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Anti-inflammatory property of quercetin through downregulation of ICAM-1 and MMP-9 in TNF-α-activated retinal pigment epithelial cells

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ABSTRACT

Quercetin is a flavonoid polyphenolic compound present in fruits and vegetables that has proven anti-inflammatory activity. The goal of the present investigation was to investigate the effects of quercetin on tumor necrosis factor-α (TNF-α)-induced inflammatory responses via the expression of ICAM-1 and MMP-9 in human retinal pigment epithelial cells (ARPE-19 cells). Real-time PCR, gelatin zymography, and Western blot analysis showed that TNF-α induced the expression of ICAM-1 and MMP-9 protein and mRNA in a time-dependent manner. These effects were attenuated by pretreatment of ARPE-19 cells with quercetin. Quercetin inhibited the TNF-α-induced phosphorylation of PKCδ, JNK1/2, ERK1/2. Quercetin, rottlerin, SP600125 and U0126 attenuated TNF-α-stimulated c-Jun phosphorylation and AP-1–Luc activity. Pretreatment with quercetin, rottlerin, SP600125, or Bay 11-7082 attenuated TNF-α-induced NF-κB (p65) phosphorylation, translocation and RelA/p65–Luc activity. TNF-α significantly increased MMP-9 promoter activity and THP-1 cell adherence, and these effects were attenuated by pretreatment with quercetin, rottlerin, SP600125, U0126, tanshinone IIA or Bay 11-7082. These results suggest that quercetin attenuates TNF-α-induced ICAM-1 and MMP-9 expression in ARPE-19 cells via the MEK1/2–ERK1/2 and PKCδ–JNK1/2–c-Jun or NF-κB pathways.

1. Introduction

Retinal pigment epithelial cells (RPECs) form the cell layer between photoreceptor outer segments and choriocapillaris [1]. RPECs have multiple functions including phagocytosis of shed photoreceptor outer segments, maintenance of the blood–retinal barrier, secretion of cytokine, chemokines, and growth and neurotrophic factors, and immune defense of the central retina [2–6]. Because of their strategic location and vital functions, RPECs play a central role in the pathogeneses of various retinal inflammatory diseases, and their dysfunction and atrophy can lead to vision loss [2,7].

Retinal inflammation contributes to vision-threatening injury and is a pathogenic factor in most retinal diseases such as uveitis [8], diabetic retinopathy [9], and age-related macular degeneration (AMD) [4]. These retinal inflammatory diseases have certain similarities, including the presence of an inflammatory response throughout the course of disease and activation of inflammatory proteins such as intercellular adhesion molecule-1 (ICAM-1) [10–13] and matrix metalloproteinases

Abbreviations: ICAM-1, Intercellular adhesion molecule-1; MMP-9, matrix metalloprotease-9; ARPE-19 cells, human retinal pigment epithelial cells; TNF-α, Tumor necrosis factor-α

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(MMPs). ICAM-1, a cell-surface glycoprotein, can facilitate recruitment of leukocytes to the site of tissue damage or infection [14] and thereby plays an important role in inflammatory processes. During retinal inflammation, ICAM-1 is strongly expressed in both retinal vascular endothelial cells and RPECs, where it contributes to severe retinal damage [15]. MMP levels are upregulated in many eye diseases including AMD, glaucoma, and diabetic retinopathy [16–18]. Among the MMPs, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) expressions are increased in Bruch's membrane in association with AMD, especially in the choroidal new vessel area [19–21]. Increased MMP-2 and MMP-9 expressions have also been observed in the retina and vitreous in diabetic retinopathy [22]. Administration of inhibitors of ICAM-1 or MMPs is an attractive potential therapy and is being investigated in several experiments [23–25]. Understanding the roles of inflammatory mediators will provide a rationale for a therapeutic approach to treating retinal inflammatory diseases.

Quercetin is a natural flavonoid that is ubiquitously present in herbs, fruits, and vegetables such as berries, onions, tea, and apples, and has proven anti-inflammatory, antioxidant, antiangiogenic, anticancer, and antiapoptotic effects [26,27]. Recently, the use of quercetin to treat eye diseases has gained attention. It has been shown that quercetin downregulates the expression of monocyte chemoattractant protein-1 (MCP-1), interleukin 6 (IL-6), and IL-8 through the p38 and ERK1/2 pathways in human retinal pigment epithelial cells (ARPE-19 cells) [28]. Quercetin also inhibits Nuclear factor kappa B (NF-κB) and caspase-3 expression, which protect against retinal neurodegeneration and oxidative stress in rats with streptozotocin-induced diabetes [29]. In addition, quercetin reduces MMP-2, MMP-9, ICAM-1, and VCAM-1 expression in a dry eye model [30]. Although the anti-inflammatory function of quercetin has been studied in several fields and the related multiple signaling molecules have received considerable attention, the mechanisms underlying the quercetin-mediated protection of RPECs against the effects of ICAM-1 and MMPs remain poorly understood.

