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Article

Anti-inflammatory activity of quercetogetin isolated from Citrus unshiu peel involves suppression of NF-κB and MAPK signaling pathways in LPS-induced RAW264.7 macrophages

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Abstract: Citrus peel has been used in Asian traditional medicine for the treatment of cough, asthma, and bronchial disorders. However, the anti-inflammatory effect of quercetogetin (QUE), a polymethoxylated flavone isolated from the peel of citrus unshui is poorly understood. We investigated the anti-inflammatory effect and the molecular mechanisms of QUE in lipopolysaccharide (LPS)-induced RAW264.7 cells. QUE inhibited the production of NO and prostaglandin E2 by suppressing the LPS-induced expression of inducible nitric oxide synthase and cyclooxygenase-2 at both the mRNA and protein levels. QUE suppressed the production of proinflammatory cytokines, such as interleukin (IL)-1β, IL-6, and tumor necrosis factor-α. QUE also inhibited the translocation of the nuclear factor kappa B subunit, p65, into the nucleus by interrupting the phosphorylation of IκB-α in LPS-induced RAW 264.7 cells. Based on the finding that QUE significantly decreased p-ERK protein expression in LPS-induced RAW264.7 cells, we confirmed that suppression of the inflammatory process by QUE was mediated through the MAPK pathway. This is the first report on the strong anti-inflammatory effects of QUE, which is a compound that can potentially be used as a therapeutic agent for inflammatory diseases.

Keywords: quercetogetin (QUE), anti-inflammatory, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), MAPK, NF-κB

1. Introduction

Inflammation is required for a healthy immune response, but it is also responsible for the pathogenesis of several diseases. Lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria, is a well-known and important proinflammatory factor that can cause endotoxemia, shock, and multiple organ dysfunction syndromes [1]. Macrophages play an important role in regulating inflammatory responses by generating proinflammatory mediators. Inflammatory stimuli, such as LPS activation of macrophages, lead to activation of the nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways, which promote the expression of several proinflammatory cytokines, including interleukin (IL)-1β and IL-6 and tumor necrosis factor (TNF)-α, as well as other inflammatory mediators, including nitric oxide (NO) and prostaglandin (PG)E_2, which are synthesized by inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2), respectively [2-4].

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NF-κB is arrested in the cytoplasm by the inhibitor kappa B (IκB) in normal conditioned cells. Upon activation by LPS, IκB-a is phosphorylated and degraded, resulting in free NF-κB, which is then translocated to the nucleus [5]. The translocation of NF-κB into the nucleus triggers the expression of proinflammatory genes, such as *iNOS* and *COX*-2 [6]. Additionally, activation of the MAPK pathway also plays an essential role in the initiation and development of inflammatory processes that are transmitted by sequential phosphorylation events. The three major groups of MAPK cascades are the extracellular-signal-regulated kinase (ERK), p38, and JNK. Each MAPK is activated by its upstream activation of MAPK kinase (MKK) and MAPK kinase kinase. Once activated by kinases, MAPK can phosphorylate transcription factors or other downstream kinases that produce the expression of proinflammatory mediators of extracellular stimuli [7, 8].

The highest concentrations of polymethoxylflavones (PMFs) are found in citrus peel, with much lower amounts found in citrus juice [9, 10]. Nobiletin (NOB) and tangeretin (TAN) are two PMFs that are relatively common in citrus peel [11]. Several studies have been performed on the PMF content of citrus fruits [12, 13]. NOB and sinensetin are isolated from sweet orange flavedo (*Citrus sinensis*), whereas TAN has been isolated from tangerine (*Citrus reticulata*). Six PMFs have been isolated from orange essential oil, and their structures have been characterized by the absence of a hydroxyl group [14]. Citrus fruit-derived flavonoids and their metabolites have been shown to participate in significant protective biological activities, including anticancer, antiviral, anti-inflammatory, and antiatherogenic activities [15-17]. However, pharmacological studies of the biological effects of PMFs compounds have been limited.

In the present study, NOB, TAN, and QUE were isolated from extracts of citrus unshiu (Satsuma mandarin) peel (CUP). Because the biological activities, including the anti-inflammatory effect of QUE (Figure 1), have not been reported, we characterized the anti-inflammatory effects and molecular mechanisms of QUE in LPS-induced RAW264.7 cells.

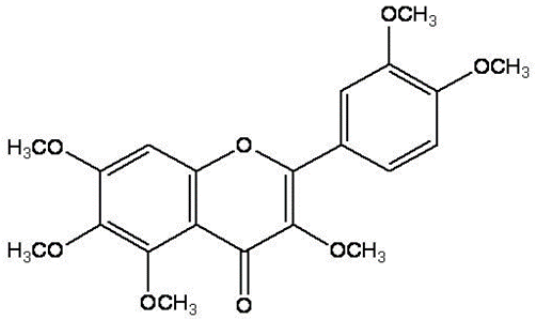

Figure 1. Chemical structure of quercetogetin (QUE, 3,5,6,7,3',4'-hexamethoxyhomoflavone).

