

Article

Citrus aurantium L. Dry Extracts Ameliorate Adipocyte Differentiation of 3T3-L1 Cells Exposed to TNF α by Down-Regulating *miR-155* Expression

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Abstract: *Citrus aurantium* L. dry extracts (CAde) improve adipogenesis in vitro. These effects are dependent from an early modulation of CCAAT/enhancer-binding protein beta (*C/EBP β*) expression and cyclic Adenosine Monophosphate (cAMP) response element-binding protein (*CREB*) activation. *C/EBP β* and *Creb* are also targets of *miR-155*. This study investigated whether CAde regulates *miR-155* expression in the early stages of adipogenesis and whether it ameliorates adipocyte differentiation of cells exposed to tumor necrosis factor-alpha (TNF α). Adipogenic stimuli (AS) were performed in 3T3-L1 pre-adipocytes treated with CAde, TNF α , or both. Gene and miRNA expression were determined by quantitative real-time PCR. Adipogenesis was evaluated by Oil-Red O staining. CAde treatment enhanced AS effects during the early adipogenesis phases by further down-regulating *miR-155* expression and increasing both *C/EBP β* and *Creb* mRNA and protein levels. At variance, TNF α inhibited 3T3-L1 adipogenesis and abolished AS effects on *miR-155*, *C/EBP β* , and *Creb* expression. However, in cells exposed to TNF α , CAde improved adipocyte differentiation and restored the AS effects on miRNA and gene expression at early time points. In conclusion, this study identified *miR-155* down-regulation as part of the mechanism through which CAde enhances adipogenesis of pre-adipocytes in vitro. Furthermore, it provides evidence of CAde efficacy against TNF α negative effects on adipogenesis.

Keywords: *Citrus aurantium* L. dry extracts; *miR-155*; tumor necrosis factor-alpha; adipogenesis

1. Introduction

Adipocyte differentiation is a highly orchestrated physiological process, which involves a large number of molecular events and whose dysregulation is relevant to human disease by contributing metabolic dysfunction in obesity [1,2]. microRNAs (miRNAs) represent a class of small non-coding RNAs (≈ 22 nt in length) involved in a variety of cellular processes, whose role is to repress target

translation and to induce target mRNA degradation [3]. Oskowitz et al. were the first to detail miRNA expression during human multipotent stromal cell differentiation toward adipogenic lineage and to demonstrate that adipogenic differentiation and lipid accumulation of these cells is disrupted by inhibition of miRNA biogenesis [4]. In the decade that followed, several miRNAs have been implicated in adipocyte fate determination and adipocyte formation from precursor cells; some of them appear to enhance, while some others appear to inhibit adipocyte differentiation [4–18]. For instance, *miR-27a* and *-27b*, belonging to the *miR-27* gene family, were identified as negative regulators of adipogenesis [7,10,11]. The expression of both is indeed down-regulated during adipogenic differentiation of 3T3-L1 cells, and their over-expression is inhibited in vitro adipocyte formation by reducing the levels of master adipogenic regulators such as peroxisome proliferator-activated receptor gamma (*PPAR γ*) and CCAAT/enhancer-binding protein alpha (*C/EBP α*) [7]. *miR-130* suppresses adipogenesis by directly targeting and inhibiting *PPAR γ* expression [17], while *miR-375* enhances adipocyte differentiation via suppression of extracellular signal-regulated kinases (ERK 1/2) signaling [18].

miR-155, initially described as the B-cell integration (*bic*) cluster gene in chickens [19], is a multifunctional miRNA involved in numerous physiological and pathological processes such as haematopoietic lineage differentiation, immunity, inflammation, cancer, and cardiovascular diseases [20,21]. Its role in the regulation of adipocyte differentiation has also been described [22]. Liu et al. indeed reported that *miR-155* expression is down-regulated at the early stage of 3T3-L1 adipogenesis and that its overexpression suppresses adipocyte differentiation by directly targeting the 3'-untranslated regions (3'-UTRs) of *C/ebp β* and cyclic Adenosine Monophosphate (cAMP) response element-binding protein (*Creb*) mRNAs [22]. In addition, it has been identified as the most responsive miRNA to inflammatory stimuli [22,23], and its expression is higher in the adipose tissue of obese compared to normal-weight subjects and correlates with both tumor necrosis factor-alpha (TNF α) expression and body mass index [23]. It follows that the modulation of the *miR-155* expression in adipocyte precursor cells may offer new ways to enhance adipose tissue homeostasis during obesity.

Nutraceuticals, which encompass all the substances derived from plants and food sources that provide medical or health benefits, have been described as modulating miRNA expression [24–26]. As well as this, nutraceuticals are known to regulate adipocyte cell line activity [27]. In line with this, we have recently demonstrated the nutraceutical properties of CAde, a dry extract preparation obtained from *Citrus aurantium* L. (CAde) fruit juice, on the regulation of 3T3-L1 cell adipocyte differentiation and function [28]. CAde enhances in vitro terminal adipocyte differentiation of 3T3-L1 pre-adipocytes in terms of increased gene expression of *Ppar γ* , glucose transporter type 4 (*Glut4*), and fatty acid-binding protein 4 (*Fabp4*), as well as the functional capacity of 3T3-L1 mature adipocytes in terms of improved insulin-induced glucose uptake [28]. Furthermore, CAde promotes the early differentiation stage as well, by anticipating the 3T3-L1 cell cycle entry and progression during mitotic clonal expansion and by activating *CREB* and nearly doubling the expression of the transcription factor *C/ebp β* a few hours later in the adipogenic induction [28].

In the present study, the hypothesis was that *miR-155* and the regulation of its expression are the missing pieces that link CAde to *C/ebp β* and *CREB* proteins. Furthermore, we also wondered whether CAde might be effective against micro-environmental insults, which affect adipogenesis and *miR-155* expression, such as TNF α .

2. Materials and Methods

2.1. *Citrus aurantium* L. Dry Extract (CAde)

Citrus aurantium L. (CAde) fruit juice dry extracts were from [28]. Lyophilized CAde was re-hydrated with distilled H₂O to a final concentration of 10 mg/mL and used for treatments at the concentration of 100 μ g/mL.

