Oleanolic acid regulates NF-κB signaling by suppressing MafK expression in RAW 264.7 cells

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INTRODUCTION

Inflammation, a physiological response to infection or tissue injury, plays a crucial role in many chronic diseases and various human cancers (1, 2). Nuclear factor-κB (NF-κB) is a transcription factor with multiple genetic targets that controls various cellular functions, including immune inflammatory responses, cell adhesion, differentiation, apoptosis, stress-induced responses, survival, and progression of most chronic diseases (3, 4). Because NF-κB activation has been shown to elicit chemoresistance in certain tumors, it is a promising novel target for tumor therapy (5-7).

Nrf2 is a member of the CNC-basic leucine zipper (CNC-bZIP) family of transcription factors and coordinately regulates the constitutive and inducible expression of antioxidant and phase II detoxification enzymes such as heme oxygenase-1 (HO-1) and NAD (P)H:quinone oxidoreductase 1 (NQO1) via cis-acting ARE DNA elements (8, 9). These cytoprotective genes can rapidly neutralize, detoxify, and remove invading xenobiotics. Accumulating evidence shows that Nrf2 is also a promising target for cancer chemoprevention (10, 11).

Recent evidence has suggested crosstalk between NF-κB and Nrf2 under oxidative stress. These two pathways are proposed to inhibit each other at their transcription level via protein-protein interactions or through secondary messenger effects. Thus, identification of factors that regulate crosstalk between NF-κB and Nrf2 has garnered recent interest (12-14). As part of those studies, we recently identified a novel function of MafK, which can act as a modulator of NF-κB by inducing CBP-mediated p65 acetylation in vitro and in vivo (15). Inactivation of the MafK pathway may represent an effective genetic approach against oxidative-stress-induced diseases.

Several anti-inflammatory or anti-oxidative phytochemicals suppress NF-κB signaling and activate the Nrf2-ARE pathway (16-18) suggesting that the suppression of NF-κB signaling and the activation of the Nrf2-ARE pathway may crosstalk with each other. In previous studies, we found that Prunella vulgaris var. lilacina contains various compounds which are flavonoids, triterpenoids, phenolic acids such as camphorol, rutin, rosmarinic acid (RA), caffeic acid (CA), ursolic acid (UA), oleanolic acid (OA) and tannins that provide a great assortment of biological properties (21).

Thus, the purpose of this study was to determine the anti-inflammatory effects of major compounds of P. vulgaris var. lilacina including their inhibitory effects on inflammatory mediators and regulatory effects on NF-κB and Nrf2 signaling. In addition, we also examined whether major compounds of P. vulgaris var. lilacina modulates MafK expression to regulate NF-κB signaling.
RESULTS

Cytotoxicity of *P. vulgaris* var. *lilacina* compounds in RAW 264.7 cells

The cytotoxicities of several compounds were measured in RAW 264.7 cells by using MTT assay. Cells were treated with 4 compounds (RA, OA, UA, and CA) at various concentrations (0, 10, 25, and 50 μM) for 1 h and then co-incubated with lipopolysaccharides (LPS; 1 μg/ml) for an additional 24 h. As shown in Fig. 1, in groups treated with 25 and 50 μM compounds, the viability range of RAW 264.7 cells after exposure was 43% to 91%. In case of 10 μM treated group, cell viability was not significantly affected by the compounds. Therefore, these compounds were used at 10 μM in subsequent experiments.

Inhibition of LPS-induced nitric oxide (NO) and prostaglandin E2 (PGE2) production in RAW 264.7 cells

To determine whether the compounds inhibit LPS-induced NO production, RAW 264.7 cells were pretreated with each compound for 24 h and then stimulated with LPS (1 μg/ml). After 24 h of stimulation, the cell medium was harvested and the production of NO was measured using a Griess assay. As shown in Fig. 2A, we found that LPS treatment significantly increased NO production to 26.1 μM; however, RA, OA, UA, and CA reduced NO production by 46.2%, 30.0%, 37.3%, and 27.3%, respectively. We also evaluated the effects of the compounds on PGE2 production in LPS-stimulated RAW 264.7 cells. As shown in Fig. 2B, stimulation with LPS (1 μg/ml) for 24 h in the absence of the compounds caused RAW 264.7 macrophages to produce PGE2 at a concentration of 3.4 ng/ml. However, RA, OA, UA, and CA significantly reduced the production of LPS-induced PGE2 by 21.8%, 42.8%, 28.4%, 38.1%, respectively.

