Adaptive immunoregulation of luteolin and chlorogenic acid in lipopolysaccharide-induced interleukin-10 expression

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Abstract

Objective: To investigate the mechanism of the adaptive effect of two compounds in Lonicerae japonica flos (LJF), luteolin (LUT) and chlorogenic acid (CGA), on the expression of interleukin (IL) IL-10 and IL-6. Materials and Methods: RAW264.7 cells receiving lipopolysaccharide (LPS) were pretreated with CGA and LJF. The expression of pro-inflammatory cytokines and IL-10 was evaluated by reverse transcription-polymerase chain reaction. Moreover, the concentrations of IL-10 and IL-6 were measured by enzyme-linked immunosorbent assay in the culture medium obtained 24 h after LPS treatment. Nuclear extracts of RAW264.7 cells, pretreated with CGA or LUT and LPS, were measured after 6 h, and C/EBPβ and C/EBPδ were measured by Western blotting. Nuclear factor-xB (NF-xB) activity was measured by electrophoretic mobility shift assay. The phosphorylated form of IkB, ERK1/2, p38, JNK, and IkB, ERK2, p38, or JNK were also measured by Western blotting. Results: CGA enhanced the LPS-induced expression of IL-10 and IL-6, and increased NF-xB, Sp1, C/EBPβ and δ. The effect of CGA is interfered with Lut by suppressing the phosphorylation of IkB and p38, and NF-xB activity. In the event, IL-6 was suppressed and IL-10 was not influenced. Conclusion: LUT and CGA, which are abundant in LJF that is one of the ingredients in Gingyo-san, have adaptive immunoregulative effect on the expression of IL-10.

Keywords: Chlorogenic acid, Interleukin-10, Lonicerae japonica flos, Luteolin

Introduction

Interleukin (IL) (IL-10) functions as a cytokine synthesis-inhibiting factor and plays a role in the activity inhibition of Th1 cells [1]. IL-10 has various biological effects on different cell types. In macrophages, IL-10 suppresses ligand-induced activation and production of pro-inflammatory cytokines from macrophages [2,3]. IL-10 inhibits the proliferation as well as cytokine synthesis of CD4+ T cells [4]. Other immunosuppressive effects on eosinophils, neutrophils, and dendritic cells have also been well documented [5]. IL-10 on the other hand has been shown to have an immunostimulation effect on cytotoxic T cells [6] and can act as a growth co-stimulator for thymocytes and mast cells [7]. The immunosuppressive functions of IL-10 are an important mechanism for protecting the host from the harmful effects of exaggerated inflammatory and immune responses under the situation of microbial infection [2,3]. Several reports suggested that lipopolysaccharide (LPS)-induced IL-10 expression was mediated through the pathway of nuclear factor-xB (NF-xB)- and mitogen-activated protein kinase (MAPK)-induced protein expression and DNA binding of C/EBPβ [8-10].

Luteolin (LUT), a flavone found in high concentrations in celery, green pepper, perilla leaf and seeds, and Lonicerae japonica flos (LJF) [11], is one of the most potent and efficacious flavonoid inhibitors of LPS-induced tumor necrosis factor-α (TNF-α) and IL-6 production and inducible nitric oxide synthase (iNOS) expression [12,13]. LUT blocks LPS-induced NF-xB and AP-1 signaling and pro-inflammatory gene expression [13,14]. Chlorogenic acid (CGA), an ester of caffeic acid, is found with quinic acid in a wide range of fruits and vegetables and is particularly abundant in coffee [15] and Lonicera Flos [16]. CGA possess antioxidant properties in vivo [17,18]. CGA has been reported to prevent different cancers and cardiovascular diseases in several experimental animal models [19-21]. CGA also possess chemopreventive effect through the suppression of NF-xB, AP-1, and MAPK activation [22]. It has been reported that CGA

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augmented the inducible cytokine messages, i.e., IL-10, IL-13, interferon-γ (IFN-γ), IL-6, and TNF-α that involve in the immunoregulation processes [23].