2.2. Cell Culture, Adipocyte Differentiation, and Treatments

Murine embryonic fibroblast 3T3-L1 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown and maintained in culture at 37 °C in a humidified atmosphere of 5% CO₂ in expansion medium consisting of Dulbecco's modified Eagle's medium (DMEM; 4.5 g/L glucose; Lonza, Walkersville, MD, USA), 10% fetal calf serum (FCS; Thermo Fisher Scientific, Waltham, MA, USA), 100 U/mL penicillin (Lonza, Walkersville, MD, USA), and 100 mg/mL streptomycin (Lonza, Walkersville, MD, USA) [29,30]. Adipocyte differentiation of 3T3-L1 cells was induced as follows. Cells were grown to confluence in expansion medium. Two days after reaching 100% confluence (day 0), cells were cultured for 48 h with the adipogenic differentiation medium (AS) consisting of DMEM (4.5 g/L glucose), 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA, USA), 100 U/mL penicillin, and 100 mg/mL streptomycin, supplemented with dexamethasone (1 µm, Sigma Aldrich, St Louis, MO), 3-isobutyl-1-methylxanthine (0.5 mm, Sigma Aldrich, St. Louis, MO, USA), and insulin (1 µg/mL, Sigma Aldrich, St. Louis, MO, USA). Forty-eight hours later, the medium was changed, and cells were maintained in culture in AS supplemented with 1 µg/mL insulin, replaced every 48 h, until day 8 post-induction. Where indicated, cells were differentiated with CAde (100 µg/mL), or TNFα (1 ng/mL; EMD Millipore, Burlington, MA) or both. CAde was added at day 0 and in every replacement. TNFα was added only at day 0. For short-term experiments, 3T3-L1 cells at day 0 were stimulated for 15, 30, 60, 120, and 240 min in AS or in AS supplemented with CAde (100 µg/mL), or TNFα (1 ng/mL) or both.

2.3. miRNA Mimic and Inhibitor Transfection in Differentiating 3T3-L1

miRNA mimic and inhibitor transfection were performed according to [31]. Briefly, 100% confluent 3T3-L1 cells (day 2) were transfected with 5 nmol·L⁻¹ of miRIDIAN mimic *mmu-miR-155-5p* (C-310430-07-0005, Dharmacon Inc., Lafayette, CO, USA) or 5 nmol·L⁻¹ of the miRIDIAN Hairpin Inhibitor *mmu-miR-155-5p* (IH-310430-08-0005, Dharmacon Inc.) using Lipofectamine 3000 Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. The non-targeting control oligonucleotide miRIDIAN microRNA Mimic negative control #1 (5 nmol·L⁻¹; CN-001000-01-05, Dharmacon Inc.) and the non-targeting control oligonucleotide miRIDIAN microRNA Hairpin Inhibitor negative control #1 (5 nmol·L⁻¹; IN-001005-01-05, Dharmacon Inc.) were used as a negative control of miRNA mimic and inhibitor transfection, respectively. Forty-eight hours after the transfection (day 0), adipogenesis was induced into cells and left to differentiate into mature adipocytes for a further 8 days, as described above in this section.

2.4. Image Acquisition, Oil-Red O Staining, and Triglyceride (TG) Quantification Assay

Images of 3T3-L1 cells at day 8 post-induction were taken using an Olympus microscope system (Olympus, Center Valley, PA, USA). Microphotographs are shown (×10 magnifications); scale bars, 30 µm. Oil-Red O staining was performed as described in [32]. Briefly, 3T3-L1 cells at day 8 post-induction were fixed and stained with Oil-Red O staining solution (Sigma-Aldrich, St. Louis, MO, USA). Lipid accumulation was then quantified by measuring the optical density of the dissolved Oil-Red O staining at 490 nm by a spectrophotometer. Cellular TG concentration was determined according to [33]. Briefly, 3T3-L1 cells at day 8 post-induction were lysed into PBS 1X by sonication. TG content per sample was measured using a TG assay kit from Sigma-Aldrich. Per sample DNA was also isolated using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Hilden, Germany), and DNA concentration was quantified and used to normalize data. The values were expressed as µg Triglyceride (TG) · µg Deoxyribonucleic acid (DNA)⁻¹

2.5. Total RNA and miRNA Purification, Reverse Transcription, and Quantitative Real-Time PCR

Total RNA, including miRNA, was isolated from 3T3-L1 cells using AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Total RNA

concentration was quantified with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Gene expression was determined as described [34]. Total RNA (1000 ng) was reverse-transcribed using the SuperScript III Reverse Transcriptase (Qiagen, Hilden, Germany). Gene expression was analyzed by quantitative real-time PCR using the iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and quantified as relative expression units. Cyclophilin was used as a housekeeping gene. Primer sequences used are the following: *C/Ebpβ*Fwd, 5'-cgcccgccgcttagac-3'; *C/Ebpβ*Rev, 5'-cgctcgtgctcgcaatgg-3'; *Creb* Fwd, 5'-agctgcactcagccgggta-3'; *Creb* Rev, 5'-tgggtcctgctgggtgctgtg-3'; Cyclophilin Fwd, 5'-gcaagcatgtggtctttggg-3'; Cyclophilin Rev, 5'-gggtaaaatgccgcaagtc-3'. miRNA expression was determined as described in [31]. Total RNA (500 ng) was reverse-transcribed using the miScript II RT Kit (Qiagen, Hilden, Germany). miRNA expression was analyzed by quantitative real time-PCR using the miScript SYBR Green PCR Kit (Qiagen, Hilden, Germany) and quantified as relative expression units. U6 small nuclear RNA (snRNA) was used as housekeeping small RNA. Primer sequences were from Qiagen: Mm_miR-155_1 miScript Primer Assay, MS00001701; Mm_miR-130a_1 miScript Primer Assay MS00001547; Mm_miR-375_2 miScript Primer Assay MS00032774; RNU6B_13 miScript Primer Assay, MS00014000.