2.1. Effect of QUE on cell viability of LPS-induced RAW264.7 cells

Raw264.7 cells were treated with various concentrations of QUE (Figure 2), and the cell viability was measured using the MTT assay. The survival curve shown in Figure 2 shows that QUE at 1, 10, and 100 μM did not exhibit cytotoxic effects on the proliferation of the Raw264.7 cells.

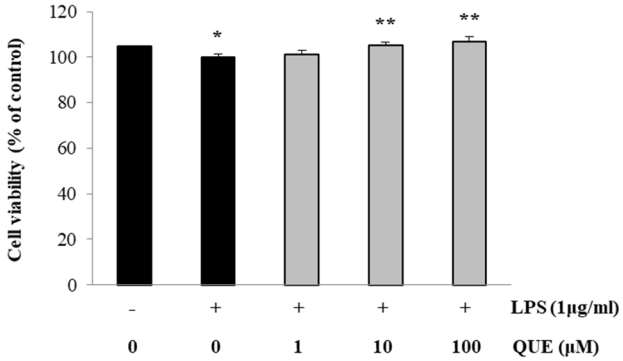

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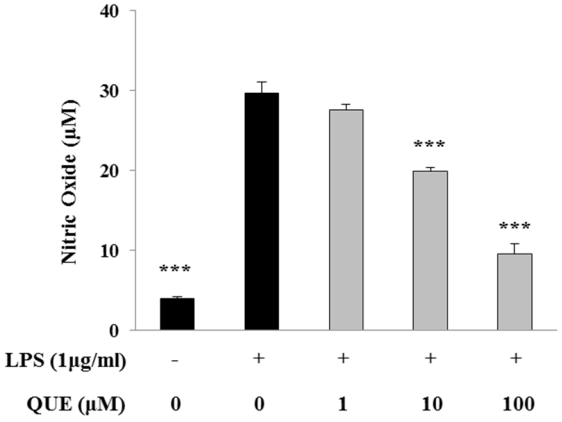
Figure 2. Effect of QUE on the cell viability in LPS-induced RAW264.7 cells. The cells were stimulated with 1μg/ml LPS and treated with various concentrations (1, 10, and 100μM) of QUE for 24h. The data shown represent the mean ± S.D. derived from three determinations. Statistically significant (* P<0.05, ** P<0.01, *** P<0.001) compared with LPS-treated group.

2.2. QUE inhibits production of NO by suppressing iNOS expression in LPS-induced RAW264.7 cells.

To examine the effects of QUE on NO production, we measured the levels of nitrite in the medium using the Griess reaction. As shown in Figure 3a, QUE (1, 10, or 100 μM) inhibited LPS-induced NO production in a dose-dependent manner. To identify the molecular mechanism by which QUE inhibited NO production in response to LPS, the mRNA and protein expression levels of iNOS were determined by reverse transcriptase-polymerase chain reaction (RT-PCR) and western blot analysis, respectively. The results showed that the iNOS mRNA and protein expression levels in RAW264.7 cells were increased via LPS induction. Conversely, QUE inhibited LPS-induced expression of iNOS mRNA and protein in a dose-dependent manner (Figure 3b and 3c). These results suggested that QUE inhibition of NO production in LPS-induced RAW264.7 cells was associated with inhibition of iNOS expression.



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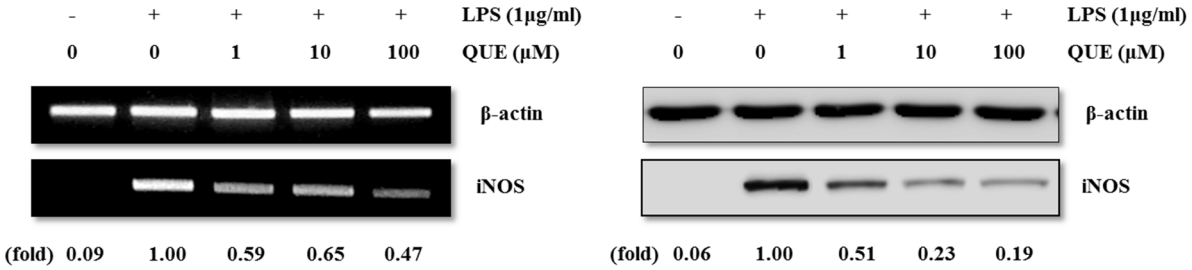

(b) (C)

Figure 3. Effects of QUE on NO production and iNOS expression in LPS-induced RAW264.7 cells. The cells were induced with 1μg/ml LPS and treated with various concentrations (1, 10, and 100μM) of QUE for 24h. (a) The nitric oxide production in culture supernatants were measured by Griess assay. The data shown represent the mean±S.D. derived from three determinations. Statistically significant (* P<0.05, ** P<0.01, *** P<0.001) compared with LPS-treated group. The expression of iNOS (b) mRNA and (c) protein was determined by RT-PCR and Western blot analysis, respectively.

