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Quercetin negatively regulates TLR4 signaling induced by lipopolysaccharide through Tollip expression

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ABSTRACT

Polyphenolic compounds have been regarded as one of the most promising dietary agents for the prevention and treatment of inflammation-related chronic diseases; however, the anti-inflammatory activities of flavonoids, such as quercetin, are not completely characterized, and many features remain to be elucidated. In this study, we showed the molecular basis for the downregulation of TLR4 signal transduction by quercetin. Quercetin markedly elevated the expression of the Toll-interacting protein, a negative regulator of TLR signaling. Lipopolysaccharide-induced expression of cell surface molecules (CD80, CD86, and MHC class I/II) and production of pro-inflammatory cytokines (tumor necrosis factor-α, IL-1β, IL-6, and IL-12p70) were inhibited by quercetin, and this action was prevented by Toll-interacting protein silencing. In addition, quercetin-treated macrophages inhibited lipopolysaccharide-induced activation of mitogen-activated protein kinases, such as extracellular signal-regulated kinase 1/2, p38, and c-Jun N-terminal kinase, and the translocation of nuclear factor-κB and p65 through Toll-interacting protein. Treatment with quercetin resulted in a significant decrease in prostaglandin E_2 and cyclooxygenase-2 levels as well as inducible nitric oxide synthase-mediated nitric oxide production induced by lipopolysaccharide. Taken together, these findings represent new insights into the understanding of negative regulatory mechanisms of the TLR4 signaling pathway and effective therapeutic intervention for the treatment of inflammatory disease.

1. Introduction

Inflammation is a process by which the human body attempts to counteract potential injurious agents, such as invading bacteria and viruses, and is an adaptive response that is triggered by noxious stimuli and conditions, such as infection and tissue injury [1–3]. Inflammation is increasingly considered to be involved in the development of many human diseases, including arteriosclerosis, obesity, diabetes, and even cancer, and the inflammatory response is coordinated by a large range of complex regulatory networks, which underlies a wide variety of physiological and pathological processes [4]. In macrophages, lipopolysaccharide (LPS) is the best stimuli characterized to induce the pro-inflammatory mediators (e.g., prostaglandin E_2 (PGE_2), cytokines, and cyclooxygenase (COX)-2) [5–8]. It has become clear that LPS shows activation of nuclear factor κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling as well as an increase in surface molecule expression (e.g., CD80, CD86, and major histocompatibility complex (MHC) class II) in macrophage [9,10], and suppression of these signaling cascades might be based on the anti-inflammation effect. Among various inflammatory mediators, toll-like receptors (TLRs) are pathogen-recognition proteins that play a central role in initiating the inflammatory response [11]. The intracellular signaling pathways activated by TLRs are mediated through several adaptor molecules, including myeloid differentiation factor 88, ultimately leads to degradation of inhibitory κB (Iκ-B) and translocation of nuclear factor κB (NF-κB) to the nucleus [11–13]. Negative regulation of TLR signaling by suppressor of cytokine signaling 1 (SOCS1), Toll-interacting protein (Tollip), and interleukin (IL)-1 receptor-associated kinase (IRAK)-M, is mediated through its ability to potently suppress the production of pro-

Abbreviations: LPS, lipopolysaccharide; Tollip, toll-interacting protein; TLR, toll-like receptor; MHC, major histocompatibility complex; Iκ-B, inhibitor of κB; NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor-α; IL, interleukin; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; SOCS1, suppressor of cytokine signaling 1; IRAK, IL-1 receptor-associated kinase; PGE_2, prostaglandin E_2; COX, cyclooxygenase; iNOS, nitric oxide synthase; NO, nitric oxide; ELISA, enzyme-linked immunosorbent assay; SDS, sodium dodecyl sulfate.

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inflammatory mediators induced by the activity of IL-1 receptor associated kinase (IRAK) [14]. Among them, Tollip, a negative regulator of TLR signaling, also associates directly with TLR4 and plays an inhibitory role in TLR-mediated cell activation induced by LPS. Various polyphenolic compounds have been found to possess a wide range of physiological activities in the fields of nutrition and medicine. One of the most important of these beneficial effects is the ability of some phenolic compounds to counter-regulate the chronic inflammatory states [15,16]. Recently, it has become clear that some polyphenolic compounds derived from grape and green tea extract are considered to be an important target for the prevention and treatment of inflammation-related chronic diseases [17–19]. In particular, several polyphenolic compounds, such as quercetin, which represents the most abundant dietary flavonoid found in a broad range of fruits, vegetables and beverages, possess anti-inflammatory action through the inhibition of NF-κB-mediated pro-inflammatory cytokines, inducible nitric oxide synthase (iNOS), and COX-2 genes [20,21]. While the above reports indicate that quercetin plays critical roles in anti-inflammatory activity through the inhibition of various pro-inflammatory mediators, exactly how these components are mechanistically interrelated with a negative regulator of TLR signaling is unclear.

