Carnosic acid inhibits TLR4-MyD88 signaling pathway in LPS-stimulated 3T3-L1 adipocytes

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BACKGROUND/OBJECTIVES: Carnosic acid (CA), found in rosemary (Rosmarinus officinalis) leaves, is known to exhibit anti-obesity and anti-inflammatory activities. However, whether its anti-inflammatory potency can contribute to the amelioration of obesity has not been elucidated. The aim of the current study was to investigate the effect of CA on Toll-like receptor 4 (TLR4) pathways in the presence of lipopolysaccharide (LPS) in 3T3-L1 adipocytes.

MATERIALS/METHODS: 3T3-L1 adipocytes were treated with CA (0-20 μM) for 1 h, followed by treatment with LPS for 30 min; mRNA expression of adipokines and protein expression of TLR4-related molecules were then measured.

RESULTS: LPS-stimulated 3T3-L1 adipocytes showed elevated mRNA expression of tumor necrosis factor (TNF-α), interleukin-6, and monocyte chemoattractant protein-1, and CA significantly inhibited the expression of these adipokine genes. LPS-induced up regulation of TLR4, myeloid differentiation factor 88, TNF receptor-associated factor 6, and nuclear factor-κB, as well as phosphorylated extracellular receptor-activated kinase were also suppressed by pre-treatment of 3T3-L1 adipocytes with CA.

CONCLUSIONS: Results of this study suggest that CA directly inhibits TLR4-MyD88-dependent signaling pathways and decreases the inflammatory response in adipocytes.

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INTRODUCTION

Accumulating evidence suggests that obesity-associated inflammation plays an important role in development of obesity-related diseases, such as metabolic syndrome, type 2 diabetes mellitus, and fatty liver disease. The mechanism for the initiation or acceleration of the inflammatory response of adipocytes has not been completely elucidated, however, there is evidence indicating partial involvement of innate pattern recognition receptors in such a process [1,2]. Toll-like receptors (TLRs) are pattern recognition molecules and their signaling pathways are associated with chronic inflammation in obesity [3]. In particular, TLR4 activation leads to up-regulation of interleukin (IL)-6 and release of monocyte chemoattractant protein (MCP)-1 via activation of the extracellular signal-regulated kinase (ERK) [4] and nuclear factor-κB (NF-κB) pathway in adipocytes [5]. In addition, lipopolysaccharide (LPS), a TLR4 ligand, is known as an early key factor that triggers inflammation in adipose tissue [6]. Therefore, LPS-induced TLR4 signaling pathways may be critical factors in adipocyte-related inflammation.

Carnosic acid (CA) is a primary phenolic compound of rosemary (Rosmarinus officinalis) leaves. CA has a wide range of biological activities, including anti-adipogenic, antioxidant, anti-cancer, anti-inflammatory, and neuroprotective effects [7-11]. CA has been shown to decrease body weight gain, fat mass gain, and several inflammatory markers in both diet-induced and genetically modified obesity models [7,8]. CA also effectively attenuates LPS-induced hepatotoxicity via anti-inflammatory and antioxidant activities [9]. CA has also been reported to inhibit adipocyte differentiation by blocking CCAAT/enhancer-binding protein α (C/EBPα) and peroxisome proliferator-activated receptor γ (PPARγ) pathways [10], tumor necrosis factor-α (TNF-α) mediated inflammation and insulin resistance via inhibition of NF-κB, activator protein-1 (AP-1), and forkhead box O1 (FoxO1) signaling in adipocytes [11]. A recent study showed that CA effectively suppresses Gaussia luciferase secretion in 3T3-L1 cells with a secretory Gaussia luciferase gene under control of a NF-κB element [12], however, little is known about the molecular mechanism responsible for its anti-inflammatory effect in adipocytes. Therefore, in this study, we investigated the question of whether CA can inhibit LPS-induced TLR4-related signaling in 3T3-L1 adipocytes.

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MATERIALS AND METHODS

Materials
CA powder was purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco’s modified Eagles medium (DMEM), bovine calf serum (BCS), antibiotic-antimycotic solution, and trypsin-EDTA were purchased from Gibco (Grand Island, NY, USA). LPS, dexamethasone (DEX), and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of the highest commercially available grade.