In this study, we investigated whether quercetin can downregulate TNF-α-induced expression of ICAM-1 and MMP-9 in RPECs. We report for the first time that quercetin reduces the TNF-α-induced expression of ICAM-1 and MMP-9 through inhibition of the Protein kinase Cδ (PKCδ)–JNK1/2, ERK1/2–c-Jun, or NF-κB (p65) pathways in human RPECs. A better understanding of these underlying mechanisms may provide important insights for understanding the development of, and possibly preventing, retinal inflammation.

2. Materials and methods

2.1. Materials

Anti-phospho-c-Jun, anti-phospho-PKCδ, anti-phospho-NF-κB (p65), anti-phospho-ERK1/2, and anti-phospho-JNK1/2 antibodies were from Cell Signaling Technology (Danvers, MA, USA). Anti-ICAM-1, anti-GAPDH, anti-NF-κB (p65) and anti-lamin B antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Bay 11-7082, tanshinone IIA, rottlerin, SP600125, SB202190, and U0126 were from Enzo Life Sciences (Farmingdale, NY, USA). Human recombinant TNF-α was from R&D Systems (Minneapolis, MN, USA). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA). Quercetin was from Biotic Chemical Co., Ltd. (New Taipei City, Taiwan). Stock solution of quercetin was prepared in a 1:9 (v/v) mixture of dimethyl sulfoxide and 99% ethanol, and then diluted to the desired final concentration with culture medium.

2.2. Cell culture

ARPE-19 cells were purchased from Bioresource Collection and Research Center (Hsinchu City, Taiwan) and maintained in DMEM/F-12 (Gibco BRL, Grand Island, NY, USA), supplemented with 10% (v/v) FBS (HyClone, Logan, Utah, USA), sodium bicarbonate, and antibiotics (50 ng/ml gentamycin, 100 U/ml penicillin G, 100 μg/ml streptomycin; all from HyClone) at 37 °C in a humidified 5% CO_2 atmosphere.

2.3. Preparation of cell extracts and Western blot analysis

Growth-arrested ARPE-19 cells were incubated with TNF-α at 37 °C for the indicated times. When 100 μM quercetin or inhibitors of JNK1/2 (SP600125), PKCδ (rottlerin), MEK1/2 (U0126), or NF-κB (Bay 11-7082) were used, they were added 1 h before addition of TNF-α. The highest concentrations used of these inhibitors did not cause any toxic effects on ARPE-19 cells (data not shown). After treatment, ARPE-19 cells were rapidly rinsed with ice-cold PBS and solubilized in lysis buffer (25 mM Tris-HCl, pH 7.4, 25 mM NaCl, 25 mM NaF, 25 mM sodium pyrophosphate, 1 mM sodium vanadate, 2.5 mM EDTA, 2.5 mM EGTA, 0.05% (w/v) Triton X-100, 0.5% (w/v) SDS, 0.5% (w/v) deoxycholate, 0.5% (w/v) NP-40, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 mM PMSF) for 30 min on ice. The lysates were centrifuged at 15,000 rpm for 10 min at 4 °C. A Pierce BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA) was used to measure the protein concentration.

Samples (30 μg protein) were denatured, subjected to SDS–PAGE using a 10% running gel, and then transferred to PVDF membranes. Membranes were blocked with blocking buffer (Visual Protein, Taipei, Taiwan) for 60 min and then incubated with specific primary antibodies, including anti-phospho-c-Jun, anti-phospho-PKCδ, anti-phospho-NF-κB (p65), anti-phospho-ERK1/2, anti-phospho-JNK1/2, anti-ICAM-1, anti-GAPDH, and anti-lamin B at a dilution of 1:1000 in blocking buffer overnight at 4 °C. After incubation, membranes were washed extensively with Tween-Tris buffered saline and then incubated with a 1:10,000 dilution of anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Finally, the immunoreactive bands developed by ECL reagents were captured and analyzed using a ChemiDoc XRS + system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.4. Total RNA extraction, RT-PCR, and real-time PCR

ARPE-19 cells were treated with or without drugs (quercetin or Bay 11-7082) for 1 h before exposure to TNF-α for the indicated times. Total RNA was extracted with TRIzol reagent (Sigma-Aldrich) according to the manufacturer's protocol. A microspectrophotometer (Nano-100; Allsheng Instruments, Hangzhou City, China) was used to measure the RNA concentration, and RNA was then reverse-transcribed into cDNA using an iScript cDNA Synthesis Kit (Bio-Rad). The synthesized cDNA was amplified using PCR, and the PCR products were assessed by agarose gel electrophoresis. The expression of β-actin was used as an internal control for the assay of a constitutively expressed gene. The primers used were as follows: 5′-CAGTGACCATCACAGCTTTCCGG-3′ (sense) and 5′-GCTGCTACCACAGTGATGATGACAA-3′ (anti-sense) for ICAM-1; 5′-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3′ (sense) and 5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′ (anti-sense) for β-actin.

