotes

the browning of 3T3-L1 cells (14), while *in vivo* approaches have demonstrated its role in decreasing weight gain, body fat, inflammatory adipokines, adipocyte hypertrophy and hyperplasia, as well as improving insulin sensitivity and glucose tolerance in high-fat diet-induced obese mice (15,16). In addition, the anti-obesogenic effects of 6-shogaol have been demonstrated by the inhibition of 3T3-L1 preadipocyte proliferation and differentiation (17), as well as accumulation of lipids in mature adipocytes (13). In a clinical trial, the mean body weight, body mass index and body fat levels were significantly lower in patients with obesity receiving capsules of 6-shogaol derived from an ethanolic extract of steamed ginger (18). A potential mechanism for its anti-obesogenic effect is decreasing the expression of peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT enhancer-binding protein α (C/EBPα), which in turn decreases the levels of key regulators of adipogenesis and lipogenesis, such as fatty acid synthase (FAS), acetyl CoA carboxylase (ACC) and fatty acid binding protein 4 (FABP4) (6,10-13). To the best of our knowledge, however, there are few studies (10-13) investigating the effects of other gingerols present in ginger, particularly on adipose tissue.

Another abundant bioactive compound in ginger root is 10-gingerol (10-G), which is notable for having high bioavailability for humans (19). Although it has been poorly studied, 10-G may be responsible for the beneficial effects of ginger, especially its anti-obesogenic effect (20). Therefore, the aim of the present study was to evaluate anti-adipogenic activity of 10-G on the 3T3-L1 cell line.

Materials and methods

3T3-L1 cell culture and differentiation. Mouse 3T3-L1 cells were donated by Dr Trinidad Garcia-Iglesias (Immunology Laboratory of the University Center of Health Sciences, University of Guadalajara (Guadalajara, Mexico). The incubation was performed at 37˚C and 5% CO_2 in DMEM (Sigma-Aldrich; Merck KGaA; cat. no. SIG-D6429) supplemented with 10% calf bovine serum (CBS; Cytiva; HyClone; cat. no. 12389812) and 1% antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin; Gibco; Thermo Fisher Scientific, Inc.; cat. no. 15140122). 3T3-L1 preadipocytes were seeded in Petri dishes at a density of $1x10^5$ cells/dish and the medium was replaced every 2-3 days until the cells reached 100% confluence. The day after cells reached confluence (day 0), media were replaced with differentiation medium composed of DMEM/F12 (Gibco; Thermo Fisher Scientific, Inc.; cat. no. 21041025) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.; cat. no. 16000044), 1% antibiotics and an adipogenic cocktail including 500 µM 3-Isobutyl-1-methylxanthine, 1 µM dexamethasone, 1.5 µg/ml insulin and 1 µM rosiglitazone (3T3-L1 Differentiation kit; Sigma-Aldrich; Merck KGaA; cat. no. SIG-DIF001-1KT) for 3 days at 37˚C. Media were replaced with a maintenance medium composed of DMEM/F12 supplemented with 10% FBS, 1% antibiotics and 5 µg/ml insulin. The maintenance medium was replaced every 2 days for a total of 8 days of differentiation.

10-G preparation. 10-G was acquired from Merck KGaA (Sigma-Aldrich; cat. no. G5798) at a standard concentration of 5 mg/ml (Fig. 1). A stock solution was prepared by dissolving 10-G in methanol. Subsequently, dilutions were made to 50 µg/ml in DMEM supplemented with 10% CBS, 1% antibiotic-antimycotic and 0.5% DMSO (Sigma-Aldrich; Merck KGaA; cat. no. 276855-1L).

Dosage determination and treatment. A dose-response curve assessment was performed with 1, 5, 10, 15, 25 and 35 µg/ml 10-G (Sigma Aldrich; Merck KGaA; cat. no. SIG-G-027-1ML) during adipogenesis to select for further experiments.

A total of three study groups was formed: Negative control (NC; 3T3-L1 preadipocytes), positive control (PC; mature 3T3-L1 adipocytes) and 10-G (3T3-L1 preadipocytes stimulated with 10-G during adipogenic differentiation). In the 10-G group, confluent preadipocytes were incubated at 37˚C and 5% CO_2 with 10-G until mature adipocytes were formed (8 days). Supernatant was collected in tubes and stored at -80˚C. Finally, 3T3-L1 adipocytes were cryopreserved at -80˚C for further experimental analysis.

