Wogonin protects human retinal pigment epithelium cells from LPS-induced barrier dysfunction and inflammatory responses by regulating the TLR4/NF-κB signaling pathway

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Received October 21, 2015; Accepted November 18, 2016

DOI: 10.3892/mmr.2017.6252

Abstract. Inflammation in the retinal pigment epithelium is an important contributor to the pathogenesis of age-related macular degeneration. Wogonin is a flavonoid isolated from the root of *Scutellaria baicalensis* and has multiple pharmacological effects, including anti-inflammatory effects. The present study sought to determine if the pharmacological effects of wogonin were relevant to the treatment of AMD. ARPE-19 cells were pre-conditioned with different concentrations of wogonin (0-50 μM) prior to induction of inflammation with LPS (2 μg/ml). Transepithelial electrical resistance analysis demonstrated that 24 h treatment with 10 and 50 μM wogonin ameliorated LPS-induced changes. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and immunofluorescence analyses revealed that wogonin restrained LPS-induced tight junction proteins, claudin-1 and ZO-1. LPS-induced upregulation of inflammatory mediators in ARPE-19 cells, including IL-1β, IL-6, IL-8, cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) and TNF-α was reduced after pre-treatment with wogonin. In addition, RT-qPCR and western blotting demonstrated that wogonin inhibited the expression of TLR4 in LPS-stimulated ARPE-19 cells. This is a novel mechanism indicating that pre-treatment with wogonin could attenuate the TLR4/NF-κB-mediated inflammatory response in LPS-stimulated ARPE-19 cells, and thus could be a potential therapy for the treatment of AMD.

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Key words: inflammation, age-related macular degeneration, retinal pigment epithelium, ARPE-19, wogonin, toll-like receptor 4, NF-κB

Introduction

Age-related macular degeneration (AMD) is the leading cause of irreversible sight loss in the elderly, with risk factors including aging, genetic characteristics, smoking, obesity and hypertension (1,2). The two clinical classifications of AMD are dry (nonneovascular) and wet (neovascular) AMD (3-5). Dry AMD affects the majority of patients with AMD, and a small number of dry AMD cases progress to become wet AMD by abnormal growth of blood vessels from the choroid into the macula. A major pathological hallmark of dry AMD is the presence of age-dependent degenerative damage to the retinal pigment epithelium (RPE), a monolayer of hexagonal epithelial cells located adjacent to, and physically interacting with, retinal photoreceptors, forming the outer blood-retinal barrier (BRB) (6). RPE is a common barrier for solutes and fluids from the choroidal vasculature that must access the inner retina (7,8). Strict control of fluids and solutes across the BRB is achieved by well-developed tight junctions, which mean that the liquid is not able to penetrate the barrier between the two cells. Zonula occludens-1 and claudin-1 are the most-studied tight junction proteins, with most attention focusing on their relation to the BRB (9,10).

RPE cells are important in retinal physiology and pathology (9). Previous studies have revealed that abnormal distribution or expression of tight junction proteins in RPE cells are involved in AMD pathogenesis (10). Increased BRB permeability allows toxic substances and microorganisms to cross the choroidal vasculature, resulting in the activation of Toll-like receptor 4 (TLR4) (9). TLRs are a family of signal transduction molecule, which are transmembrane proteins usually expressed by sentinel cells and recognize structurally conserved molecules from micro organisms. TLR4-mediated signaling pathways have been revealed to activate nuclear transcription factor-κB (NF-κB), suggesting that TLR-4 is critical to the regulation of multiple proinflammatory genes, including cytokines, chemokines, cyclooxygenase-2 (COX-2), interleukin (IL)-6, IL-8 and inducible nitric oxide synthase (iNOS) (11). Therefore, the activation of NF-κB has been proposed as a cause of ocular inflammatory disease. AMD is a multifactorial disease that has several risk factors, including aging, genetic characteristics and smoking. The involvement

of oxidative stress and inflammatory changes have been highlighted in multiple studies (4). Therefore, inhibiting inflammation and improving BRB function is the focus of pathophysiology research regarding AMD.

