Carnosic Acid Inhibits Lipid Accumulation in 3T3-L1 Adipocytes Through Attenuation of Fatty Acid Desaturation

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Background: Excess body fat accumulation contributes to the development of metabolic disorders that can cause adverse health effects. Carnosic acid (CA), a major bioactive component of rosemary (Rosmarinus officinalis), has been suggested to possess anti-adipogenic properties. The present study was conducted to elucidate the mechanism underlying the anti-adipogenic effects of CA.

Methods: 3T3-L1 pre-adipocytes were treated with CA (0.1, 1, and 10 μM) from day 0 to day 8 of differentiation. On day 8, biochemical markers of lipid accumulation and the degree of fatty acid desaturation were measured.

Results: Oil Red O staining results, triglyceride (TG) accumulation, and glycerol 3-phosphate dehydrogenase activity suggested that CA significantly inhibited lipid accumulation in 3T3-L1 adipocytes. CA significantly decreased mRNA expression of peroxisome proliferator-activated receptor-γ, sterol regulatory element-binding protein 1, and CCAAT/enhancer binding protein-α in a dose-dependent manner. Moreover, it decreased the ratio of both C16:1/C16:0 and C18:1/C18:0, with reduced expression of stearoyl CoA desaturase 1 mRNA and protein.

Conclusions: These results suggest that CA efficiently suppressed adipogenesis in 3T3-L1 adipocytes and its action, at least in part, is associated with the downregulation of adipogenesis-related genes and the fatty acid composition of TG accumulated in adipocytes. (J Cancer Prev 2015;20:41-49)

Key Words: 3T3-L1 adipocytes, Carnosic acid, Differentiation, Fatty acid

INTRODUCTION

Obesity is characterized by excess accumulation of fat in the adipose tissue. Adipocytes are highly specialized cells that play a critical role in maintaining cellular energy homeostasis by storing excess energy as triglycerides (TG). However, it has been well documented that enlarged adipocytes contribute to dysregulation of lipid metabolism, thereby contributing to insulin resistance and chronic inflammation.¹² Therefore, obesity-associated metabolic disorders may be prevented by decreasing the number of adipocytes and by suppressing lipid accumulation within adipocytes. Peroxisome proliferator-activated receptor-γ (PPARγ), sterol regulatory element-binding protein1 (SREBP1), and CCAAT/enhancer binding protein-α (C/EBPα) are key transcriptional factors involved in lipid accumulation in differentiating 3T3-L1 adipocytes. These genes promote the expression of adipogenesis-related genes.⁴

Recent studies have suggested that increased desaturation of stearate is also associated with adipogenesis. In obese humans, stearoyl-CoA desaturase 1 (SCD1), which catalyzes the conversion of palmitate (C16:0) and stearate (C18:0) to palmitoleate (C16:1) and oleate (C18:1), respectively, is shown to be upregulated.⁵ In mice, adipose-specific deletion of SCD1 induced upregulation of GLUT1, which plays a critical role in glucose utilization.⁶ Moreover, high ratios of monounsaturated to saturated fatty acids (SFA) (C16:1/C16:0 and 18:1/C18:0) in adipose tissue TG have been used as an index of obesity-related hyperlipidemia.⁷ Adipose tissue stearic acid (C18:0) content was positively related...
to insulin sensitivity, but negatively associated with adipocyte size. These findings suggest that increased adipocyte desaturation indexes (C16:1/C16:0 and C18:1/C18:0) may be closely related to lipid accumulation in adipocytes and obesity-related metabolic complications.

Carnosic acid (CA) is a major phenolic compound of rosemary (Rosemarinus officinalis L) leaves. A previous study indicated that CA inhibited mouse 3T3-L1 adipocyte differentiation and hepatic TG accumulation in ob/ob mice; however, the molecular mechanisms underlying these effects have not been explored. In a previous study, a 0.02% CA diet significantly decreased the hepatic content of oleic acid (C18:1), in an animal model of high-fat diet-induced hepatic steatosis. In this study, we examined the effect of CA on lipid accumulation in differentiating 3T3-L1 adipocytes. Additionally, the molecular mechanisms involved in the regulation of lipid accumulation were investigated with special emphasis on the ratio between C16:1/C16:0 and C18:1/C18:0 as an important regulatory factor of lipid accumulation.