MTT assay. 3T3-L1 cells were seeded at a concentration of $5x10^3$ cells/well in 96-well plates. Cells were differentiated as aforementioned in the presence or absence of 10-G. Next, 1 mg/ml MTT (Invitrogen; Thermo Fisher Scientific, Inc.; cat. No. M6494) solution was added to the wells and cells were incubated for 1 h at 37˚C. The formazan crystals were dissolved using an extraction buffer (20% SDS and 50% dimethylformamide) followed by spectrophotometric measurement at 570 nm using a microplate reader (MultiScan GO; Thermo Fisher Scientific, Inc.; cat. no. 51119300).

Oil red O staining. Fully differentiated 3T3-L1 cells were fixed with 10% (v/v) formaldehyde solution for 60 min at room temperature and washed with distilled water. Then, lipid content in mature adipocytes was stained with oil red O (Sigma-Aldrich; Merck KGaA; cat. no. O0625) solution for 15 min and washed with distilled water at room temperature. Briefly, the stained oil red O was dissolved in 100% 2-propanol (Sigma-Aldrich; Merck KGaA; cat. no. I9516-1L) followed by spectrophotometric measurement at 515 nm using a microplate reader (MultiScan GO; Thermo Fisher Scientific, Inc.; cat. no. 51119300).

Reverse transcription-quantitative (RT-q)PCR. Total RNA was isolated from cells groups using the RNeasy Mini kit according to the manufacturer's instructions (Qiagen GmbH; cat. no. 74104). Then, cDNA was reverse-transcribed from 1 µg total RNA using M-MLV Reverse Transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.; cat. no. 28025013) according to the manufacturer's instructions. Gene expression was evaluated by qPCR (LightCycler 96 Thermocycler; Roche Diagnostics) with the OneTaq® Hot Start Master Mix (NEB-R; cat. no. N01-M0484L) and TaqMan® probes (Thermo Fisher Scientific, Inc.) as follows: 18S rRNA (*Rn18s;* cat. no. Mm03928990_g1), *C/ebpα* (cat. no. Mm00514283_s1), *Pparγ* (cat. no. Mm00440940_m1), *Fabp4* (cat. no. Mm00445878_m1), acetyl-coenzyme A carboxylase (*Acaca*; cat. no. Mm01304257_m1), sterol regulatory element binding transcription factor 1 (*Srebf1*; cat. no. Mm00482136_m1) and mechanistic target of rapamycin complex (*Mtor;* cat. no. Mm00444968_m1). The thermocycling

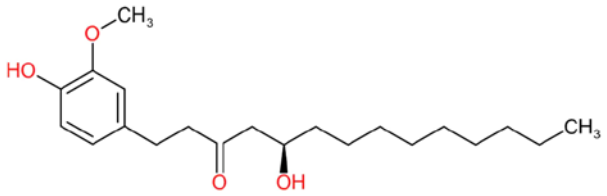

Figure 1. Molecular structure of 10-gingerol.

conditions for qPCR were as follows: Initial denaturation at 95˚C for 300 sec, followed by 30 cycles of denaturation at 95˚C for 20 sec and amplification at 60˚C for 60 sec and final extension at 68˚C for 300 sec. Relative gene expression was determined based on the $2^{-\Delta\Delta Cq}$ method (21) and normalized against the mRNA expression of *Rn18S*. mRNA expression levels were determined at days 4 and 8 of differentiation. All quantifications were independently performed three times.