2.3. QUE inhibits production of PGE_2 by suppressing COX-2 expression in LPS-induced RAW264.7 cells.

As PGE_2 is another important inflammatory mediator, we examined the effects of QUE on PGE_2 production in LPS-induced RAW264.7 cells. QUE inhibited LPS-induced PGE_2 production in a

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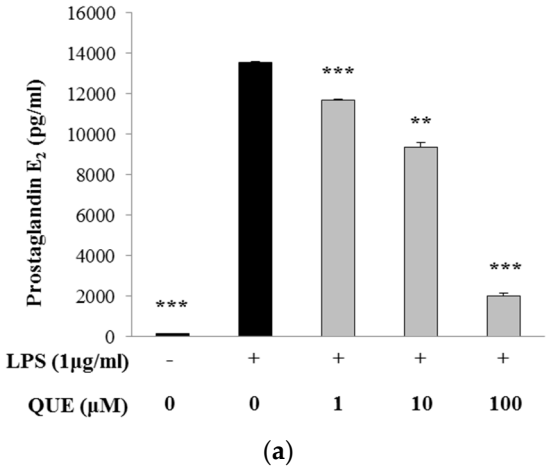
dose-dependent manner (Figure 4a). To identify the molecular mechanism by which QUE inhibited PGE_2 production in response to LPS, the mRNA and protein expression levels of COX-2 were determined by RT-PCR and western blot analysis, respectively. As shown in Figure 4b and 4c, the COX-2 mRNA and protein expression levels in RAW264.7 cells were increased by LPS induction. Conversely, QUE inhibited the LPS-induced expression of COX-2 mRNA and protein in a dose-dependent manner. These results suggested that the QUE-induced inhibition of PGE_2 production in LPS-induced RAW264.7 cells was associated with the inhibition of COX-2 expression.



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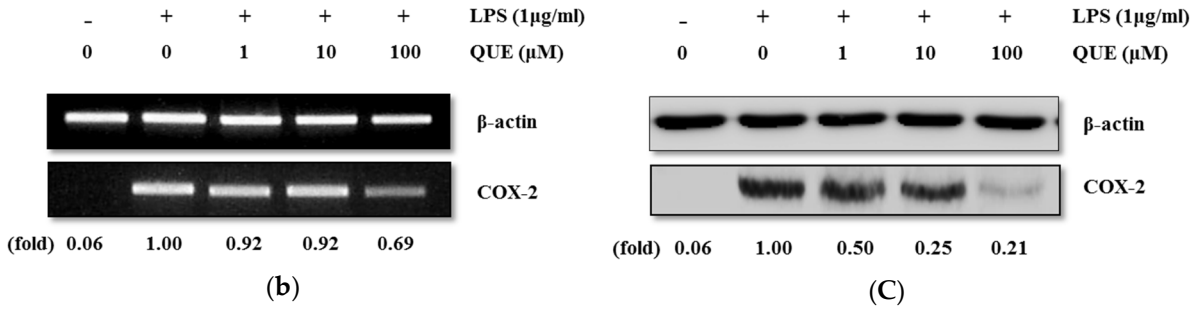

(b) (C)

Figure 4. Effects of QUE on PGE2 production and COX-2 expression in LPS-induced RAW264.7 cells. The cells were induced with 1ug/ml of LPS and treated with various concentrations (1, 10, or 100uM) of QUE for 24h. (a) The PGE2 production in cultures supernatants were measured by enzyme-immunoassay. The data shown represent the mean±S.D. derived from three determinations. Statistically significant (* P<0.05, ** P<0.01, *** P<0.001) compared with LPS-treated group. The expression of COX-2 (b) mRNA and (c) protein was determined by RT-PCR and Western blot analysis, respectively..