Using the cDNA templates, real-time PCR was performed using SYBR Green PCR reagents (Bio-Rad). The ΔΔCt values were calculated, and the results are expressed as the ratio of the target gene mRNA copies to β-actin copies. All data are expressed as the fold change in mRNA expression relative to that in the control cells. The primers for real-time PCR spanning exon-exon boundaries were designed using a PrimerQuest® Tool software (Integrated DNA Technologies Pte. Ltd., Singapore, Republic of Singapore). The primers used were as follows: 5′-TGCATAAGGACGACGTGAAT-3′ (sense) and 5′-GTGTGGTGGTGGTTGGAG-3′ (anti-sense) for MMP-9; 5′-ACCATCTACAGCTTTCCGGC-3′ (sense) and 5′-CTGAGACCTCTGGCTTCGTC-3′ (anti-sense) for ICAM-1.

2.5. Cell viability assay

Viability of ARPE-19 cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich). Cells were seeded into 96-well plates and then treated with quercetin (0.001–1000 μM) for 24 h, after which, the 0.5 mg/ml MTT solution was added to each well. The wells were incubated at 37 °C for 30 min, and the plates were analyzed using a SpectraMax i3x microplate reader (Molecular Devices, CA, USA) at 570 nm. Cell viability was calculated as OD (quercetin)/OD (control) × 100%. The assay was performed in triplicate at each concentration.

2.6. Immunofluorescence staining

ARPE-19 cells were plated into six-well culture plates with coverslips. When inhibitors were used, they were added 1 h before application of 15 ng/ml TNF-α. After treatment, cells were washed with warm PBS, fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.3% Triton X-100, blocked with PBS containing 5% bovine serum albumin, and stained with an anti-p65 antibody. The coverslips were mounted with aqueous mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA). The images were observed using a fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

2.7. Monocyte adhesion assay

ARPE-19 cells were seeded into six-well culture plates. When quercetin or inhibitors were used, they were added 1 h before application of TNF-α for 6 h at 37 °C in a humidified 5% CO_2 atmosphere. THP-1 cells (human acute monocytic leukemia cell line, obtained from ATCC) were labeled with a fluorescent dye (5 μM calcein AM) at 37 °C for 30 min in RPMI-1640 medium (Gibco) in the dark. The labeled THP-1 cells (5×10^5 cells/ml) were added to plates containing ARPE-19 cells, and the plates were incubated at 37 °C for 1 h and then washed gently with PBS three times to remove nonadherent THP-1 cells. The numbers of fluorescently labeled adherent THP-1 cells were counted in five random fields using a fluorescence microscope (Leica Microsystems).

2.8. Gelatin zymography

The conditioned medium was collected and mixed with 5× non-reducing sample buffer and electrophoresed on a 10% polyacrylamide gel containing 0.15% gelatin as the MMP substrate. After electrophoresis, the gel was washed in 2.5% Triton X-100 for 30 min to remove SDS and then incubated in developing buffer on a rotary shaker for 96 h at 37 °C. After incubation, the gel was stained with staining buffer (30% methanol, 10% acetic acid, and 0.5% (wt/vol) Coomassie brilliant blue) for 30 min and then destained. Gelatinolytic activity was manifested as horizontal white bands on a blue background.

2.9. Measurement of AP-1–, NF-κB– and MMP-9–luciferase (Luc) activity

To prepare the AP-1–Luc, RelA/p65–Luc and MMP-9 plasmids, the human AP-1 promoter region (−861 to +146 bp), RelA/p65 promoter region (−620 to +323 bp) and MMP-9 promoter region (−720 to −11) were inserted between *Mlu*I and *Bgl*II sites of the pLightSwitch_Prom vector (SwitchGear Genomics, Inc, Carlsbad, CA, USA) All plasmids were prepared using an EasyPrep EndoFree Maxi Plasmid Extraction Kit (Biotools Co., Ltd, New Taipei City, Taiwan). Plasmid transient transfection of ARPE-19 cells was performed according to the protocol for Lipofectamine 3000 reagent (Thermo Fisher Scientific, Rockford, IL, USA). Briefly, ARPE-19 cells were plated in six-well culture plates. At 70–80% confluence, the cells were washed with PBS and cultured in 1 ml of Opti-MEN (Gibco BRL). The reporter DNA (2 μg) and β-galactosidase DNA (0.5 μg) were mixed with 5 μl of Lipofectamine 3000 reagent in 250 μl of Opti-MEN for 10 min at room temperature. The mixture was gently added to plates containing ARPE-19 cells at 37 °C and, 4 h later, 1 ml of antibiotics-free DMEM/F-12 containing 10% FBS was added for 24 h. After transfection, the cells were shifted to DMEM/F-12 containing 1% FBS medium for 24 h. TNF-α was added to the medium for the indicated times. When quercetin or inhibitors were used, they were added 1 h before application of TNF-α. Cell extracts were prepared, and luciferase and β-galactosidase activities were measured. RelA/p65–Luc and MMP-9–Luc activity was determined using a luciferase assay system (Biotools) according to the manufacturer's instructions. The luciferase activities were standardized to β-galactosidase activity.

2.10. Statistical analysis

Data is combined from three independent experiments with three replicate samples per group in each experiment. The intensity of the bands on the Western blotting and gelatin zymography were assessed by Image Lab software (Bio-Rad). THP-1 monocyte adhesion assay was quantified using Image J software (W. Rasband, NIH, USA). Quantitative data were estimated using the GraphPad Prism program (GraphPad, San Diego, CA, USA) and are expressed as mean ± SD. One-way ANOVA followed by Tukey's post hoc test was used to identify significant differences between multiple groups. A P-value of < 0.05 was considered significant.