5,7-dihydroxy-8-methoxyflavone, or wogonin, is a naturally-derived ingredient isolated from the roots of *Scutellaria baicalensis* Georgi, commonly known as Huang-Qin. In traditional Chinese medicine, this substance has been used to treat allergies, inflammatory diseases and tumors (11-13). Previous studies have demonstrated that wogonin suppresses LPS-induced expression of iNOS, tumor necrosis factor-α (TNF-α), NO, and IL-1β in microglia via inhibition of NF-κB activation (14,15). It has also been revealed that wogonin (10^{-6}-10^{-5} M) inhibits IL-6 and IL-8 gene expression and down-regulates the inflammation-associated protein COX-2 through suppression of NF-κB binding in a murine skin inflammation model (16,17). This evidence demonstrates the benefit of wogonin treatment in inflammatory diseases, but little is known about the function of wogonin in relation to AMD.

The human RPE cell line ARPE-19 has been demonstrated to show structural and functional properties that are characteristic to RPE cells *in vivo*, and so is ideal to use for *in vitro* studies (18). The present study investigated both the anti-inflammatory function of wogonin in LPS-induced ARPE-19 cells, and the molecular mechanisms through which it modulates inflammation.

Materials and methods

Cell culture and treatments. ARPE-19 cells (American Type Culture Collection, Manassas, VA, USA) were seeded in Dulbecco's modified Eagle's medium/F-12, a human amniotic membrane nutrient mixture (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.). Cultures were grown in a humidified incubator at 37˚C in an atmosphere of 5% CO_2. Culture medium was changed every 2 days. An additional 2 μg/ml lipopolysaccharide (LPS; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) was added to the medium for the LPS groups for 24 h, as previously described (19). ARPE-19 cells ($5x10^4$ cells/well) were cultured in 96-well plates for 24 h at 37˚C, then pre-treated with different concentrations of wogonin (0-50 μM) for 24 h, followed by 24 h LPS stimulation. The concentrations of wogonin used to treat ARPE-19 cells were based on the results of previous studies (19-21).

Measurement of transepithelial electrical resistance (TEER). TEER was used to measure the paracellular permeability of cell monolayers. ARPE-19 cells ($5x10^4$ cells/well) were cultured on microporous filter membranes (0.4 μm pore size and 6.5 mm diameter; Corning Incorporated, Corning, NY, USA) of apical chambers until the confluent monolayer achieved a TEER >300 Ωcm^2 (~15-18 days), indicating a tight monolayer. A voltmeter (Millicell-ERS; Merck Millipore) was used to measure TEER as previously described (22): TEER (Ωcm^2)=[total resistance-blank resistance (Ω)]x[area(cm^2)]. Measurements were repeated at least three times for each well and each experiment was repeated for at least five different wells.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Following 24 h of LPS exposure, RT-qPCR was performed to detect the mRNA expression levels of tight junction components ZO-1 and claudin-1 in ARPE-19 cells. mRNA expression levels of biological markers of inflammation, COX-2, iNOS and TNF-α, were also determined. Total RNA was isolated from ARPE-19 cells using TRIzol (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. First-strand cDNA was synthesized using the PrimeScript RT kid (cat. no. DRR0375, Takara Bio Inc., Otsu, Japan). qPCR was performed using an ABI Prism 7500 Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.) with thermocycling conditions as follows: An initial denaturation step at 95˚C for 5 min, followed by 40 cycles of denaturation at 95˚C for 15 sec, annealing at 60˚C for 60 sec and extension at 72˚C for 30 sec. The expression of each PCR product was analyzed using the $2^{-\Delta\Delta Cq}$ method relative to β-actin (23). The SYBR Green real-time PCR master mixes (cat. no. PA-012 and cat. no. PA-011) were from SuperArray Bioscience Corporation (Frederick, MD, USA). The primers of the target genes were as follows: ZO-1, forward 5'-AGC CTGCAAAGCCAGCTCA-3' and reverse 5'-AGTGGCCTG GATGGGTTCATAG-3'; claudin-1, forward 5'-GCATGA AGTGTATGAAGTGCTTGGA-3' and reverse 5'-CGATTC TATTGCCATACCATGCTG-3'; TLR4, forward 5'-GAG CCGTTGGTGATCTTTG-3' and reverse 5'-TGCCGTTTC TTGTTCTTCC-3'; β-actin, forward 5'-GGCGGACTATGA CTTAGTTG-3' and reverse 5'-AAACAACAATGTGCAATC AA-3'; iNOS, forward 5'-AGAGAGATCGGGTTCACA-3' and reverse 5'-CACAGAACTGAGGGTACA-3'; COX-2, forward 5'-TTAAAATGAGATTGTCCGAA-3' and reverse 5'-AGA TCACCTCTGCCTGAGTA-3'.