Modulation of innate immunity by natural plant products may represent an attractive strategy to prevent lung inflammation associated with dysregulated innate immune responses. In our previous study, Gingyo-san (GGS) is a powder preparation of traditional Chinese medicine and Lonicerae Japonica Flos is one of its ingredients; it reduced the LPS-induced lung inflammation with the effects of down-regulating inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-8) and anti-inflammatory cytokines (IL-12 and IL-4) and up-regulating IL-10 in vivo [24]. In this study, we tried to investigate the mechanism that the adaptive effect of two compounds in LJF, LUT and CGA, on the expression of IL-10 and IL-6.

**MATERIALS AND METHODS**

CGA (CAS number 327-97-9), LUT (CAS number 491-70-3) and LPS were purchased from Sigma-Aldrich Inc. (Darmstadt, Germany) DMEM medium was purchased from Invitrogen (Waltham, Massachusetts, USA). Fetal bovine serum, gentamicin, and L-glutamine were purchased from Invitrogen. All antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). SP-1, C/EBPβ, C/EBPδ, and enzyme-linked immunosorbent assay (ELISA) kits of IL-10 and IL-6 were obtained from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA.

Preparation of the extract of gingyo-san and Lonicerae japonica flos

The medicinal plants used to prepare GGS were provided by Koda Pharmaceuticals Ltd., Taoyuan, Taiwan. Gingyo-san is commercially available in Taiwan and Japan. The extraction procedure and its components were reported [24]. LJF was prepared by the same procedure. Brief procedure: Raw herbs of LJF were soaked and rinsed in running water repeatedly to wash any dirt or impurities off. After drying, slicing, and precise scaling, Koda Pharmaceuticals attains optimal extraction via cyclic-style extraction under controlled temperature, pressure, water, and duration using fully automated and computerized equipment under vacuum and low-temperature conditions. After extraction, the residue and extract were separated by filtration, to remove dregs from the extracts. Low-temperature vacuum system is used to remove part of the decoction liquid, and then concentrate the filtered decoction liquid into herbal liquid extracts. These extracts of GGS and LJF were dissolved in pyrogen-free isotonic saline (YF Chemical, Taipei, Taiwan) and filtered through a 0.2-mm filter (Microgen, Laguna Hills, CA, USA) before use.

High-performance liquid chromatography analysis

Samples (0.5 g) of the herbal mixtures were extracted at three separate times during boiling and were mixed with 10 ml of methanol in a rotating cultivator (200 rpm, 60 min). The extracted liquid was filtered, and the final volume was adjusted to 10 ml with methanol. After passing the filtrate through a 0.45-μm membrane filter, the samples were subjected to analysis by high-performance liquid chromatography (HPLC). Koda Pharmaceuticals Ltd. offered this HPLC. The chromatographic system consisted of a Hitachi D-7000 Interface, L-7100 pump, L-7455 DAD, L-7200 Autosampler, Mightysil RP-18, and a GP 250 mm × 4.6 mm (5 μm) column. Methanol was used as the mobile phase, and components were detected at 330 nm. The injection volume was 10 μl, and the flow rate was 0.8 mL/min. A standard solution was prepared by dissolving 1.0 mg CGA and 1.0 mg LUT 10 mL of 70% methanol. The solution was injected into the HPLC system after passage through a 0.45-μm membrane filter [25].

Measurement of tumor necrosis factor-α, interleukin-1β, interleukin-6, inducible nitric oxide synthase, and interleukin-10 mRNA by reverse transcription-polymerase chain reaction

Reverse transcription-polymerase chain reaction (RT-PCR) was conducted to determine the expression of mRNA in RAW264.7 cells. Cells were collected at 24 h following various treatments. Total RNA was isolated from all RAW264.7 cell preparations by the TRizol method (Invitrogen) and according to the manufacturer’s instructions. The sequences for the TNF-α, IL-1β, IL-6, IL-10, iNOS, and β-actin PCR primers were as follows: TNF-α (antisense: 5'-AGAAGAGGCACCTCCCCAAA-3; sense: 5'-CCGAAGTTGATGACAGAAGGCG-3'); IL-1β (5'-AAGCTCTCACCTCAATGGA-3' and 5'-TGCTGAGAGGTCTGATGTT-3'); IL-10 (sense: 5'-CGTCGGATCCGCATGCTGGCTACACTGTCF-3' and antisense: 5'-CTGTCTTCTAGTATGCTTTTATTTGATCA-3'); IL-6 (sense: 5'-GACACTTTGGCATTGTTGAGGAGATGAGGATGAG-3' and antisense: 5'-ATCGAGGATGATGTCTTCTTTGATCA-3'); iNOS (sense: 5'-ACCAACTGACGGGAGATGAG-3'; antisense: 5'-ATAGCGGATGATGTTCTCGAGGATATGAGGATGAG-3'); β-actin (sense: 5'-ATAGCGGATGATGTTCTCGAGGATATGAGGATGAG-3'; antisense: 5'-CTTCATCTAGGCTAGAGCAACCG-3').

