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Anti-Inflammatory Effects of *p*-coumaric Acid in LPS-Stimulated RAW264.7 Cells: Involvement of NF-κB and MAPKs Pathways

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Abstract

P-coumaric acid (*p*-CA), which was widely found in nutritious plant foods, has various anti-inflammatory effects *in vivo*. In order to clarify the anti-inflammatory mechanisms, the effects on lipopolysaccharide (LPS)-stimulated inflammatory responses in RAW264.7 macrophage cells were examined by pretreated with *P*-CA (10-100 μg/ml). *P*-CA significantly inhibited iNOS, COX-2, IL-1β and TNF-α expression at mRNA and/or protein level. Furthermore, *P*-CA suppressed the phosphorylation of IκB and ERK1/2. The above results suggest that *P*-CA may inhibit the production of inflammatory cytokines induced by LPS through blocking NF-kB and MAPKs signaling pathways, which further support the anti-inflammatory and immunomodulatory potential of *P*-CA in different models of inflammation.

Keywords: *P*-coumaric acid; Inflammation; Anti-inflammatory; Phosphorylation

Introduction

P-coumaric acid (*p*-CA) is a hydroxyderivative of cinnamic acid widely found in fruits, vegetables and plant products, including propolis, rice, cranberry syrups, tremella fuciformis, apple cider, grape juices, tomatoes, etc. [1-6], which is also a main metabolite from other biological phenolic acids such as rosmarinic acid and chlorogenic acid [7]. *P*-CA has been reported to possess various activities such as anti-platelet, anti-UV damage, anti-angiogenic, antioxidant, antimicrobial, anti-Alzheimer's disease, anti-metabolic disorders, and immunomodulatory activities [8-17]. The anti-inflammatory activities of *P*-CA were also verified in various animal modes *in vivo* [14-17]. However, the specific mechanisms involved in its anti-inflammatory activities remain unclear. Since inflammation is a complex process mediated by activated immune cells like macrophages [18], and they plays critical role in the ignition, maintenance, and resolution of inflammation [19].

Lipopolysaccharide (LPS)-mediated activation of macrophages leads to initiates a cascade of signaling events, including both Nuclear transcription factor kappa-B (NF-κB) and MAP kinase pathways, as well as the production of various proinflammatory cytokines, such as tumor necrosis factor-a (TNF-a), interleukin (IL)-6, IL-1β, and nitric oxide (NO) [20]. NF-κB plays a fundamental role in the inflammatory and acute response [21]. In addition, mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinases (ERK) 1 and 2, c-Jun N-terminal kinase (JNK), and p38, also regulate inflammatory gene transcription, such as proinflammatory cytokines, COX-2 and iNOS [22]. In this study, we sought to study the anti-inflammatory effects of *P*-CA in LPS-stimulated macrophage and elucidate the potential anti-inflammatory mechanism through NF-κB and MAPK signaling pathways.

Materials and Methods

Materials

RAW264.7 mouse macrophages were ordered from American Type Culture Collection (Rockville, MD, USA). *P*-CA (purity>98%) was purchased from Pure One Biotechnology (Shanghai, China). High-glucose Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (NY, USA). Phosphate buffer saline (PBS) was purchased from Thermo Scienfic HyClone (Logan, UT). Lipopolysaccharide (LPS), dimethylsulfoxide (DMSO) and 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemicals Co. (MO, USA). TRIZOL Reagent was purchased from Invitrogen (Carlsbad, CA, USA). Hypoderm Molecular Biology Grade Water, Thermo RevertAid First Strand cDNA Synthesis Kit and BCA Protein Assay Kit were purchased from Thermo Scientific. Complete Protease Inhibitor Cocktail Tablets. Phos STOP Protease Inhibitor Cocktail Tablets and FastStart Universal SYBR Green Master (Rox) were purchased from Roche Applied Science (Mannheim, Germany). Rragents ECL Western blotting kit was purchased from Millipore. The antibodies against COX-2, iNOS, p38, JNK, ERK (1/2), p-IkBα, NF-κB (p65), loading buffer, and RIPA Buffer were purchased from Cell Signaling Technology (Beverly, MA). GAPDH was purchased from Santa Cruz Biotech (Santa Cruz, CA). The goat anti-rabbit IgG-HRP were purchased from Asbio.