Western blot analysis. Preadipocytes and adipocytes were obtained by washing cells with PBS buffer (Sigma-Aldrich; Merck KGaA; cat. no. P3813) and lysed with standard buffer containing 100 mM HEPES, KCl and ethylenediaminetetraacetic acid (EDTA), 1% NP-40 cell lysis buffer, 100 mM dithiothreitol, 1 M NaF, 10 mM Na_3VO_4 and 100 mM phenylmethylsulfonyl fluoride (PMSF). Total protein was obtained by centrifuging the lysate at 15,000 x g for 20 min at 4˚C. Protein extract was quantified by the Bradford method. 20 ug of protein/lane was resuspended in SDS-containing Laemmli buffer, heated for 5 min at 85˚C and separated by 10% SDS-PAGE under reducing conditions (2-mercaptoethanol). Proteins were then transferred to PVDF membranes (Bio-Rad Laboratories, Inc.; cat. no. 1620177), blocked for 2 h at room temperature with non-fat dry milk and incubated overnight at 4˚C with specific antibodies against C/EBPα (1:1,000; Santa Cruz Biotechnology, Inc.; cat. no. sc-166258). Afterward, membranes were incubated with HRP-conjugated goat anti-mouse secondary antibody (1:20,000; LI-COR Biosciences; cat. no. 926-80010) for 90 min at room temperature. Bands were detected using Immobilon Western Chemiluminescent HRP substrate (EMD Millipore; cat. no. WBKLS05000). Chemiluminescence was digitized using the C-Digit Blot Scanner (LI-COR Biosciences; cat. no. 3600) and analyzed using Image Studio Digits version 5.5.4 processing software (licor.com/bio/image-studio/). All band density quantifications were normalized against β-actin (1:1,000; Thermo Fisher Scientific, Inc.; cat. no. 31464) as loading control.

Statistical analysis. Experiments were performed in triplicates. Data are presented as the mean ± SEM. Data were analyzed by one-way ANOVA followed by Tukey's post hoc test. Data were analyzed with GraphPad Prism 9 (GraphPad Software, Inc.; Dotmatics). P<0.05 was considered to indicate a statistically significant difference.

Results

Cell viability dose-response curve with 10-G. A dose-response curve with 10-G at 1, 5, 10, 15, 25 and 35 μg/ml was performed on the 3T3-L1 cell line to establish the

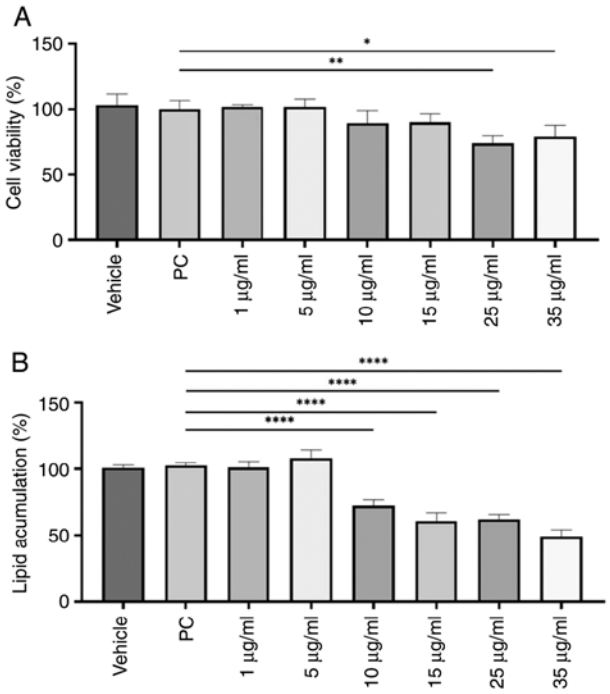

Figure 2. Dose-response curve of 10-gingerol on cell viability and lipid content. (A) 3T3-L1 cells were treated with 10-gingerol during adipogenic differentiation. Cell viability was determined using MTT assay. (B) Lipid content was measured spectrophotometrically. *P<0.05, **P<0.01, ****P<0.0001. NC, negative control; PC, positive control.

treatment dose. Subsequently, cell viability was evaluated by MTT assay. Treatment of immature 3T3-L1 cells with ≥25 μg/ml 10-G during the differentiation process significantly decreased the percentage of living cells (Fig. 2A). Thus, doses of 25 and 35 μg/ml were excluded for subsequent experiments.

Lipid content dose-response curve with 10-G. Amount of stored lipids was estimated by Oil Red O staining. Except for 1 and 5 μg/ml, 10-G significantly reduced the lipid content compared with the PC group (Fig. 2B). The dose of 35 μg/ml achieved the most significant decrease in lipid content, by 50.73%. However, this dose was not used for subsequent experiments due to low cell viability.