2. Materials and methods

2.1. Materials

Quercetin was purchased from Sigma–Aldrich (St. Louis, MO). LPS from *Escherichia coli* O111:B4 was purchased from Invivogen (San Diego, CA). The fluorescein isothiocyanate (FITC)-annexin V/propidium iodide (PI) kit was purchased from R&D Systems (Minneapolis, MN). iNOS polyclonal anti-body (Ab), COX-2 polyclonal Ab, anti-TLR4 polyclonal Ab, anti-CD14 polyclonal Ab, anti-phosphorylated extracellular signal-regulated kinase (ERK)1/2 monoclonal Ab, anti-phosphorylated c-Jun N-terminal kinase (JNK) monoclonal Ab, anti-phosphorylated p38 monoclonal Ab, anti-NF-κB (p65) polyclonal Ab, anti-phosphorylated inhibitor of κB (IκB)-α monoclonal Ab, anti-SOCS1 polyclonal Ab, anti-IRAK-M monoclonal Ab, anti-Tollip monoclonal Ab, horseradish peroxidase (HRP)-conjugated anti-goat donkey IgG Ab, and anti-lamin B polyclonal Ab were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). HRP-conjugated anti-mouse IgG Ab and HRP-conjugated anti-rabbit Ab were obtained from Calbiochem (San Diego, CA), and anti-β-actin monoclonal Ab (AC-15) was purchased from Sigma–Aldrich. APC-conjugated monoclonal Ab to F4/80, phycoerythrin (PE)-conjugated monoclonal Ab to CD80, CD86, and MHC class I/II were purchased from eBioscience (San Diego, CA). IL-6, IL-1β, IL-12p70, and TNF-α enzyme-linked immunosorbent assay (ELISA) kits were obtained from BD Biosciences (San Diego, CA).

2.2. Cell culture

RAW264.7 cells were purchased from the Korean Cell Line Bank (Seoul, Republic of Korea). RAW264.7 cells were maintained in DMEM (GIBCO, Carlsbad, CA, USA) containing 10% FBS (GIBCO), 100 U/mL penicillin and 100 U/mL streptomycin (complete medium) under humidified condition at 37 °C and 5% CO_2 in an incubator. In all experiments, cells were allowed to acclimate for 24 h before any treatments.

2.3. Construction of Tollip-suppressed cells

Tollip short hairpin RNA expression vector was purchased from Santa Cruz Biotechnology. shRNA plasmids consist of a pool of three to five entiviral vector plasmids each encoding target-specific 19–25 nt (plus hairpin) shRNAs designed to knockdown gene expression. For each transfection, we added 0.8 mL shRNA plasmid transfection medium to well and then incubated the cells for 7 h. We further performed the neomycin selection for obtaining stably transfected cells.

2.4. Cytotoxicity analysis

The quercetin (100 μM) was added to cultures of RAW264.7 cells in 12-well plate (0.5×10^6 cells/mL). To investigate the cytotoxic effect of quercetin on RAW264.7 cells, the cell death pattern of RAW264.7 cells was analyzed after treatment with quercetin. After 24 h of treatment, harvested RAW264.7 cells were washed with PBS and stained by FITC-annexin V and propidium iodide (BD Biosciences). Thereafter, cytotoxicity of RAW264.7 cells was analyzed by FACSCanto flow cytometry (BD Biosciences).

2.5. Measurement of nitric oxide (NO) production

The concentration of NO in culture supernatants was determined by measuring its oxidation product, nitrite, using the Griess method. Briefly, supernatants from experimental macrophage cultures were mixed with the Griess reagent (1:1) and incubated at room temperature for 15 min, and then, the absorbance of the solution at 517 nm was measured using a microplate reader (Zenyth 3100; Anthos Labtec Instruments GmbH). $NaNO_2$ freshly prepared in deionized water was used to generate a standard curve (0–100 μM) to calculate the nitrite concentration in cell culture supernatants.

2.6. ELISA

Supernatants from experimental RAW264.7 cells cultures were collected and stored at −70 °C until use. The levels of IL-12p70, IL-6, IL-1β, and TNF-α in the supernatants were determined using cytokine detection ELISA kits (BD Biosciences) according to the manufacturer's instructions, with detection at 450 nm using a microplate reader. The concentration of PGE_2 in cell culture supernatants was determined by using a PGE_2 detection ELISA kit (Cayman Chemicals, Ann Arbor, MI, USA) following the manufacturer's instructions.

