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Antiadipogenic effect of carnosic acid, a natural compound present in *Rosmarinus officinalis*, is exerted through the C/EBPs and PPARγ pathways at the onset of the differentiation program

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ABSTRACT

Background: Obesity is a serious health problem all over the world, and inhibition of adipogenesis constitutes one of the therapeutic strategies for its treatment. Carnosic acid (CA), the main bioactive compound of *Rosmarinus officinalis* extract, inhibits 3T3-L1 preadipocytes differentiation. However, very little is known about the molecular mechanism responsible for its antiadipogenic effect.
Methods: We evaluated the effect of CA on the differentiation of 3T3-L1 preadipocytes analyzing the process of mitotic clonal expansion, the level of adipogenic markers, and the subcellular distribution of C/EBPβ.
Results: CA treatment only during the first day of 3T3-L1 differentiation process was enough to inhibit adipogenesis. This inhibition was accompanied by a blockade of mitotic clonal expansion. CA did not interfere with C/EBPβ and C/EBPδ mRNA levels but blocked PPARγ, and FABP4 expression. C/EBPβ has different forms known as LIP and LAP. CA induced an increase in the level of LIP within 24 h of differentiation, leading to an increment in LIP/LAP ratio. Importantly, overexpression of LAP restored the capacity of 3T3-L1 preadipocytes to differentiate in the presence of CA. Finally, CA promoted subnuclear de-localization of C/EBPβ.
Conclusions: CA exerts its anti-adipogenic effect in a multifactorial manner by interfering mitotic clonal expansion, altering the ratio of the different C/EBPβ forms, inducing the loss of C/EBPβ proper subnuclear distribution, and blocking the expression of C/EBPα and PPARγ.
General significance: Understanding the molecular mechanism by which CA blocks adipogenesis is relevant because CA could be new a food additive beneficial for the prevention and/or treatment of obesity.

1. Introduction

Obesity is a serious health problem both, in developed and developing countries [1,2]. During the last two decades, it was well established that the adipose tissue is an endocrine organ responsible for the secretion of numerous adipokines (i.e. leptin, adiponectin, resistin, among others) that overall play a key role not only in the control of energy balance but also in the maintenance of the metabolic homeostasis [3,4]. Due to these important functions, deregulation of the adipose tissue, like in obese patients, constitutes a risk factor for the development of diseases like type 2 diabetes, dyslipidemias, atherosclerosis and even certain cancers [5–8]. Weight loss is increasingly recognized to have major health benefits for obese as well as for overweight people. However, weight loss and weight control drugs provided by the diet industry have failed in the long-term maintenance of weight control. Therefore, it is a challenge in the field to find new types of drugs that could be beneficial for the prevention and treatment of obesity. Since obesity is a consequence of an increase in adipocyte size and the formation of new mature adipocytes from undifferentiated precursors [9,10], drugs that may control adipogenesis could be beneficial for the treatment of obesity.

Adipocyte differentiation has been studied mainly using cells that are already committed, as for example murine 3T3-L1 pre-adipocytes, which are comparable to native committed precursors, since they have the ability to differentiate into cells that accumulate lipids, respond to

Abbreviations: CA, carnosic acid; RE, *Rosmarinus officinalis* extract; COH, carnosol; RA, rosmarinic acid; C/EBP, CCAAT/Enhancer Binding Protein; PPARγ, peroxisome proliferator-activated receptor γ; IBMX, isobutyl-3-methylxanthine; MTS, 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; FBS, fetal bovine serum; DAPI, 6-diamino-2-phenylindole; IIF, indirect immunofluorescence

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insulin and secrete leptin [9–11]. The molecular events that take place during the process of adipogenesis have been extensively studied. In this regard, the cascade of genetic and signaling events that take place during adipocyte differentiation are well characterized [10]. The bZIP family of transcription factors CCAAT/Enhancer Binding Proteins (C/EBPs) and the peroxisome proliferator-activated receptor (PPAR) γ are central transcriptional regulators for cell to acquire the adipocyte phenotype [10]. At the onset of adipogenesis, the expression of C/EBPβ and C/EBPδ is induced and these transcription factors concentrate mainly in pericentromeric heterochromatin [12–15]. C/EBPβ has different forms, LAP (*Liver Activating Protein*) corresponding to p35 and p32C/EBPβ, and LIP (*Liver Inhibitory Protein*) that lacks most of the N-terminal transactivation domain [16]. LAP and LIP form homo- and heterodimers that have differential transcriptional capacities [16,17]. Interestingly, LAP and LIP homodimers are differentially distributed in the nucleus possibly as a mean to control their bioavailability for regulating target genes. LAP homodimers localize in pericentromeric heterochromatin and has a fraction distributed in euchromatic domains, the latter possibly corresponding to the transcriptionally active pool [15]. In contrast, LIP homodimers are exclusively located in pericentromeric heterochromatin and, in this way, being "sequestered" from euchromatin [15]. C/EBPβ and C/EBPδ are important factors in the adipogenic transcriptional network inducing the expression of C/EBPα and PPARγ [18–20]. Their requirement for adipogenesis was demonstrated by a severe reduction in adipose tissue observed in C/EBPβ-C/EBPδ double knockout mice [21]. Moreover, expression of C/EBPα and PPARγ genes is impaired during *in vitro* differentiation of the C/EBPβ and C/EBPδ double knockout embryonic fibroblasts [21]. C/EBPα null mice, as expected, also exhibit defect of adipose tissue but accompanied by defects in carbohydrate metabolism that causes the death of these mice due to severe hypoglycemia within 8 h after birth [10]. As for PPARγ, it is a key regulator of adipogenesis [10,22,23]. Even C/EBPα −/− fibroblasts acquire morphological characteristics of adipocytes upon ectopic expression and activation of PPARγ [24]. PPARγ is not only critical for adipogenesis to proceed but also required for the maintenance of the differentiated state. In this regard, expression of a dominant negative form of PPARγ in 3T3-L1 adipocytes causes their dedifferentiation with loss of lipid accumulation and a decrease in expression of adipocyte markers [25]. Two PPARγ isoforms, PPARγ1 and PPARγ2, which are identical except for additional 31 amino acids at the N terminus of PPARγ2, are expressed in adipose tissues [10,26]. Studies from PPARγ knockout mice have further shown PPARγ central role not only in adipogenesis but also in the control of insulin sensitivity [23,27–29]. Therefore, PPARγ has received considerable attention due to the fact that its ligands are potent drugs for treating insulin resistance and dyslipidemia [30–32]. However the limitations and side effects of these drugs have driven researchers to look for a new type of drugs that could be beneficial for the treatment of metabolic disorders [32].

Rosemary, *Rosmarinus officinalis* L. is an evergreen perennial shrub native to Europe cultivated in many parts of the world. A number of studies have reported its therapeutic potentials as antioxidant, hepatoprotective, and anti-inflammatory [33,34]. We have previously reported that an acetone rosemary extract (RE) containing a high amount of the diterpene carnosic acid (CA) not only has antioxidant but also antimicrobial activity [35]. Recent reports showed that a rosemary leaf extract limits weight gain and liver steatosis in mice fed with a high-fat diet [36]. Further, ob/ob mice fed with standard chow diet supplemented with CA experienced significant weight loss and reduced visceral obesity [37]. It has been also reported that CA inhibition of 3T3-L1 cell differentiation seemed to be mediated by activation of the antioxidant-response element and induction of phase II enzymes involved in the metabolism of glutathione (GSH) leading to an increase of the intracellular level of GSH [38]. Thus, the aim of this study was to investigate the effect of RE and CA, the main diterpene present in RE, in the regulation of key differentiation markers in order to gain insight into the molecular mechanism of CA-dependent inhibition of adipocyte differentiation. Here, we report for the first time that the RE and particularly CA, exerts their anti-adipogenic effect acting at multiple levels. CA blocks the mitotic clonal expansion, alters the ratio of C/EBPβ forms, promotes the subnuclear delocalization of C/EBPβ and inhibits the expression of C/EBPα and PPARγ.

2. Materials and methods

2.1. Materials

Carnosol (COH), carnosic acid (CA) and rosmarinic acid (RA) were purchased from Alexis Biochemicals (San Diego, California, USA). Isobutyl-3-methylxanthine (IBMX), dexamethasone, insulin, and Oil red O, were obtained from Sigma–Aldrich Chemical Co (St. Louis, MO, USA). 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) was purchased from Promega (Madison, WI, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), LipidTOX and 4′,6-diamino-2-phenylindole (DAPI) were purchased from Life Technologies from Invitrogen (Carlsbad, CA, USA).