2.6. Western Blot (WB) Analysis

WB analysis was performed as described in [28]. 3T3-L1 cell lysates were obtained by lysing cells in buffer containing 20 mM Tris-HCl, pH 7.5; 5 mM Ethylenediaminetetraacetic acid (EDTA); 150 mM NaCl; 1% Nonidet P40 (NP40), 10 μM phenylmethylsulfonyl fluoride (PMSF); 5 μg/mL aprotinin; and 5 μg/mL leupeptin. Protein concentration was determined by Coomassie blue protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of proteins lysates were then analyzed by SDS-PAGE, and then electrophoretically transferred to a Polyvinylidene fluoride (PVDF) membrane. Membranes were probed with antibodies to total CREB (48H2, #9197; Cell Signaling Technology, Danvers, MA, USA), *C/EBPβ* (C-19, sc-150; Santa Cruz Biotechnology, Dallas, TX, USA), and VINCULIN (7F9, sc-73614; Santa Cruz Biotechnology, Dallas, TX, USA), and successively re-probed with secondary mouse or rabbit antibodies (Bio-Rad Laboratories) before signal detection with Enhanced chemiluminescence (ECL) plus (GE Healthcare, Chicago, IL, USA).

2.7. Statistical Procedures

Data are given as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) followed by Bonferroni correction post-hoc test was used for comparisons with three or more groups. Data were analyzed by using the GraphPad Software (version 6.00 for Windows, La Jolla, CA, USA).

3. Results

3.1. CAde Down-Regulated the Expression of miR-155 and Enhanced *C/Ebpβ* and *Creb* Levels during the Early Stage of Adipogenesis in 3T3-L1 Cells

To test the hypothesis that CAde may potentially exert its pro-adipogenic effects in vitro by modulating *miR-155* expression, the early stage of fat cell differentiation was investigated in 3T3-L1 pre-adipocytes. In control cells, AS induced at 15 min a 10% reduction of the *miR-155* expression, whose levels remained steadily lowered to about 30%–35% at 30 min, 1, 2, and 4 h upon the adipogenic induction (Figure 1a). In AS + CAde-treated cells, the expression of *miR-155* resulted in levels being decreased by 31% already by 15 min, and its levels further declined by 58% at 30 min, 51% at 1 h, 54% at 2 h, and 54% at 4 h upon adipogenic induction compared to control cells at time 0 (Figure 1a). Interestingly, compared to AS-treated cells, CAde treatment enhanced the effect of AS on *miR-155* down-regulation of by 25%–40% at each time point (Figure 1a and Figure S1a). Additionally, we evaluated CAde effect on *miR-130a* and *miR-375*, whose role in adipocyte fate determination has already been demonstrated [17,18]. In control 3T3-L1 cells, AS caused a time-dependent down-regulation of

the anti-adipogenic *miR-130a* (Figure 1b) and up-regulation of the pro-adipogenic *miR-375* (Figure 1c) compared to control cells at time 0. Of note, *CAde* treatment did not affect the expression of these two miRNAs. Indeed, in AS + *CAde*-treated cells, the expression levels of both *miR-130a* (Figure 1b and Figure S1b) and *miR-375* (Figure 1c and Figure S1c) at each time point were comparable to those levels observed in AS-treated control cells. Altogether, these findings suggest that *CAde* specifically modulated the expression of *miR-155* in the early stage of fat cell differentiation.