2.7. Measurement of cell surface molecules by flow cytometry

RAW264.7 cells were harvested, washed with PBS, and resuspended in washing buffer (2% FBS and 0.1% sodium azide in PBS) for fluorescence-activated cell sorting (FACS) on the FACSCanto flow cytometer (BD Biosciences). The cells were pre-incubated with 0.5% BSA in PBS for 30 min and washed with PBS. The cells were then stained with PE-conjugated anti-I-Ab (MHC class I/II), anti-CD80, and anti-CD86, along with APC-conjugated anti-F4/80 (BD Biosciences) for 45 min at 4 °C. All antibodies were diluted 100-fold before use. Cells were washed 3 times and resuspended in a final volume of 500 μL of PBS. Fluorescence was measured by flow cytometry, and the data were analyzed using the CellQuest data analysis software.

2.8. Immunoblotting analysis

Cells were lysed in 100 μL lysis buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Triton-X100, 1 mM EDTA, 50 mM NaF, 30 mM Na_4PO_7, 1 mM phenylmethanesulfonyl fluoride (PMSF), 2 μg/mL aprotinin, and 1 mM pervanadate. Whole-cell lysates were resolved by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and then transferred onto a nitrocellulose membrane. The membranes were blocked in 5% skim milk and incubated with the respective Ab for 2 h, followed

by incubation with HRP-conjugated secondary Ab for 1 h at room temperature. Epitopes on target proteins, including mitogen-activated protein kinases (MAPKs) and NF-κB, recognized specifically by the used Abs were visualized using the ECL advance kit (GE Healthcare, Little Chalfont, UK).

2.9. Nuclear extract preparation

Nuclear extracts from cells were prepared as follows. Cells were treated with 100 μL lysis buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol (DTT), 0.5 mM PMSF) on ice for 10 min. Following centrifugation at 4000 rpm for 5 min, the pellet was re-suspended in 100 μL extraction buffer (20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF) and incubated on ice for 30 min. After centrifugation at 12,000 rpm for 10 min, the supernatant containing nuclear extracts was collected and stored at −80 °C until required.

2.10. Statistical analysis

All experiments were repeated at least 3 times with consistent results. The levels of significance for comparison between samples were determined by Tukey's multiple comparison test distribution using statistical software (GraphPad Prism Software, version 4.03; GraphPad Software, San Diego, CA). The data in the graphs are expressed as the mean ± SEM. Each value of $^{*}p < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$ was considered to be statistically significant.

3. Results

3.1. Cytotoxicity of quercetin

We examined quercetin-induced cytotoxicity in RAW264.7 cells. As shown in Fig. 1A, treatment with quercetin did not result in any cellular toxicity against macrophages. This finding suggests that, at concentrations below 100 μM, quercetin is not cytotoxic to RAW264.7 cells and does not contain significant amounts of endotoxin that would potentially interfere with our studies using concentrations below 100 μM.

3.2. Quercetin up-regulates Tollip protein

To elucidate additional mechanisms underlying the quercetin-mediated anti-inflammatory activity, we examined whether