Immunofluorescence. Immunostaining of ARPE-19 cells were performed as described (24). ARPE-19 cells were fixed with 4% PFA for 1 h, washed with PBS containing 0.1% Triton X-100 (PBST), and blocked at 37˚C for 30 min in PBST supplemented with 10% FBS (Thermo Fisher Scientific, Inc.). ARPE-19 cells were incubated overnight at 4˚C with ZO-1 antibody (cat. no. 8193; dilution, 1:100, Cell Signaling Technology, Inc., Danvers, MA, USA) or claudin-1 antibody (cat. no. 4933; dilution, 1:100, Cell Signaling Technology, Inc.), then incubated with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G secondary antibody (cat. no. sc-2357; dilution, 1:5,000, Santa Cruz Biotechnology, Inc.) at 37˚C for 2 h. This was followed by DNA staining using 4',6-diamidino-2-phenylindole. Fluorescent signals were visualized with the Leica TCS SP2 Confocal Spectral Microscope (Leica Microsystems, Inc., Buffalo Grove, IL, USA).

Enzyme-linked immunosorbent assay (ELISA). ARPE-19 cells were plated in 24-well culture plates at a density of $5x10^4$ cells per well and treated as they were to measure the TEER. ARPE-19 cells were collected and centrifuged at 1,500 x *g* at 4˚C for 5 min following 24 h LPS treatment in the presence or absence of wogonin. ELISA was performed to measure IL-1β (cat. no. 432601), IL-6 (cat. no. 430506) and IL-8 (cat. no. 431506) according to the manufacturer's instructions (BioLegend ELISA MAX™ Deluxe kit; BioLegend, Inc., San

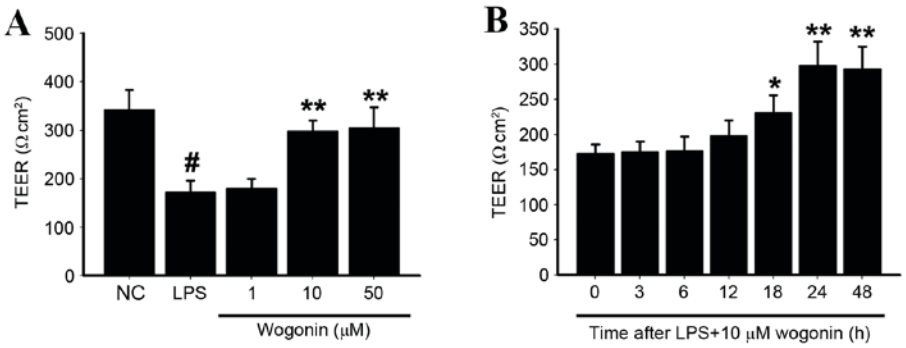

Figure 1. Effect of wogonin on TEER in LPS-stimulated ARPE-19 cells (A) TEER in LPS-stimulated ARPE-19 cells, incubated with 1, 10 and 50 μM wogonin for 24 h. (B) TEER in LPS-stimulated ARPE-19 cells treated with wogonin for different durations. Values are presented as the mean + standard deviation (n=3) $^{\#}$P<0.001 vs. NC group; *P<0.05 and **P<0.001 vs. LPS group. TEER, transendothelial electrical resistance; LPS, lipopolysaccharide; NC, unstimulated negative control cells.