3. Results

3.1. TNF-α induced the expression of ICAM-1 and MMP-9 in ARPE-19 cells

The expression of ICAM-1 and MMP-9 proteins is associated with eye diseases such as dry eye [30]. To explore the expression of TNF-α-induced ICAM-1 and MMP-9 in ARPE-19 cells, cells were treated with different concentrations of TNF-α for designated times (2, 4, 6, 18, and 24 h for ICAM-1 protein level; 1 and 4 h for ICAM-1 mRNA level; 48 and 72 h for MMP-9 mRNA and protein levels). TNF-α induced ICAM-1 protein expression and MMP-9 enzymatic activity in a positive time- and concentration-dependent manner, as shown in Fig. 1A, B, D and E. Real-time PCR was used to measure the levels of ICAM-1 and MMP-9 mRNA in TNF-α-stimulated ARPE-19 cells. After the TNF-α stimulation, the levels of ICAM-1 and MMP-9 mRNA were significantly upregulated in a time-dependent manner within 4 h and 72 h, respectively (Fig. 1C and F). These observations suggest that the ICAM-1 and MMP-9 expression was induced by TNF-α at both the translational and transcriptional levels in ARPE-19 cells.

3.2. Quercetin inhibited the expression of ICAM-1 and MMP-9 induced by TNF-α in ARPE-19 cells

Quercetin, a flavonoid polyphenolic compound, possesses antioxidant and anti-inflammatory properties in RPECs [28,31,32]. Before examining the effect of quercetin on TNF-α-induced ICAM-1 and MMP-9 expression, the viability of ARPE-19 cells was measured after quercetin treatment. As shown in Fig. 2A, quercetin at 0.001–100 μM had almost no influence on the cell viability of ARPE-19 cells, although quercetin at 1000 μM significantly reduced ARPE-19 cell viability. Therefore, quercetin was used at concentrations ≤100 μM in the following study to explore the pharmacological mechanisms of quercetin's effects on TNF-α-induced ICAM-1 or MMP-9 expression. Before incubation with TNF-α (15 ng/ml), ARPE-19 cells were treated with quercetin at different concentrations (10, 50, or 100 μM). Pretreatment with quercetin significantly inhibited ICAM-1 protein expression in a concentration-dependent manner (Fig. 2B) and reduced the mRNA level (Fig. 2C).

To investigate how quercetin affects TNF-α-induced MMP-9 expression, ARPE-19 cells were pretreated with quercetin at different