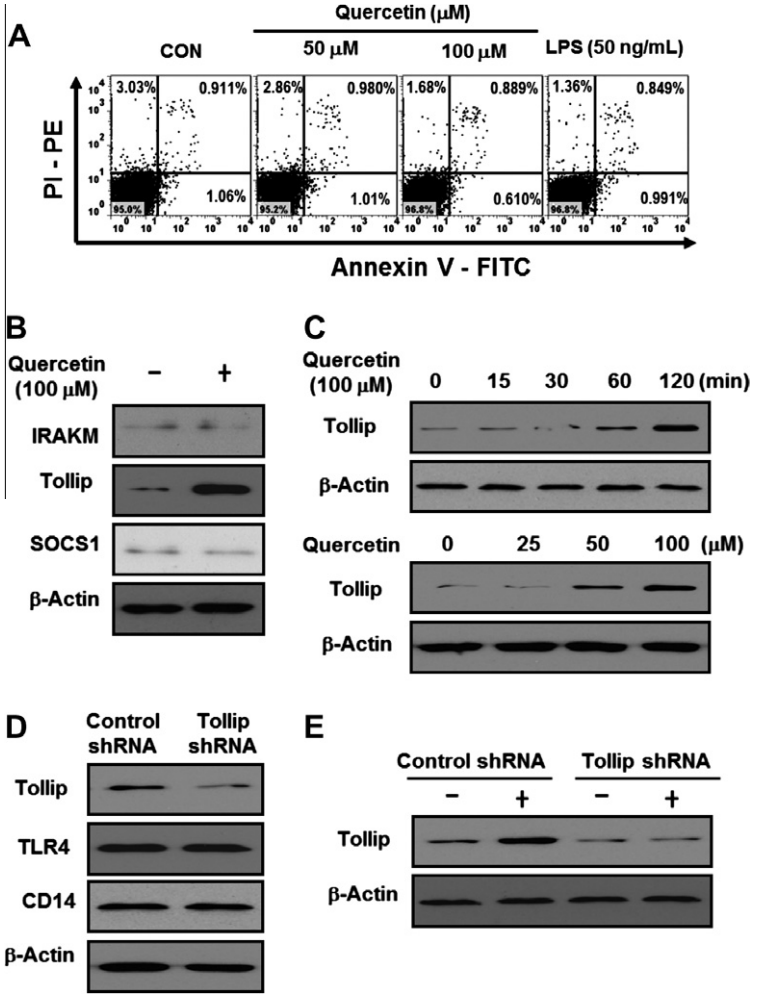

Fig. 1. Quercetin upregulates Tollip protein expression. (A) RAW264.7 cells were treated with quercetin for 24 h, and cell viability was analyzed by flow cytometry. RAW264.7 cells were stained with annexin V, and PI. The percentage of positive cells (annexin V- and PI-stained cells) in each quadrant is indicated. The results are representative of three experiments. (B) RAW264.7 cells were treated with 100 μM quercetin for 1 h. Total cellular proteins were resolved by SDS–PAGE, and the separated proteins were transferred to nitrocellulose membranes and detected using specific SOCS1, IRAK-M, or Tollip Abs. The data are shown as mean ± SD ($n = 3$) values. (C) RAW264.7 cells without LPS stimulation were treated with quercetin at 100 μM for the indicated period and dose. (D) RAW264.7 cells were transfected with the Tollip shRNA vector. Protein levels of Tollip, TLR4, and CD14 were detected by immunoblotting. (E) Cells were treated with 100 μM quercetin for 1 h. Total cellular proteins were resolved by SDS–PAGE, and the separated proteins were transferred to nitrocellulose membranes and detected with anti-Tollip Ab.

quercetin induces negative TLR regulators. We found that the protein level of Tollip in control cells was upregulated by quercetin treatment, whereas the levels of other proteins were not affected (Fig. 1B). Furthermore, we found that quercetin elevated the expression of Tollip in a time- or dose- dependent manner in LPS-untreated control cells (Fig. 1C). These results indicate that 100 μM quercetin transcriptionally enhances the expression of Tollip, a negative regulator of TLR4, and the anti-inflammatory actions of quercetin may be due, in part, to the upregulation of Tollip protein. Next, to establish whether Tollip is indeed involved in the suppressive effect of quercetin on LPS-induced inflammatory responses, we used stable RNAi to silence Tollip expression in RAW264.7 cells. Immunoblot analysis indicated that stable RNAi against Tollip specifically silenced Tollip protein expression without affecting the expression of TLR4 and CD14 (Fig. 1D). This result suggested that the silencing of Tollip did not affect the expression