Diego, CA, USA). ELISA was performed in triplicate in three independent experiments.

Western blot. Total protein was extracted from ARPE-19 cells that had been lysed with a buffer (1 M Tris-HCl pH 7.5, 1% Triton X-100, 10% sodium dodecyl sulfate [SDS], 0.5% sodium deoxycholate, 0.5 M EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethanesulfonyl fluoride or phenylmethylsulfonyl fluoride [PMSF]) at 4˚C for 2 h. The soluble proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride filter membranes (Merck Millipore). Primary antibodies were incubated overnight at 4˚C, including inhibitor of NF-κB (IκB; cat. no. 9242; dilution, 1:500; Cell Signaling Technology, Inc.), phospho-IκB (cat. no. 9246; dilution, 1:500; Cell Signaling Technology, Inc.), TLR4 (cat. no. sc-M300; dilution, 1:500, Santa Cruz Biotechnology, Inc.) or β-actin antibody (cat. no. sc-1616; dilution, 1:500, Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Horseradish peroxidase-labelled secondary antibody (cat. no. sc-3901; dilution, 1:10,000, Santa Cruz Biotechnology, Inc.) was added at 37˚C for 1 h. Following this the membranes were washed extensively in Tris-buffered saline+Tween-20 (25 mmol/l Tris, pH 7.5, 150 mmol/l NaCl and 0.1% Tween-20) for 1 h. The films were visualized using femto LUCENT® (Geno Technology Inc., Saint Louis, MO, USA). All experiments were repeated independently four times in triplicate.

Statistical analysis. Statistical analysis was performed using GraphPad Prism software (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA). All values were expressed as the mean ± standard deviation. Data was analyzed with one-way ANOVA tests followed by Tukey's range tests. P<0.05 was considered to indicate a statistically significant difference.

Results

The effect of wogonin on TEER changes in LPS-stimulated ARPE-19 cells. TEER was significantly reduced in cells stimulated with LPS compared with the unstimulated negative control (NC) cells (P<0.05; Fig. 1A). The effect of different concentrations of wogonin on LPS-stimulated ARPE-19 cells was then investigated. Significant increases in TEER were observed in cells treated with 10 and 50 μM wogonin compared with the LPS-only group (P<0.05 and P<0.05 respectively; Fig. 1A). Treatment with 10 μM wogonin following LPS-stimulation resulted in increasing TEER as time increased, peaking at 24 h (P<0.05 at 18, 24 and 48 h; Fig. 1B).

The effect of wogonin on the expression of ZO-1 and claudin-1 in LPS-stimulated ARPE-19 cells. ZO-1 and claudin-1 mRNA expression levels were significantly increased in ARPE-19 cells stimulated with LPS compared with NC (P<0.05 and P<0.05, respectively; Fig. 2A and B, respectively). Treatment with 10 and 50 μM wogonin for 24 h resulted in significantly reduced ZO-1 and claudin-1 expression in LPS-stimulated ARPE-19 cells compared with LPS stimulated/wogonin untreated cells (P<0.05; Fig. 2A and B). The changes observed in immunofluorescence staining demonstrated similar trends to those observed in RT-qPCR analysis: The groups treated with wogonin following LPS stimulation exhibited higher ZO-1 and claudin-1 expression than the group treated with LPS alone (Fig. 2C). These results indicated the protective effects of wogonin against inflammation in the LPS-induced ARPE-19 cells.