The acetone rosemary extract (RE) was obtained as previously described [35]. In brief, rosemary leaves were chopped into small pieces with a blender and placed in deionized water. The solution was steam-distilled for 1 h in a Clevenger type apparatus to oil isolation and the RE was obtained by extracting the residue with acetone using a Soxhlet apparatus. The phenolic content of RE was: 13.86 ± 2% of CA, 8.81 ± 1.2% of COH and 0.83 ± 0.1% of RA, percentages that are equivalent to 5 μg/ml of CA, 2.5 μg/ml of COH and 0.3 μg/ml of RA, respectively.

2.2. 3T3-L1 cell culture

Murine 3T3-L1 cells (from ATCC) were grown in DMEM containing 10% FBS in an atmosphere of 10% CO_2, 90% air at 37 °C. Adipocyte differentiation was induced as previously described [15]. Briefly, two days post-confluence cells were switched to differentiation medium (MDI) containing 10% FBS, 1 μM dexamethasone, 520 μM IBMX, and 167 nM of insulin. Two days after, media was replaced and insulin was added. Forty-eight hours later, cells were kept in DMEM with 10% FBS. RE, CA, COH, and RA were dissolved in ethanol and added to the medium at the indicated concentrations and periods of time. One percent of ethanol (vehicle) was added to the medium as a vehicle control.

For over-expression assays, 3T3-L1 preadipocytes were grown on coverslips to 80% confluence and transfected with 5 μg of the CMV-LAP (a kind gift from Dr. J. Schwartz from the Univ. of Michigan Medical School, MI, USA) encoded p32C/EBPβ [17] or empty vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Twenty four hours later cells were induced to differentiate in the absence or the presence of 5 μg/ml of CA during the first day of adipogenesis. At the sixth day post-induction of differentiation, lipid vesicles were stained using LipidTOX (see below), nuclei were counterstained with DAPI and the percentage of adipocytes was determined using Image-J program (v.1.42) from the NIH.

2.3. MTS and Tripan blue assays

After the indicated treatment, cells were washed with phosphate buffer saline (PBS) once, and incubated with 8% MTS and 0.8% phenazine methosulfate in DMEM, 10% FSB for 1 h at 37 °C. Then, absorbance of the medium was measured at 595 nm in a Beckman coulter to determine cell viability. Trypan blue exclusion test of cell viability was performed as previously described [18,39].

2.4. Oil Red O, LipidTOX staining

For Oil red O staining, cells were washed with PBS, fixed with 4% formaldehyde (PFA) at room temperature for 1 h, washed twice with water, and stained with Oil Red O, as previously described [18,39]. After washes in PBS, nuclei were counterstained with DAPI [14]. For the staining of lipid vesicles, cells were grown on coverslips, fixed with 4% PFA, permeabilized with PBS 0.5% Triton-X100, nuclei were counterstained with DAPI, then coverlips were inverted onto 25 μl of LipidTOX diluted 1/300 in PBS and incubated 30 min at room temperature. Images were obtained with a fluorescence microscope (Axiovert 35 M, Zeizz). All measurements were performed at least twice in triplicate. To measure the content of lipids, the lipid droplets stained by Oil red O were dissolved in isopropanol containing 4% Nonidet P-40, and then quantified by measuring the absorbance at 520 nm.

2.5. [^{3}H] Thymidine incorporation

3T3-L1 preadipocytes grown in 96-well plates until confluence were induced to differentiate in the absence or the presence of RE (30 μg/ml) or CA (7.5 μg/ml), and DNA synthesis was evaluated by the incorporation of tritiated thymidine ([^{3}H]TdR). 0.4 μCi/ml of [^{3}H] TdR was added at the time of induction, and after 24 h cells were frozen. Cell lysates were thawed and DNA was collected by filtration onto GF/C glass microfiber filters (Whatman), washed with 95% ethanol, dried and counted, as previously described [40,41].

2.6. Flow cytometric analysis

3T3-L1 preadipocytes were induced to differentiate in the absence or the presence of 30 μg/ml of RE or 7.5 μg/ml of CA for 24 h. Cells were trypsinized, resuspended in 0.4 ml, collected by centrifugation, fixed with 70% ethanol for at least 2 h on ice, washed twice with PBS and incubated in a solution containing 50 μg/ml of propidium iodine (PI) and 60 μg/ml of RNase A for 1 h at 37 °C. A BD FACSCalibur flow cytometer (BD, Biosciences, USA) was used to assess the percentage of cells in G0/G1, S and G2/M phase by fluorescence-activated cell sorting (FACS) analysis.

2.7. RT-PCR analysis

Total RNA was prepared from 3T3-L1 cells using the Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's directions. RNA quantification and purity were assessed by measuring absorbance at 260 and 280 nm. RT-PCR was performed to evaluate mRNAs. Primer sequences (Integrated DNA Technologies, Coralville, IA, USA), were as follows (5′ to 3′):

C/EBPβ	Fw: GGGGTTGTTGATGTTTTTGG
	R: CGAAACGGAAAAGGTTCTCA
C/EBPδ	Fw: CAGACAGTGGTGAGCTTGGC
	R: CAGAGTCTCAAAGGCCCACG
PPARγ	Fw: CGCTGATGCACTGCCTATGA
	R: AGAGGTCCACAGAGCTGATTCC
FABP4	Fw: CATGGCCAAGCCCAACAT
	R: CGCCCAGTTTGAAGGAAATC
β-Actin	Fw: TGTCCACCTTCCAGCAGATGT
	R: AGCTCAGTAACAGTCCGCCTAGA

PCRs were performed in the presence of 2.5 mM $MgCl_2$ and 0.25 mM dNTPs using the following conditions: denaturation at 95 °C for 10 min; 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; and elongation at 72 °C for 5 min. The PCR amplified products were visualized after ethidium bromide staining on a 2% agarose gel. The mRNA levels of all genes were normalized using β-actin as internal control.

2.8. Western blot analysis

Cultured cells were washed twice with ice-cold PBS and harvested in RIPA buffer [1% Nonidet P-40, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EGTA (pH 7.8), 1 mM $NaVO_4$ and 1 mM PMSF]. After 30 min of incubation on ice, whole cell lysates were centrifuged at 20,000 *g* for 10 min at 4 °C and supernatants, containing the protein fraction, were collected and stored at −20 °C until use. Fifty μg of protein were separated by 13% SDS-PAGE and transferred to PVDF membranes. Membranes were incubated with the indicated antibodies (PPARγ (E8), C/EBPα (SC-9314), C/EBPβ (SC-150), C/EBPδ (SC-636) from Santa Cruz, CA, USA) and signals were detected by ECL (Amersham, Little Chalfont Buckinghamshire, England), as previously described [15]. Intensity of bands was analyzed by densitometry using Scion Image for Windows software.

2.9. Indirect immunofluorescence assays

Indirect immunofluorescence (IIF) was performed as previously described [14,15]. Briefly, 3T3-L1 cells were grown on coverslips and then induced to differentiate as indicated above, in the presence or the absence of RE and CA at indicated concentrations. Cells were simultaneously fixed and permeabilized by immersion in cold methanol (−20 °C) for 2 h. Coverslips were washed three times with cold PBS and inverted onto 25 μl drop of PBS 1% BSA with anti-C/EBPβ (dilution 1/100 (Santa Cruz, CA)) and anti-HP1α (dilution 1/100, (Millipore, CA)) overnight at 4 °C. Then, coverslips were incubated with secondary antibodies labeled with Alexa Fluor 488 or Rhodamine for 1 h at room temperature. Finally, nuclei were stained with DAPI and coverslips were mounted in Vectashield. Laser-scanning confocal microscopy was performed with Meta (Carl Zeiss).

2.10. Statistical analysis

Unless otherwise indicated, all values are expressed as means ± standard error (S.E.). All data were analyzed by ANOVA followed by Tukey test. Differences were considered to be significant at $p < 0.05$.