2.4. QUE inhibits production and mRNA expression of proinflammatory cytokines in LPS-induced RAW264.7 cells.

LPS induction increases the production of proinflammatory cytokines, such as IL-1b, IL-6, and TNF-a, which play important roles in pathogen-induced inflammatory responses. We used an ELISA and RT-PCR analysis to characterize the effects of QUE on the production of proinflammatory cytokines. Figure 5 shows that treatment with 100 μM QUE resulted in a significantly reduction in LPS-induced IL-1b, IL-6, and TNF-a cytokine levels. Additionally, we also determined whether QUE regulated cytokine production at the level of transcription. The mRNA levels of cytokines increased in LPS-induced cells, but treatment with QUE decreased this increase. Taken together, these results suggested that QUE prevented production of proinflammatory cytokines by suppressing gene expression.

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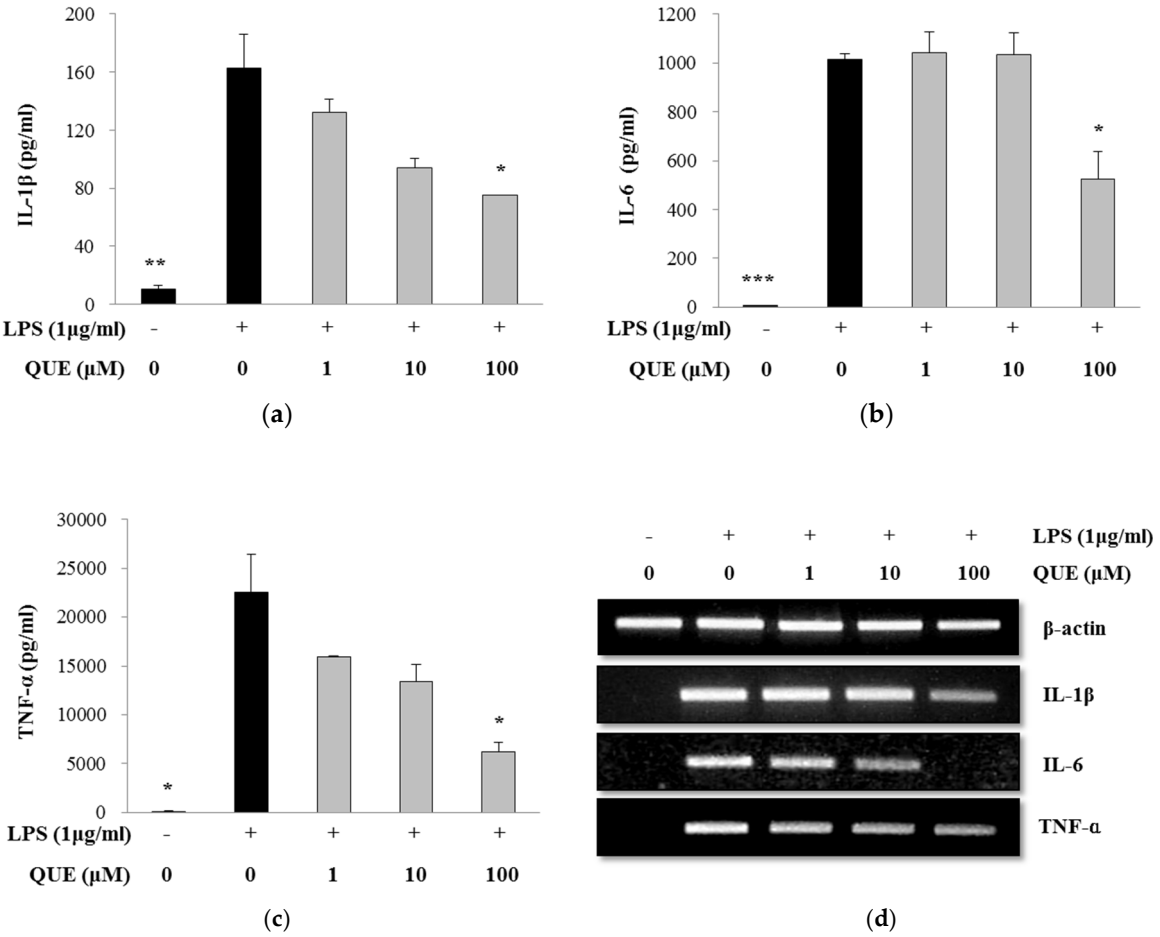

Figure 5. Effects of QUE on IL-1β, IL-6, and TNF-α production on their mRNA expressions in LPS-induced RAW264.7 cells. The cells were induced with 1ug/ml LPS and treated with various concentrations (1, 10, or 100uM) of QUE for 24h. The production of (a) IL-1β, (b) IL-6, and (c) TNF-α in the culture media of LPS-stimulated cells were measured using the ELISA kit. The data shown represent the mean ± S.D. derived from three determinations. Statistically significant (* P<0.05, ** P<0.01, *** P<0.001) compared with LPS-treated group. (d) The expression of IL-1β, IL-6, and TNF-α mRNA was determined by RT-PCR.