Key inflammatory mediators in LPS-stimulated ARPE-19 cells are inhibited by wogonin. Expression levels of COX-2, iNOS and TNF-α mRNA were significantly increased in the LPS-stimulated cells compared with NC (P<0.05, P<0.05 and P<0.05; Fig. 3A-C). COX-2, iNOS and TNF-α mRNA expression levels in LPS-stimulated cells were slightly decreased with 1 μM wogonin treatment compared with the group stimulated with LPS alone, and significantly decreased by 10 μM and 50 μM wogonin treatments (P<0.05; Fig. 3A-C). The ELISA results indicated that protein expression levels of IL-1β, IL-6 and IL-8 were also significantly increased in LPS-stimulated ARPE-19 cells compared with NC, and significantly reduced in LPS-stimulated cells treated with 10 and 50 μM wogonin compared with LPS-stimulated/wogonin-untreated cells (P<0.05; Fig. 3D-F). This suggests that wogonin suppresses inflammatory activity in LPS-stimulated ARPE-19 cells.

Effect of wogonin on NF-κB activation in LPS-stimulated ARPE-19 cells. NF-κB is central to the regulation of several

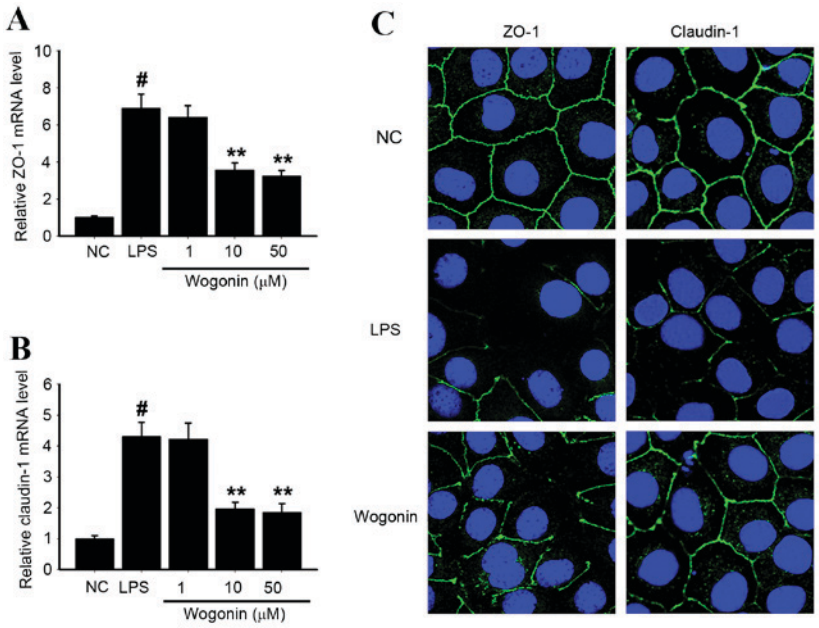

Figure 2. Effects of wogonin on expression of ZO-1 and claudin-1 in LPS-stimulated ARPE-19 cells. (A) RT-qPCR analysis of ZO-1 mRNA expression levels, relative to β-actin. (B) RT-qPCR analysis of claudin-1 expression levels, relative to β-actin. Values are presented as the mean + standard deviation (n=3) $^{\#}$P<0.001 vs. NC group; **P<0.001 vs. LPS group. (C) Immunofluorescent staining of ZO-1 and claudin-1. The nuclear phenotype was investigated by 4',6-diamidino-2-phenylindole staining. Scale bar=25 μm. ZO-1, zonula occludens-1/tight junction protein-1; LPS, lipopolysaccharide; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; NC, unstimulated negative control cells.