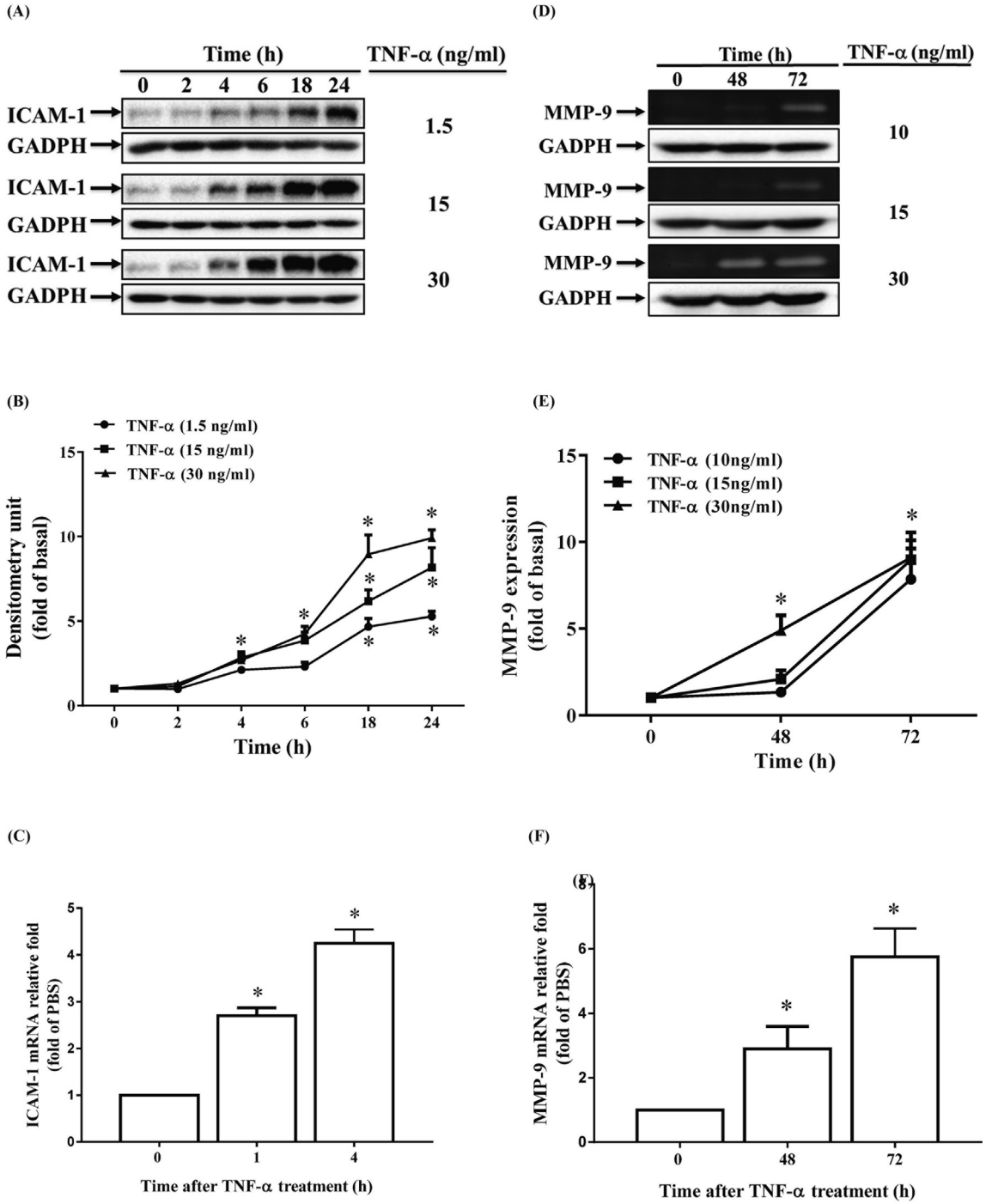

Fig. 1. TNF-α induces the expression of ICAM-1 and MMP-9 in ARPE-19 cells. TNF-α was used at different concentrations to stimulate ARPE-19 cells for designated times (2, 4, 6, 18 and 24 h for ICAM-1 protein level; 1 and 4 h for ICAM-1 mRNA level; 48 and 72 h for MMP-9 mRNA and protein levels) (A) ICAM-1 protein expression was detected by Western blotting. (B) ICAM-1 protein expression in cells used in the experiments shown in (A) was quantified by an image lab software. (C) ICAM-1 RNA transcripts were analyzed by real-time PCR (D) MMP-9 expression was analyzed by gelatin zymography. (E) MMP-9 expression in cells used in the experiments shown in (D) was quantified by an Image Lab software. (F) MMP-9 RNA transcripts were analyzed by real-time PCR. Data were obtained from at least three independent experiments and are expressed as mean ± SD. $^{*}P < 0.05$ compared with the basal level.

concentrations (5, 10, or 50 μM) for 1 h and then incubated with TNF-α (30 ng/ml) for 48 h. Quercetin significantly attenuated both TNF-α-induced MMP-9 expression and mRNA level (Fig. 2D and E).

3.3. Quercetin attenuated ICAM-1 and MMP-9 expression induced by TNF-α in ARPE-19 cells via the PKCδ–JNK1/2–c-Jun pathway

Lee et al. reported that TNF-α-induced ICAM-1 expression was mediated through the PKCδ–JNK1/2–c-Jun cascade in human RPECs [14]. To investigate whether quercetin inhibits TNF-α-induced ICAM-1 and MMP-9 expression through this pathway in ARPE-19 cells, the cells were pretreated with quercetin for 1 h and then incubated with TNF-α for 5, 10, 15, 30 and 60 min. As shown in Fig. 3A–C, PKCδ phosphorylation decreased, but this effect was not statistically significant. Quercetin also significantly attenuated TNF-α-induced JNK1/2 and c-Jun phosphorylation.