**MATERIALS AND METHODS**

1. **Materials**

CA was obtained from GBLS Corporation Ltd. (Hwasung, Korea). Dulbecco’s modified Eagle’s medium (DMEM), bovine calf serum (BFS), antibiotics-antimycotics solution, and trypsin-ethylene-diaminetetraacetic acid (EDTA) were purchased from Gibco Co. (Grand Island, NY, USA). Dexamethasone (DEX), 3-isobutyl-1-methylxanthine (IBMX), insulin, and Oil Red O were purchased from Sigma-Aldrich (St. Louis, Mo, USA). Fatty acid methyl ester (FAME) standards were purchased from Supelco (Bellefonte, PA, USA). All solvents and reagents used were of analytical grade. Boron trifluoride methanol solution (14% BF3), sodium hydroxide, and sodium chloride were purchased from Sigma-Aldrich. Chloroform, normal hexane, and methanol were purchased from J. T. Baker (Philipsburg, N, USA).

2. **3T3-L1 cell differentiation**

Mouse 3T3-L1 fibroblast cells, obtained from Korean Cell Line Bank (KCLB, Seoul, Korea), were cultured in six-well tissue culture plates. The cells were maintained in DMEM supplemented with 10% heat-inactivated BCS, 100 units/mL penicillin, and 100 μg/mL streptomycin. One day after confluency (designated as day 0), cell differentiation was induced by adipogenic agents (0.5 mM IBMX, 2 μM DEX, and 0.7 μM insulin), which were added to the culture medium. CA (0.1, 1, and 10 μM) was added to the culture medium at the initiation of differentiation and with every subsequent medium change for 8 days.

3. **Cell viability (MTT assay)**

After the indicated treatment, cells were washed with PBS once, the cells were incubated with 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution for 4 hours at 37°C. The purple formazan crystals were then dissolved with dimethyl sulfoxide (DMSO). After incubation for 15 minutes, absorbance was measured at 540 nm using a microplate reader (Molecular Devices, CA, USA).

4. **Oil Red O staining**

After 8 days of adipogenic differentiation from the point of confluency (day 0), cultured preadipocytes were fixed with 3.7% formalin for 1 hour, and stained with Oil Red O (three parts 0.5% Oil Red O dye in isopropanol to two parts water) for 40 minutes. The stained cells were washed three times with water. Oil Red O was then eluted with DMSO and quantified by measuring the optical density (OD) at 490 nm.

5. **Triglyceride accumulation**

3T3-L1 adipocytes were harvested 8 days after the initiation of differentiation. The cells were washed twice with cold phosphate-buffered saline (PBS), collected, and lysed in lysis buffer (1% Triton X-100 in PBS). The total TG content in the cells was determined using a TG kit (Wako Chemical, Osaka, Japan). The protein concentration was determined using BioRed DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Inhibition was expressed as the percentage (%) decrease in TG content against a control (0%).

6. **Glycerol 3-phosphate dehydrogenase activity**

3T3-L1 adipocytes were harvested 8 days after the initiation of differentiation. The cells were washed twice with cold PBS, scraped into 200 μL of an enzyme extract buffer (provided with the kit), and sonicated. Glycerol 3-phosphate dehydrogenase (GPDH) activity was determined using a GPDH kit (Takara Bio, Shiga, Japan) according to the manufacturer’s protocol.