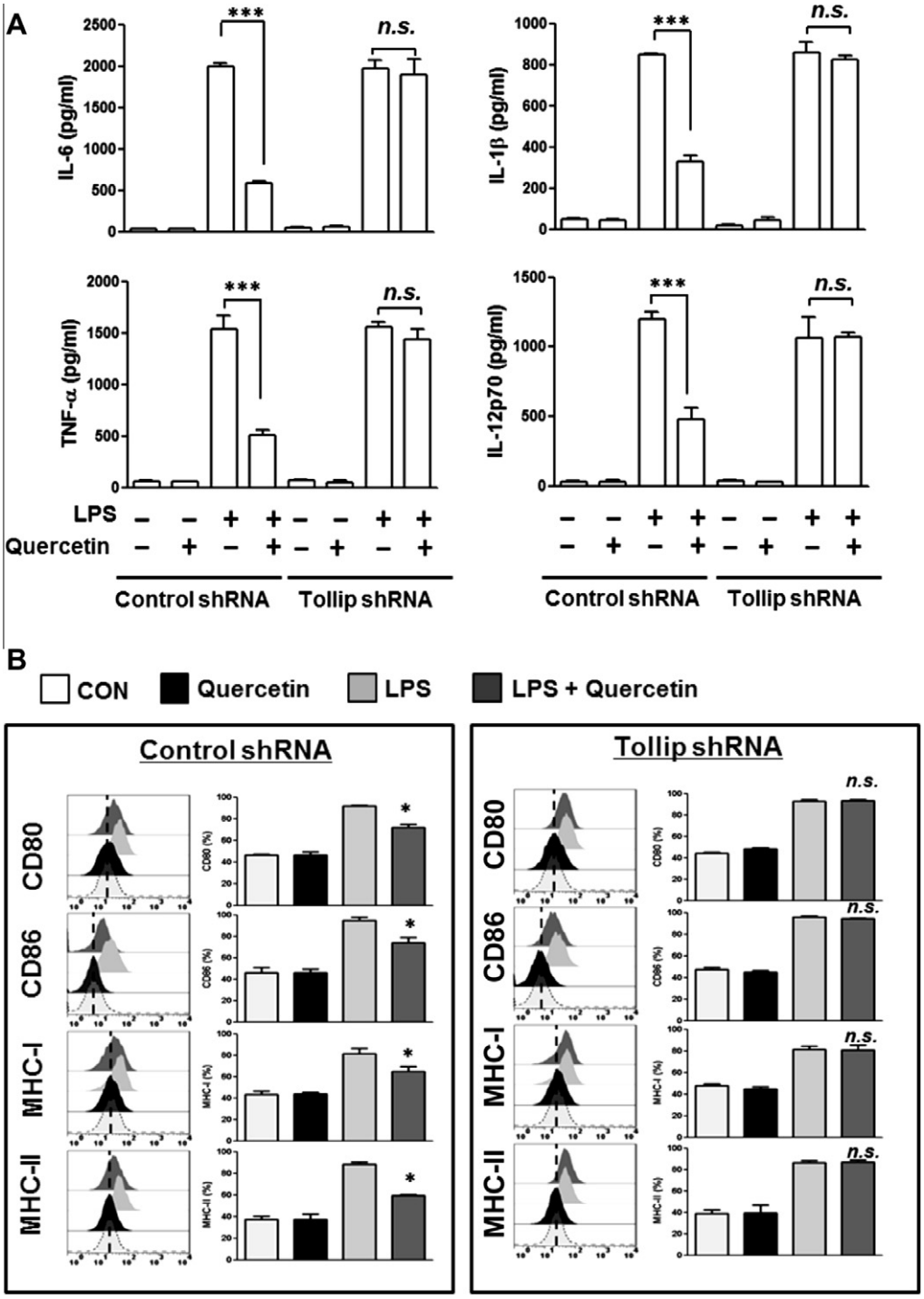

Fig. 2. Quercetin suppresses the activation of RAW264.7 cells through Tollip. (A and B) RAW264.7 cells were pretreated with quercetin (100 μM) for 1 h before exposure to LPS (50 ng/mL) for 24 h. (A) The concentrations of TNF-α, IL-6, IL12p70, and IL-1β in the culture medium were measured by ELISA. (B) The cells were gated for F4/80. RAW264.7 cells were stained with anti-CD80, anti-CD86, or anti-MHC class I/II. All the data are expressed as the mean ± SD ($n = 3$) values, and statistical significance ($^{*}p < 0.05$ or $^{***}p < 0.001$) is shown for treatments compared to LPS only. The value of *n.s.* has been defined as no significant effect.

of TLR4 or CD14 and that the binding of LPS to TLR4 may be equal in both the Tollip-downregulated cells and the control cells. In addition, in control cells treated with quercetin for 1 h, the level of Tollip protein markedly increased, whereas silencing of Tollip prevented quercetin-induced upregulation of Tollip (Fig. 1E).

3.3. Tollip mediates the TLR4 inhibitory action of quercetin

To examine the effect of quercetin mediated through Tollip on the production of inflammatory cytokines from macrophages, we stimulated Tollip-downregulated RAW264.7 cells with LPS. As shown in Fig. 2A, LPS-induced production of TNF-α, IL-6, IL-12p70, and IL-1β was significantly inhibited upon treatment with 100 μM quercetin. However, this inhibitory effect was not observed in Tollip-downregulated cells.

3.4. Quercetin suppresses LPS-induced maturation of macrophages through Tollip

To investigate whether quercetin suppresses macrophage maturation through Tollip, we measured the expression of macrophage maturation markers such as CD80, CD86, and MHC classes I/II. Tollip-downregulated cells and control cells were pretreated for 1 h with 100 μM quercetin prior to exposure to LPS and analyzed for the expression of surface markers. LPS was used as the positive control. Quercetin was found to downregulate the LPS-induced expression of the macrophage maturation markers; however, quercetin did not affect Tollip-downregulated cells (Fig. 2B).