3. Results

3.1. R. officinalis extract inhibits 3T3-L1 cells adipogenesis at the onset of the differentiation program

To investigate the effect of RE on adipocyte differentiation, 3T3-L1 preadipocytes were induced to differentiate in the absence or the presence of increasing concentrations of RE. We observed that in the presence of 20 μg/ml of RE there was a marked decreased in lipid accumulation as revealed by Oil Red O staining (Fig. 1A), and almost a complete inhibition of differentiation was observed in the presence of 30 μg/ml of RE (Fig. 1A). Due to these results, next we evaluated whether RE could affect cell viability. Preadipocytes were differentiated for 6 days in the absence or the presence of increasing concentrations of RE and cell viability was evaluated by MTS assay. We found that RE had no cytotoxic effects at the concentrations that inhibited adipogenesis (20 μg/ml and 30 μg/ml) (Fig. 1B). Likewise, a slight increment in cell viability was observed at low concentrations perhaps due to the antioxidant activity displayed by rosemary polyphenols (Fig. 1B). The effect of the RE on differentiation was confirmed through analysis of cell morphology by light microscopy. In the absence of RE, a high percentage of cells had large lipid droplets (Fig. 1C); in contrast, when cells were induced to differentiate in the presence of 20–30 μg/ml of RE, a low percentage to almost no cells containing lipid droplets were observed (Fig. 1C).

To investigate at which stage of the differentiation process RE inhibits adipogenesis, we triggered of 3T3-L1 preadipocytes differentiation in the presence of RE at different periods of time during the

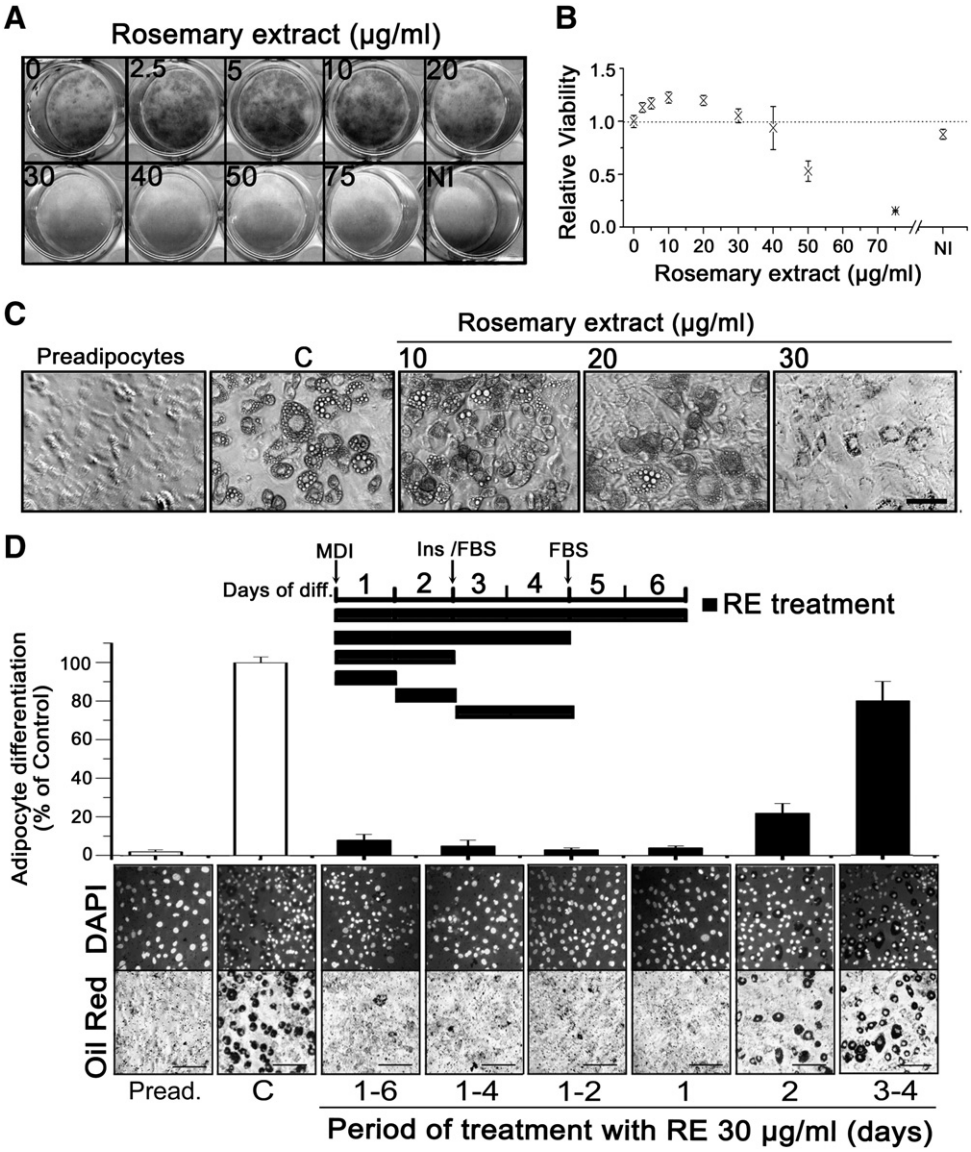

Fig. 1. RE inhibits 3T3-L1 preadipocytes differentiation without cytotoxic effects. A — 3T3-L1 preadipocytes were induced to differentiate in the presence of the indicated concentrations of RE, and at day 6 cells were stained with Oil red O. NI: Non-differentiated cells. B — Cells were induced to differentiate 6 days in the absence or in the presence of the indicated concentrations of RE, and then viability was determined by MTS assay. The viability of 3T3-L1 cells differentiated in the absence of RE was considered as 1. C — Cell morphology 6 days post-induction of differentiation in the absence (C) or the presence of increasing concentrations of RE was assessed by light microscopy. Scale bar: 50 μm. D — 3T3-L1 preadipocytes were induced to differentiate in the absence or the presence of 30 μg/ml of RE during the indicated periods of time represented in the scheme of the insert. Differentiated cells were evaluated by Oil Red O staining and total cells by DAPI staining. Values of percentage of differentiation are expressed as the mean of three independent experiments and error bars represent the SE compared with the number of cells differentiated under no treatment (C). MDI: differentiation medium, Ins: insulin, FBS: fetal bovine serum. Scale bar: 100 μm.

process of adipogenesis, as shown in the insert of Fig. 1D. When 3T3-L1 preadipocytes were in the presence of RE only during the initial 4 days of differentiation, and then continued the process in the absence of RE, almost no cells with lipid droplets were observed (Fig. 1D, 1–4 *vs.* C), similarly as the inhibition obtained in the presence of RE for the whole process (Fig. 1D, 1–4 *vs.* 1–6). The presence of RE during the first 2 days of differentiation also efficiently blocked 3T3-L1 preadipocytes differentiation (Fig. 1D, 1–2 *vs.* C). Importantly, when adipogenesis is induced in the presence of RE only during the first day, it was enough to completely inhibit the differentiation process even more efficiently than RE addition on day 2 of the differentiation process (Fig. 1D, 1 *vs.* 2). Further, the presence of RE at day 1 blocks adipogenesis as efficiently as RE treatment during the whole process (Fig. 1D, 1 vs. 1–6). In contrast, addition of RE during days 3 and 4 of the differentiation process did not significantly interfere with adipogenesis (Fig. 1D, 3–4 *vs.* C). Taken together these results demonstrate that RE exerts its inhibitory effect at the onset of the adipogenic program.