7. **RNA extraction and real-time polymerase chain reaction**

Total RNA was isolated from the 3T3-L1 cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s recommendations. Real-time quantitative polymerase chain reaction (PCR) was performed with an SYBR Green™ kit
(Quantitect™ SYBR Green PCR. QIAGEN, Valencia, CA, USA). The cycling conditions were 15 minutes at 95°C, 40 cycles of 15 seconds at 94°C, 30 seconds at 51°C, and 30 seconds at 72°C. Relative quantification was performed using the Delta-Delta method. The primer sequences of the genes examined are shown in Table 1.

8. Fatty acid analyses

Cellular lipids were extracted using a previously reported procedure. The fatty acid composition of adipocytes was analyzed according to the method described by Kramer et al. The methyl ester of total fatty acid was obtained using the reaction in a Techno DB-3D heating block (Barloworld Scientific US Ltd., Burlington, NJ, USA), with BF3 as a catalyst. FAMEs were determined using a gas chromatograph (Agilent 6890N GC; Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionization detector (FID) and an HP-FFAP capillary column (30 m × 0.32 mm × 0.25 μm film thickness; Agilent Technologies). The injector temperature was 230°C with a split ratio of 10:1 and the FID temperature was 250°C. The oven temperature program involved 100°C for 1 minute, 100°C to 230°C at a rate of 3°C per minute, and 230°C for 10 minutes. Helium was used as the carrier gas with a constant flow rate of 1.0 mL/min and the injection volume was 1 μL. Hydrogen flow to the detector was 40 mL/min, the air flow was 300 mL/min, and the helium make-up gas flow rate was 20 mL/min.

9. Western blotting

For the detection of SCD1, the total protein from the 3T3-L1 cells was extracted using a protein extraction buffer (50 mM NaF, 50 mM Tris HCl, 5 mM Na2VO4, 1% NP-40, 1 mM EDTA). For each sample, 40 μg of protein was denatured in Laemmli Buffer for 5 minutes at 95°C. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. After blocking with a blocking buffer (5% skim milk in PBS), the membranes were incubated with the SCD1 antibody (Cell Signaling Technology, Inc., Danvers, MA, USA). The proteins were visualized using enhanced chemiluminescence with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G. Blots were scanned and analyzed using a multiple image analyzer and the Quantity One program (Bio-Rad Laboratories).

10. Statistical analysis

Statistical analysis was performed using the SAS package release 9.3 (SAS Institute Inc., Cary, NC, USA). Data were expressed as means ± standard deviation. Data were analyzed using one-way analysis of variance followed by Duncan’s multiple range tests. A P-value of <0.05 was considered statistically significant.

RESULTS

1. Effects of carnosic acid on 3T3-L1 adipocytes viability

3T3-L1 adipocytes viability was assessed by MTT assay. As shown Table 2, CA in the concentrations used did not affect viability of 3T3-L1 cells.

2. Effects of carnosic acid on lipid accumulation and glycerol 3-phosphate dehydrogenase activity in 3T3-L1 adipocytes

The effects of CA on the accumulation of intracellular lipid droplets in 3T3-L1 adipocytes are shown in Figure 1. Compared to the OD value of control cells, the OD values of Oil Red O in the cells treated with 1 μM and 10 μM CA significantly decreased by 19.1% and 33.4%, respectively, indicating reduced intracellular lipid accumulation (P < 0.05; Fig. 1B). Cellular TG was quantified, and adipocytes treated with CA at 1 μM and 10 μM showed

Table 1. Gene-specific primers used for real-time polymerase chain reaction

| Gene   | Direction | Sequence                               |
|--------|-----------|----------------------------------------|
| PPARγ  | Forward   | 5'-TTT TCA AGG GTG CCA GTT TC-3'       |
|        | Reverse   | 5'-AAT CCT TGG CCC TCT GAG AT-3'       |
| SREBP1 | Forward   | 5'-TGT TGG CAT CCT GCT ATC TG-3'       |
|        | Reverse   | 5'-AGG GAA AGC TTT GGG GTC TA-3'       |
| C/EBPα | Forward   | 5'-TCA CAA CAG GCC AGG TTT CC-3'       |
|        | Reverse   | 5'-GCC TGG CGA CAT ACA GTA CA-3'       |
| SCD1   | Forward   | 5'-GGA TCA GGT TTT GTG GTG CT-3'       |
|        | Reverse   | 5'-TTG TGG CCC ATA AAG TCC CC-3'       |
| β-Actin| Forward   | 5'-CCA CAG CTG AGA GGG AAA TC-3'       |
|        | Reverse   | 5'-AGG GAA GGG TGG AAA AGA GC-3'       |