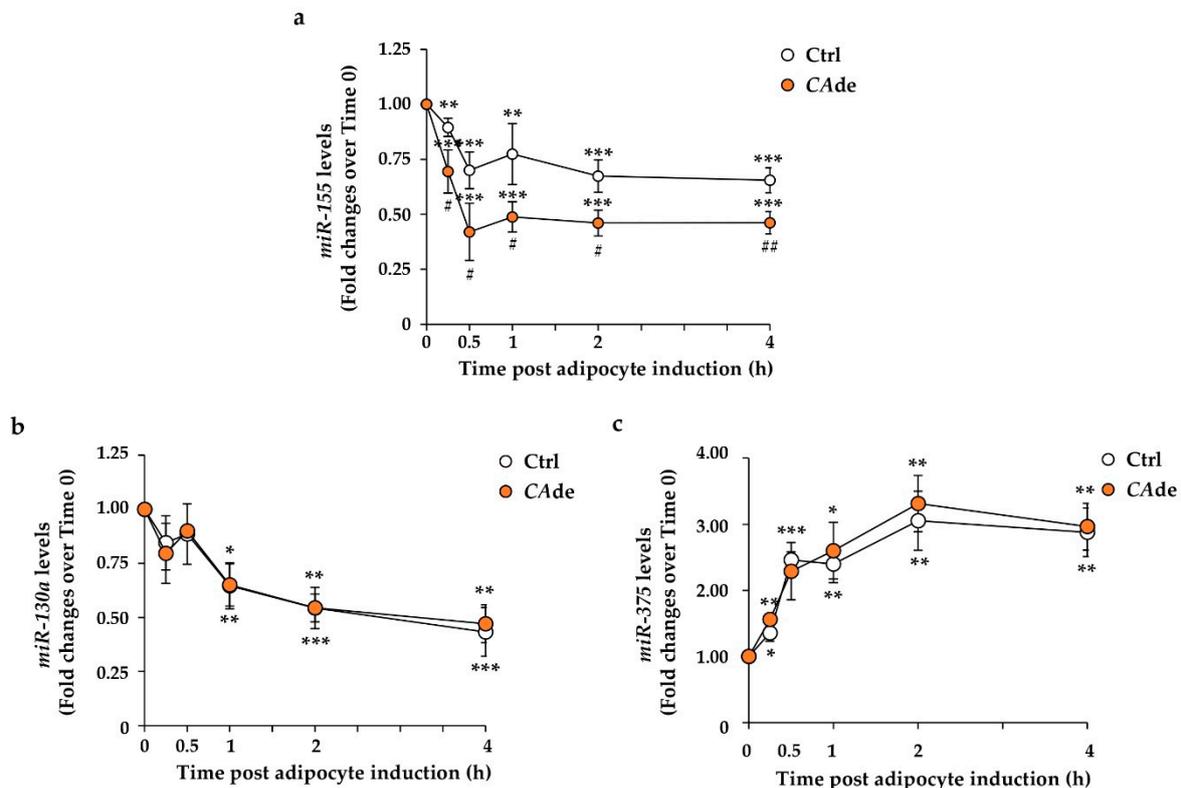


Figure 1. Effect of *Citrus aurantium* L. dry extracts (*CAde*) on the expression of *miR-155* during the early stage of adipogenesis in 3T3-L1 cells. 3T3-L1 pre-adipocytes were cultured for 0.25, 0.5, 1, 2, and 4 h with adipogenic differentiation medium (AS) (Ctrl) or AS + *CAde* (100 µg/mL). Time course of *miR-155* (a), *miR-130a* (b), and *miR-375* (c) levels, evaluated by quantitative real-time PCR, in Ctrl and *CAde*-treated cells relative to untreated 3T3-L1 cells at time 0. Values are means ± Standard Error of the Mean (SEM) of three independent experiments. Control value at time 0 was set as 1.00. Statistical significances among groups were tested by one-way ANOVA followed by Bonferroni correction post-hoc test (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. untreated 3T3-L1 cells at time 0; # $p < 0.05$, and ## $p < 0.01$ vs. control 3T3-L1 cells).

We thus investigated whether *CAde* may consequently affect the expression of the *miR-155* target genes—*C/Ebpβ* and *Creb* [21]. In the control cells, AS led to a time-dependent up-regulation of both *C/Ebpβ* (Figure 2a) and *Creb* (Figure 2b) mRNA levels compared to control cells at time 0. It is worth noting that, compared to AS-treated cells, *CAde* treatment furtherly up-regulated the expression of *C/Ebpβ* by about 65% and 45% at 2 and 4 h, respectively (Figure 2a and Figure S2a), while the combined stimulation of AS and *CAde* up-regulated *Creb* mRNA levels by about 40%, 55%, and 25% at 1, 2, and 4 h, respectively, as compared to treated control cells (Figure 2b and Figure S2b). Coherently with the gene expression data, the protein levels of *C/EBPβ* isoform liver-enriched activating protein (LAP) resulted as being up-regulated at 2 and 4 h from AS in *CAde*-treated cells compared to control cells (Figure 2c). Additionally, the protein levels of *CREB* were increased, starting from 0.25 h from AS in *CAde*-treated cells (Figure 2c). Altogether, these findings at the early stage of fat cell differentiation indicated that *CAde* may exert its function on the adipogenic induction in 3T3-L1 cells, at least in part,

by specifically lowering the expression of *miR-155* at the early time points and thus up-regulating the mRNA and protein expression of its target genes *C/Ebpβ* and *Creb*.

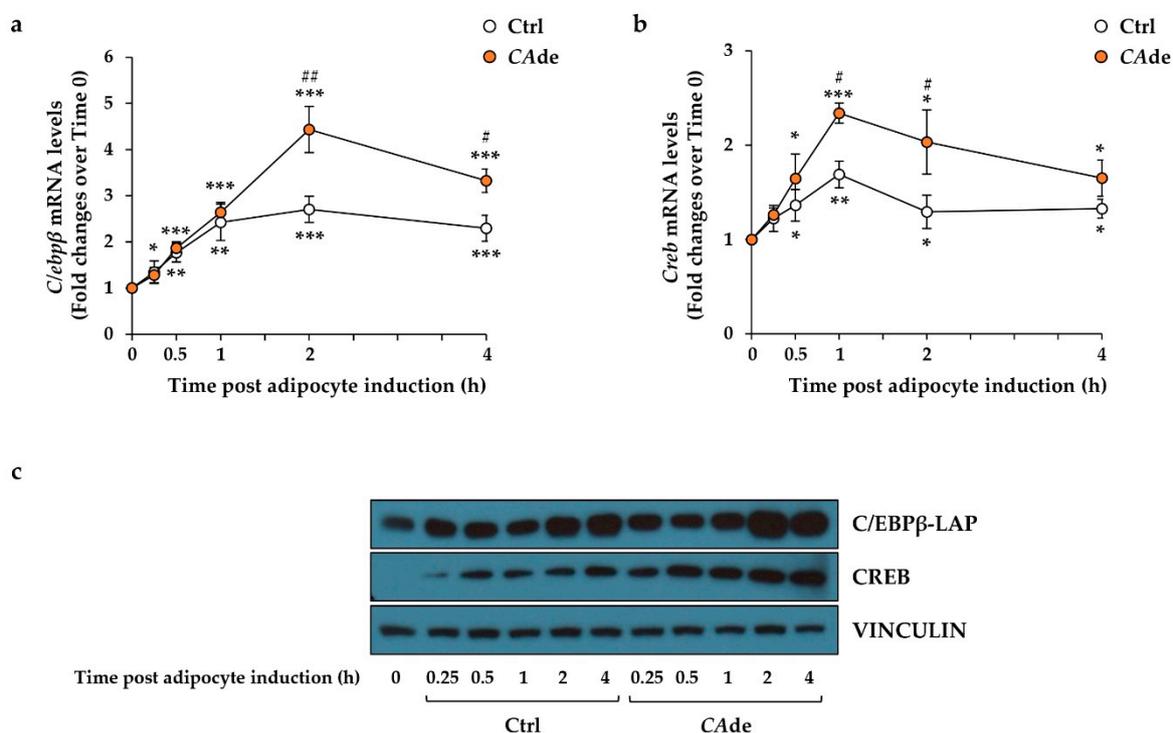


Figure 2. Effect of CAde on the expression of the *miR-155* target genes, CCAAT/enhancer-binding protein beta (*C/Ebpβ*) and cyclic Adenosine Monophosphate (cAMP) response element-binding protein (*Creb*), during the early stage of adipogenesis in 3T3-L1 cells. 3T3-L1 pre-adipocytes were cultured for 0.25, 0.5, 1, 2, and 4 h with AS (Ctrl) or AS + CAde (100 μg/mL). The *C/Ebpβ* and *Creb* levels were evaluated by quantitative real-time PCR. Time course of *C/Ebpβ* (a) and *Creb* (b) levels in Ctrl and CAde-treated cells relative to untreated 3T3-L1 cells at time 0. Values are means ± SEM of three independent experiments. Control value at time 0 was set as 1.00. Statistical significances among groups were tested by one-way ANOVA followed by Bonferroni correction post-hoc test (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. untreated 3T3-L1 cells at time 0; # $p < 0.05$ and ## $p < 0.01$ vs. control 3T3-L1 cells). (c) Representative Western blots of the total form of the *C/Ebpβ*-liver-enriched activating protein (LAP), CREB, and VINCULIN proteins in control cells and CAde-treated cells at 0.25, 0.5, 1, 2, and 4 h after adipogenic induction. *C/Ebpβ*-LAP and CREB protein expression levels at the basal (0) are also reported.