3.5. Effect of Tollip downregulation on the expression of LPS signaling mediators and anti-inflammatory action of quercetin

NO and PGE_2 as well as iNOS or COX-2 are major mediators involved in systemic inflammation, and these mediators are induced readily in response to LPS [22–24]. To test whether the Tollip silencing affects LPS-induced inflammatory responses, Tollip downregulated cells and control cells were pretreated for 1 h with quercetin prior to exposure to LPS. As shown in Fig. 3A and B, production of No and PGE_2 and expression of iNOS and COX-2 proteins were significantly inhibited upon treatment with quercetin. However, this inhibitory effect was not observed in Tollip-downregulated cells, suggesting that quercetin suppresses the production

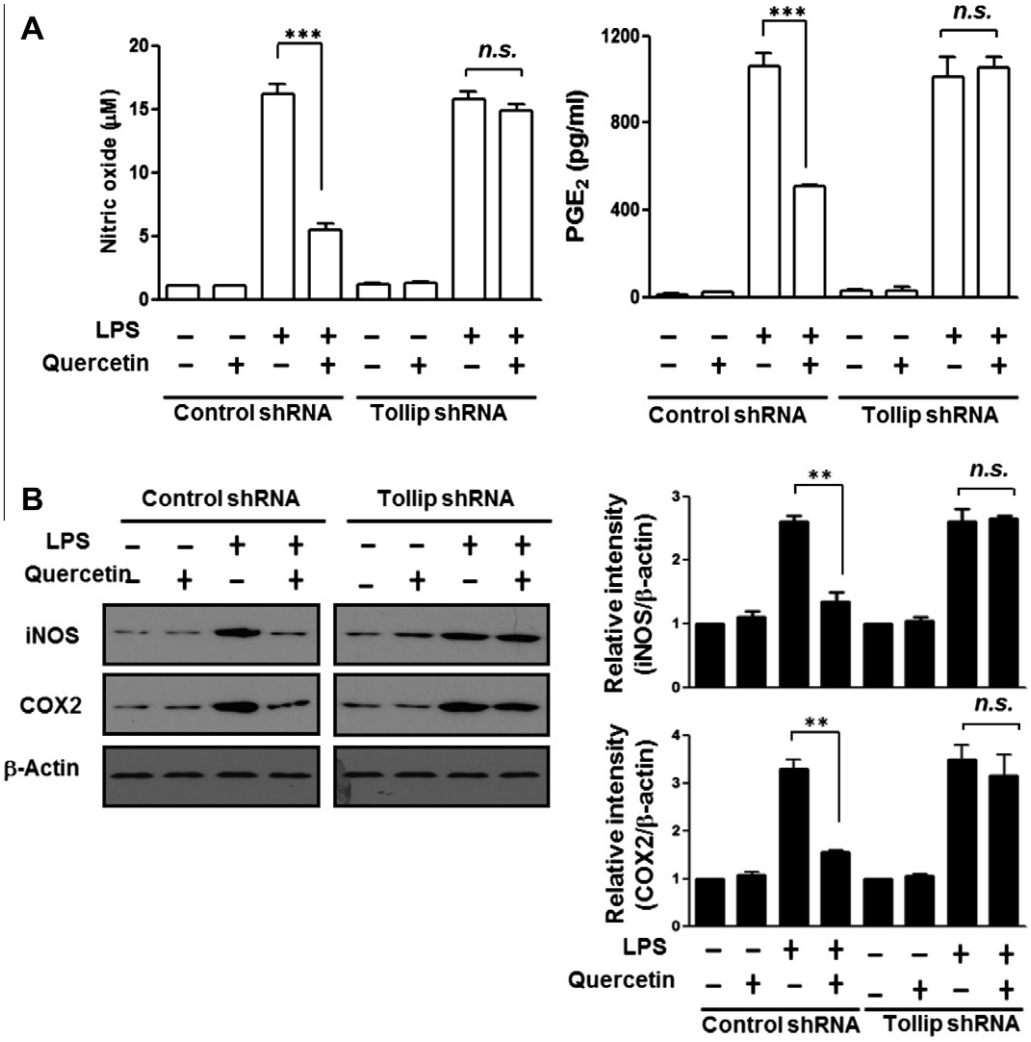

Fig. 3. Tollip is indispensable for anti-inflammatory action of quercetin in RAW264.7 cells. (A and B) RAW264.7 cells were incubated with quercetin (100 μM) for 1 h and then treated with LPS (50 ng/mL) for 24 h. (A) The amount of PGE_2 in culture medium was measured by ELISA. NO level in the culture medium was measured by the Griess assay. (C) RAW264.7 cells were incubated with quercetin (100 μM) for 1 h and then treated with LPS (50 ng/mL) for 12 h. Whole-cell lysates were used for analysis of the amount of iNOS, COX-2, and β-actin by immunoblotting using each specific Ab, as described in Section 2. Relative band intensity of each protein was normalized for β-actin. All data were expressed as the mean ± SD ($n = 3$). $^{**}p < 0.01$ or $^{***}p < 0.001$.