Therefore, 15 μg/ml was chosen for treatment of 3T3-L1 preadipocytes during differentiation since it showed a significant decrease in lipid content compared with PC without significantly affecting the number of living cells.

Effects of 10-G on cell viability and adipocyte differentiation. Cell viability and adipocyte differentiation in the presence or absence of 10-G were assessed. No significant differences were in the percentage of viable cells between the control and 10-G groups. Therefore, 10-G did not affect viability of 3T3-L1 cells (Fig. 3A).

Regarding the adipocyte differentiation, Oil red O staining revealed lipid content differences between groups. NC cells showed no staining, while staining was observed in the PC and 10-G groups. The largest amount of stained lipid vacuoles was observed in the PC, which indicated successful differentiation of adipocytes (22). On the other hand, the 10-G group showed

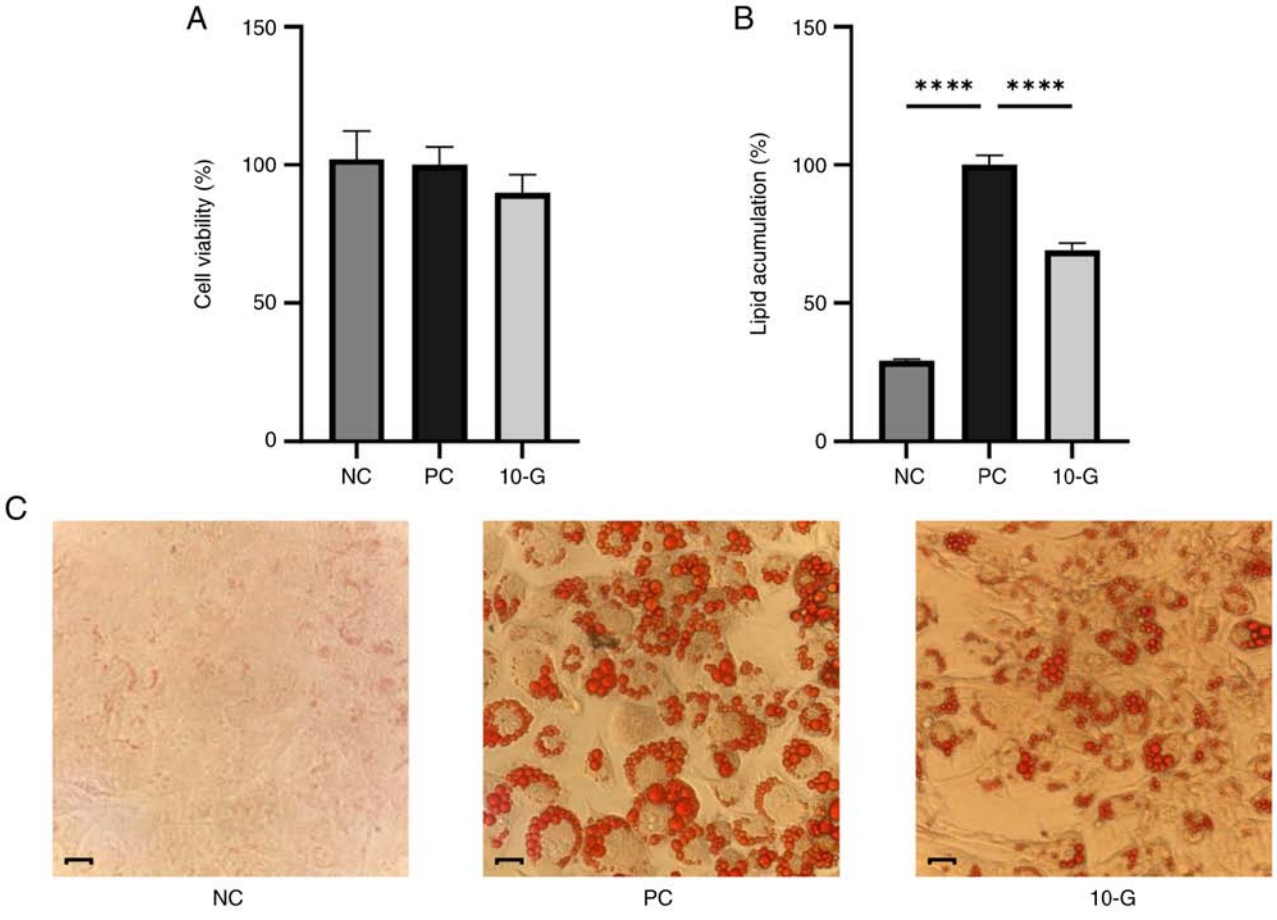

Figure 3. Effect of 10-G on cell viability and lipid content. (A) Cells were treated with 15 μg/ml 10-G and viability was determined using MTT assay. (B) Lipid content was determined with (C) Oil Red O staining. Magnification, x40; scale bar, 100 μm. ****P<0.0001. NC, negative control; PC, positive control; 10-G, 10-gingerol.

less staining compared with the PC group, which indicated lower levels of stored lipids (Fig. 3C). Lipid content of 10-G group was 71.17% and significantly decreased compared with the PC group (Fig. 3B).

Effects of 10-G on pro-adipogenic and lipogenic genes in 3T3-L1 adipocytes. Expression of genes related to the regulation of adipogenesis and lipogenesis was assessed. The 10-G group showed significantly decreased expression of *C/ebpα*, *Pparγ*, *Mtor, Acaca,* and *Fabp4* on day 4 compared with the PC group (Fig. 4A). The 10-G group, on day 8, showed higher mRNA expression of *C/ebpα* and *Pparγ*, but the latter was not significant