3.2. CAde Improved Terminal Adipocyte Differentiation of Both 3T3-L1 Cells Over-Expressing *miR-155* by Mimic Transfection and 3T3-L1 Cells Exposed to TNFα

Over-expression of *miR-155*, by a gain of function approach [22] or by pro-inflammatory cytokine induction [22,23], has been reported to impair in vitro adipocyte differentiation of 3T3-L1 cells. We then hypothesized that CAde might preserve adipogenesis of 3T3-L1 cells, where *miR-155* was over-expressed by mimic transfection or induced by the pro-inflammatory cytokine TNFα.

3.2.1. *miR-155* Gain and Loss of Function Studies

Fat cell differentiation of 3T3-L1 cells was firstly investigated in cells transfected with the *miR-155* mimic or the *miR-155* inhibitor. The specific over-overexpression of mimic *miR-155* reduced the number of 3T3-L1 cells able to differentiate into adipocytes by about 50%, as shown by light microscopy images (Figure 3a) and Oil-Red O lipid accumulation (Figure 3b). In cells treated with CAde, the intracellular lipid accumulation was increased by about 1.4-fold compared to *miR-155* over-overexpressing cells

(Figure 3a,b). On the other hand, the specific loss of function of *miR-155* by hairpin inhibitor transfection increased adipogenesis of 3T3-L1 cells by about 90% (Figure S3); no further increase of adipogenesis was observed in 3T3-L1 cells transfected with *miR-155* hairpin inhibitor upon CAde treatment (Figure S3). These findings indicated that CAde treatment partially prevents the inhibitory effects of *miR-155* on adipocyte differentiation of 3T3-L1 cells.

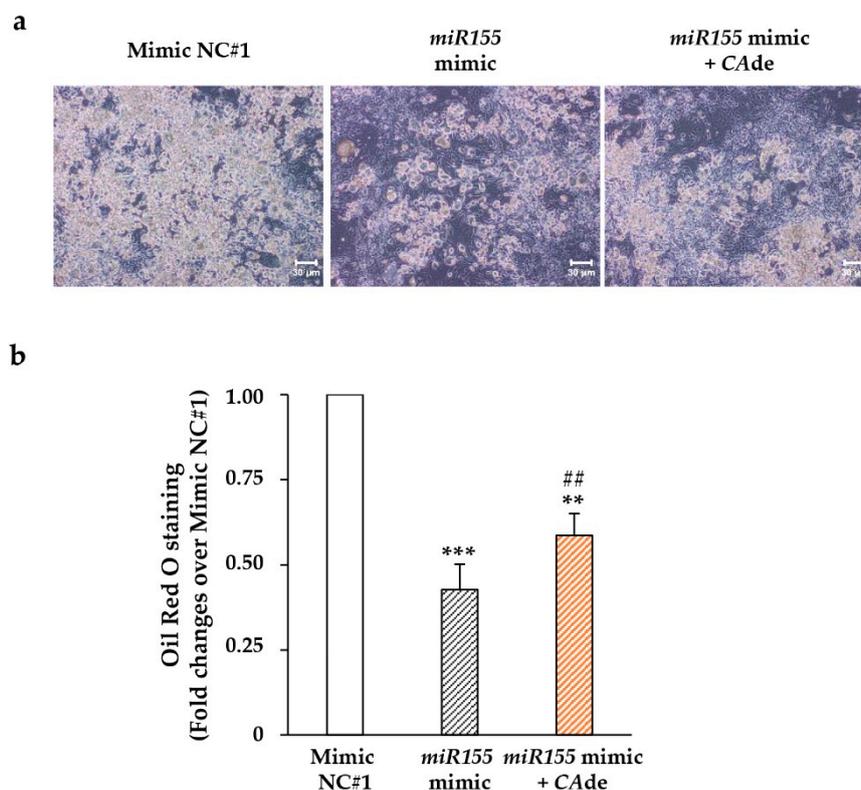


Figure 3. Effect of CAde on adipocyte differentiation and lipid accumulation of 3T3-L1 adipocytes over-expressing *miR-155*. 3T3-L1 pre-adipocytes were transfected with the mimic negative control (NC) #1 or with the *miR-155* mimic, and were cultured to reach adipocyte differentiation for 8 days with AS. Cells transfected with the *miR-155* mimic were also differentiated with AS supplemented with CAde (100 µg/mL). (a) Representative microphotographs of adipocytes transfected with mimic negative control (NC) #1 or with the *miR-155* mimic ± CAde are shown (X10 magnifications); scale bars, 30 µm. (b) Oil-Red O staining of adipocytes transfected with mimic negative control (NC) #1, or with the *miR-155* mimic ± CAde. Values are mean ± SEM of determinations from three independent experiments. Statistical significances among groups were tested by one-way ANOVA followed by Bonferroni correction post-hoc test (** $p < 0.01$, and *** $p < 0.001$ vs. mimic negative control (NC) #1; ## $p < 0.01$, *miR-155* mimic + CAde vs. *miR-155* mimic).