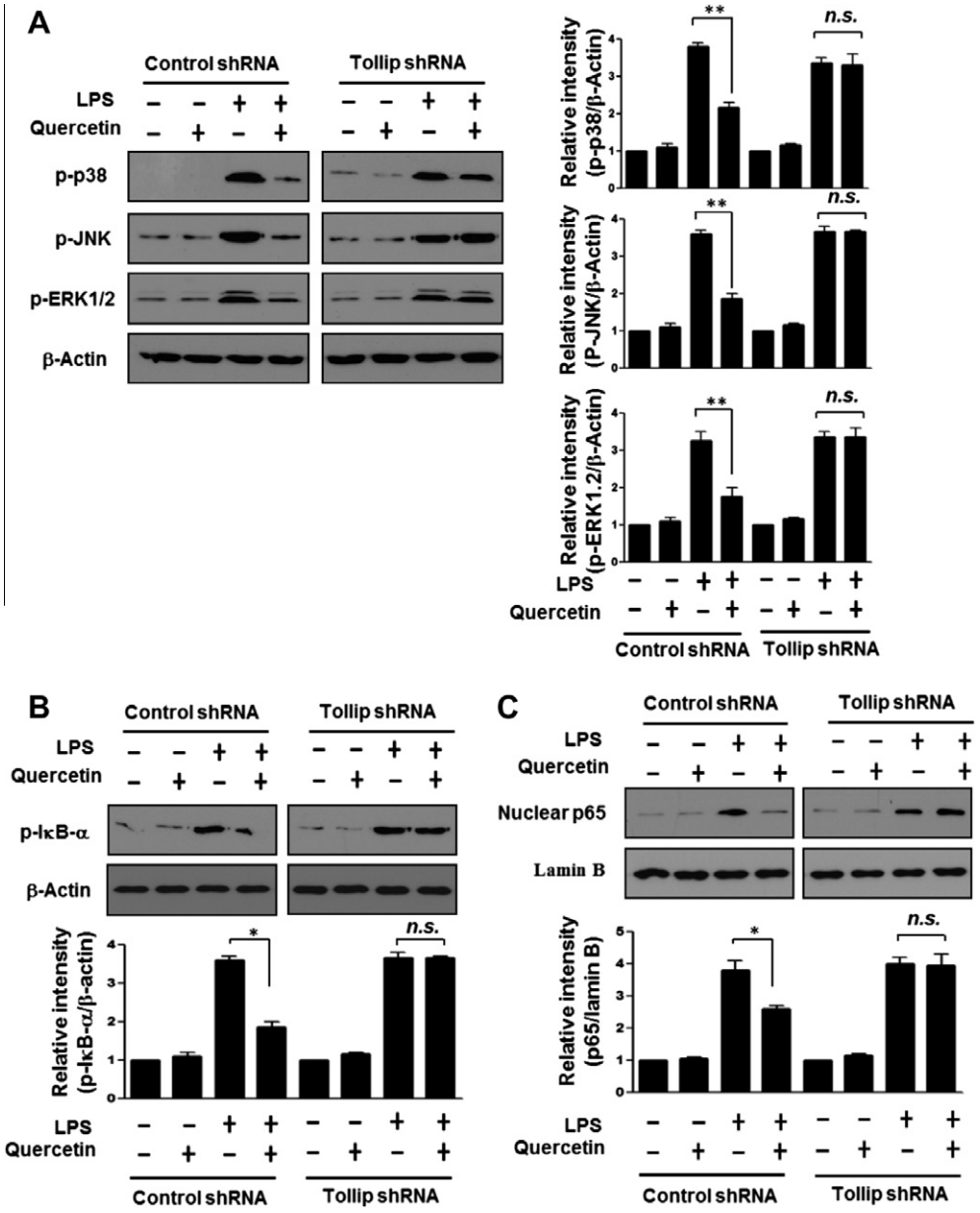

Fig. 4. Quercetin inhibits LPS-induced activation of MAPK and NF-κB signaling pathways through Tollip. The cells were pretreated with the indicated concentrations of quercetin (100 μM) for 1 h before exposure to LPS (50 ng/mL) for 45 min. Cells lysates were subjected to SDS–PAGE, and immunoblot analysis was performed using specific Abs to p-p38, p-ERK1/2, p-JNK, p-IκB-α, and p65 NF-κB. β-Actin and lamin B were used as loading controls for cytosolic and nuclear fractions, respectively. The results shown are representative of three experiments conducted under each condition. The data are shown as mean ± SD ($n = 3$) values. Relative band intensity of each protein was expressed. Statistical significance ($^{*}p < 0.05$ or $^{**}p < 0.01$) was indicated for LPS only versus LPS plus quercetin. The value of *n.s.* was defined as no significant effect.

or expression of LPS-induced inflammatory mediators through Tollip.