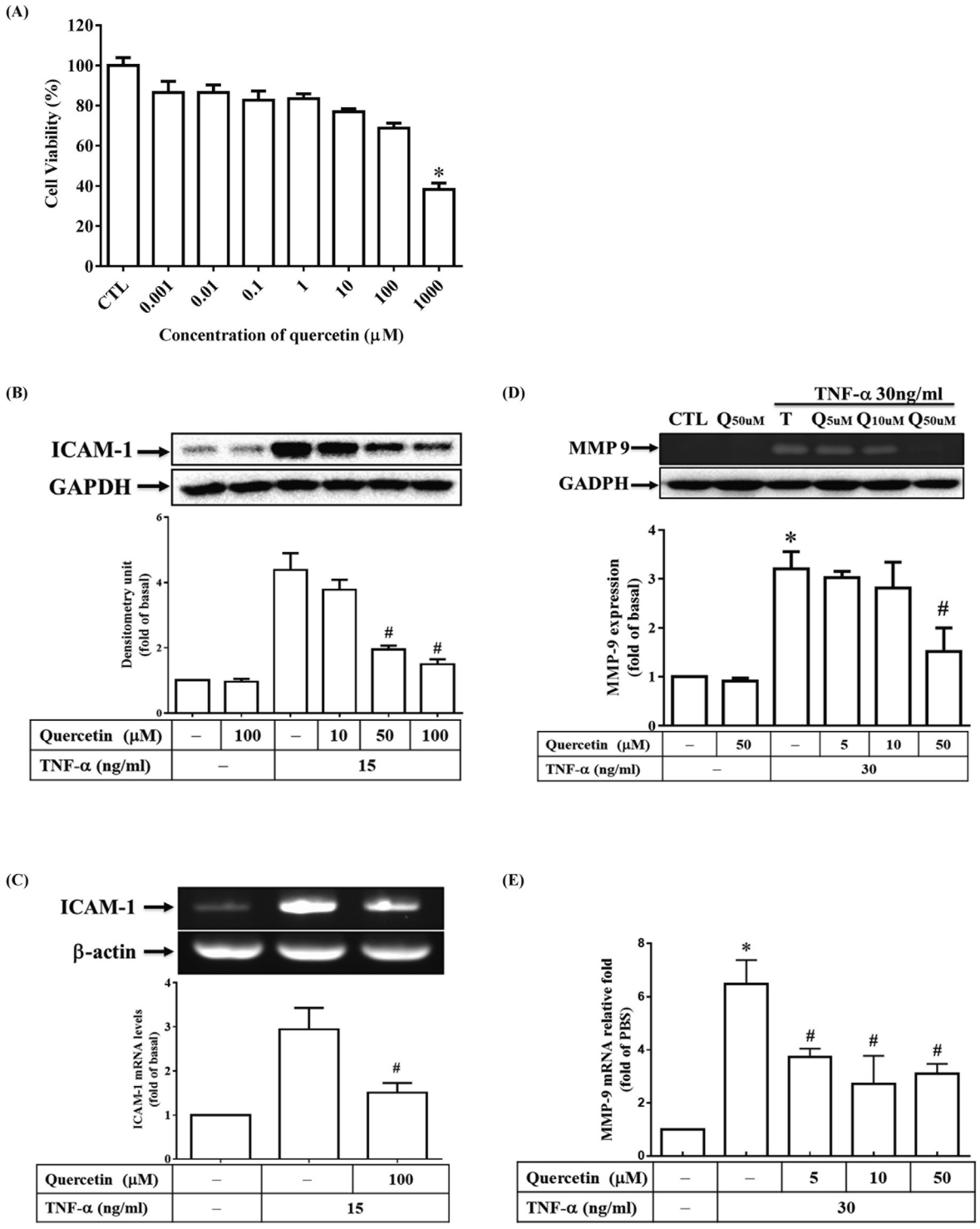

Fig. 2. Quercetin attenuates the expression of ICAM-1 and MMP-9 induced by TNF-α in ARPE-19 cells. (A) Effects of quercetin on the viability of ARPE-19 cells. ARPE-19 cells were treated with different concentrations of quercetin (0.001–1000 μM) for 24 h and the cell viability was analyzed using the MTT assay. ARPE-19 cells were incubated with TNF-α in the presence of quercetin for (B) 6 h, (C) 4 h, and (D, E) 48 h. (B, C) ICAM-1 protein expression and mRNA level were measured by (B) Western blotting and (C) RT-PCR and real-time PCR, respectively. (D, E) MMP-9 zymogen activity and mRNA level were analyzed by (D) gelatin zymography and (E) real-time PCR, respectively. (B) ICAM-1 protein expression was quantified using Image Lab software. (D) MMP-9 proteolytic activity was manifested as white bands on a blue background, and GADPH expression was used as an internal control. Data were obtained from at least three independent experiments and are expressed as mean ± SD. *P < 0.05 vs. control cells. #P < 0.05 vs. TNF-α-stimulated cells.

Next, SP600125 (a JNK1/2 inhibitor) and rottlerin (a PKCδ inhibitor) were used to investigate the relationship between PKCδ, JNK1/2, and c-Jun in the TNF-α-induced response. As shown in Fig. 3D and E, SP600125 and rottlerin downregulated JNK1/2 and c-Jun phosphorylation in TNF-α-stimulated ARPE-19 cells. These results suggest that quercetin decreased TNF-α-induced ICAM-1 and MMP-9 expression via the PKCδ–JNK1/2–c-Jun pathway.