Instruments

Multi label Plate Reader (VICTOR™ X5, PekinElmer, USA); NanoDrop 2000C Spectrophotometer (Thermo Scientific); ABI 7500 Real-Time PCR System; Trans -Blot Cell (Bio-Rad); Mini-PROTEAN Tetra Electrophoresis System (Bio-Rad); ChemiDoc XRS (Bio-Rad).

Total RNA extraction and RT-PCR

RAW264.7 macrophages were cultured at a density of 2×10^5 cells/ml in 6-well plates overnight. The cells were incubated for 2 h in combination with different concentrations of P-CA. The cells were

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further cultured for 12 h on treatment with LPS (1 μg/mL). The cells were rinsed with cold PBS, and total cellular RNA from RAW264.7 cells was extracted using a TRIzol Reagent kit according to instructions of the manufacturer. Total RNA (1 μg) was converted to cDNA using a Thermo Revert Aid First Strand cDNA Synthesis Kit. The PCR primers used in this study are listed below and were purchased from Life Technology: Mouse IL-1β (Forward TGGGATAGGGCCTCTCTTGC and Reverse CCATGGAAT CCG TGTCTTCCT); iNOS (Forward TGAGTTCCGAAGCAAGCCAA and Reverse AGACCTCAACA GAGCCCTCA; TNF-α (Forward GTGTC CCA ACATTCA TATTGTCAGT and Reverse TGGGAAGAGAAACCAGGGAGA; COX-2 (Forward TCTCCAACCT CTCCTACTAC and Reverse GCACGTA GTCTTCGATCACT; and GADPH (Forward GTTTTCAGGGATGAAGCGGC, and Reverse TGGGATAGGG CCTCT CTTGC). The gene expressions of TNF-α, iNOS, IL-1β, and COX-2 were amplified from the synthesized cDNA. Real-time PCR was performed using Roche FastStart Universal SYBR Green Master. GADPH mRNA levels were used as internal controls. The PCR reactions were carried out as the following: 95°C for 10 min; 40 cycles of 95°C for 15 s. Final extension was performed at 60°C for 1 min.

Western blot analysis

RAW 264.7 macrophages (1×10^6 cells/mL) were seeded in 6-well plates and incubated for 24 h, then pretreated with 1.64, 8.2 and 16.4 μM of *P*-CA for 2 h. After LPS (1 μg/ml) stimulation for 1 h (ERK1/2, JNK and P38 analysis) and 8 h (iNOS, COX-2 IκBα and NF-κB analysis), the cells were collected and washed twice with cold PBS. The cells were lysed in RIPA buffer containing protease inhibitors. After lysis, the lysates were clarified by centrifugation at 10,000×g for 5 min at 4°C; the protein concentration in the supernatants was determined using a BCA Protein Assay Kit. Proteins (35 μg/lane) were separated by 10% acrylamide SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked using 5% skim milk and sequentially incubated with specific primary antibody at 4°C overnight. With the use of rabbit peroxidase-conjugated secondary antibodies, membrane-bound antibodies were detected using ECL plus. Rabbit anti- iNOS, COX-2, JNK, ERK1/2, IκBα, NF-κB (p65) and p38 were utilized as primary antibodies and HRP-goat anti-rabbit IgG was used as a secondary antibody, and detected with ECL reagent (Millipore, USA).

Statistical analysis

Data are shown as mean ± SD. Differences between mean values of normally distributed data were assessed by the one-way ANOVA (Dunnett's *t*-test). Values were considered significantly different at $p<0.01$ or $p<0.05$.

Results

P-CA suppresses COX-2, iNOS, TNF-α and IL-1β mRNA expression in LPS-stimulated RAW264.7 macrophages

P-CA has been reported to inhibit NO production in LPS-stimulated RAW264.7 macrophages [7]. Since inflammatory mediators (COX-2, iNOS) and cytokines (TNF-α, IL-1β) are important factors in inflammation. To understand whether *P*-CA can inhibit LPS-induced activation of pro-inflammatory genes expression, a semi-quantitative RT-PCR was performed. Treatment of RAW264.7 cells with LPS resulted in significantly increased pro-inflammatory genes expression ($p<0.01$ versus control; Figure 1), while pre-treatment with *P*-CA (10-100 μg/ml) significantly inhibited COX-2, iNOS, TNF-a, IL-1β mRNA expression in a dose-dependent manner, compared with LPS-treated cells ($p<0.01$).