3.6. Effect of Tollip down-regulation on quercetin-induced inactivation of the MAPK and NF-κB signaling pathway

We examined whether quercetin suppresses LPS-induced MAPK activation through Tollip. LPS-induced phosphorylation of ERK1/2, p38, and JNK was inhibited by treatment with quercetin, whereas in Tollip-downregulated cells, the inhibitory effect of quercetin on LPS-induced up-regulation of phosphorylation was attenuated (Fig. 4A). Next, we also examined whether quercetin inhibits NF-κB signaling pathway through Tollip. As shown in Fig. 4B, quercetin inhibited LPS-induced phosphorylation of IκB-α in control cells, whereas the inhibitory effect of quercetin was attenuated in Tollip-downregulated cells, suggesting that quercetin inhibits LPS-induced phosphorylation and degradation of IκB-α through Tollip. Furthermore, we investigated the effect of quercetin on LPS-induced nuclear translocation of p65. We also found that quercetin significantly suppressed LPS-induced nuclear translocation of p65 in control cells, whereas this effect was not observed in Tollip-downregulated cells (Fig. 4C). These results indicate that quercetin inhibits the MAPK and NF-κB signaling pathway in macrophage through Tollip.

4. Discussion

Numerous epidemiological studies have shown that phenolic compounds from the flavonoid group have a wide range of bio-

logical activities, such as anticancer, antimicrobial, anti-inflammatory, immunomodulatory, antithrombotic activities, and cardiovascular disease protection [25–29]. In particular, many investigations of quercetin on anti-inflammatory effect have been reported to regulate inflammatory action through the inhibition of MAPKs and NF-κB-mediated pro-inflammatory cytokines, iNOS, and COX-2 genes [20,21]. Although this research has proposed various different mechanisms for the anti-inflammatory action of quercetin, it is still not clear which quercetin induced molecular events are responsible for the inhibition of inflammation. Macrophage activation in response to bacterial products, such as LPS, can lead to local inflammation through TLR signaling. Their activation in response to microbial infection and inflammation triggers NF-κB and MAPK signaling, and involved in the production of numerous cytokines, chemokines, adhesion molecules, and enzymes [30]. Recently, it was reported that inhibitory proteins, such as SOCS1, IRAKM, and Tollip are one of the most important molecules for the targeting various stages of the TLR signaling pathways. Among the negative regulators of TLRs, among the negative regulators of TLRs, Tollip regulates the activation of LPS-induced pro-inflammatory responses through TLR4-triggered NF-κB and MAPK signaling pathways [31,32]. Furthermore, Tollip is mediated through its ability to potently suppress the production of pro-inflammatory mediators induced by the activity of IRAK [14]. Tollip also interacts with IRAK-1 prior to stimulation and suppresses IRAK-1's kinase activity [33], indicating that Tollip negatively regulates TLR4 signaling.

In this study, quercetin markedly elevated the expression of the Tollip, a negative regulator of TLR signaling. LPS-induced expression of cell surface molecules (CD80, CD86, and MHC class I/II) and production of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-12p70) were inhibited by quercetin, and this action was prevented by Tollip silencing. It has also been described that MAPK activation followed by NF-κB translocation from the cytosol to the nucleus was particularly important in the LPS-induced secretion of pro-inflammatory cytokines such as TNF-α, IL-6, IL-12p70, and IL-1β [11]. As reported by others, quercetin-treated macrophages inhibited LPS-induced activation of MAPKs, such as ERK 1/2, p38, and JNK, and the NF-κB and p65 through Tollip. In addition, quercetin induced a significant decrease in PGE_2 and COX-2 levels as well as iNOS-mediated NO production induced by LPS through Tollip. In agreement with our observations, previous studies have shown that COX-2 and PGE_2 control the production of LPS-induced pro-inflammatory cytokines in macrophages [24]. As it has been demonstrated by the previous report [34], the induction of iNOS is mainly triggered by a series of signaling pathway including NF-κB and MAPKs. Although the molecular mechanisms involved in the anti-inflammatory action of quercetin are largely unknown, similar findings for quercetin reported the inhibitions of iNOS mRNA and NO production in LPS-induced inflammatory action of macrophage [22,23].

In summary, our findings demonstrate that quercetin suppresses TLR4 signaling through the upregulation of Tollip expression. We have obtained novel first-hand insights highlighting the potential therapeutic importance.

Acknowledgments

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