2.5. QUE inhibits degradation of IκB-α and NF-κB binding activity in LPS-induced RAW264.7 cells.

NF-κB is an important transcription factor complex that controls the expression of proinflammatory mediators, such as iNOS, COX-2, TNF-α, and ILs [18]. To determine whether QUE regulated the NF-κB pathway, we determined the phosphorylation and degradation of IκB-α using western blot analysis. The results showed that QUE inhibited the phosphorylation and degradation of IκB-α in LPS-induced RAW264.7 cells (Figure 6a). Because p65 is the major subunit of the NF-κB heterodimer, we investigated the translocation of p65 from the cytoplasm to the nucleus after release from IκB-a. As shown in Figure 6b, QUE significantly attenuated the LPS-induced nuclear translocation of p65. Overall, these results suggested that the NF-κB signaling pathway might be involved in the regulation of inflammatory factors by QUE in RAW264.7 cells.

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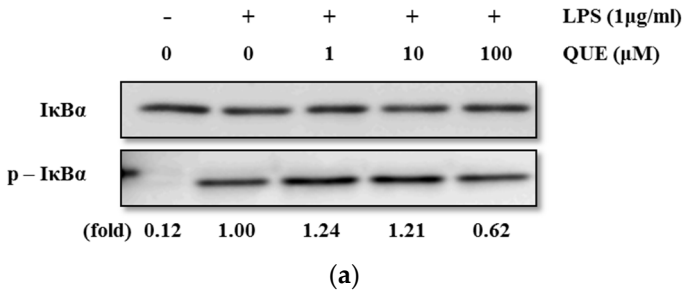

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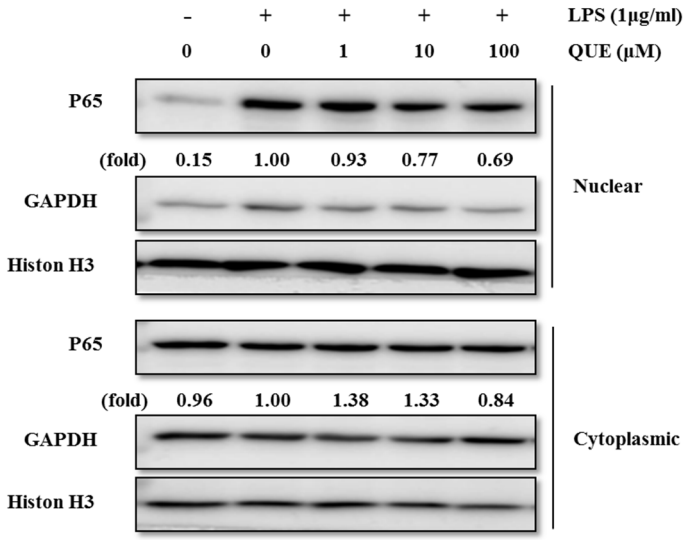

(b)

Figure 6. Effects of QUE on the phosphorylation of IκBα and the nuclear translocation of NF-κB in LPS-induced RAW264.7 cells. The cells were pre-treated for 2h with various concentrations (1, 10, or 100uM) of QUE, and induced for 30min with 1ug/ml of LPS. (a) Whole-cell lysates were prepared and subjected to western blot analysis with anti-IκBα antibody, and anti-phospho-IκBα. (b) Cytosolic and nuclear extracts were prepared and subjected to western blot analysis with anti-p65 antibody for NF-κB p65 subunit..

2.6. QUE inhibits phosphorylation of ERK and MAPK in LPS-induced RAW264.7 cells.

MAPK is an important regulator of inflammatory mediators, including NO and proinflammatory cytokines [19]. LPS activates MAPK cell signaling pathways in RAW264.7 cells [20]. To determine whether the suppression of inflammatory reactions by QUE was mediated through the MAPK pathway, we determined the effects of QUE on the LPS-induced phosphorylation of ERK, JNK, and p38 MAPK in LPS-induced RAW264.7 cells. Figure 7 show that LPS increased the activation of all three MAPK molecules. However, the phosphorylation of ERK was significantly and dose-dependently decreased by QUE, whereas the phosphorylation of p38 and JNK was not affected.