PCR reaction was performed under an annealing temperature of 61°C for TNF-α and IL-1β, 65°C for iNOS and IL-6, and 60°C for β-actin. The intensities of gel bands were calculated with a software Gel-Pro analyzer (Meyer Instruments).

Western blot analysis

RAW264.7 cells were treated with LPS and CGA and/or LUT s and lysed with 250-μL sample buffer (62.5 mM Tris-HCl, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 50 mM dithiothreitol, 0.1% bromophenol blue, pH 6.8). Nuclear extracts were also prepared from the same samples. The nuclear concentration was determined with a BCA protein assay kit (Pierce, Rockford, IL, USA). Proteins (10 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis, and protein bands were electrophoretically transferred to nitrocellulose membranes. Membranes were probed with polyclonal antibodies against IκB, JNK, p38, and ERKs (in cytosol), and SP-1, C/EBPβ, and C/EBPδ (in nuclear extracts). Bound antibodies were detected with peroxidase-conjugated anti-rabbit antibodies followed by chemiluminescence (ECL system, Amersham, Buckinghamshire, UK) and autoradiographic exposure. The intensities of dark bands were calculated with a Gel-Pro analyzer [25].

Electrophoretic mobility shift assay

The nuclear extracts were prepared from the same RAW264.7 cells used for electrophoretic mobility shift assay
EMSA) [26] with a biotin-labeled NF-κB consensus sequence probe (Promega, Madison, WI, USA). After nuclear extraction, NF-κB DNA-binding capacity was determined using an NF-κB EMSA kit (Panomics Inc. Fremont, CA, USA) according to the manufacturer’s instructions. Briefly, the nuclear extracts (10 μg) were mixed with a biotin-labeled NF-κB probe and incubated at 37°C for 30 min. DNA protein complexes were separated in a 6% nondenaturing polyacrylamide gel in a >0.5 tris-borate-EDTA (TBE) buffer at 120 V for 60 min and then transferred to a Biodyne B nylon membrane (Pierce, Rockford, IL, USA) at 300 mA for 40 min. The membrane was fixed using an ultraviolet crosslinker, blocked with a blocking buffer, and incubated with a streptavidin-horseradish peroxidase mixture for 15 min at room temperature. After incubation, the membrane was washed and detected using an imaging system (Princeton Instruments, NJ, USA).

Statistical analysis
A one-way ANOVA was used to determine if the means were statistically significantly different (P < 0.05). If means were significantly different, a Tukey–Kramer post hoc multiple group comparison test was used to compare individual groups. Error bars in figures represent ± standard error of mean.

Results
Effect of the herbs on lipopolysaccharide-induced interleukin-10 expression

In our previous study, GGS was demonstrated to upregulate LPS-induced IL-10 [24]. The GGS is a mixture of ten crude plant ingredients. We tried to investigate the effect of GGS and ten crude plants on LPS-induced IL-10 at RAW264.7 cell line by ELISA [Figure 1]. The result showed that GGS with the dosage more than 100 μg/mL and 10 μg/mL of LJF have significantly upregulated IL-10 expression at 24-h in a dose-dependent manner. However, the difference between another nine herb groups and the LPS-induced group was not statistically significant.

Effects of gingyo-san and Lonicerae japonica flos on pro-inflammatory cytokines and interleukin-10 mRNA expression in lipopolysaccharide-stimulated RAW264.7 cells

We compared 100 μg/mL GGS with the same dosage of LJF in the mRNA of pro-inflammatory cytokines and IL-10 by RT-PCR [Figure 2]. The data demonstrated that GGS and LJF significantly suppressed the pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, and iNOS) and upregulated IL-10. GGS is more effective than LJF in the expression of pro-inflammatory cytokines. However, LJF obviously promoted IL-10 mRNA more than GGS. The results indicated that LJF could be a major herbal ingredient of GGS on promoting the LPS-induced IL-10. In our previous publication, HPLC was employed to examine the methanol-soluble components of the LJF preparation [25]. The principal components were identified as CGA (0.0528 mg/mL) and LUT 0.00456 mg/mL. The concentration ratio of CGA and LUT is almost 10:1.