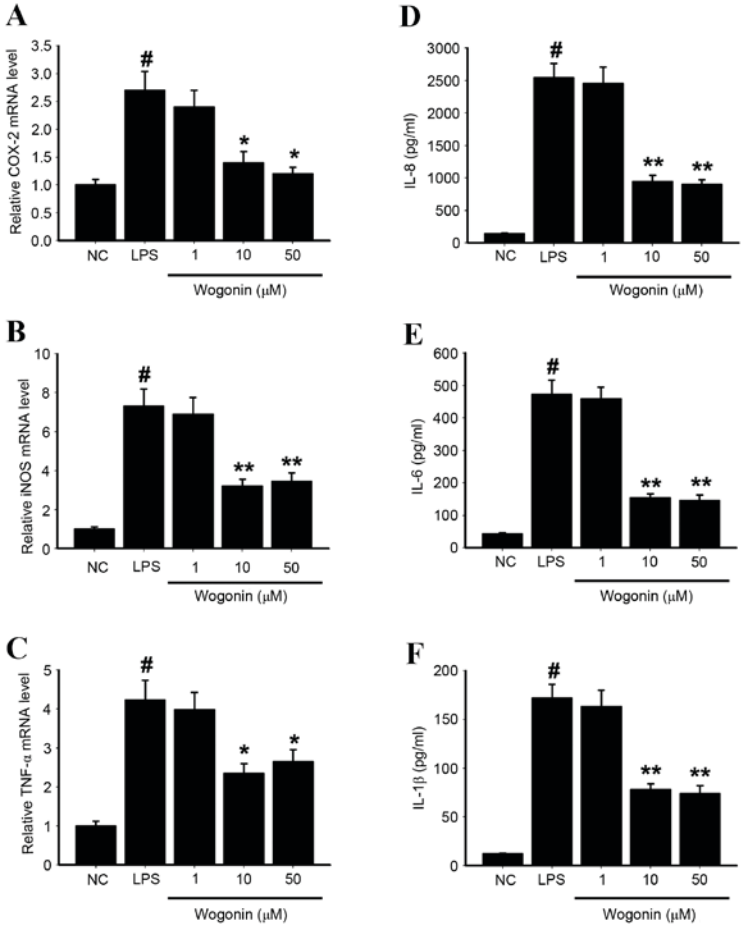

Figure 3. Effect of wogonin on the expression of inflammatory mediators in LPS-stimulated ARPE-19 cells. Reverse transcription-quantitative polymerase chain reaction analysis of mRNA expression levels of (A) COX-2, (B) iNOS and (C) TNF-α in ARPE-19 cells. Enzyme-linked immunosorbent assay analysis of protein expression levels of (D) IL-8, (E) IL-6 and (F) IL-1β in ARPE-19 cells. Values are presented as the mean + standard deviation (n=3). $^{\#}$P<0.001 vs. NC group; *P<0.05 and **P<0.001 vs. LPS group. LPS, lipopolysaccharide; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; TNF-α, tumor necrosis factor-α; IL, interleukin; NC, unstimulated negative control cells.