Cell culture and treatment
Mouse 3T3-L1 fibroblasts were obtained from the Korean Cell Line Bank (Seoul, Korea) and cultured in DMEM supplemented with 10% heat-inactivated BCS, 100 U/ml penicillin, and 100 μg/ml of streptomycin. Adipocytic differentiation was induced at 24 h after confluence by adipogenic agents (0.5 mM IBMX, 2 μM DEX, and 1.7 μM insulin) that were added to the culture medium. At day 6-8 of differentiation, 3T3-L1 adipocytes were pre-treated with 5-20 μM CA for 1 h and then stimulated with 100 ng/ml LPS for 30 min. CA was dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 20 μM (or an equivalent volume of DMSO as control) in serum-free medium.

Cell viability (MTT assay)
3T3-L1 cells were seeded at a density of 1 × 10⁵ cells/well in six-well plates. After adipocytic differentiation, mature adipocytes were cultured with various concentrations of CA for 1 h and treated with or without LPS (100 ng/ml) for 30 min. Cells treated with 0.1% DMSO were used as controls. After treatment, 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).

Real-time RT-PCR analysis
Total RNA was isolated from 3T3-L1 adipocytes using Trizol reagent (Invitrogen, CA, USA), following the manufacturer’s recommendations. Real-time quantitative PCR was performed using a SYBR Green™ kit (Quantitect™ SYBR Green PCR, Qiagen). The cycling conditions were 15 min at 95°C and then 40 cycles of 15 s at 94°C and 30 s at 72°C. Relative quantification was calculated using the ΔΔCT method [13]. Specific primer sets were as follows: 5′-aatccaaccttcccaaacg-3′ (for IL-6), 5′-tccacagagaaacttcccaagacg-3′ (for actin). The intensities of the bands were measured using a LAS3000 (Fujifilm, Tokyo, Japan). Antibodies used included anti-ERK (Cell Signaling, Beverly, MA, USA), anti-TLR4 (Santa Cruz Biotechnology, Dallas, TX, USA), and anti-tumor necrosis factor receptor-associated factor 6 (TRAF6) (Santa Cruz Biotechnology, Dallas, TX, USA). β-actin (Cell Signaling, Beverly, MA, USA) was used as the loading control.

Quantification of NF-κB activation
For quantification of NF-κB activity, nuclear extracts were prepared using a Nuclear Extract kit (Active Motif, Carlsbad, CA, USA) and analyzed using a PathScan Total NF-κB p65 Sandwich ELISA kit (Cell Signaling Technology) according to the manufacturer’s instructions.

Statistical analysis
Data are expressed as the mean ± standard deviation (SD). All analyses were performed using SAS statistical software version 9.1 (SAS Institute, Cary, NC, USA). Data were analyzed by one-way analysis of variance followed by Duncan’s multiple range tests. P < 0.05 was considered statistically significant.

RESULTS

Cell treatment conditions and viability
LPS induced dose-dependent stimulation of IL-6 mRNA expression, which reached significance at a concentration of 100 ng/ml (Fig. 1A). This concentration was chosen for all further incubations in this study for stimulation of IL-6 and ERK and to demonstrate possible inhibitory effects of CA. IL-6 showed high mRNA expression from 30 min of incubation with LPS (Fig. 1B) and ERK was also maximally phosphorylated at 30 min of incubation with LPS (Fig. 1C). Other previous studies have shown that CA has anti-inflammatory effects at 1-30 μM in vitro study [11,12,14,15]. From these references we selected 10 μM and showed the time-dependent inhibitory effects of CA on IL-6 mRNA expression. As shown in Fig. 1D, incubation with CA (10 μM) effectively inhibited the LPS-induced expression of

![Fig. 1](image-url)
IL-6 mRNA from 1 hr of incubation. Based on these data we determined the LPS stimulation and the CA treatment conditions. Cell viability was assessed by MTT assay. As shown in Fig. 2, CA in the concentrations used had no effect on viability of 3T3-L1 cells with or without LPS treatment.

Proinflammatory cytokine mRNA levels

Because TNF-α, IL-6, and MCP-1 are major inflammatory cytokines that are overexpressed in adipocytes, we first analyzed the possibility of their induction in response to LPS. As shown in Fig. 3, mRNA expression of pro-inflammatory cytokines was significantly increased by LPS stimulation (P < 0.05). Treatment with CA resulted in a dose-dependent decrease in LPS-induced expression of TNF-α, IL-6, and MCP-1 mRNA compared with that in the LPS control group (Fig. 3A-C, P < 0.05). TNF-α, IL-6, and MCP-1 mRNA expression was significantly decreased by 84.27%, 80.26%, and 72.94%, respectively, in the 20 μM CA group (Fig. 3A-C, P < 0.05).