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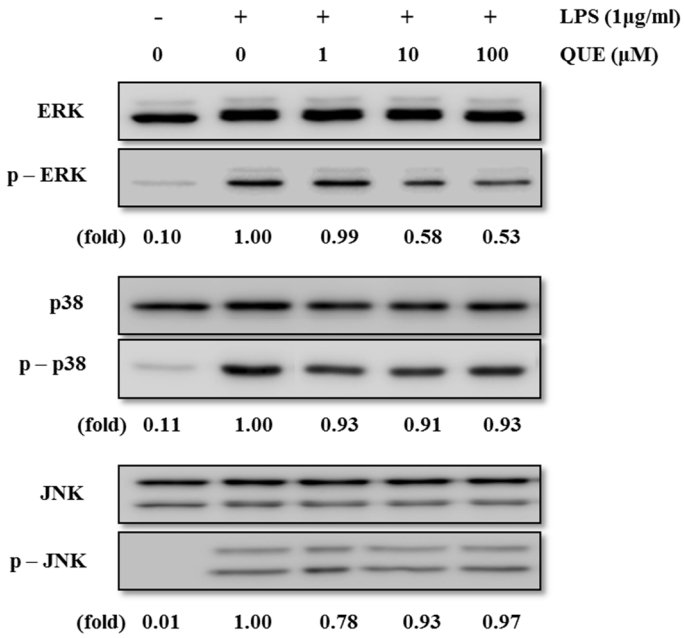

Figure 7. Effects of QUE on the phosphorylation of MAPK molecules in LPS-induced RAW264.7 cells. The cells were pre-treated for 2h with various concentrations (1, 10, or 100μM) of QUE, and induced for 30min with 1μg/ml of LPS. Total proteins from cells were used for detection of phosphorylated or total forms of three MAPK molecules, ERK, p38, and JNK MAPK..

3. Discussion

CUP has been used in Asian traditional medicine for the treatment of cough, asthma, and bronchial disorders. It is a rich source of flavanones and many PMFs, which are found in smaller quantities in other plants [21].

In the present study, we isolated and characterized compounds from extracts of CUP that inhibited NO production and had anti-inflammatory effects, and we identified the underlying molecular mechanisms in LPS-induced RAW 264.7 cells. By screening NO production from CUP extracts, we isolated PMF compounds, such as NOB, TAN, and QUE, from chloroform layers. These compounds inhibited NO production in LPS-induced RAW264.7 cells in a dose-dependent manner.

PMFs are usually flavone aglycones with ≥ four methoxyl substituents. NOB and TAN belong to this class of flavonoids, which are commonly found in CUP. These compounds not only play important physiological and ecological roles but are also of commercial interest because they have multiple applications in the food and pharmaceutical industries. Recent studies have reported that some PMFs participate in potent biological activities, including anti-cancer and anti-inflammatory activities [22-24]. However, the human health-related activities of one of these compounds, QUE, including its anti-inflammatory effects, has not been previously characterized. In the present study, we therefore characterized the anti-inflammatory mechanism of this compound.

The inflammation response is a complex reaction of the immune system that is regulated by many inflammatory mediators, such as NO, PGE2, and cytokines. Overproduction of iNOS-derived NO and COX-2-derived PGE2 can cause chronic diseases involving pathological processes, especially in inflammatory and autoimmune disorders [25]. These mediators play a regulatory role in a variety of physiological and pathological processes following immune responses and inflammation [26]. The inhibition and control of inflammatory mediators are therefore useful targets for the development of novel anti-inflammatory agents. In the present study, we showed that QUE (10–100 μM) inhibited iNOS and COX-2 expression, thereby suppressing iNOS-induced NO and COX-2-induced PGE2 production. Moreover, QUE reduced LPS-induced IL-1β, IL-6, and TNF-α expression in RAW264.7 cells. These results suggested that QUE exerted its anti-inflammatory effects via the suppression of inflammatory enzymes and mediators, such as iNOS, COX-2, NO, and PGE2, and of proinflammatory cytokines, such as IIL-1β, IL-6, and TNF-α.

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The key anti-inflammatory targets include enzymes, cytokines, and transcription factors, such as NF-κB and MAPKs [27]. Several studies have reported that NF-κB has a significant role in regulating the expression of inflammation-associated enzymes and cytokine genes that contain NF-κB binding motifs within their respective promoters [28]. The transcription factor, NF-κB, has been implicated in the regulation of many genes that code for mediators of immune, acute-phase, and inflammatory responses, including iNOS and COX-2 [29]. The p50/p65 heterodimer is the most common dimer found in the NF-κB signaling pathway [30]. During basal conditions, NF-κB is sequestered in the cytoplasm by inhibitor proteins, usually IκB. But when released, NF-κB dimers translocate to the nucleus to activate target genes by binding with high affinity to κB elements in their promoters [31]. We examined the effects of QUE on IκB-α phosphorylation and NF-κB (p65) nuclear translocation in RAW264.7 cells. As shown in Figure 4, the phosphorylation and degradation of IκB-α and the nuclear translocation of p65 induced by LPS were significantly reduced after pretreatment with QUE. These results suggested that QUE regulated inflammatory reactions through the inhibition of NF-κB signaling.