P-CA suppresses COX-2 and iNOS protein expression in LPS-stimulated RAW264.7 macrophages

To understand whether *p*-CA can inhibit LPS-induced activation of iNOS and COX-2 protein, the levels of COX-2 and iNOS protein expression were examined by Western blot analyses. LPS treatment significantly induced iNOS and COX-2, but the pre-treatment of *p*-CA (50-100 μg/ml) for 12 h resulted in decreased iNOS ($p<0.01$) and COX-2 ($p<0.05$) protein expression (Figure 2).

P-CA suppresses LPS-induced NF-κB pathway

NF-κB is a critical key transcription factor that expresses the genes involved in inflammation and has attracted attention as a new target for treating inflammatory diseases. The activation of NF-κB plays critical roles in the LPS-induced expression of inflammatory mediators and cytokines such as COX-2, iNOS, TNF-α, IL-1β and IL-6. NF-κB and IκBα protein were examined by Western blot to test whether the inhibition of inflammatory response by *P*-CA is mediated through the NF-κB pathway. As shown in Figure 3, phosphorylation of of IκB-α and nuclear translocation of p65 was increased after treatment with LPS (1 h). However, the pretreatment with *P*-CA for 12 h, at 10 to 100 μg/ml, markedly inhibited LPS-induced phosphorylation of IκB-α, thereby preventing the translocation of p65 into the nucleus.

P-CA suppresses LPS-induced MAPK pathway

In order to determine whether the suppression of inflammatory reactions by *p*-CA was mediated through a MAPK pathway, we assessed the effects of *p*-CA on the LPS-induced phosphorylation of ERK, JNK and p38 MAPKs in RAW 264.7 cells. The phosphorylation of ERK, JNK and p38 were enhanced after LPS stimulation, but the expression levels of ERK, JNK and p38 were unaffected. However, pre-treatment with *p*-CA for 2 h, at 10 to 100 μg/ml, obviously inhibited the LPS-induced phosphorylation of ERK1/2 and JNK (Figure 4).

Discussion

Macrophages are centrally involved in acute and chronic inflammatory responses. The NO enhances the ability of macrophages to kill microorganisms [23]. In addition, studies found that COX-2 expression was related to the production of PGE2, which is known to play a key role in inflammatory processes including pain, fever, swelling and tenderness [24]. IL-1β, IL-6 and TNF-α were proinflammatory cytokines secreted by activated macrophages [25]. High levels of TNF-α could injure tissues, and even caused sepsis and death [26]. IL-1β acted as playing a pivotal role in the pathogenic mechanism of periodontal tissue destruction [27]. NF-κB regulates cell survival and coordinates the expression of proinflammatory mediators, including iNOS, COX-2 and proinflammatory cytokines [28]. The MAPK pathways, including ERK1/2, JNK and p38, were thought to modulate NF-κB activation, and in turn promote the expression of pro-inflammatory cytokines and inflammatory process [29-31]. Our study indicated that *P*-CA decreased the production of iNOS, COX-2, IL-1β and TNF-α expression both at mRNA and/or protein levels in LPS-stimulated RAW264.7 cells. The decreased level of these cytokines will reduce further activation of the MAPK and NF-κB pathway and thus alleviate immune response. Based on all above, our finding provides an important proof-of-principle for understanding the anti-inflammatory mechanisms of *P*-CA.

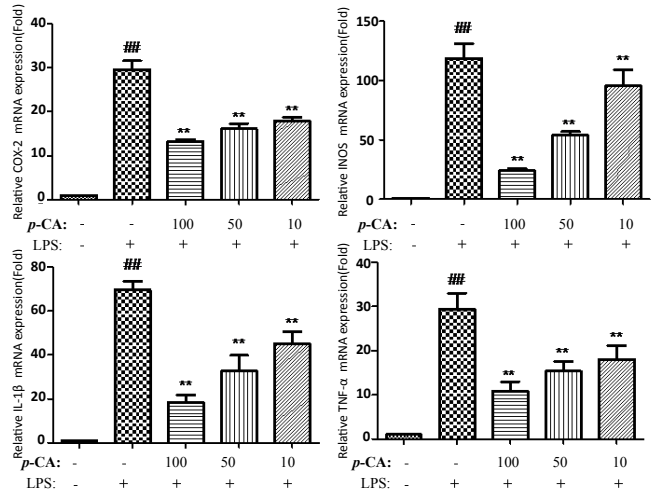

Figure 1: Effects of *P*-CA on inflammation-related gene expression in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. The mRNA levels (A: COX-2; B: iNOS, C: IL-1β; D: TNF-a) were analyzed using real-time PCR; ++p<0.01, control group vs LPS-stimulated group;**P<0.01, LPS vs LPS plus *P*-CA-treated group. Values shown in the graphs are mean ± standard deviation (SD).