compared with the PC group. On the other hand, the gene expression of *Mtor* and *Srebf1* showed a significant decrease in the 10-G compared with the PC group. In addition, mRNA levels of the lipid metabolism-associated genes *Acaca* and *Fabp4* were significantly decreased at day 8 of adipocyte differentiation in the 10-G compared with the PC group (Fig. 4B).

Effect of 10-G on the protein expression of adipocyte transcription factor C/EBPα. Treatment with 15 μg/ml 10-G notably decreased C/EBPα protein levels in the 3T3-L1 adipocytes at day 8 of differentiation compared with the PC group (Fig. 4C).

Discussion

The present study demonstrated that 10-G, one of the most abundant phenols in ginger, decreased adipogenesis and accumulation of cytoplasmic lipid droplets in 3T3-L1 cells via mRNA downregulation of adipogenic transcriptional factors and lipid metabolism-associated genes.

The proliferation of adipocytes and excessive fat accumulation are associated with development of obesity and metabolic complications (23). Decreased adiposity is related to a decrease in number of adipocytes and lipid content (10). Ginger possesses abundant phenols, such as 6-gingerol and 6-shogaol, that exert beneficial health effects on obesity by decreasing the intracellular lipid accumulation in 3T3-L1 preadipocyte cells (13,20,24).

Here, stimulation with 10-G during the differentiation process significantly decreased lipid content in adipocytes without affecting cell viability. Similar effects have been described for other ginger phenols, primarily 6-gingerol (10,14-16,20), 6-shogaol (13,17,18) and galanolactone (25). Thus, 10-G, similar to other gingerols, serves a critical role in the process of adipogenesis and accumulation of cytoplasmic lipid droplets during 3T3-L1 cell differentiation (7).

Adipogenesis is a complex process that is regulated by sequential activation of transcriptional factors. In the initial