PPARγ: peroxisome proliferator-activated receptor-γ. SREBP1, sterol regulatory element-binding protein 1; C/EBPα, CCAAT/enhancer binding protein-α. SCD1, stearoyl CoA desaturase 1.

Table 2. Effect of carnosic acid on 3T3-L1 viability

| Carnosic acid (mM) | Viability (% control) |
|--------------------|-----------------------|
| 0                  | 100                   |
| 0.1                | 98.7 ± 3.8            |
| 1.0                | 96.4 ± 8.9            |
| 10.0               | 95.9 ± 4.2            |

Values are presented as number only or mean ± SD.
Figure 1. Effects of carnosic acid (CA) on adipocyte differentiation, triglyceride (TG) content, and glycerol 3-phosphate dehydrogenase (GPDH) activity in 3T3-L1 cells. Preadipocytes were grown to confluency (day 0) and induced to differentiate with an optimized adipocyte differentiation medium in the presence of CA (0.1, 1, and 10 μM) throughout differentiation. (A) Morphological observation and Oil Red O staining of 3T3-L1 cells. After differentiation (day 8), the cells were fixed and stained with Oil Red O and photographed using a microscope (×200). (B) Quantification of intracellular Oil Red O staining using spectrophotometry. (C) Quantification of TG content in 3T3-L1 cells. (D) GPDH activity in 3T3-L1 cells. Data are expressed as mean ± standard deviation (n = 4). a,b,cMeans with the different letters are significantly different from each other (P < 0.05) using Duncan’s multiple-range test.

reduced TG content by 15.5% and 39.8%, respectively, compared to that in the control cells (P < 0.05; Fig. 1C). The activity of cytosolic GPDH, a key regulatory enzyme involved in TG synthesis was also significantly decreased in CA-treated adipocytes compared to that in the control cells (P < 0.05; Fig. 1D).

3. Effect of carnosic acid on adipogenesis-related gene expression in 3T3-L1 adipocytes

To gain a better understanding of the molecular mechanism(s) underlying the anti-adipogenic effects of CA, we examined the expression levels of key transcriptional factors. Consistent with the decreases in TG accumulation and GPDH activity, CA significantly decreased the mRNA expression of PPARγ, C/EBPα, and SREBP1 by 63.54%, 51.05%, and 47.91%, respectively, compared to the corresponding values in the control cells (P < 0.05; Fig. 2).

4. Effect of carnosic acid on the ratio of monounsaturated fatty acids to saturated fatty acids in 3T3-L1 adipocytes

Adipocytes treated with 1 and 10 μM CA had a significantly lower monounsaturated fatty acids (MUFA) to SFA ratio compared to that in the control cells. Treatment with 10 μM CA significantly decreased the ratios of C16:1/C16:0 and C18:1/C18:0 by 42.86% and 41.25% of the control cells, respectively (P < 0.05; Fig. 3).
Figure 2. Effect of carnosic acid (CA) on mRNA expression levels of peroxisome proliferator-activated receptor-γ (PPARγ) (A), CCAAT/enhancer binding protein-α (C/EBPα) (B), and sterol regulatory element-binding protein 1 (SREBP1) (C) in 3T3-L1 adipocytes. Preadipocytes were grown to confluency (day 0) and induced to differentiate with an optimized adipocyte differentiation medium in the presence of CA (0.1, 1, and 10 μM) throughout differentiation. After differentiation (day 8), real-time polymerase chain reaction (RT-PCR) was performed using specific primer pairs for PPARγ, C/EBPα, and SREBP1. The relative RT-PCR values were corrected to β-actin expression levels and normalized with respect to the control. Data are expressed as mean ± standard deviation (n = 4). a, b, c Means with the different letters at each sample are significantly different from each other (P < 0.05) using Duncan’s multiple-range test.