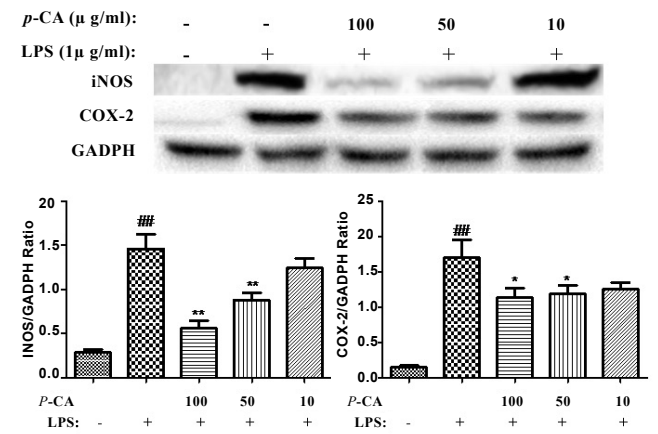

Figure 2: Effects of *p*-CA on LPS-induced iNOS and COX-2 protein expression. iNOS protein expression levels were determined by Western blot analysis. RAW264.7 cells cultured in 6-well plates were incubated with 10, 50 and 100 μg/ml of *p*-CA or not for 2 h, and then were stimulated with LPS (1 μg/ml) for 8 h .Cell lysates were prepared and subjected to Western blot by using anti-iNOS and COX-2 antibodies. GAPDH expression was used as control. Equal loading protein was confirmed by GAPDH. The data are represented as the mean ± SEM (n=3) (++p<0.01, control group vs LPS-stimulated group;**P<0.01, LPS vs LPS plus *P*-CA-treated group;*P<0.05, LPS vs LPS plus *P*-CA-treated group).

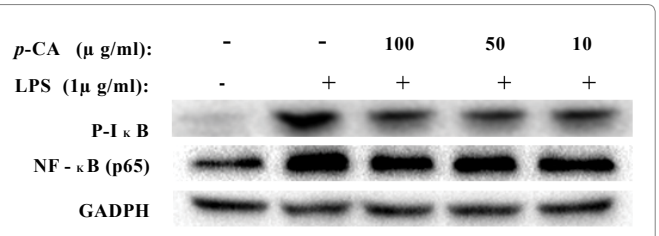

Figure 3: Effect of *p*-CA on LPS-induced p-IκBα and NF-κB nuclear translocation in cultured RAW 264.7 cells. RAW264.7 cells cultured in 6-well plates were incubated with 10, 50 and 100 μg/ml of *p*-CA or not for 2 h, followed by incubation with LPS (1 μg/ml) for 8 h. Cells were then lysed and p-IκBα, and NF-κB protein levels were analyzed by Western blotting. Equal loading protein was confirmed by GAPDH.

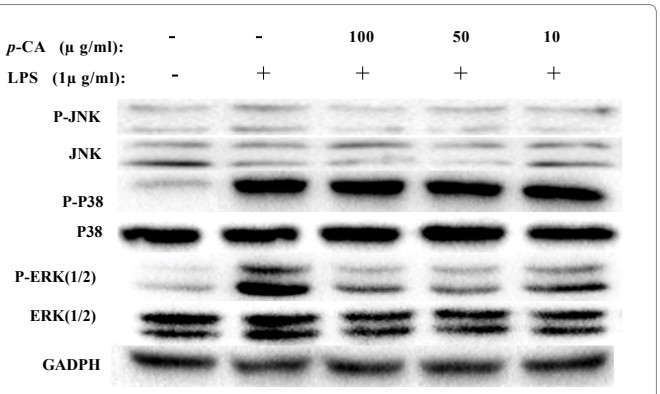

Figure 4: Effects of *p*-CA on the phosphorylation of MAP kinases. RAW264.7 cells were pretreated with 10, 50 and 100 μg/ml of *p*-CA for 2 h and then stimulated with LPS (1 μg/ml) for 1 h. Phosphorylation of ERK, JNK and p38 MAP kinases was determined by specific antibodies against the phosphorylated form of MAP kinases. Equal loading protein was confirmed by GAPDH.

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