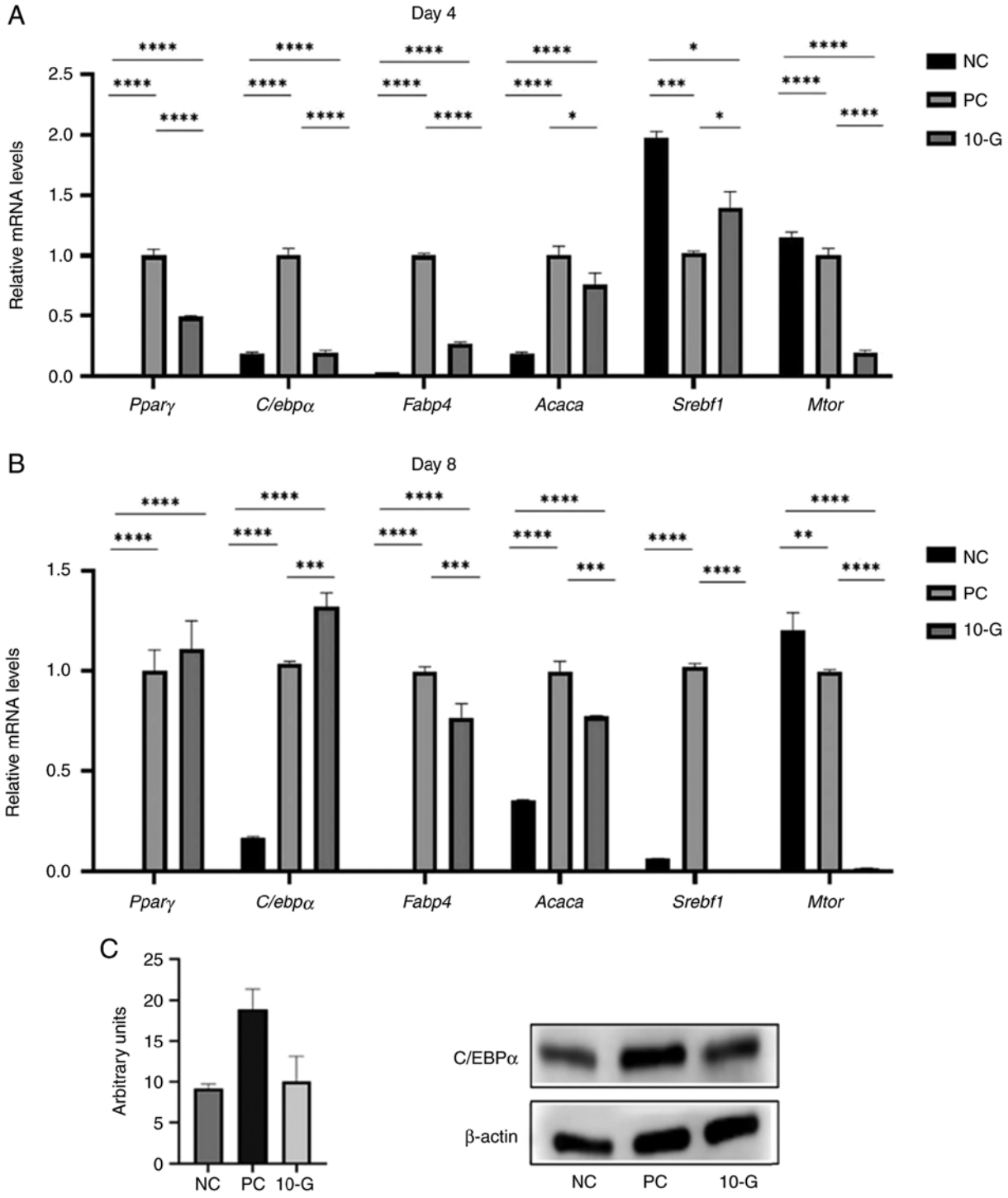

Figure 4. Effect of 10-G on pro-adipogenic and lipogenic markers and C/EBPα protein expression. *Pparγ*, *C/ebpα*, *Fabp4*, *Acaca*, *Srebf1* and *Mtor* mRNA levels in 3T3-L1 cells were examined using quantitative PCR on day (A) 4 and (B) 8 of differentiation. The relative gene expression of each sample was normalized to *Rn18S*. (C) Protein levels of C/EBPα were determined by western blot analysis after 8 days of differentiation. β-actin was used as a loading control. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. *Pparγ*, peroxisome proliferator-activated receptor γ; *C/ebpα*, CCAAT enhancer-binding protein α; *Fabp4*, fatty acid binding protein 4; *Acaca*, acetyl-coenzyme A carboxylase; *Srebf1*, sterol regulatory element binding transcription factor 1; *Mtor*, mechanistic target of rapamycin complex; NC, negative control; PC, positive control; 10-G, 10-gingerol.

stage, PPARγ and C/EBPα are required to trigger transcriptional changes that lead to an adipose phenotype (26). Here, treatment with 10-G decreased mRNA expression of *Ppary* and *C/ebpa* on day 4 of differentiation, consistent with previous results for 6-gingerol (10). Similarly, key fat cell genes such as *Acaca* and *Fabp4* were also decreased. These results suggest that 10-G could act via inhibition of adipogenic differentiation.