3.2. Identification of the bioactive compound(s) present in RE responsible for the inhibition of adipocyte differentiation

Based on HLPC analysis, 30 μg/ml of the RE, which completely blocked 3T3-L1 adipocyte differentiation, contained 5 μg/ml of CA, 2.5 μg/ml of COH and 0.3 μg/ml of RA. To identify the specific bioactive compound(s) present in RE responsible of the inhibition of adipogenesis, 3T3-L1 preadipocytes were induced to differentiate in the presence of different combinations of the main bioactive compounds at the same concentrations as they were present in the plant extract. The combination of CA (5 μg/ml) and COH (2.5 μg/ml) in the presence or absence of RA (0.3 μg/ml) resulted in a similar inhibitory effect on adipogenesis as RE (Fig. 2A). Further, RA had no

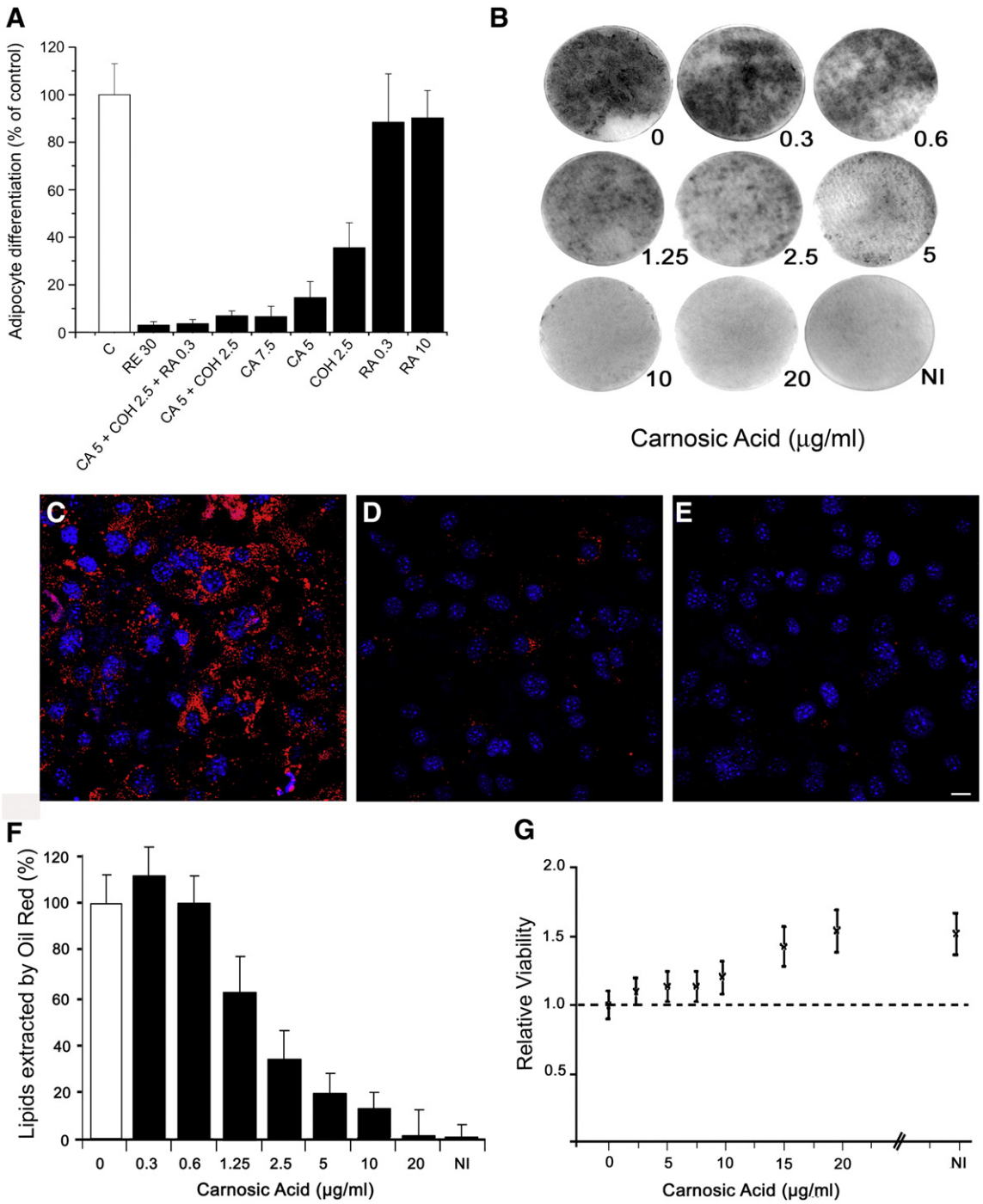

Fig. 2. Identification of the bioactive compound(s) present in the RE responsible for inhibiting adipogenesis. A — 3T3-L1cells were incubated during the first day of differentiation in the absence (C) or presence of the indicated compounds, and their differentiation was determined as described in Fig. 1. Results were confirmed by three independent experiments and error bars represent the SE. B — 3T3-L1 cells were treated with increasing concentrations of CA during the first day of differentiation and stained with Oil red O at day 6. NI: Non-differentiated cells. C–E — Lipid vesicles were stained with LipidTOX in 3T3-L1 cells induced to differentiate in the absence (panel C) and the presence of 5 and 10 μg/ml of CA (panels D and E, respectively). Nuclei were counterstained with DAPI. Scale bar: 10 μm. F — Lipid accumulation was assessed by the quantification of OD_{520} as described in Materials and Methods. Results are given as the lipid content of each experimental group relative to cells differentiated in the absence of CA (designated as 100%). G — 3T3-L1 preadipocytes were induced to differentiate in the absence or presence of increasing concentrations of CA and at day 6 cell viability was determined by MTS staining. The viability of cells differentiated in the absence of CA was considered as 1.

effect on adipocyte differentiation even at the highest concentration tested. Importantly, CA alone was as effective as RE to inhibit adipogenesis (Fig. 2A). In the presence of COH, that is a derivative of CA, differentiation of 3T3-L1 preadipocytes was also inhibited to an extent comparable to the blockade obtained in the presence of CA (Fig. 2A).

CA treatment inhibited 3T3-L1 adipocyte differentiation in a dose-dependent manner, exhibiting a dramatic inhibition of lipid accumulation at concentrations higher than 5 μg/ml, as shown by Oil Red O staining (Fig. 2B). Further, inhibition of adipocyte differentiation by increasing concentrations of CA was clearly observed by the almost lack of cells stained by LipidTOX (Fig. 2D and E) compare to

Table 1
Effect of CA on 3T3-L1 viability determined by Tripan blue.

Carnosic acid (μg/ml)	Viability (% control)
0	100
1.25	95 ± 5.9
2.50	94 ± 9.1
5.00	91 ± 3.5
10.00	97 ± 6.7
20.00	92 ± 5.7

3T3-L1 preadipocyte differentiated in the absence of CA (Fig. 2C) and also by the decrease in lipids cell content in the presence of increasing concentrations of CA (Fig. 2F). Importantly, when preadipocytes were induced to differentiate for 6 days in the presence of increasing concentrations of CA and cell viability was assessed by MTS assay (Fig. 2G) or Tripan blue (Table 1), no cytotoxic effect of CA was detected at concentrations that inhibited adipogenesis. Moreover, an increment in cell viability was observed in the presence of CA possibly due to its antioxidant activity. Therefore, these results demonstrated that CA is the bioactive compound present in RE responsible for the inhibition of adipocyte differentiation of 3T3-L1 cells.

3.3. Carnosic acid inhibits 3T3-L1 mitotic clonal expansion

It has been reported that 3T3-L1 cells undergo two rounds of mitotic clonal expansion (MCE) when adipogenesis is triggered [10,13]. Thus, we tested whether RE or CA might interfere with 3T3-L1 cells clonal expansion when they were induced to differentiate. Cell proliferation was evaluated by the incorporation of [^{3}H]TdR and the progression through the cell cycle was monitored by changes in the cellular DNA content using FACS analysis. It was observed that when 3T3-L1 cells were induced to differentiate in the presence of RE or CA the incorporation of [^{3}H]TdR was completely abrogated (Fig. 3A, *columns* RE and CA *vs.* C). In addition, when 3T3-L1 cells differentiation was induced for 24 h in the presence of RE or CA, FACS analysis showed that cells did not progress into S phase compared with preadipocytes differentiated in the absence of RE or CA (Fig. 3B). Quantification of cell cycle phases, showed that 1% and 74% of CA and RE-treated cells were in S and G0/G1 phases respectively in marked contrast to the percentages obtained for 3T3-L1 cells induced to differentiate in the absence of RE or CA (59.6% in S phase and 25% in G0/G1) (Fig. 3C). Similar results were obtained after treatment with RE and CA at the same concentrations using 3T3-L1 preadipocytes (data not shown). Therefore, these results show that RE and CA blocked the mitotic clonal expansion arresting the cells in G0/G1 phase.