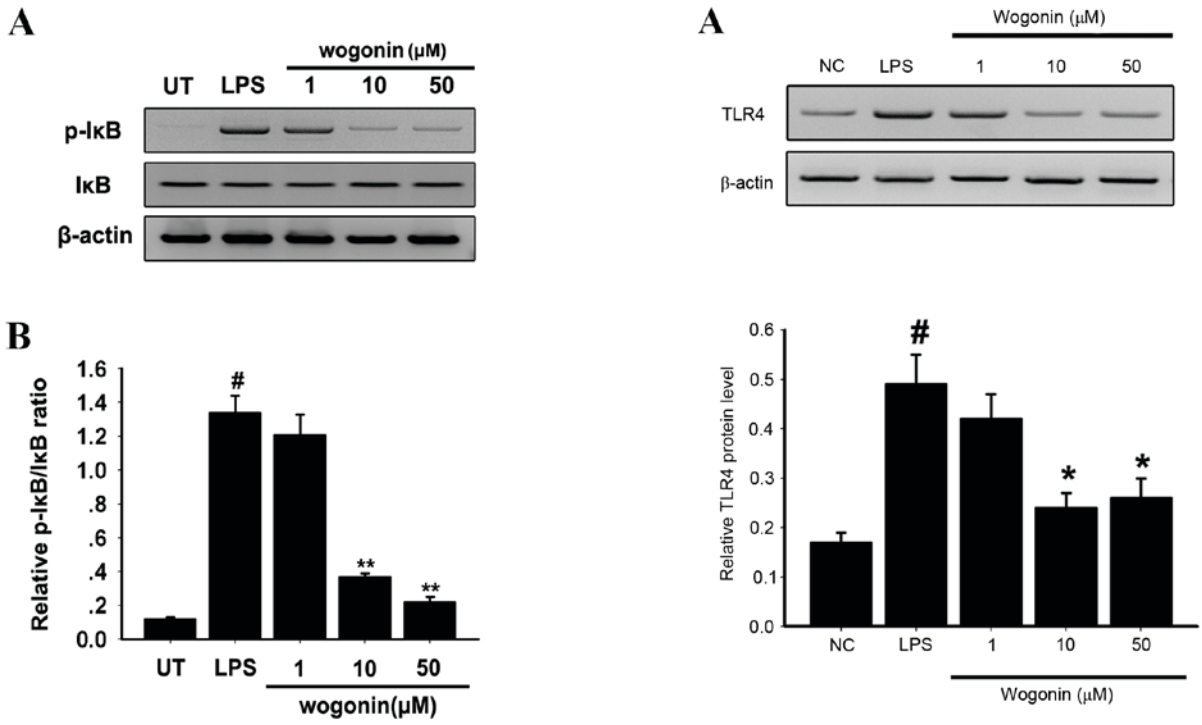

Figure 4. Effect of wogonin on NF-κB activation in LPS-stimulated ARPE-19 cells. (A) Protein expression levels of IκB, p-IκB and β-actin were analysed by western blot analysis. Densitometric analysis of (B) p-IκB/IκB ratio, with quantification relative to the β-actin control. Values are presented as the mean ± standard deviation (n=3). #P<0.001 and ##P<0.05 vs. NC group; *P<0.05 and **P<0.001 vs. LPS group. NFκB, nuclear transcription factor κB; LPS, lipopolysaccharide; p-, phosphorylated; IκB, inhibitor of NF-κB; NC, unstimulated negative control cells.

Figure 5. Effect of wogonin on mRNA and protein expression levels of TLR4 in LPS-stimulated ARPE-19 cells. (A) Western blot analysis of TLR4 protein expression levels, with quantification relative to β-actin. (B) Reverse transcription-quantitative polymerase chain reaction analysis of mRNA expression levels of TLR4 relative to β-actin. Values are presented as the mean + standard deviation (n=3). #P<0.001 vs. NC group; *P<0.05 and **P<0.001 vs. LPS group. TLR4, toll-like receptor 4; LPS, lipopolysaccharide; NC, unstimulated negative control cells.

genes involved in the inflammatory response (24). Activation of NF-κB by LPS is induced by a cascade of events leading to the activation of IκB. The rate of tyrosine phosphorylation of p65 and degradation of IκB could measure the effects of wogonin on LPS-induced NF-κB activation. LPS stimulation was observed to significantly increase the serine phosphorylation of IκB (Fig. 4). Treatment of LPS-stimulated ARPE-19 cells with 10 and 50 μM wogonin significantly decreased IκB activation in response to LPS compared with the cells stimulated with LPS alone (P<0.05; Fig. 4). Wogonin may, therefore, significantly inhibit LPS-induced NF-κB transcriptional activity in ARPE-19 cells.