The effect of chlorogenic acid and luteolin on lipopolysaccharide-induced interleukin-10 and interleukin-6 expression

LPS induced a massive increase of IL-10 and IL-6 by ELISA. Pretreatment of LUT significantly suppressed the LPS-induced IL-6, but had no effect on IL-10 [Figure 3b and d]. In contrast, CGA can upregulate the LPS-induced IL-10 and IL-6 in a dose-dependent manner [Figure 3a and c].
The synchronizing action of chlorogenic acid and luteolin on lipopolysaccharide-induced interleukin-10 and interleukin-6 expression

We investigated the synchronizing action of CGA (10 µM) and LUT (1 µM) on LPS-induced IL-10 and IL-6 expression by ELISA. CGA and CGA+ LUT promoted the LPS-induced IL-10 expression, whereas no effect was observed in the LUT-treated group. The levels of IL-6 in the CGS-treated group seem increased, but the difference between the LPS-treated group was not statistically significant [Figure 4a]. Respectively, IL-6 decreased significantly in LUT and CGA+ LUT groups compared with the LPS group [Figure 4b]. By integrating the two results, it can be concluded that CGA could promote LPS-induced IL-10 and IL-6 and LUT could suppress IL-6 only.

The synchronizing action of chlorogenic acid and luteolin on lipopolysaccharide-induced nuclear factor-κB, SP-1, C/EBPβ, and δ activation

The expression of Sp1 and C/EBPβ and δ, in nuclear extracts prepared from RAW264.7 cells treated with LPS for 6 h, was investigated by using immunoblot analysis. No difference was observed in Sp1 expression between control and LPS-treated cells [Figure 5a]. CGA at 1 and 10 µM enhanced LPS-induced Sp1 expression, but the effect of LUT on Sp1 expression was not different between CGA-treated cells [Figure 5a]. Increase of C/EBPβ and δ expression was observed in cells treated with LPS for 6 h [Figure 5a]. CGA at 1 and 10 µM enhanced LPS-induced C/EBPβ and δ expression but LUT inhibited LPS-induced C/EBPβ and δ expression. The synchronizing action of CGA and LUT on C/EBPβ and δ expression in LPS-treated cells demonstrated that LUT reduced CGA and LPS-enhanced C/EBPβ and δ expression in a dose-dependent manner [Figure 5a].

We also investigated the synchronizing action of CGA and LUT on LPS-induced NF-κB activation. Our data showed that LPS significantly induced NF-κB activation and CGA enhanced LPS-induced NF-κB activation [Figure 5b]. However, LUT has an inhibitory effect on LPS-induced and CGA-enhanced NF-κB activation in a dose-dependent manner [Figure 5a].

The synchronizing action of chlorogenic acid and luteolin on lipopolysaccharide-induced IκB pathway and mitogen-activated protein kinase pathway

For understanding the mechanism of the adaptive action of CGA and LUT, we studied the IκB and MAPK pathways that could affect NF-κB, SP-1, C/EBPβ, and δ activation. CGA enhanced LPS-induced phosphorylation of IκB, ERK1/2, p38, and JNK in a dose-dependent manner; however, LUT reduced the phosphorylation of IκB, ERK1/2, p38, and JNK in a dose-dependent manner [Figure 6a]. In the adaptive action of CGA and LUT, LUT could abolish the LPS-induced or CGA-enhanced phosphorylation of IκB and p38. LUT did not influence the LPS-induced and CGA-enhanced phosphorylation of ERK1/2 and JNK [Figure 6b].

Discussion

In this study, we analyzed the immunomodulatory effect of GGS and its components which enhanced LPS-induced IL-10 expression. In the event, L. japonica, a component of GGS, could induce the expression of IL-10. In addition, we found that CGA and LUT, the ingredients of L. japonica, have an adaptive effect on the increased LPS-induced IL-10 expression and the
decreased LPS-induced IL-6 expression. We also demonstrated that LUT could abolish the LPS-induced or CGA-enhanced phosphorylation of IκB, ERK1/2, p38, and JNK. As a consequence, LUT has inhibitory effect on LPS-induced and CGA-enhanced NF-κB and C/EBPβ and δ activation.