3.4. Carnosic acid blocks PPARγ and FABP4 gene expression without altering C/EBPβ and C/EBPδ expression

To determine whether RE and CA inhibit adipocyte differentiation by blocking the expression of key transcriptional regulators, we induced the differentiation of 3T3-L1 preadipocytes in the absence or the presence of RE or CA, and examined the mRNA levels of C/EBPβ and C/EBPδ by RT-PCR. We observed no significant changes in C/EBPβ or C/EBPδ mRNA levels (Fig. 4A and B, respectively). In contrast, there was almost no detectable PPARγ and FABP4 mRNAs (Fig. 4C and D, respectively) when 3T3-L1 cells were induced to differentiate in the presence of RE or CA. Taken together, these results demonstrate that the anti-adipogenic effect of RE and CA resulted in the inhibition of *pparγ*, and *fabp4* expression.

3.5. Carnosic acid regulates the differential expression of C/EBPβ forms, LAP and LIP

Next, we investigate whether the inhibitory effect of RE or CA on adipocyte differentiation of 3T3-L1 cells may be consequence of changes in the pattern of protein expression of C/EBPβ, and/or C/EBPδ. When 3T3-L1 preadipocytes were induced to differentiate, C/EBPβ was rapidly increased (Fig. 5A, *lanes 2* and *4 vs. 1*), and from day 4 its expression level decreased (*lanes 6* and *8*), as previously shown [12]. Interestingly, when 3T3-L1 preadipocytes were induced to differentiate in the presence of RE or CA, a significant increase in the expression level of LIP compared to LAP was observed (Fig. 5A, *lane 3 vs. 2*, and *5 vs. 4*), and consequently resulting in a 4 fold increase in the LIP/LAP ratio as shown upon densitometric analysis of the bands (Fig. 5B). The level of C/EBPδ increased rapidly and transiently after induction of differentiation (Fig. 5C, *lane 2 vs. 1*), as previously described [12]; however, RE and CA treatments did not modify its expression pattern (Fig. 5C). Since C/EBPα and PPARγ are both targets of C/EBPβ [19], we next analyzed their expression by western blot. In the presence of RE or CA, no increase in C/EBPα and PPARγ protein levels was observed at days 4 and 6 of adipogenesis

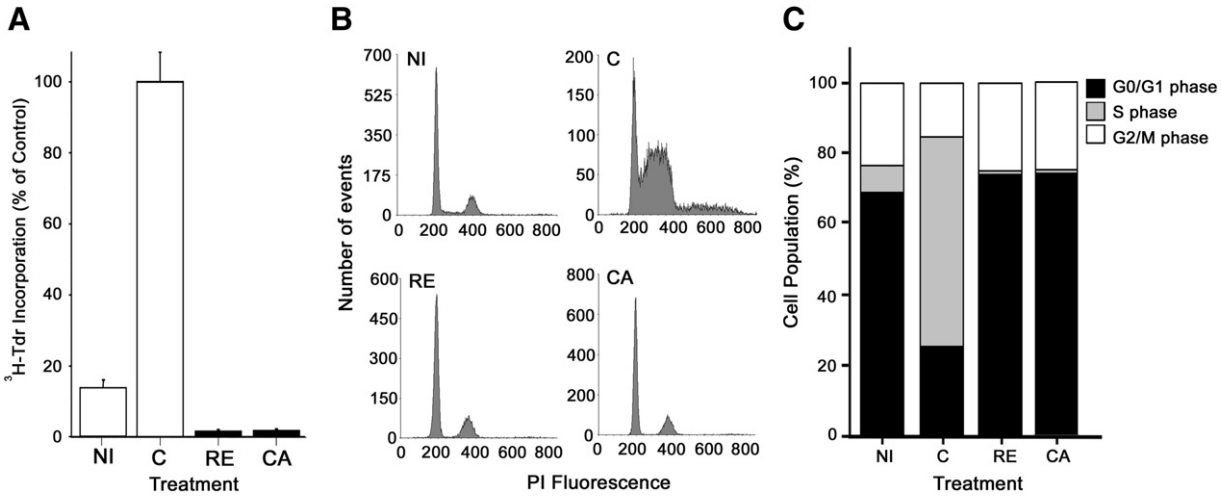

Fig. 3. RE and CA block mitotic clonal expansion when 3T3-L1 preadipocytes are induced to differentiate. Cells were grown in 96-well plates to full confluence and induced to differentiate in the absence (C) or presence of RE 30 μg/ml or CA 7.5 μg/ml for 24 h. NI: Non-differentiated cells. A — [3H]TdR was added at the time of induction and after 24 h its incorporation was measured. [^{3}H]TdR incorporation is shown as the percentage of incorporation with respect to the values obtained in the cells induced to differentiate in the absence of RE or CA. Data are presented as mean ± SE (n = 4). B — FACS analysis of 3 T3-L1 cells 24 h post induction of adipocyte differentiation. C — Quantitative analysis of percentage of cells in the different phases of cell cycle.

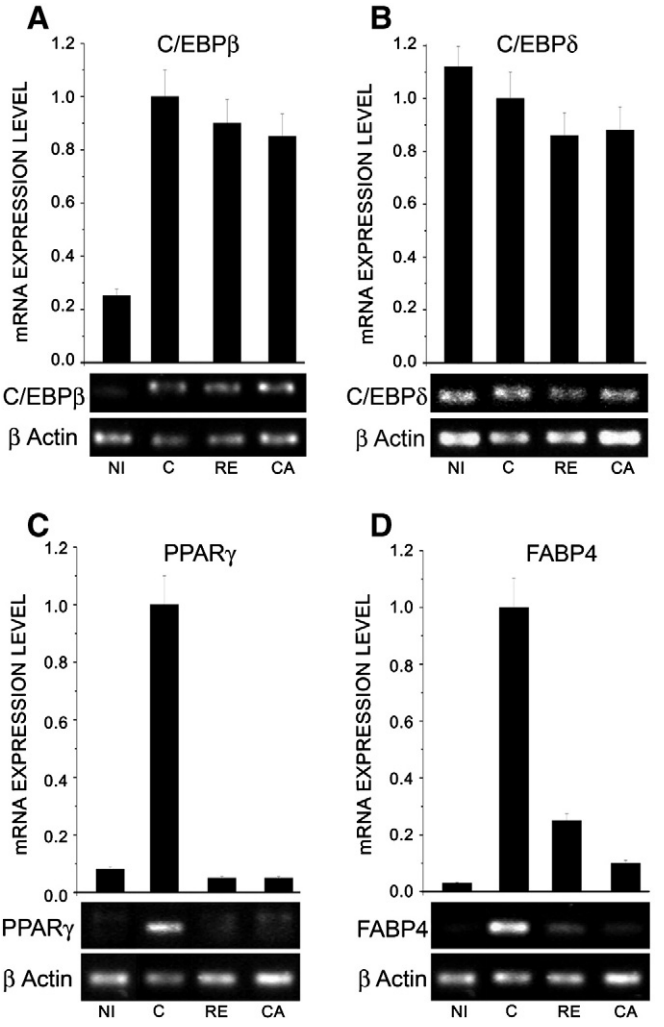

Fig. 4. RE and CA block the expression of late adipocyte related genes. 3T3-L1 cells were treated with RE 30 μg/ml or CA 7.5 μg/ml during the first day of differentiation. RT-PCR results indicate the mRNA levels of C/EBPβ and C/EBPδ (day 1) (panels A and B, respectively) and PPARγ and FABP4 (day 5) (panels C and D, respectively) relative to the level in cells differentiated under no treatment (C). β actin was used as an internal control. The results represent at least three independent experiments and data are presented as mean ± S.E. NI: Non-differentiated cells.