The MAPKs perform critical roles in controlling cellular responses to stress and activating NF-κB [32]. The MAPKs are a family of protein serine/threonine kinases, including ERK, p38, and JNK. The MAPKs react to extracellular stimuli and control a variety of cellular activities, including gene expression, differentiation, apoptosis, and inflammation. MAPKs regulate inflammatory and immune responses [33, 34], and the MAPK signaling pathways are known to be involved in expressing iNOS and COX-2 as well as in producing proinflammatory cytokines in LPS-induced macrophages [35]. The inhibition of any one of the ERK, p38, and JNK pathways would be sufficient to block the induction of proinflammatory mediators by LPS [36]. The ERK pathway plays a key role in transducing chronic inflammatory articular pain [37]. Because ERK is known to be involved in the regulation of IL-6, IL-12, IL-23, and TNF-α synthesis [38], the results suggested the possible involvement of ERK in the joint damage associated with the proinflammatory cytokine production by macrophages. The p38 is generally considered to be the most promising MAPK therapeutic target for inflammatory diseases, because p38 isoforms have been implicated in the regulation of many inflammatory processes, such as the production of cytokines and proinflammatory mediators [39]. JNK is a member of the MAPK family, is activated by environmental stress and some proinflammatory cytokines, and has an important role in immune system signaling [40]. To investigate whether the suppression of inflammatory reactions by QUE was mediated through the MAPK signaling pathway, we assessed the effect of QUE on the LPS-induced phosphorylation of ERK, JNK, and p38 in RAW264.7 cells. QUE significantly suppressed the LPS-induced phosphorylation of ERK in a dose-dependent manner, but it did not suppress the phosphorylation of p38 and JNK (Figure 7).

In conclusion, QUE was isolated from CUP, and its anti-inflammatory effects, which were mediated through the inhibition of the NF-κB and ERK MAPK signaling pathways, were demonstrated in RAW264.7 macrophage cells. These findings suggested that QUE may be a potential therapeutic agent for the treatment of inflammatory diseases.

4. Materials and Methods

4.1. Chemicals and reagents

The RAW264.7 murine macrophage cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). Dulbecco's Modified Eagle's Medium (DMEM), 100 U/mL penicillin, 100 μg/mL streptomycin, and fetal bovine serum (FBS) were purchased from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). LPS from *Escherichia coli* 0111: B4), Griess reagent, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The ELISA kits for PGE2, IL-1β, IL-6, and TNF-α were purchased from R&D Systems (Minneapolis, MN, USA). The antibodies against iNOS, COX-2, β-actin, and anti-IgG-HRP rabbit polyclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibodies against Nf-κB (p65), IκB-α, p-IκB-α, ERK, p-ERK, p38, p-p38, JNK, and p-JNK were purchased from Cell Signaling Technology (Danvers, MA, USA).

4.2. Extraction and isolation of quercetogetin (3,5,6,7,3',4'-hexamethoxyhomoflavone, QUE)

C. unshiu peel (CUP) was harvested along the coast of Jeju Island, Korea. in 2010. The dried of CUP (3 kg) were extracted three times with 95% (v/v) ethyl alcohol overnight at room temperature. The EtOH (838g)

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extracts was evaporated and suspended in distilled water and divided into two fractions with chloroform as the nonaqueous phase. The chloroform fraction (155.9g) was separated by liquid chromatographic column (15 × 40 cm) packed with silica gel (230-400 mesh) using gradient mixtures of CHCl3-MeOH gradients (from CHCl3-MeOH =100/1 to 1:1, v/v) as mobile phases to give 9 fractions, F01 – F09. Nine collective fractions were obtained after TLC monitoring. F04 (50.2g) was subjected by liquid chromatographic column (15 × 40 cm) packed with silica gel (230-400 mesh) using gradient mixtures of Hexan-EtOAc gradients (from Hexan:EtOAc=80:1 to 1:3, v/v) to afford 14 sub-fractions (F04-1~14). The active fraction F04-11 (271mg) were fractionated by solid-phase extraction (SPE) using a MeOH 100% as a solvent system to afford on active compound 3 (3,5,6,7,3',4'-hexamethoxyhomoflavone, 9.48mg). The structure of the isolated QUE was elucidated by spectroscopic (^{1}H-NMR and ^{13}C-NMR) data after comparison with published data [11] (Supplemental Figure S1-S3).

4.3. Cell culture

The RAW264.7 murine macrophage cells were incubated at 37°C in 5% CO_2 in DMEM supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% FBS. The cells were also treated with 0.02% DMSO as a vehicle control.