3.2.2. Treatment with TNF α

Secondly, we investigated whether CAde may preserve adipogenesis of cells exposed to the pro-inflammatory cytokine TNF α , which exerts profound inhibition of adipocyte differentiation by *miR-155* induction [21,22]. As expected, the number of 3T3-L1 pre-adipocytes able to achieve full differentiation was strongly reduced by TNF α treatment, as shown by the light microscopy images (Figure 4a). Consistent with this, TNF α reduced the intracellular lipid accumulation (Figure 4b) by about 60% and the TG deposition by about 70% (Ctrl, 41.5 ± 0.3 ; TNF α , 11.2 ± 0.3 , µg TG · µg DNA $^{-1}$; $p < 0.001$) compared to control adipocytes. It is worth noting that in cells co-treated with TNF α and CAde, the number of adipocytes was increased compared to TNF α -treated cells (Figure 4a). In addition, upon CAde treatment, the intracellular lipid accumulation (Figure 4b) and TG deposition were increased by 1.5- and about 2.2-fold, respectively, compared to TNF α -treated adipocytes (TNF α +

CAde, 26.4 ± 5.8 ; $\text{TNF}\alpha$, 11.2 ± 0.3 , $\mu\text{g TG} \cdot \mu\text{g DNA}^{-1}$; $p < 0.05$). Altogether, these data suggest that CAde treatment partially protected the adipogenesis of 3T3-L1 cells from the $\text{TNF}\alpha$ inhibitory effect.

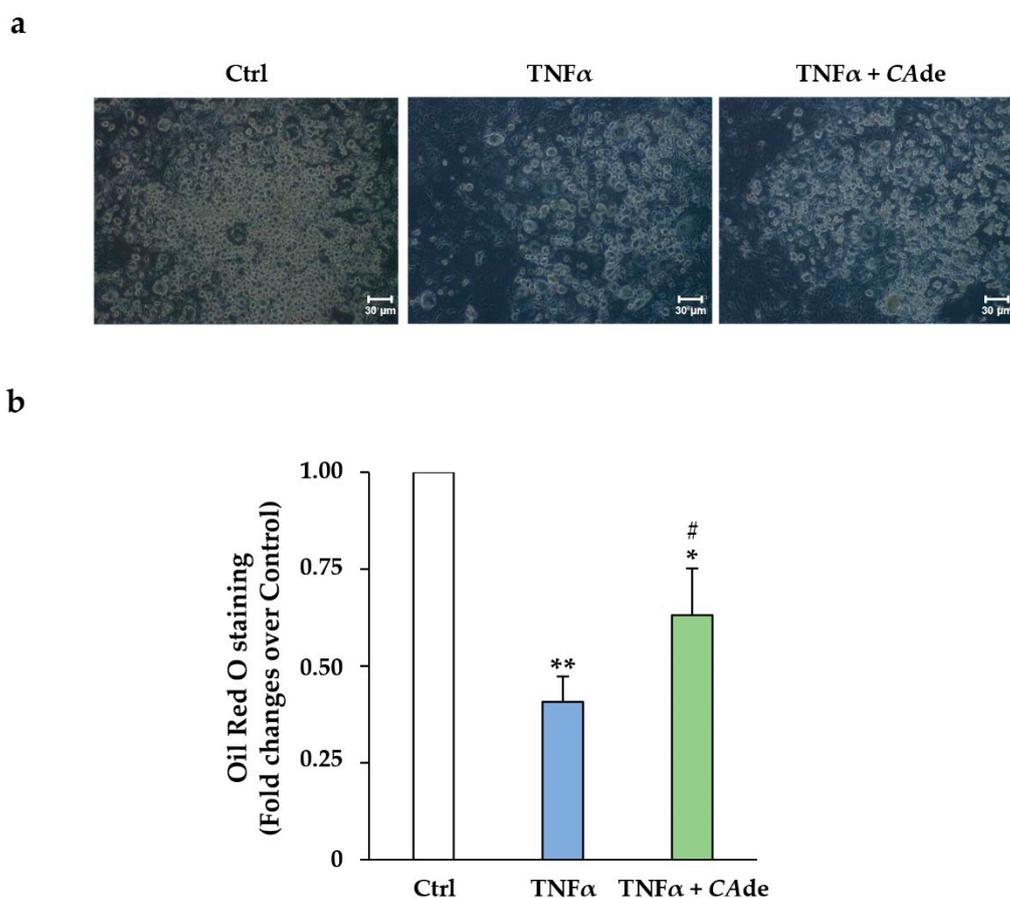


Figure 4. Effect of CAde on adipocyte differentiation and lipid accumulation of 3T3-L1 adipocytes treated with tumor necrosis factor- α ($\text{TNF}\alpha$). 3T3-L1 pre-adipocytes were cultured to reach adipocyte differentiation for 8 days with AS (Ctrl) or AS supplemented with $\text{TNF}\alpha$ (1 ng/mL) \pm CAde (100 $\mu\text{g/mL}$). (a) Representative microphotographs of Ctrl, $\text{TNF}\alpha$ -, and $\text{TNF}\alpha$ + CAde-treated adipocytes are shown (X10 magnifications); scale bars, 30 μm . (b) Oil-Red O staining of Ctrl, $\text{TNF}\alpha$ -, and $\text{TNF}\alpha$ + CAde-treated adipocytes. Values are mean \pm SEM of determinations from three independent experiments. Statistical significances among groups were tested by one-way ANOVA followed by Bonferroni correction post-hoc test. (* $p < 0.05$, and ** $p < 0.01$ vs. Ctrl; # $p < 0.05$, $\text{TNF}\alpha$ + CAde vs. $\text{TNF}\alpha$).

3.3. CAde Prevented $\text{TNF}\alpha$ -Induced Dysregulation of *miR-155*, *C/EBP β* , and *Creb* Expression during the Early Stage of Adipogenesis in 3T3-L1 Cells