Inhibition of NF-κB activity and Nrf2 and NF-κB target gene expression

NF-κB activation is necessary for pro-inflammatory responses (22, 23). Thus, we next examined the effects of the compounds on NF-κB activation by performing a luciferase activity assay. As shown in Fig. 3A, LPS treatment in the absence of the compounds induced NF-κB activity, while pretreatment with RA, OA, UA, and CA decreased NF-κB activity.

Because Nrf2 is known to suppress inflammation and NF-κB is known to induce inflammation (24-27), we examined the possibility that the compounds activate and inhibit Nrf2 and NF-κB signaling, respectively. First, we examined whether the compounds induced the expression of Nrf2 target genes such as...
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Fig. 3. Effect of major compounds from Prunella vulgaris var. lilacina on NF-κB activity (A) and Nrf2 (B) and NF-κB (C)-dependent gene expression in LPS-induced RAW 264.7 cells. (A) The NF-κB-responsive-element-Luc reporter was transfected in RAW 264.7 cells. After 24 h, cells were treated with LPS and 10 μM of compounds. After 24 h, cells were lysed for the luciferase assay. (B and C) RAW 264.7 cells were treated with compounds 10 μM. After treatment for 24 h, RNA was isolated and reverse-transcribed for RT-PCR analyses. The bars represent the means ± SEM of 3 experiments performed in triplicate. Statistical significance was determined relative to a control by the Student’s t-test (*P < 0.05 compared with the control group). RA, rosmarinic acid; OA, oleanolic acid; UA, ursolic acid; CA, caffeic acid.

NQO1 and HO-1. The RAW 264.7 cells were treated with each of the 4 compounds. Total RNA was extracted from the treated cells and Nrf2, NQO1, and HO-1 mRNA expression was analyzed using real-time reverse-transcription polymerase chain reaction (RT-PCR). As shown in Fig. 3B, expression of these 3 genes was induced by each of the 4 compounds. Next, we determined the effect of the compounds on NF-κB target gene expression. As shown in Fig. 3C, each of the 4 compounds inhibited the expression of NF-κB target genes, including those encoding TNFα, cIAP2, and IkBα. Taken together, these results show that the 4 compounds are associated with Nrf2 activation and NF-κB suppression.

The effect of the compounds on MafK-mediated NF-κB signaling
In a previous study, we showed that MafK is a novel modulator of NF-κB. Inactivation of the MafK expression would be regulating the NF-κB signaling (15). Here, we investigated whether the compounds from P. vulgaris var. lilacina could modulate MafK expression. As shown in Fig. 4A, OA significantly decreased MafK expression, while the other compounds had no effect. Therefore, among major compounds of P. vulgaris var. lilacina, OA could be expected to regulate the expression of MafK as a NF-κB inhibitor.

To determine whether the OA-mediated inhibition of MafK proteins affected NF-κB signaling, we examined IkBα phosphorylation and its subsequent degradation. As shown in Fig. 4B, OA-treated group and knockdown of MafK group did not significantly inhibit TNFα-induced IkBα degradation or phosphorylation. However, we found that OA-treated group in MafK knockdown cell inhibited IkBα phosphorylation, which was synergistically inhibited by the OA. And as previously reported, we confirmed that MafK knockdown cell were decreased the p65 acetylation. Because OA reduced the MafK expression, OA-treated group would be reducing the p65 acetylation by reduced MafK. In addition, when simultaneously OA and siMafK treat to cell, p65 acetylation did not observe. Taken together, we suggest that OA regulates NF-κB signaling via regulation of MafK expression.