(Fig. 5C, *lanes 9* and *10 vs. 8*, and *lanes* 12 and 13 *vs.* 11, respectively). Densitometric analysis of the bands corroborated this observation (Fig. 5D). Overall these results suggest that RE and CA increases the LIP/LAP ratio that also contributes to the blockade of adipocyte differentiation. The different forms of C/EBPβ are translated from the same mRNA; consequently it is not possible to knock down the expression of LIP without interfering LAP. Thus, to test the possibility that the increase of LIP/LAP ratio may play a role in CA-dependent inhibition of adipogenesis, we analyzed whether ectopic expression of LAP may rescue the capacity of preadipocytes to differentiate in the presence of CA. As expected, overexpression of LAP in 3T3-L1 preadipocytes restored their capacity to differentiate in the presence of CA demonstrated by the presence of cells that accumulate lipid vesicles (Fig. 6 *panel C vs. B*). The percentage of differentiated cells was the same as the obtained when preadipocytes were differentiated in the absence of CA (Fig. 6D, *column* LAP + CA *vs.* C); result that is in marked contrast to the lack of cells differentiated in the presence of CA (*column* CA). In 3T3-L1 preadipocytes overexpressing LAP and differentiated in the presence of CA (LAP + CA), the level of PPARγ increased similarly as in cells differentiated in the absence of CA (LAP) (Fig. 6E, *lanes* 5–6 *vs.* 2–3). Notably, PPARγ expression is similar to the level observed in cells transfected with empty vector (EV) (Fig. 6E, *lane* 5 *vs.* 8), demonstrating that adipocyte markers are properly expressed. In contrast and as expected, when adipogenesis is triggered in cells transfected with EV in the presence of CA, PPARγ expression is blocked (Fig. 6E, *lane 9 vs. 8*). Ectopic expression of LAP also normalizes the level of expression of C/EBPα when cells are induced to differentiate in the presence of CA (data not shown). Taken together these results demonstrate that CA blocks adipogenesis, in part, by increasing the LIP/LAP ratio that ultimately may affect the expression of other adipogenic markers such as PPARγ and C/EBPα.

3.6. Carnosic acid promoted changes in the subnuclear distribution of C/EBPβ

C/EBPβ concentrates in pericentromeric heterochromatin co-localizing with HP1α when 3T3-L1 preadipocytes are induced to differentiate [15]. To determine whether CA treatment may affect the subnuclear distribution of C/EBPβ, we analyzed by IIF and confocal microscopy its localization in 3T3-L1 cells 24 h after induction of differentiation. As shown in Fig. 7, C/EBPβ was detected diffusely distributed throughout the nucleus of 3T3-L1 cells, and in foci (*panel A*) that correspond to pericentromeric heterochromatin intensely stained by DAPI (*panel C*), where co-localized with HP1α (*panel B* and merged in *panel D*), as previously shown [14,15]. In contrast, when 3T3-L1 preadipocytes were induced to differentiate in the presence of CA, C/EBPβ was detected in tiny speckles throughout the nucleus (Fig. 7, *panel E*). C/EBPβ was almost absent from chromocenters intensely stained by DAPI and enriched in HP1α (Fig. 7, *panels F, G,* and *H*). Thus, subnuclear delocalization of C/EBPβ induced by CA treatment may also contribute to inhibition of adipocyte differentiation.

4. Discussion

In the present study we show for the first time that carnosic acid, the main bioactive compound of *R. officinalis* extracts, exerts its anti-adipogenic effect by a complex mechanism. We found that CA: 1 — blocks mitotic clonal expansion (MCE), 2 — alters the ratio of C/EBPβ forms, 3 — promotes the subnuclear delocalization of C/EBPβ, and 4 — inhibits the expression of the C/EBPα and PPARγ, key regulators not only of differentiation but also the maintenance of the adipocyte phenotype (Fig. 8). Thus, the inhibitory effect of CA on adipocyte differentiation is multifactorial and takes place at early stages of the adipogenic program.

CA is very effective in blocking the adipocyte differentiation of 3T3-L1 preadipocytes (Fig. 2) [38,42]. However, how this diterpene exerts its anti-adipogenic effect is poorly understood. It was reported that CA inhibition of 3T3-L1 cells differentiation seemed to be mediated by activation of the antioxidant-response element and induction of phase II enzymes leading to an increase in of the intracellular level of GSH (Fig. 8) [38]. ROS production is markedly increased during differentiation of 3T3-L1 preadipocytes, suggesting that ROS production increases in parallel with fat accumulation in adipocytes [43]. Thus, it was proposed that one possibility of CA-dependent inhibition of adipogenesis is the increase of intracellular level of GSH neutralizing the increase in ROS production during adipogenesis [38]. In the present study, we provide evidence that the mechanism of CA action is even more complex.

It is well known that when 3T3-L1 preadipocytes are induced to differentiate, the cells initiate several rounds of mitotic clonal expansion, and then become quiescent when the coordinated transcription of adipogenic genes is initiated [44]. Here, we show that CA blocks the entrance of 3T3-L1 preadipocytes into S-phase and their proliferation (Fig. 3), being this one of the mechanisms through which CA blocks the progression of the cell differentiation program. This effect of CA blocking the mitotic clonal expansion and differentiation of 3T3-L1 preadipocytes is similar to the antiadipogenic action of roscovitine,

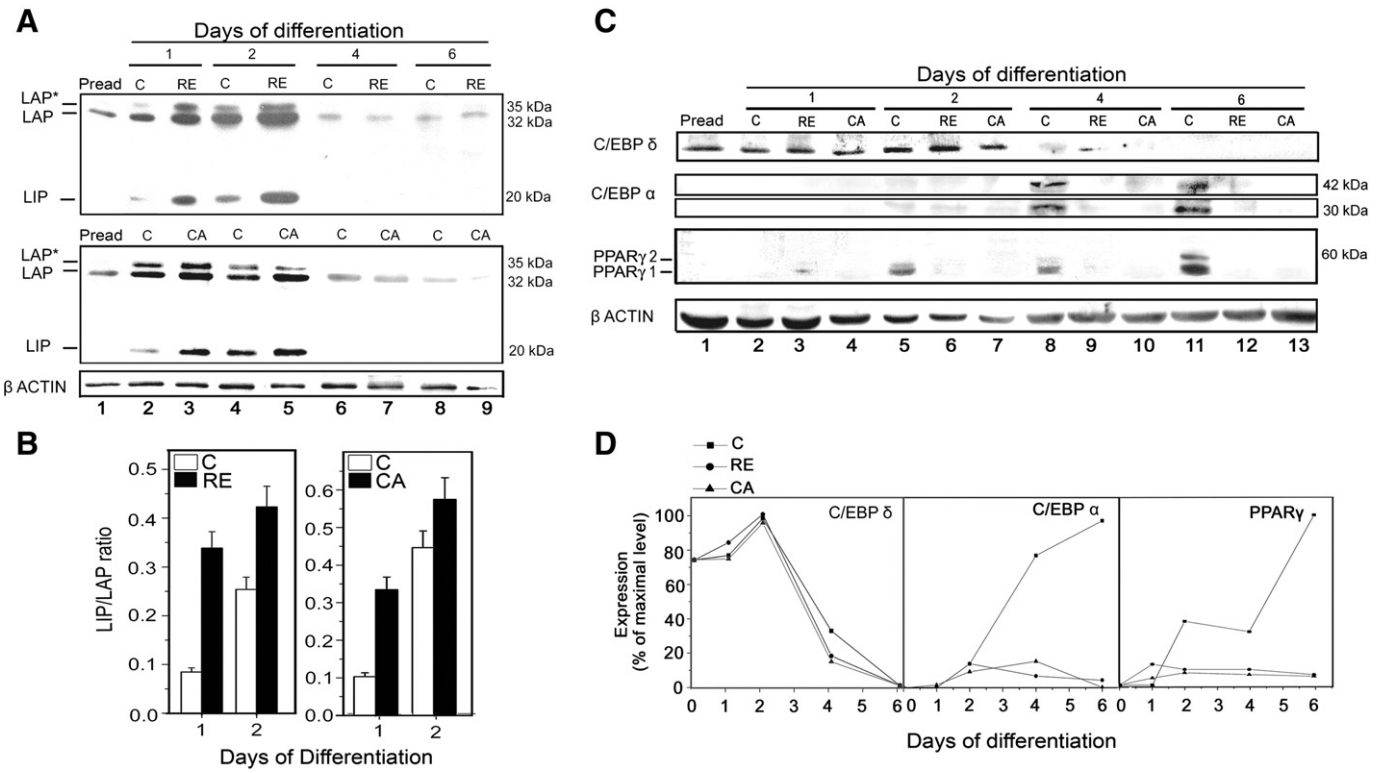

Fig. 5. RE and CA increase p20-C/EBPβ and inhibit the expression of C/EBPα and PPARγ. A — Representative immunoblot analyses of C/EBPβ performed with the lysates of 3T3-L1 cells that were differentiated in the absence (C) or in the presence of 30 μg/ml RE or 7.5 μg/ml CA for the indicated times. β actin was used as an internal control. B — Densitometric analysis of the western blots. The LIP/LAP ratio was calculated at day 1 and 2 post-induction of adipogenesis in the absence or presence of RE or CA. Data represent the mean ± S.E of two independent experiments ($p < 0.01$). C — Immunoblot analysis of C/EBPδ, C/EBPα and PPARγ as indicated in (A). β actin was used as an internal control. D — Densitometric analysis of the western blots during the course of differentiation. Values, mean of at least two independent experiments, are given as a percentage of the maximal expression of each factor.

