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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].