LJF, also called Jinyinhua, is a widely used herb prescribed in many Chinese formulas. It has latent heat-clearing, antipyretic, detoxicant, and anti-inflammatory actions [27]. LJF not only significantly promotes blood neutrophil activity but also increases the neutrophil phagocytosis at its proper concentrations [28]. Some studies suggested that the aqueous extract of LJF flower may function as a therapeutic agent for inflammatory disease through a selective regulation of NF-κB activation [29]. In a previous study, we also demonstrated that GGS, containing L. japonica, enhances LPS-induced IL-10 expression and suppresses IL-6 expression [24]. In this study, we showed that the LJF may play a major role in the inhibitory effect of TNF-α, IL-6, iNOS and in the enhancement of IL-10.

It has been reported from in vivo and in vitro experiments that CGA mostly presents antioxidant and anticarcinogenic activities [22,30]. Some reports demonstrated that CGA inhibited the peripheral synthesis/release of inflammatory mediators, such as TNF-α and NO, but it did not inhibit the febrile response induced by LPS [31]. On the other hand, CGA has proven to augment the inducible cytokine messages, i.e., IL-10, IL-13, IL-6, TNF-α, and IFN-γ in mast cells [23]. Another compound, LUT, has been reported to decrease the production of pro-inflammatory mediators in LPS-stimulated macrophages, fibroblasts, and intestinal epithelial cells [12,14,32], and it also suppressed LPS-stimulated TNF-α and IL-6 in a murine macrophage cell line [12]. We identified two compounds (CGA and LUT) from LJF. We demonstrated that CGA enhanced LPS-induced IL-10 and IL-6, and LUT inhibited IL-6, but did not influence the expression of IL-10. Our results also showed that the adaptive action of CGA and LUT inhibited LPS-induced IL-6 and enhanced LPS-induced IL-10. Those results of the adaptive action of CGA and LUT may explain the effect of LJF on the increased LPS-induced IL-10 expression and the decreased LPS-induced IL-6 expression.

A previous study reported that the transcription factors Sp1 and C/EBPβ and δ are critical for LPS-induced expression of IL-10 in mouse macrophages, and the increase in protein and DNA binding of C/EBPβ and δ could activate IL-10 gene expression [10]. On the other hand, NF-κB is an important response element located upstream of the IL-6 transcriptional initiation site, and the inhibition of NF-κB led to a remarkable downregulation of IL-6 gene and protein expression [33]. Those transcription factors are regulated by NF-κB and MAPK signal pathways, including ERK, JNK, and p38 [10,33]. Our data show that CGA enhanced the LPS-induction of the transcription factor Sp1, C/EBPβ and δ. Consequently, the expression of IL-10 and IL-6 increased. LUT suppressed the
LPS-induced NF-κB binding ability and reduced the expression of IL-10. LUT did not influence the transcription factors Sp1 and C/EBPβ and δ. We also demonstrated the adaptive action of CGA and LUT on the suppression of the phosphorylation of IκB and p38, but not of ERK1/2 and JNK.

**CONCLUSION**

As illustrated in Figure 7, our present results indicate that CGA enhanced the LPS-induced expression of IL-10 and IL-6, and increased NF-κB, Sp1, C/EBPβ, and δ. The effect of CGA was interfered with LUT by suppressing the phosphorylation of IκB and p38 and downregulated NF-κB activity. In the event, IL-6 was suppressed and IL-10 was not influenced.

**Figure 7:** Schematic of adaptive immunoregulation of LUT and chlorogenic acid in LPS-induced IL-10 expression. CGA enhanced LPS-induced expression of IL-10 and IL-6 and increased the expression of NF-κB, Sp1, C/EBPβ, and δ. The effect of CGA was interfered with LUT by suppressing the phosphorylation of IκB and p38 and downregulated NF-κB activity. In the event, IL-6 was suppressed and IL-10 was not influenced. LPS: Lipopolysaccharide, CGA: Chlorogenic acid, LUT: Luteolin, NF-κB: Nuclear factor-κB

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**Conflict of interest**

There are no conflicts of interest.

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