a potent and specific cdk inhibitor [41]. We found that CA induces cell cycle arrest of 3T3-L1 cells predominantly at G2/M phase. It has been previously reported that CA treatment induces cell cycle arrest at G2/M phase of human colonic adenocarcinoma Caco-2 cells by causing a reduction in cyclin A levels without affecting cyclin B1 [45]. Therefore, CA may block MCE required for adipogenesis to proceed

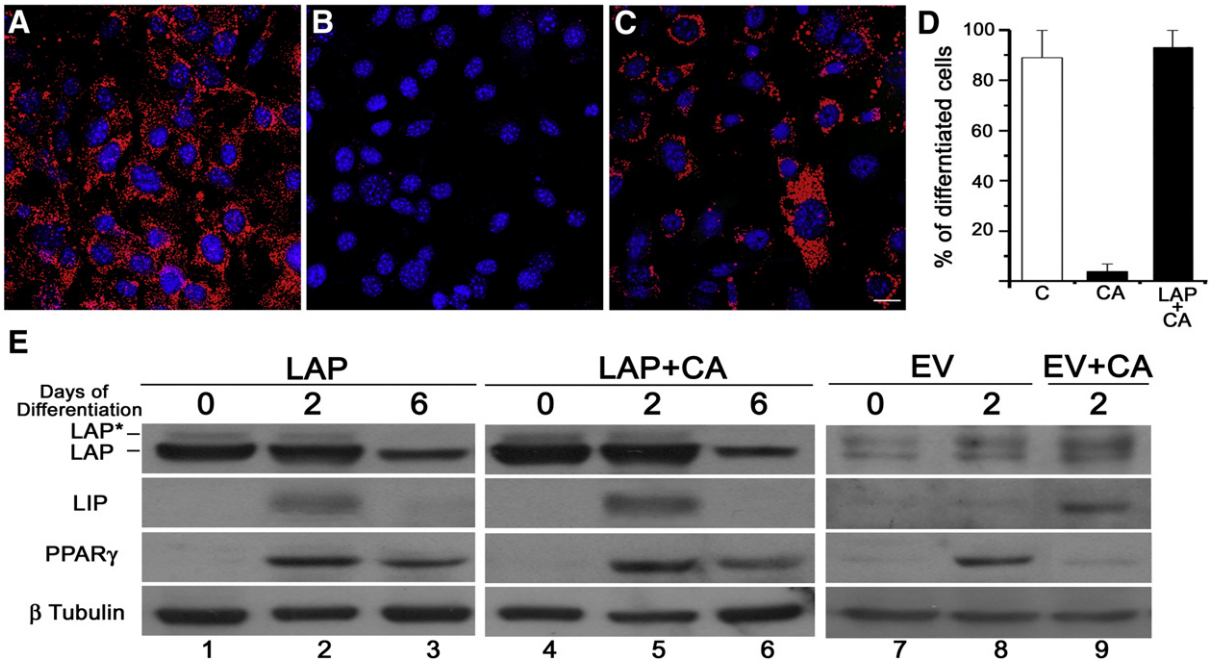

Fig. 6. 3T3-L1 cells transfected with empty vector (panels A and B) or CMV-LAP (panel C) were induced to differentiate in the absence (A) or presence of CA (5 μg/ml) (B and C) for 6 days, and lipid vesicles were stained with LipidTOX. Nuclei were counterstained with DAPI. Scale bar: 10 μm. D — Percentage of differentiated cells were evaluated from three independent experiments. Data are presented as means ± SE. E — 3T3-L1 cells were transfected with 5 μg of LAP or empty vector (EV), induced to differentiate in the absence or presence of CA (5 μg/ml), and cell lysates were obtained at the indicated time points. Proteins were solved by SDS-PAGE and analyzed by immunoblotting. β tubulin was used as an internal control. Results are representative of five independent experiments.

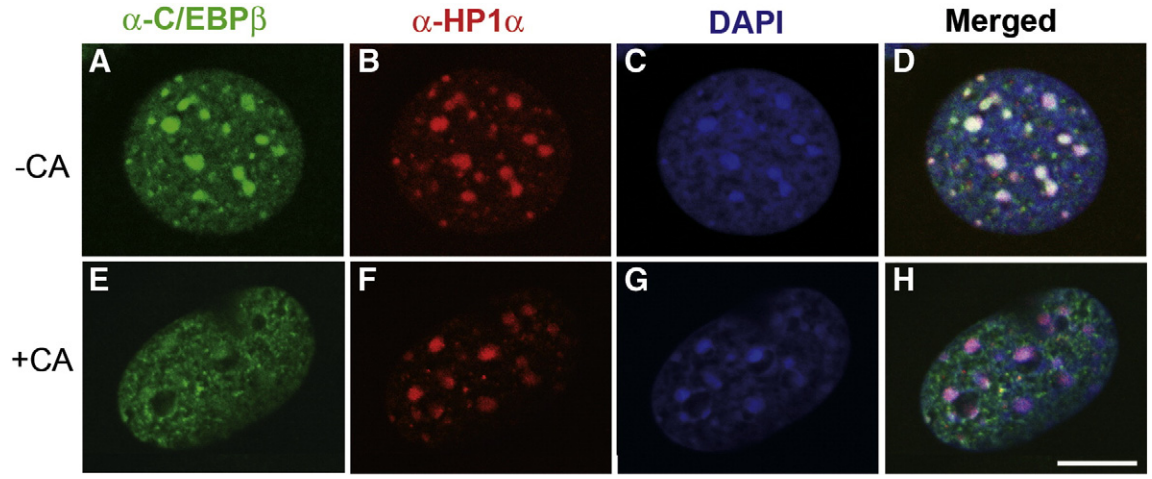

Fig. 7. Subnuclear localization of C/EBPβ is altered by CA treatment. Cell monolayers were grown on coverslips and adipocyte differentiation was induced in the absence or presence of CA 7.5 μg/ml for 24 h. Cells were fixed and subjected to IIF as described in Materials and Methods using anti-C/EBPβ, anti-HP1α, and DAPI for nuclei counterstaining. The right panel shows the merged of the confocal images. Results are representative of at least three independent experiments. Scale bar: 5 μm.

possibly by causing a decrease in cyclin A; however further studies are required to address the mechanism by which CA influences cyclin levels.

Interestingly, we also found that in preadipocytes differentiated in the presence of CA, C/EBPβ mRNA level is normal (Fig. 4) however we detected marked changes in the level of expression of the different C/EBPβ forms. Particularly, in the presence of RE and CA an increase in LIP/LAP ratio was observed (Fig. 5). Since LAP and LIP have antagonistic functions in the transcriptional control of C/EBPβ target genes, it is reasonable to expect that the biological outcome depends on the precise control of LIP/LAP ratio. LIP inhibits the expression of C/EBPβ target genes, for example it represses the activity of the albumin D promoter [16], as well as interferes with of growth hormone dependent c-*fos* promoter activity [17,46]. In the case of 3T3-L1 preadipocytes induced to differentiate, the presence of RE or CA cause a marked increase in the expression of LIP that may deregulate the expression of C/EBP target genes and consequently promote a blockade of adipogenesis. These results are in agreement with previous reports showing that ectopic expression of LIP blocks adipogenesis of 3T3-L1 preadipocytes [20,47,48]. Importantly, ectopic expression of LAP rescues the capacity of 3T3-L1 preadipocytes to differentiate in the presence of CA (Fig. 6), supporting the notion that the increase in the LIP/LAP ratio promoted by CA is one of the causes of the inhibition of adipogenesis.