DISCUSSION
In our previous study, we demonstrated that P. vulgaris var. lilacina has significant anti-inflammatory effects through inhibiting the release of inflammatory mediators and cytokines, which might be due to its blocking of the NF-κB signaling pathway (20). And also, we identified that a novel function of MafK, which can act as a modulator of NF-κB (15). As a following study, we focused on anti-inflammatory and MafK regulating effect of major compounds of P. vulgaris var. lilacina.

The Nrf2- NF-κB pathway is a viable therapeutic target for protection against oxidative stress in various diseases. Several Nrf2
activating and NF-κB inhibiting small molecules have been identified (28-33). Curcumin, a polyphenolic compound that acts via Nrf2, is suggesting that curcumin-activated Nrf2 modulates the expression and transactivation of NF-κB p65 protein levels, DNA-binding activity and promoter activity of NF-κB, increase in Nrf2 overexpressing cells in the presence of curcumin (28). Phenethyl isothiocyanate (PEITC) and sulforaphane attenuated LPS-induced NF-κB activation via inhibit IκBα phosphorylation and p65 NF-κB subunit nuclear translocation, consequently alleviating NF-κB signaling (29) and it activates Nrf2, possibly by modifying the sensor cysteines present in Keap1 (30, 31). The EGCG inhibited activation of NF-κB and AP-1 thereby suppressing the COX-2 induction in mouse skin in vivo and/or cultured human mammary epithelial (MCF10A) cells (32). EGCG also upregulated antioxidant enzymes by activating the Nrf2-ARE signaling pathway (33). In the present study, we evaluated the regulatory effects of major compounds such as RA, OA, UA and CA of P. vulgaris var. lilacina on these signaling pathways and the expression of pro-inflammatory mediator, such as NO and PGE2 in LPS-stimulated RAW 264.7 macrophages. The data obtained from our current study indicated that RA, OA, UA and CA significantly inhibited LPS-induced NO and PGE2 production in RAW 264.7 cells. In addition, we found that RA, OA, UA and CA exert their inhibitory effects on these inflammatory responses via the suppression of NF-κB in the LPS-stimulated RAW 264.7 cells. Taken together, these data suggest that RA, OA, UA and CA from P. vulgaris var. lilacina contributes to anti-inflammatory effects by suppressing NF-κB activity.

The multitude of possible interactions in the Nrf2- NF-κB pathway makes the design of novel Nrf2- NF-κB pathway regulators especially difficult. Nevertheless, we identified MafK as the new regulator of Nrf2- NF-κB pathway. To find new chemicals for regulation of Nrf2- NF-κB pathway via inhibiting the MafK expression, based on the results of the present study, we evaluated the inhibition of MafK expression by RA, OA, UA and CA compounds. In the results, we confirmed a single compound “OA” with greatly inhibition than other compounds. OA significantly suppressed MafK expression and subsequently activate IκBα phosphorylation, inhibit the p65 acetylation. Taken together, these results suggest that the suppression of MafK expression by OA is mediated by the inhibition of NF-κB activation.

In conclusion, our results suggest that OA, a major compound of P. vulgaris var. lilacina, exerts potent anti-inflammatory effects by inhibiting LPS-induced NO, PGE2 in RAW 264.7 cells. These effects are mediated by inhibition of NF-κB activity via suppression of Nrf2 expression. These findings suggest that OA as NF-κB inhibitors can potentially be used in therapeutic applications for the treatment of oxidative stress-induced diseases.

MATERIALS AND METHODS

Reagents
Rosmarinic acid, oleanolic acid, ursolic acid, caffeic acid and LPS from Escherichia coli O55:B5 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco’s Modified Eagle’s Medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin were obtained from Invitrogen (Carlsbad, CA, USA). Antibodies against IκBα, p-IκBα, and β-actin were purchased from Cell Signaling Technology (Danvers, MA, USA). Monoclonal antibodies against MafK and Acetyl-p65 (K310) were purchased from Promega (Madison, WI, USA). All other chemicals were purchased from Sigma unless otherwise specified.