Effect of wogonin on TLR4 expression in LPS-induced ARPE-19 cells. As activation of TLR4 stimulates the activation of NF-κB, the effects of wogonin on TLR4 expression were examined. Western blotting revealed that basal TLR4 expression was low in the NC group (Fig. 5A). The protein expression levels of TLR4 were significantly increased in LPS-stimulated ARPE-19 cells compared with NC cells (P<0.05; Fig. 5A). However, treatment of LPS-stimulated cells with 10 and 50 μM wogonin resulted in significantly lower TLR4 protein expression levels than LPS-stimulated cells (P<0.05 and P<0.05, respectively; Fig. 5A). RT-qPCR analyses of mRNA expression levels were consistent with the protein results (Fig. 5B). This suggests that wogonin has a significant inhibitory effect on LPS-stimulated TLR4 expression in ARPE-19 cells. Therefore, wogonin may inhibit the activation of inflammation-associated cytokines through the TLR4/NF-κB pathway, which may be the molecular mechanism underlying the protective effect of wogonin on RPE cells.

Discussion

The present study presents three novel findings. First, wogonin inhibits inflammation in LPS-stimulated ARPE-19 cells, which results in the protection of the tight junction proteins ZO-1 and claudin-1. By protecting endothelial tight junctions, wogonin treatment helps maintain an intact BRB. Secondly, wogonin attenuated the LPS-induced inflammatory response via the

inhibition of IL-1β, IL-6, IL-8, COX-2, iNOS and TNF-α gene expression. This is consistent with the findings of previous studies, that demonstrated the ability of wogonin to inhibit IL-1β-induced IL-6 and IL-8 expression via the suppression of NF-κB binding activities (19). Thirdly, wogonin inhibited the activation of the TLR4/NF-kB pathway, which is also associated with the inflammatory response. Previous studies have demonstrated that wogonin acts as a potent inhibitor of several other kinases involved in signal transduction (23). This is consistent with the findings of the present study, with wogonin revealed to attenuate AMD.

Lipopolysaccharide (LPS), also known as endotoxin, is the major cell wall constituent of gram-negative bacteria, and functions as a microglia activator via induction of TLR4 (25). *In vitro* assays have demonstrated that LPS induces inflammation in RPE, followed by subsequent destruction of the outer BRB (25,26). Therefore, cultured ARPE-19 cells were exposed to LPS to induce inflammation. Inflammation depends largely on gene expression and shares key regulators, including COX-2, iNOS and TNF-α (24). TLR4-mediated NF-κB signaling is thought to be central to the regulation of numerous inflammatory responses, and considered to be one of the indicators for ocular inflammatory disease (24). Activation of the NF-κB transcription pathway is considered to be essential for expression of pro-inflammatory genes encoding enzymes such as COX-2, iNOS and TNF-α (27). It has previously been demonstrated that suppression of TLR4/NF-kB signaling by anti-inflammatory agents reduces RPE cell damage (23). The present study also observed that TLR4/NF-kB signaling pathway activation in AMD was attenuated by wogonin treatment. The obtained data were in agreement with previous studies demonstrating that wogonin suppresses neutrophil infiltration and reduces injury-induced IL-1β, IL-6, IL-8 and COX-2 expression, thereby ameliorating RPE damage (24).

The most abundant activated form of NF-κB is a heterodimer of p50 and p65, çontaining transcriptional activation domains necessary for gene induction (28). In resting cells, the NF-κB heterodimer is held in the cytosol through interaction with IκB inhibitory proteins. Following exposure to pro-inflammatory stimuli IκB becomes phosphorylated, ubiquitinated, and then degraded (28,29). The present study provides further support for targeting this mechanism of NF-kB activation to provide protection from inflammation. Wogonin reduced the stimulation effect of LPS on the phosphorylation of IκB and the expression of iNOS, COX-2 and TNF-α together. This suggests that NF-κB was activated via phosphorylation of IκB.

In conclusion, these findings indicate that the upstream factors (TLR4/NF-kB) and downstream factors (COX-2, iNOS, TNF-α activity and IL-1β, IL-6, IL-8 expression) of the TLR4/NF-kB pathway were associated with the neuroprotective effects of RPE cells and may be involved in the pathogenesis of AMD. Further studies are required to explore the potential efficacy of wogonin in other ocular diseases against inflammatory responses.

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