In addition, several reports have shown that C/EBPβ contributes to growth arrest but in a highly specific cell context. It was reported that

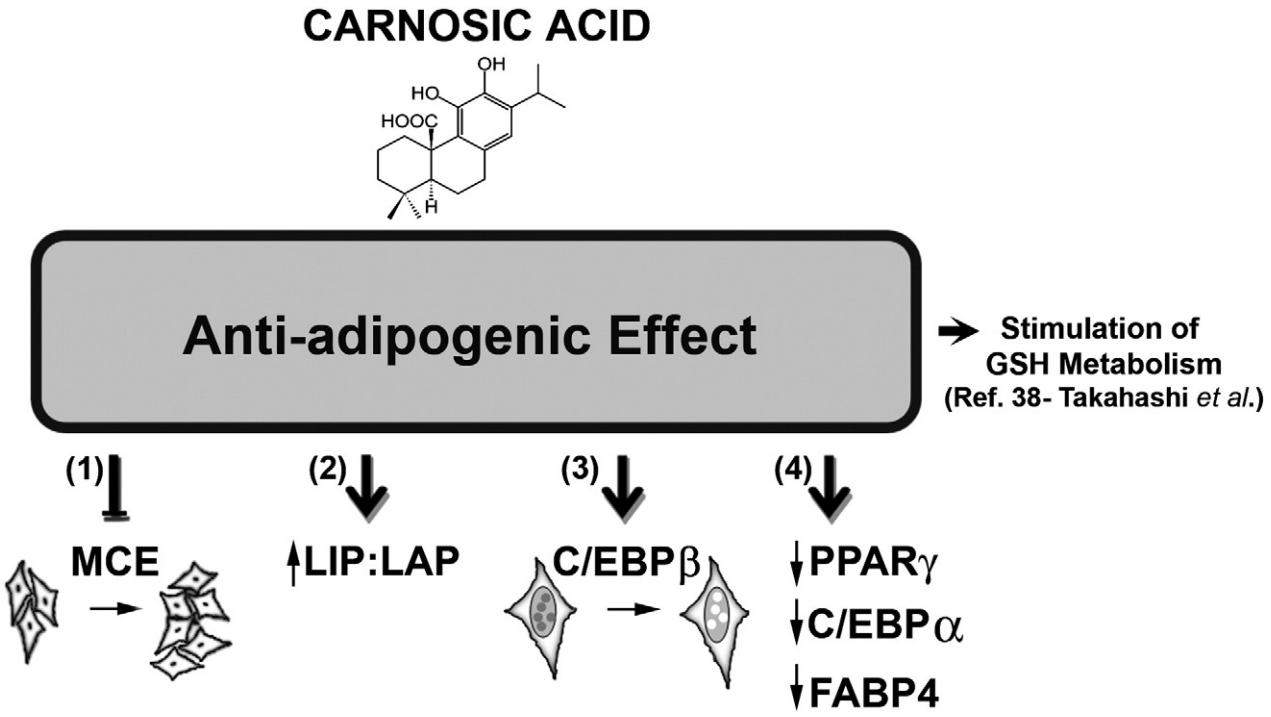

Fig. 8. CA exerts its anti-adipogenic effect at multiple levels. CA inhibits the early event of mitotic clonal expansion (MCE) (1), alters the ratio of the different C/EBPβ forms (2), induces the subnuclear delocalization of C/EBPβ (3) and blocks the expression of the adipogenic markers PPARγ, C/EBPα and FABP4 (4).

MEFs (Mouse Embryonic Fibroblasts) obtained from mice C/EBPβ −/− neither undergo mitotic clonal expansion nor differentiate into adipocytes [49]. Ectopic expression of LAP but not of LIP rescued the capacity of MEFs C/EBPβ −/− to undergo mitotic clonal expansion, and differentiate [49]. Therefore, the increased level of LIP observed in 3T3-L1 cells induced to differentiate in the presence of RE or CA may also contribute to the inhibition of the mitotic clonal expansion (Fig. 3), interfering with the progress of the adipogenic program.

The expression level of the different forms of C/EBPβ changes during different developmental processes as well as in malignant transformation [50–53]. A single C/EBPβ mRNA produces three forms through alternative translation from three in frame AUG codons. Besides the integrity of evolutionary conserved uORF in C/EBPβ mRNA, signal transduction pathways that regulate the function of the translation initiation factors eIF-2 and eIF-4E play a role in controlling the ratio of C/EBPβ forms [48]. Future experiments will elucidate whether RE and CA may regulate eIF activity, and in this way possibly control LIP/LAP ratio. In addition, CUG triplet repeat-binding protein-1 (CUGBP-1), a RNA binding protein that regulates translation of proteins, was detected bound to C/EBPβ mRNA in the liver of old animals leading to an increase in LIP/LAP ratio [54]. In this regard, an increase in LIP/LAP ratio dependent on CUGBP-1 explains the decreased capacity of preadipocytes obtained from old rats to differentiate [55]. Then, it is tempting to speculate that the increase in LIP/LAP ratio induced by RE and the bioactive component CA may dependent on CUGBP-1, possibility that needs to be explored.

The expression of PPARγ, a nuclear hormone receptor that is critical for adipogenesis and insulin sensitivity, is blocked when differentiation of 3T3-L1 preadipocytes is induced in the presence of CA (Figs. 4 and 5). Ligands for PPARγ include synthetic antidiabetic agents as thiazolidinediones and a number of natural substances [30]. It has been reported that PPARγ ligands are transiently produced, when 3T3-L1 cells are induced to differentiate [56]. This ligand is required for proper adipogenesis, since addition of PD0668235, a PPARγ antagonist, effectively blocked adipocyte differentiation when added at very early time points (days 1–2) [56]. It has been proposed that C/EBPβ, and the effectors that control its expression (i.e. SREBP-1 [57]), may possibly regulate the production of PPARγ ligands based on the observation that 3T3-L1 cells overexpressing LIP require an exogenous PPARγ ligand to overcome the blockade in their differentiation [20]. Thus, it is tempting to speculate that the increase in LIP/LAP ratio promoted by RE and CA may also interfere with the production of PPARγ agonist and in this way may also contribute to the blockade of adipogenesis.

It is accepted that the nucleus is highly organized in different compartments, however how nuclear organization relates to genomic function is not fully understood [58,59]. Nuclear compartments are not limited by membranes, are very dynamic and depend on the transcriptional status of the cell. We have recently shown that in 3T3-L1 cells the different forms of C/EBPβ are localized in different nuclear compartments, LAP homodimers are detected in euchromatic and heterochromatic domains while LIP homodimers are exclusively detected in heterochromatin [15]. This subnuclear distribution mirrors the site where LAP and LIP interact with the non-histone protein HP1α that restrains C/EBPβ transcriptional capacity [15]. The importance of proper C/EBPβ subnuclear distribution was further demonstrated by the fact that 3T3-L1 preadipocytes differentiation was blocked by Trichostatin A, an inhibitor of deacetilases that causes subnuclear delocalization of C/EBPβ [15]. Therefore, the lack of proper subnuclear distribution of C/EBPβ promoted by CA (Fig. 7) is another level at which CA interferes with the progress of the adipogenic program by possibly contributing to deregulation of the expression of C/EBPβ target genes, i.e. *c/ebpa* and *pparg*.

Obesity is a serious health problem that significantly reduces the average life expectancy due to the risks of cardiovascular disease, type 2 diabetes, arthritis, and some types of cancers. The prevalence of obesity has dramatically increased mainly due to high-fat diet and a sedentary life style; however reducing dietary fat combined with increased physical exercise was shown to failed in the long-term maintenance of weight loss in obese patients [60]. Treatment of obesity is a lifelong task, and even a modest weight loss of 5–15% significantly reduces obesity-related health risks, for these reasons it is necessary to characterize new drugs that may be useful for its treatment without undesirable long-term side effects. Dietary bioactives derived from natural products that inhibited differentiation of preadipocytes or adipogenic precursors may provide suitable therapeutic approach for the treatment of obesity [61–64]. In particular, it was reported that retinoids inhibited the differentiation of adipocytes in cultured cells through the suppression of PPARγ2 only when they were added within 24 to 48 h of the exposure of preadipocytes to differentiating conditions [65,66]. The actual treatments for weight loss and weight control of obese patients have failed in the long-term maintenance of weight loss [67]. Nowadays, several natural compounds used for the treatment of obesity included metabolic stimulants, appetite suppressants, lipid metabolism and adipocyte-specific effects. In this sense, our data demonstrate that CA, and RE are effective in the inhibition of preadipocyte differentiation as well as the proliferation of undifferentiated preadipocytes. Therefore, CA that interferes with the process of adipocyte differentiation at multiple levels (Fig. 8) may be valuable dietary tool not only for the treatment of obesity but also for its prevention.

Acknowledgments

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