Cell culture and viability
The RAW 264.7 macrophage cell line was purchased from the Korean Cell Line Bank (Seoul, Korea). RAW 264.7 cells were cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin at 37°C in 5% CO2. To investigate the cell vi-
ability of compounds cells (1 × 10^5 cells/well) were added to duplicate 96-well plates and incubated for 24 h, then treated with various concentrations (10, 25, and 50 μM) of compounds for 24 h. Cell viability was measured using MTT assay.

NO Measurement
Nitrite was measured as an indicator of NO production after 48 h of treatment and LPS induction. The culture supernatant (100 μl) was placed in a 96-well plate, and an equal amount of Griess reagent (2% sulfanilamide and 0.2% N-1-naphthyl) ethylenediamine dihydrochloride in 5% H₃PO₄ was added. The plate was then incubated for 10 min and the absorbance measured at 540 nm. The amount of NO was calculated using a sodium nitrite standard curve.

PGE2 Measurement
After 12 h of treatment and LPS stimulation, the culture supernatant was collected. PGE2 was measured using a PGE2 ELISA kit following the manufacturer’s instructions (Abcam). Briefly, the diluted cell supernatant (100 μl) was placed in a 96-well goat anti-mouse IgG-coated plate and incubated for 2 h. After incubation, the plate was washed using the provided washing buffer, and the color was developed by adding PNP (200 μl) substrate after 45 min. The amount of PGE2 was calculated using a PGE2 standard curve.

Transfection of RAW 264.7 cells with pNF-κB luciferase vector
RAW 264.7 cells were transiently transfected with a pNF-κB-luciferase vector by using Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. Briefly, 5 × 10^5 cells were placed in a 24-well plate and allowed to grow to 80-90% confluence for 24 h. The cells were then treated with DNA-Transfect reagent mixture (50 μl) and incubated for 2 h. The transfected cells were collected after 24 h, and the luciferase activity was measured using a PGE2 standard curve.

Real-time RT-PCR analysis
To determine the expression levels of Nrf2, NQO1, HO-1, TFNα, cIAP2, and IkeB, mRNAs, real-time RT-PCR was performed using a Qiagen Rotorgene Q thermal cycler (Valencia, CA, USA) according to the manufacturer’s instructions. Primers were designed using Primer3 software. Primer sequences were as follows: GAPDH, 5′-GAGGACAGTGAATTTCACCATG-3′ (forward), 5′-TTGGGGA ATGTGGGCAAC-3′ (reverse); NQO1, 5′-CAGCTCGCCAGGAC CGTATG-3′ (forward), 5′-GAGTGACCCCTGACATCAGT-3′ (reverse); HO-1 5′-GGGTGATAGAAGAGGCCAAGA-3′ (forward), 5′-AGCTCCTGCAACTCTCCTAAA-3′ (reverse).

Immunoblotting
Cells were lysed in radioimmunoprecipitation assay buffer (150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 7.5, and 2 mM EDTA) on ice for 30 min. After centrifugation at 4°C for 20 min (12,000 × g), the supernatant was collected. Protein concentrations were determined using a BCA assay (GenDEPOT; Rockford, IL USA) according to the manufacturer’s instructions.

Transfection of small interfering RNA (siRNA)
Human siRNA oligonucleotides were purchased from Integrated DNA Technologies. RAW 264.7 cells were transfected with siRNA oligos by using Lipofectamine RNAi max (Invitrogen). Twenty-four hours post-transfection, cells were treated with LPS (1 μg/ml), and were then harvested. The siRNA sense oligo sequences were as follows: control, 5′-UUCUCCGAACGUGUCUCGUCA CGUTT-3′; siMafK, 5′-GCCATACGCGUUUGTCAUTT-3′.

Statistical analysis
Statistical analyses were performed with SPSS statistical software (version 12.0). The data represent means ± standard errors of the mean from 3 independent experiments unless otherwise stated. Statistical analyses were performed by the Student’s t-test at a significance level of P < 0.05.

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