Up-regulation of *miR-155* and suppression of *C/EBP β* and *Creb* expression were identified as one of the mediators of the $\text{TNF}\alpha$ -dependent inhibition of adipogenesis [22,23]. We thus evaluated whether CAde may counteract these anti-adipogenic effects of $\text{TNF}\alpha$ during the early time points upon adipogenic induction of 3T3-L1 pre-adipocytes. As expected, $\text{TNF}\alpha$ already at 15 min from adipogenic induction induced a 30% increase in *miR-155* expression, whose level remained elevated at 30 min, 1, 2, and 4 h upon AS stimulation compared to control 3T3-L1 cells at time 0 (Figure 5a). Consistent with this, $\text{TNF}\alpha$ treatment also decreased the expression of *C/EBP β* by about 50% at 30 min, 42% at 1 h, 66% at 2 h, and 35% at 4 h upon AS stimulation (Figure 5b) and by *Creb* by about 52% at 30 min and 62% at 1 h upon AS stimulation (Figure 5c). Interestingly, the co-treatment with $\text{TNF}\alpha$ and CAde down-regulated *miR-155* expression during the early time points of adipogenic induction to levels comparable to AS-treated control cells (Figure 5a and Figure S4a). At the same time points upon adipogenic induction, an up-regulation of both *C/EBP β* (Figure 5b and Figure S4b) and *Creb* (Figure 5c and Figure S4c) mRNA

expression, to levels comparable to those in AS-treated control cells, was observed in the TNF α + CAde-treated cells. Altogether, these findings suggest that CAde weakened the inhibitory effect of TNF α on the adipogenesis of 3T3-L1 cells by restoring the AS effects on *miR-155* down-regulation and *C/Ebp β* and *Creb* gene up-regulation at the early differentiation time points.

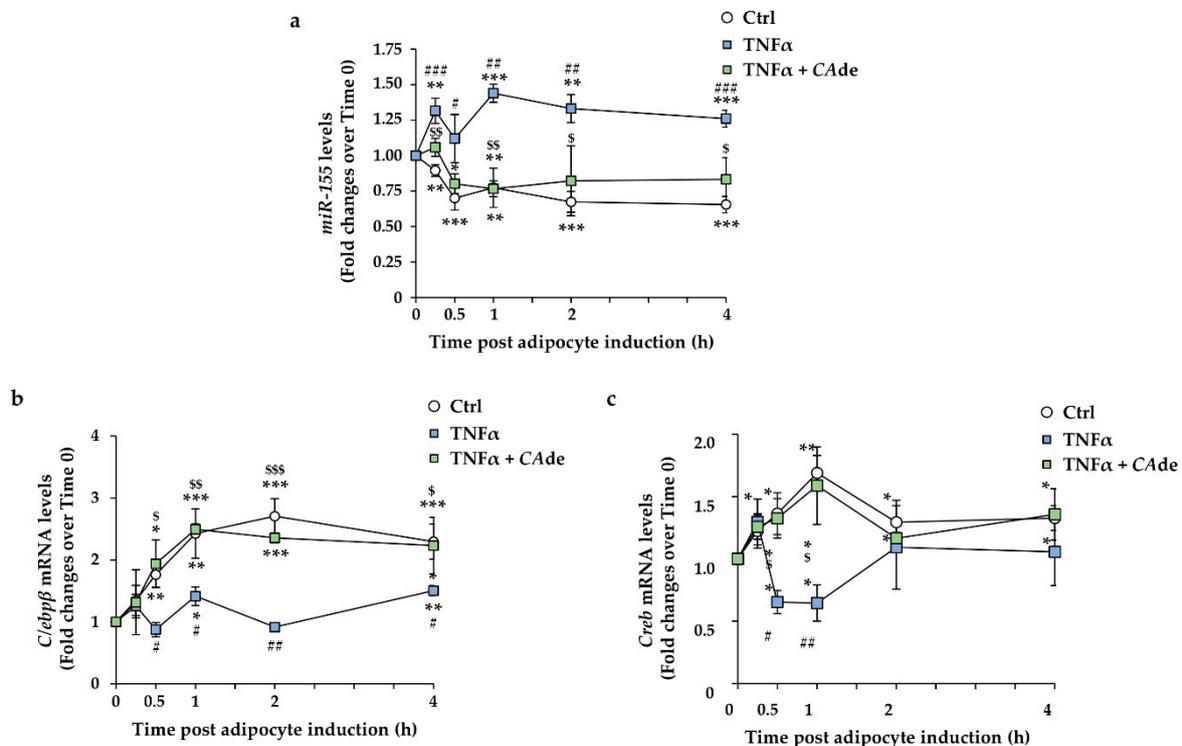


Figure 5. Effect of CAde on the expression of *miR-155* and its target genes, *C/Ebp β* and *Creb*, during the early stage of adipogenesis in 3T3-L1 cells exposed to TNF α . 3T3-L1 pre-adipocytes were cultured for 0.25, 0.5, 1, 2, and 4 h with AS (Ctrl) or AS supplemented with TNF α (1 ng/mL) \pm CAde (100 μ g/mL). The miRNA and gene expressions were evaluated by quantitative real-time PCR. Time course of *miR-155* (a), *C/Ebp β* (b), and *Creb* (c) levels in Ctrl, TNF α -, and TNF α + CAde-treated cells are shown relative to untreated 3T3-L1 cells at time 0. Values are means \pm SEM of three independent experiments. Control value at time 0 was set as 1.00. Statistical significances among groups were tested by one-way ANOVA followed by Bonferroni correction post-hoc test (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. untreated 3T3-L1 cells at time 0; # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ vs. control 3T3-L1 cells; \$ $p < 0.05$, \$\$ $p < 0.01$, \$\$\$ $p < 0.001$ vs. TNF α -treated 3T3-L1 cells).

4. Discussion

miRNAs have emerged as critical regulators of a variety of biological processes in eukaryotic cells, and the deregulation of their function is associated with an increasing number of human diseases [3]. Furthermore, it has been reported that miRNAs may contribute to the metabolic abnormalities associated with obesity and obesity-related complications by particularly affecting the function of the white adipose tissue (WAT) [35,36]. Indeed, in human WAT, numerous miRNAs are expressed, obesity influences their expression, and their predominant function is to stimulate or inhibit the differentiation of pre-adipocytes into adipocytes, as well as to regulate specific metabolic and endocrine functions, as revealed through loss or gain of function studies on the obesity-associated miRNAs [35–41]. This makes miRNAs a tangible target for the treatment of adipocyte dysfunction and its related disorders.