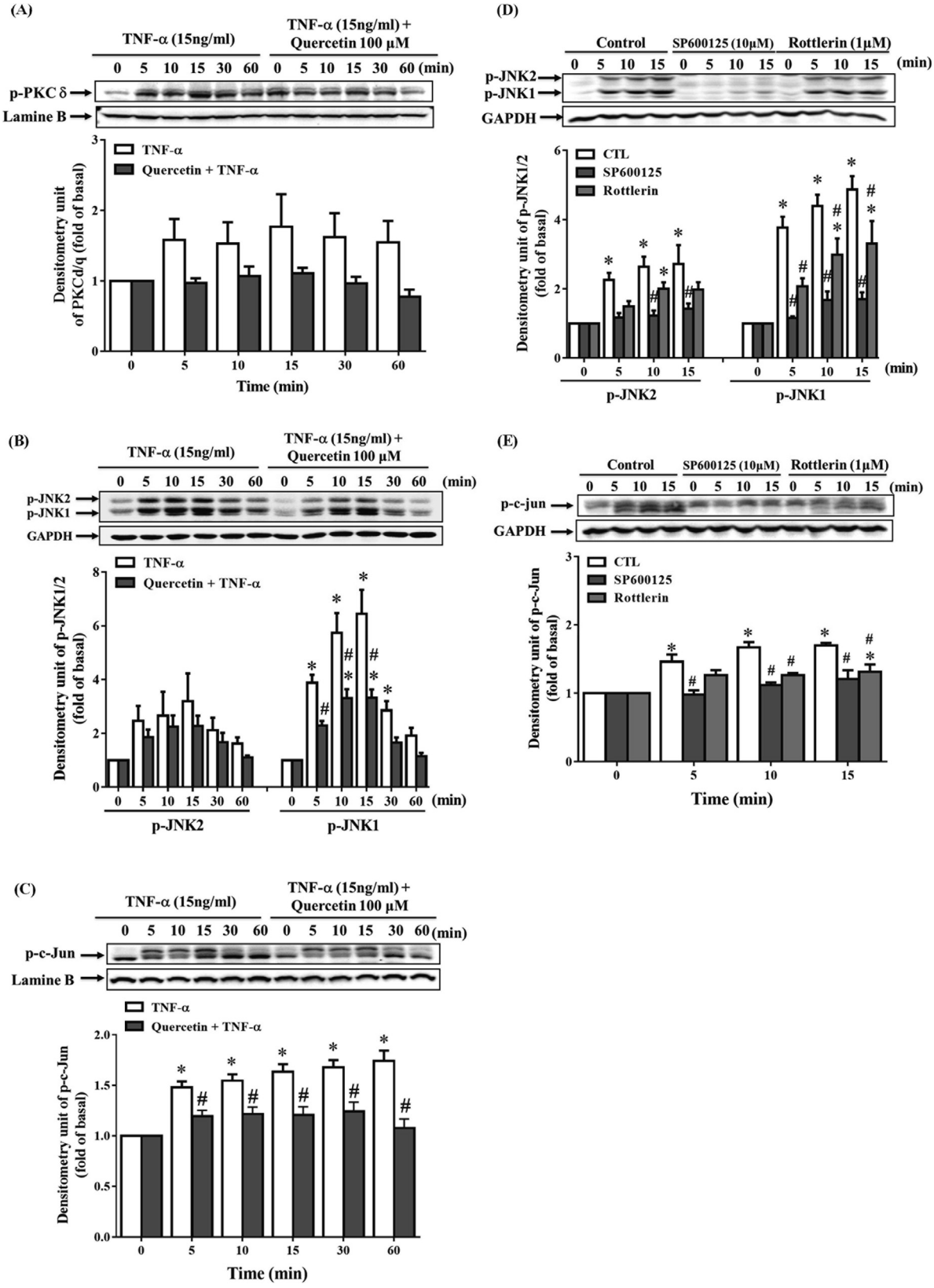

Fig. 3. Quercetin attenuates TNF-α-induced PKCδ–JNK1/2–c-Jun activation in ARPE-19 cells. ARPE-19 cells were pretreated with (A–C) quercetin, (D, E) SP600125 or rottlerin for 1 h, and the cells were then stimulated with 15 ng/ml TNF-α for designated times (5, 10, 15, 30 and 60 min for Quercetin; 5, 10 and 15 min for SP600125 and rottlerin). Phosphorylation of (A) PKCδ, (B, D) JNK1/2, or (C, E) c-Jun were analyzed by Western blotting. Data were obtained from at least three independent experiments and are expressed as mean ± SD. $^{*}P < 0.05$, vs. control cells. #P < 0.05 vs. TNF-α-stimulated cells.