The expression levels of mechanistic target of rapamycin complex (mTORC), a key factor that mediates increased cell number and size (27), was downregulated in cells treated with 10-G. Studies have shown that mTORC is an important regulator for the formation of adipose tissue and its function as lipid storage (24,28). mTORC activation is implicated in the regulation of adipocyte precursors, induction of preadipocyte

differentiation into mature adipocytes, as well as triglyceride synthesis (28,29). Conversely, decreased expression and activation of mTORC is associated with decreased expression of PPAR-γ and C/EBPα (24,30). Consequently, there is decreased adiposity and impaired adipogenesis (24). The present results showed a higher expression of *mTOR* but lower expression of *Ppary* and *C/ebpa* in NC cells that did not receive hormonal stimuli to initiate the adipogenic differentiation process (31). However, after 8 days of differentiation, 10-G decreased the expression of *mTOR* but did not decrease the expression of *Pparγ* and *C/ebpα* in adipocytes. Although the present gene expression results do not reflect those observed in other studies reporting a decrease in *Pparγ* and *C/ebpa* (11,13,20,25), decreased C/EBPα protein expression was consistent with that reported in other studies (10,13,20). Therefore, protein expression of these pro-adipogenic markers may be decreased.

mTOR is involved in a complex transcriptional network. Ginger extract rich in 6-gingerol and 6-shogaol controls mTOR expression through the activation of AMP-activated protein kinase (AMPK) in a model of obesity (20,24). AMPK expression was not evaluated in the present study and further studies are needed to clarify the molecular mechanism by which 10-G downregulates adipogenic differentiation of 3T3-L1 cells and whether AMPK is also affected.

Moreover, mTOR has been implicated in the regulation of triglyceride synthesis (28,29) via phosphorylation of lipin 1, an inhibitor of sterol regulatory element binding protein (SREBP). This enhances the nuclear translocation of SREBP and promotes transcription of lipogenic genes (27). Decreased *mTOR* expression downregulates lipogenic pathways via decreased activation of SREBP (27,29,30). Here, following 8 days of differentiation, 10-G decreased the mRNA expression of *Srebf1, Acaca* and *Fabp4*. *Acaca* encodes ACC, a lipogenic enzyme that regulates endogenous fatty acid synthesis and triglyceride storage (11,32). The FABP4 gene is a marker of terminal adipocyte differentiation and facilitates cellular uptake of long-chain fatty acids for their metabolism (20). Downregulation of *Srebf1* decreases the expression of *Acaca* and *Fabp4*, which decreases the synthesis and transport of fatty acids and, consequently, lipid content in 3T3-L1 cells (32).

The present data suggested that 10-G could suppress adipocyte differentiation via inhibition of *mTOR* expression, which may lead to decreased expression of lipid metabolism-associated genes, ultimately leading to decreased lipid accumulation.

The present study did not assess expression and phosphorylation status of the proteins involved in the mTOR pathway. Therefore, further studies are necessary to confirm its involvement in the anti-obesogenic effect of 10-G. Furthermore, it is important to evaluate gene expression at multiple time points since it can vary throughout differentiation process of preadipocyte 3T3-L1 cells. The present study only performed western blot analysis for C/EBPα at one time point. In addition, protein expression of PPARγ, SREBP, mTORC, ACC and FABP4 should be determined to confirm the mechanism of action of 10-G during adipogenesis. Further studies are needed to address these limitations.

A promising approach to test the therapeutic properties of 10-G is to combine it with nanomedicine and novel delivery systems designed for controlled and adjustable release of the drug. This approach has the potential to improve the efficiency of drug administration and targeting and to enable personalized treatment *in vivo* (33,34).

In conclusion, the present study demonstrated the antiadipogenic effects of 10-G during differentiation of 3T3-L1 cells to adipocytes. Further *in vivo* studies are necessary to provide more complete data on its anti-obesity effect and potential clinical use for the prevention and treatment of obesity.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

JRV and EML designed the experiments. GGO, MPO and MPR performed the experiments. RRE and EML analyzed data. RRE, MPO and JRV wrote the manuscript. EML and JRV confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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