Figure 3. Effect of carnosic acid (CA) on the ratio of palmitoleate : palmitate (C16:1/C16:0) (A) and oleate : stearate (C18:1/C18:0) (B) in 3T3-L1 adipocytes. Preadipocytes were grown to confluency (day 0) and induced to differentiate with an optimized adipocyte differentiation medium in the presence of CA (0.1, 1, and 10 μM) throughout differentiation. After differentiation (day 8), cellular lipids were extracted and the fatty acid composition was analyzed using gas chromatography. Data are expressed as mean ± standard deviation (n = 4). a, b, c Means with the different letters at each sample are significantly different from each other (P < 0.05) using Duncan’s multiple-range test.
5. Effect of carnosic acid on the mRNA and protein levels of stearoyl-CoA desaturase 1 in 3T3-L1 adipocytes

We measured the mRNA and protein levels of SCD1, which converts SFA to MUFA. As shown in Figure 4, CA led to a reduction of both the mRNA and protein expression levels of SCD1, suggesting that the decreased MUFA/SFA ratio is partly mediated through decreased SCD1 expression.

**DISCUSSION**

CA is a major biologically active component of rosemary and several studies have examined its biological activities including the anti-obesity, anti-inflammatory, anti-cancer, anti-viral, anti-oxidant properties. Rosemary extract has also been shown to suppress weight gain and hepatic TG accumulation in mice fed a high-fat diet. The observed weight reduction by CA-enriched rosemary extract is mediated by the suppression of gastric lipase and alteration of gut microbiota composition. Anti-adipogenic effect of CA is also known to be due to stimulation of glutathione metabolism in 3T3-L1 adipocytes. However, current evidence is insufficient to fully explain the anti-adipogenic activity of CA.

In this study, CA was found to suppress lipid accumulation in differentiating 3T3-L1 adipocytes. Oil Red O staining and cellular TG content are regarded as primary indicators of lipid accumulation in differentiating or differentiated adipocytes. Several glycerolipid-synthesizing lipogenic enzymes are highly induced during the differentiation process of 3T3-L1 preadipocytes to adipocytes. GPDH is an enzyme that catalyzes the conversion of dihydroxyacetone phosphate to sn-glycerol 3-phosphate, which is a backbone of TG and its activity is often used as a late marker of preadipocyte differentiation. We found that CA significantly inhibited GPDH activity, which indicates that CA possibly suppresses adipocyte differentiation, resulting in a reduction in the number of fat cells.

To gain a better understanding of the molecular mechanism(s) underlying its inhibitory effects on adipocyte lipid accumulation, we examined the effect of CA on the expression of major transcription factors of the adipogenesis pathway. During adipocyte differentiation, the accumulation of lipids is regulated by a complex network of various transcription factors and adipocyte-specific genes. In particular, PPARγ and C/EBPα act as master regulators that control the growth arrest of preadipocyte proliferation and induce differentiation and adipogenesis by regulating a large number of adipocyte-specific genes. SREBP1