TLR4 protein expression level

For analysis of the effect of CA on TLR4 signals, we analyzed the protein expression level of TLR4. Protein expression of TLR4 was significantly increased by LPS in 3T3-L1 cells, and CA significantly suppressed the protein expression level of TLR4 stimulated with LPS in a dose-dependent manner (Fig. 4, P < 0.05).

MyD88 and TRAF6 protein expression levels

The LPS/TLR4 complex ultimately activates downstream NF-κB via MyD88- and TRAF6-dependent signaling [16,17]. Results of Western blot analysis showed that LPS treatment caused significantly increased expression of both MyD88 and TRAF6, and this enhancement was suppressed by CA in a dose-dependent manner (Fig. 5A,B, P < 0.05).
adipose tissue [20]. Other studies have shown that increased NF-κB activation was significantly decreased in the 10 μM CA groups (Fig. 6). LPS treatment resulted in a dose-dependent decrease in LPS-induced TLR4 protein expression. These data suggest that CA can control TLR4 signaling pathways and these effects may contribute to its inhibitory effect on cytokine gene expression. There is little data on the effect of CA on TLR expression. Recently, an ethanolic rosemary extract containing CA was found to suppress the mRNA expression of TLR2 in Propionibacterium acnes-stimulated mononuclear THP-1 cells [22], and TLR4-related indicators were suppressed in RAW264.7 cells [23].

To determine whether the inhibitory effect of CA is mediated by MyD88-dependent pathways, we measured the protein expression levels of MyD88. CA treatment effectively diminished the LPS-induced MyD88 protein up-regulation and caused a significantly decrease in activation of NF-κB and the protein level of TRAF6, which is known to induce phosphorylation of IκB. These data showed that CA can directly suppress MyD88-dependent TLR4 signaling in adipocytes. Furthermore, CA-mediated inhibition of NF-κB activity may affect mitogen-activated protein kinase (MAPK) signaling. The signaling transduction pathway of MAPK plays an important role in transfer of external stimuli into the nucleus through the cytoplasm. The most well known MAPK signaling pathways are ERK, JNK, and p38 [24]. ERK is more strongly phosphorylated than JNK and p38 by LPS treatment in 3T3-L1 adipocytes [25]. Finally, we attempted to determine whether the inhibition of ERK phosphorylation by CA showed an inhibitory pattern similar to that of NF-κB activity. According to the result, treatment with CA resulted in significantly suppressed phosphorylation of ERK, suggesting that the anti-inflammatory action of CA might be mediated by a TLR4-dependent ERK signaling pathway in adipocytes. In adipocytes and adipose tissues, the phosphorylation status of ERK plays a role in modulation of insulin resistance. In obese patients with insulin resistance, pERK and pJNK are up-regulated in adipose tissue [26]. Furthermore, a clinical study demonstrated that injection of LPS into healthy volunteers resulted in increased levels of TNF-α and IL-6 in adipose tissue [20]. Other studies have shown that increased endotoxin levels trigger inflammation and body weight gain in an obesity animal model [6]. In addition, during LPS-induced systemic inflammation in mice, adipose tissue was the major source of systemic IL-6 levels [21]. These data suggest that LPS-induced TLR4-related signaling may be a critical factor in obesity-related inflammation. All TLR family members (TLR1-10) are expressed in all human adipose tissue [4]. In particular, TLR4 activation causes upregulation of IL-6 and release of MCP-1 via activation of both the ERK and NF-κB pathways [5]. To examine the mechanism of CA in the inhibition of cytokine gene expression, TLR4 protein expression was measured in LPS-treated 3T3-L1 adipocytes. CA treatment resulted in a dose-dependent decrease in LPS-induced TLR4 protein expression. These data suggest that CA can control TLR4 signaling pathways and these effects may contribute to its inhibitory effect on cytokine gene expression. There is little data on the effect of CA on TLR expression. Recently, an ethanolic rosemary extract containing CA was found to suppress the mRNA expression of TLR2 in Propionibacterium acnes-stimulated mononuclear THP-1 cells [22], and TLR4-related indicators were suppressed in RAW264.7 cells [23].

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vitro) [11-14] and 1-60 mg/kg (in vivo) [9,27,28]. Therefore, CA may effectively control TLR4-mediated inflammatory disorders in obesity, and the anti-inflammatory effects of CA need to be confirmed in animal models.

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