4.4. Cell viability

The number of viable cells was determined by the ability of mitochondria to convert MTT to formazan dye. The cells were treated with various concentrations (0, 1, 10, or 100 µM) of QUE for 24 h. At the end of the incubation, 10 µL of MTT solution [5 mg/mL in phosphate-buffered saline (PBS)] was added to each well. After incubation at 37°C for 4 h, the medium was gently removed, and 100 µL of DMSO was added. The absorbance was then determined at 570 nm (Tecan, Salzburg, Austria).

4.5. NO measurements

The nitrite that accumulated in the culture medium was measured as an indicator of NO production based on the Griess reaction. The cells were induced with 1 µg/mL of LPS and treated with various concentrations (0, 1, 10, or 100 µM) of QUE for 24 h. Briefly, 100 µL of cell culture medium was mixed with 100 µL of Griess reagent (Sigma-Aldrich) and incubated at room temperature for 20 min. The absorbance was measured at 550 nm, and the nitrite concentration was determined using sodium nitrite as a standard.

4.6. Prostaglandin E_2, IL-1β, IL-6, and TNF-α assays

RAW264.7 cells seeded in 24-well plates were treated with various concentrations of QUE and/or LPS (1 µg/mL) for 24 h. The concentrations of PGE2, IL-1β, IL-6, and TNF-α in the culture medium were determined using ELISA kits (R&D Systems) according to the manufacturer's instructions.

4.7. Preparation of cytosolic and nuclear extracts

Macrophage cells (1.8×10^6) were plated in 60-mm cell culture plates, pretreated with different concentrations of QUE or DMSO for 1 h, and then stimulated with LPS for 30 min. The cells were washed twice with ice-cold PBS, scraped in PBS, and centrifuged at 12,000 × *g* for 5 min at 4°C. The pellets were suspended in 180 µL of hypotonic buffer A (10 mM Tris-HCl, 10 mM NaCl, 3 mM MgCl2, 0.02% NaN3, 0.5 mM DTT, and 1 mM PMSF, pH 7.4), and 20 µL of 5% Nonidet P-40 was added and incubated for 5 min on ice. The mixture was centrifuged at 1,800 × *g* for 5 min, and the supernatant (cytosolic extract) was collected. The pellets were washed with hypotonic buffer and resuspended in hypertonic buffer C [20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.02% NaN3, 0.5 mM DTT, and 1 mM PMSF, pH 7.4] for 1 h on ice and then centrifuged at 14,000 × *g* for 10 min. The supernatant containing nuclear proteins was collected and stored at a -70°C after determination of the protein concentration.

4.8. Western blotting

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To determine if the effects of QUE on NO and PGE2 production were related to the modulation of iNOS and COX-2 induction and expression levels, we evaluated the effects of QUE on the activation of the NF-κB and MAPK signaling pathways. The medium was removed, the cells were washed with PBS, and they were then scraped in 300 mL of 2× Laemmli sample buffer. Cell debris was removed by micro-centrifugation, followed by quick freezing of the supernatants. The protein concentration was determined using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Prior to polyacrylamide gel electrophoresis, lysates were boiled for 10 min, followed by centrifugation at 12,000 rpm for 5 min at 4°C. They were separated by 12% SDS-polyacrylamide gel electrophoresis, and the separated proteins were transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% (w/v) nonfat skim milk powder in TBST (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20) for 1 h at room temperature. After three washes in TBST, the membranes were incubated overnight with primary antibodies at a dilution of 1:1,000 in TBST containing 5% nonfat milk at 4°C. After three washes in TBST, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody at a 1:5,000 dilution. Bound antibodies were detected using an enhanced chemiluminescence kit (Amersham, Little Chalfont, UK) following the manufacturer's instructions. The loading amount of each well was normalized using anti-actin antibody.

4.9. RT-PCR

Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RT-PCR was carried out by using an Access RT-PCR System kit (Invitrogen) with indicated primers (iNOS, COX-2, and proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α, and β-actin). PCR was performed for 30 cycles in 25 μL of reaction mixture, and the products were visualized in 1.0% agarose gels stained with ethidium bromide. β-actin was used as a housekeeping gene where indicated.

4.10. Statistical analysis

The data are expressed as the mean ± standard deviation (SD) from at least three independent experiments. One-way analysis of variance was used to determine the statistical significance of differences between different groups. P values < 0.05 were considered statistically significant.

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/xxx/s1.

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Abbreviations

LPS	lipopolysaccharide
IL	interleukin
iNOS	inducible nitric oxide synthase
COX-2	cyclooxygenase-2
NF-κB	nuclear factor kappa B
MAPK	mitogen-activated protein kinase
TNF-α	tumor necrosis factor alpha
IκB	inhibitor kappa B
PMFs	polymethoxylflavones
QUE	quercetogetin, 3,5,6,7,3',4'-hexamethoxyhomoflavone

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