Figure 4. Effect of carnosic acid (CA) on the mRNA and protein expression levels of stearoyl-CoA desaturase 1 (SCD1) in 3T3-L1 adipocytes. Preadipocytes were grown to confluency (day 0) and induced to differentiate with an optimized adipocyte differentiation medium in the presence of CA (0.1, 1, and 10 μM) throughout differentiation. After differentiation (day 8), whole cell lysates were prepared and analyzed by immunoblotting using antibody-recognizing SCD1, and real-time polymerase chain reaction (RT-PCR) was performed using specific primer pairs for SCD1. The relative protein and RT-PCR values were corrected to β-actin expression levels and normalized with respect to the control. Data are expressed as mean ± standard deviation (n = 4). Means with the different letters at each sample are significantly different from each other (P < 0.05) using Duncan’s multiple-range test.
Figure 5. The hypothetical scheme of a mechanism in which carnosic acid (CA) inhibits lipid accumulation in 3T3-L1 adipocytes. CA effectively inhibits lipid accumulation by blocking adipocyte-associated gene expression and glycerol 3-phosphate dehydrogenase (GPDH) activity, leading to changes in fatty acid composition. PPARγ, peroxisome proliferator-activated receptor-γ; C/EBPα, CCAAT/enhancer binding protein-α; SREBP1, sterol regulatory element-binding protein 1; TG, triglycerides; SCD1, stearoyl-CoA desaturase 1; DHAP, dihydroxyacetone phosphate; C16:1, palmitoleate; C18:1, oleate.

regulates lipid homeostasis by controlling genes associated with fatty acid synthesis and its over expression contributes to the generation of PPARγ ligands. In this study, mRNA expression of PPARγ, C/EBPα, and SREBP1 was reduced by CA treatment, suggesting that CA inhibits lipid accumulation through PPARγ, SREBP1, and C/EBPα mediated-adipogenesis. Although the underlying mechanisms involved in anti-adipogenic activity of CA is not fully understood, studies have suggested that CA increased the ratio of the C/EBPβ LIP/LAP which interfere with the production of PPARγ agonist in 3T3-L1 cells and CA decreased both the protein expression and activity of hepatic PPARγ by the activation of the epidermal growth factor receptor/mitogen-activated protein kinase (EGFR/MAPK) cascade which can inhibit lipid accumulation in hepatocytes. In our previous study, CA also effectively inhibited the expression of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1) in lipopolysaccharide-stimulated 3T3-L1 adipocytes. These results suggest that CA may exert anti-adipogenic effects through multiple mechanistic routes and attenuates the inflammatory response in adipocytes.

The size of the lipid droplets in an adipocyte is influenced by its fatty acid composition, which is partly regulated by SCD1. SCD1, a PPARγ-dependent enzyme, converts saturated palmitate (C16:0) and stearate (C18:0) to their monounsaturated forms palmitoleate (C16:1) and oleate (C18:1), respectively. Several studies have shown that SCD1 is upregulated in obese human and animal models. Since MUFA are the major substrates for the synthesis of TGs and cellular membrane phospholipids in adipocytes, an increased ratio of monounsaturated to SFA (C16:1/C16:0 and C18:1/C18:0) and an increased SCD1 level indicates increased de novo lipogenesis. In humans, a lower desaturation index (C16:1/C16:0 and C18:1/C18:0) is related to lower body mass index and reduced subcutaneous adipocyte cell size, and is used as a negative predictor of metabolic syndrome. In the present study, CA (1 and 10 μM) significantly decreased the ratio of both C16:1/C16:0 and C18:1/C18:0 (Fig. 3), which was possibly accompanied by the down-regulation of SCD1 mRNA and protein expression (Fig. 4). In our previous study, CA diet significantly decreased the ratio of C18:1/C18:0 fatty acids in adipose tissue and down-regulated the hepatic liver-type fatty acid-binding protein, SCD1 and fatty acid synthases mRNA expression in C57BL/6J-ob/ob mice. These results indicate that CA down-regulates lipogenesis by blocking the SCD pathways. Previous studies have demonstrated that decreased SCD1 expression was related to reduction of weight gain and improvement of insulin sensitivity.

Taken together, the diverse effects of CA on adipocyte lipid accumulation are associated with adipocyte-related gene expression as well as fatty acid composition (Fig. 5). These changes could lead to improvements in insulin resistance and abnormal glucose control induced by obesity, although further in vivo studies are needed to confirm this relationship.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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