Nutraceuticals have been used for decades now in weight management in obese individuals and are described to modulate adipogenesis and to have other positive effects on obesity pathogenesis [27]. Growing evidence sustains the hypothesis that dietary modulation of miRNA expression may explain

in part some of the beneficial effects of nutraceuticals on health [24–26]. Indeed, an increasing number of studies have reported that several natural food-derived compounds modulate miRNA expression in different animal cells and tissues [24–26]. Extracts from *Citrus aurantium* L. have been recently used for investigating adipocyte differentiation [28,42,43]. However, quite different results have been obtained, and whether or not extracts from *Citrus aurantium* L. have pro- or anti-adipogenic properties is still unclear. Kim et al. have indeed reported on the anti-adipogenic effect of preparation from the fruit peel of *Citrus aurantium* L. in 3T3-L1 cells, where the flavonoids naringin, hesperidin, and nobiletin are predominant [42]. Park et al. have instead demonstrated in 3T3-L1 cells and primary cultured adipocytes the anti-adipogenic and pro-thermogenic actions, respectively, of preparation from the immature dried fruit of *Citrus aurantium* L., which is abundant in naringin and neohesperidin [43]. We have recently shown that CAde, a dry extract preparation obtained from the fruit juice of *Citrus aurantium* L., mainly enriched in hesperidin, narirutin, and vicenin-2, increased adipocyte differentiation and function of 3T3-L1 cells [28]. Therefore, reasons for these controversial findings could probably be found on the specificity and the amounts of flavonoids within each of these *Citrus aurantium* L. extracts, which also depends on their origin.

In the present study, we have further reported new evidence that sustains nutraceutical beneficial effects of CAde on the regulation of miRNA expression and function in vitro in 3T3-L1 pre-adipocytes. In particular, we demonstrated that the treatment of cells with CAde enhanced the down-regulation of the adipogenic suppressor *miR-155* [22,23], as early as 15 min upon induction of adipogenesis in 3T3-L1 pre-adipocytes. This results in an up-regulation of the mRNA and protein levels of the *miR-155* target genes, *C/Ebp β* and *Creb* [22]. Specifically, CAde treatment further increased the expression of *C/Ebp β* mRNA and of the pro-adipogenic *C/EBP β -LAP* protein isoform upon 2 h from the adipogenic induction [44]. Additionally, CAde further up-regulated *Creb* mRNA and protein levels.

It is worth noting that CAde specifically modulated *miR-155* expression during the early stage of adipogenesis. Indeed, CAde treatment during the first 4 h post-adipogenic induction with AS did not affect the expression of other miRNAs, such as *miR-130a* and *miR-375*, whose role in adipocyte fate determination has been already demonstrated [17,18,41]. *miR-155* is biologically relevant to the regulation of adipocyte differentiation, and its perturbation is associated with obesity [22,23]. In addition, *miR-155* expression in cells is modulated by nutraceutical compounds [45–47]. Indeed, Eseberri et al. have shown that the stilbenoid resveratrol and its metabolites, trans-resveratrol-3-*O*-glucuronide and trans-resveratrol-4-*O*-glucuronide, exert their anti-adipogenic effect on 3T3-L1 cells by up-regulating *miR-155* expression [45]. On the contrary, others reported the down-regulation of the same miRNA by flavonoid treatment [46,47]. Boesch-Saadatmandi et al. have indeed demonstrated that the flavonoid quercetin and its metabolite isorhamnetin partially neutralize lipopolysaccharide-induced increase of *miR-155* in murine RAW264.7 macrophages and state that *miR-155* inhibition possibly contributes to the anti-inflammatory properties of both flavonoids [46]. Additionally, Arango et al. have reported that the flavonoid apigenin and a celery-based apigenin-rich diet exert effective anti-inflammatory activity in vivo by reducing expression of *miR-155* [47]. As reported above in this section, flavonoids, such as hesperidin, narirutin, and vicenin-2, are very abundant in our dry extract preparation [28] and their presence might be thus responsible for the observed down-regulation of *miR-155* expression in CAde-treated cells.

Here, we have also reported that CAde partially preserved adipogenesis of 3T3-L1 cells, where the expression of *miR-155* was up-regulated by mimic transfection. CAde did not further enhance adipogenesis of 3T3-L1 cells, where *miR-155* activity was abolished by specific hairpin inhibitor. These findings led us to suppose that the treatment with CAde may be effective against any micro-environmental insults, which impair adipocyte differentiation by up-regulation of *miR-155*. In accordance with this, we indeed found that the CAde treatment counteracted the detrimental effects of TNF α on adipogenesis. Indeed, terminal adipocyte differentiation of 3T3-L1 cells exposed to TNF α was improved by almost 50% by CAde and was associated with a restoring of the expression of *miR-155*, *C/Ebp β* , and *Creb* during the early stage of adipogenesis. TNF α is a pleiotropic cytokine that exerts

homeostatic and pathogenic bioactivities [48]. High TNF α levels are observed in the WAT during obesity, and they have profound effects on adipocyte metabolism by impairing triglyceride synthesis and storage and inhibiting adipocyte differentiation [22]. Liu et al. have also demonstrated in 3T3-L1 pre-adipocytes that *miR-155*, whose expression is up-regulated by TNF α as early as 5 min via NF κ B-p65 (nuclear factor kappa-light-chain-enhancer of activated B cells) binding to the *miR-155* promoter, mediates at least in part the TNF α -induced suppression of adipogenesis by down-regulating early adipogenic transcription factors [22]. These findings, therefore, provide the first piece of evidence for the efficacy of CAde treatment in vitro against micro-environment insults deleterious for the functional capacity of adipose cells. In this scenario, CAde may ameliorate, in the early stage, the differentiation process by blocking NF κ B-p65 into the cytosol and thus preventing the NF κ B-p65-mediated transcription of *miR-155*. This hypothesis was indeed sustained by our preliminary data in 3T3-L1 pre-adipocytes short-term treated with TNF α , where the TNF α -induced NF κ B-p65 translocation from cytosol to the nucleus was prevented by CAde treatment.

In conclusion, this study demonstrated that *miR-155* down-regulation is part of the mechanism through which CAde enhances adipocyte differentiation of pre-adipocytes in vitro. In addition, we herein provide substantial evidence of the efficacy of this nutraceutical compound against micro-environment insults, which are harmful to adipose cell functionality and affect *miR-155* expression, such as TNF α , suggesting that the development of CAde-derived compounds may be an effective strategy for the treatment of adipocyte dysfunction and its related disorders.

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