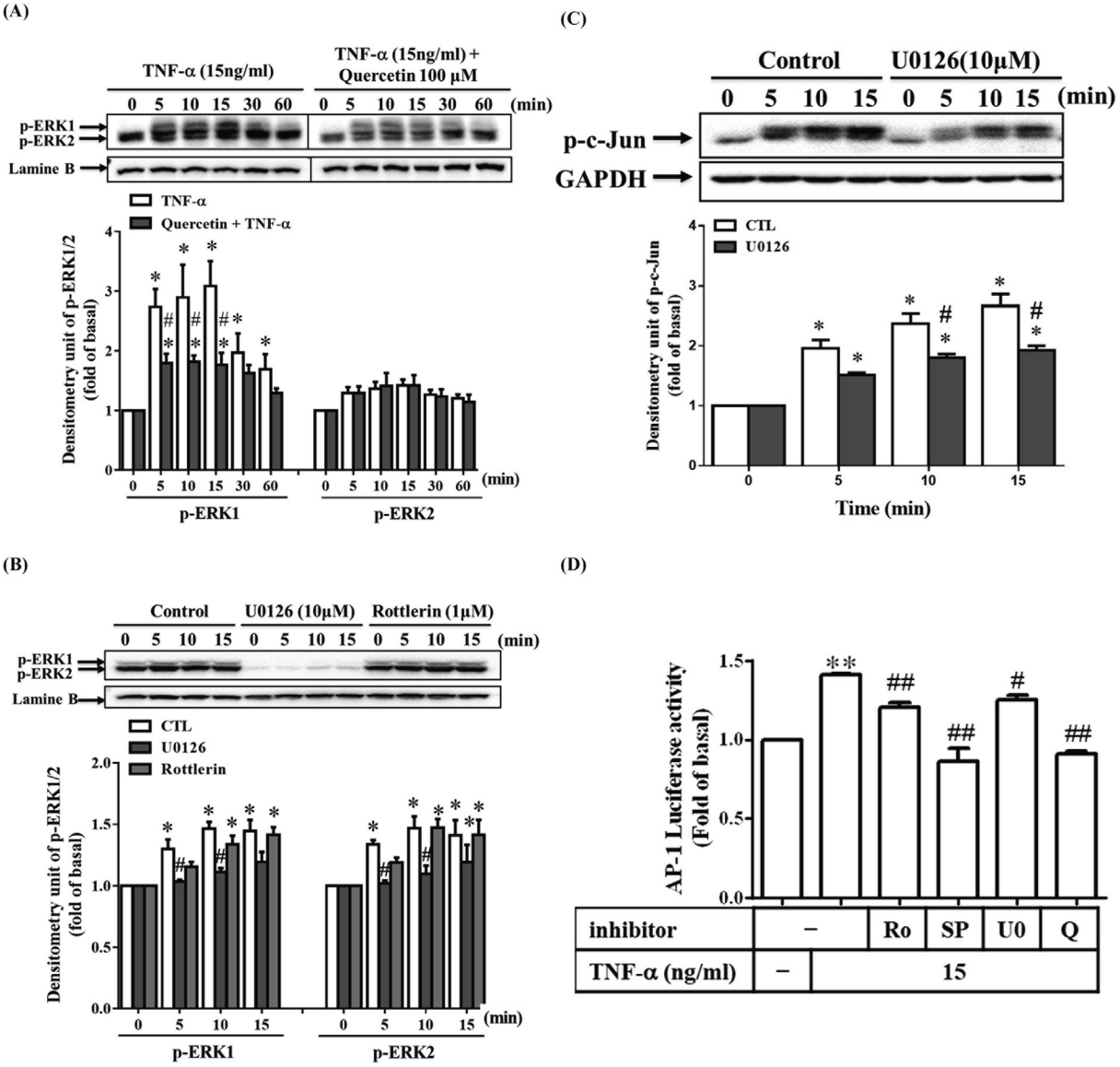

Fig. 4. Quercetin inhibits the TNF-α-activated MEK1/2–ERK1/2 pathway in ARPE-19 cells. ARPE-19 cells were pretreated with (A) quercetin, (B) rottlerin, or (B, C) U0126 for 1 h before stimulation with TNF-α for designated times. Phosphorylation of (A, B) ERK1/2 or (C) c-Jun was analyzed by Western blotting (D) To determine AP-1 promoter activity, ARPE-19 cells were transfected with AP-1–Luc and β-galactosidase plasmids. The cells were then pretreated with rottlerin (1 μM), SP600125 (10 μM), U0126 (10 μM), or quercetin (100 μM) for 1 h and then exposed to TNF-α for 1 h. Luciferase activity was determined. Data were obtained from at least three independent experiments and are expressed as mean ± SD. $^{*}P < 0.05$ vs. control cells. $^{\#}P < 0.05$ vs. TNF-α-stimulated cells.

3.4. Quercetin reduced ICAM-1 and MMP-9 expression induced by TNF-α in ARPE-19 cells via the MEK1/2–ERK1/2–c-Jun pathway

Previous studies have shown that TNF-α promoted ICAM-1 production through ERK1/2 phosphorylation in different cell types such as human lung epithelial alveolar and umbilical vein endothelial cells [33–35]. We first investigated the effects of quercetin on TNF-α-stimulated ERK1/2 phosphorylation. As shown in Fig. 4A, quercetin noticeably decreased TNF-α-induced ERK1/2 phosphorylation. We then examined the effect of a MEK1/2 inhibitor, U0126, on TNF-α-stimulated ERK1/2 phosphorylation. In ARPE-19 cells pretreated with U0126 (10 μM) for 1 h, the TNF-α-induced ERK1/2 phosphorylation was almost completely inhibited (Fig. 4B).

To determine whether TNF-α-induced PKCδ phosphorylation is related to ERK1/2 activation, ARPE-19 cells were preincubated with rottlerin (1 μM) before exposure to TNF-α. As shown in Fig. 4B, ARPE-19 cells pretreated with rottlerin was not decreased ERK1/2 phosphorylation. Fig. 4C shows the relationship between ERK1/2 and c-Jun in the TNF-α-mediated responses. Pretreatment with U0126 inhibited TNF-α-induced c-Jun phosphorylation.

Next, a luciferase gene activity assay was used to confirm whether quercetin can attenuate AP-1 promoter activity and, if so, whether this occurs through the signaling pathways mentioned above. As shown in Fig. 4D, AP-1 promoter activity was significantly affected by TNF-α and reached its maximum at 1 h (Data not shown). Quercetin attenuated this AP-1 promoter activity, which might happen through the PKCδ–JNK1/2–ERK1/2 signaling pathway.

Taken together, these results suggest that quercetin reduced TNF-α-induced ICAM-1 and MMP-9 expression in ARPE-19 cells via the MEK1/2–ERK1/2–c-Jun pathway.

3.5. Quercetin attenuated ICAM-1 and MMP-9 expression induced by TNF-α via NF-κB (p65) in ARPE-19 cells

Inflammation caused by stimulation of cytokines, such as TNF-α, depends on activation of NF-κB. We next investigated the role of NF-κB

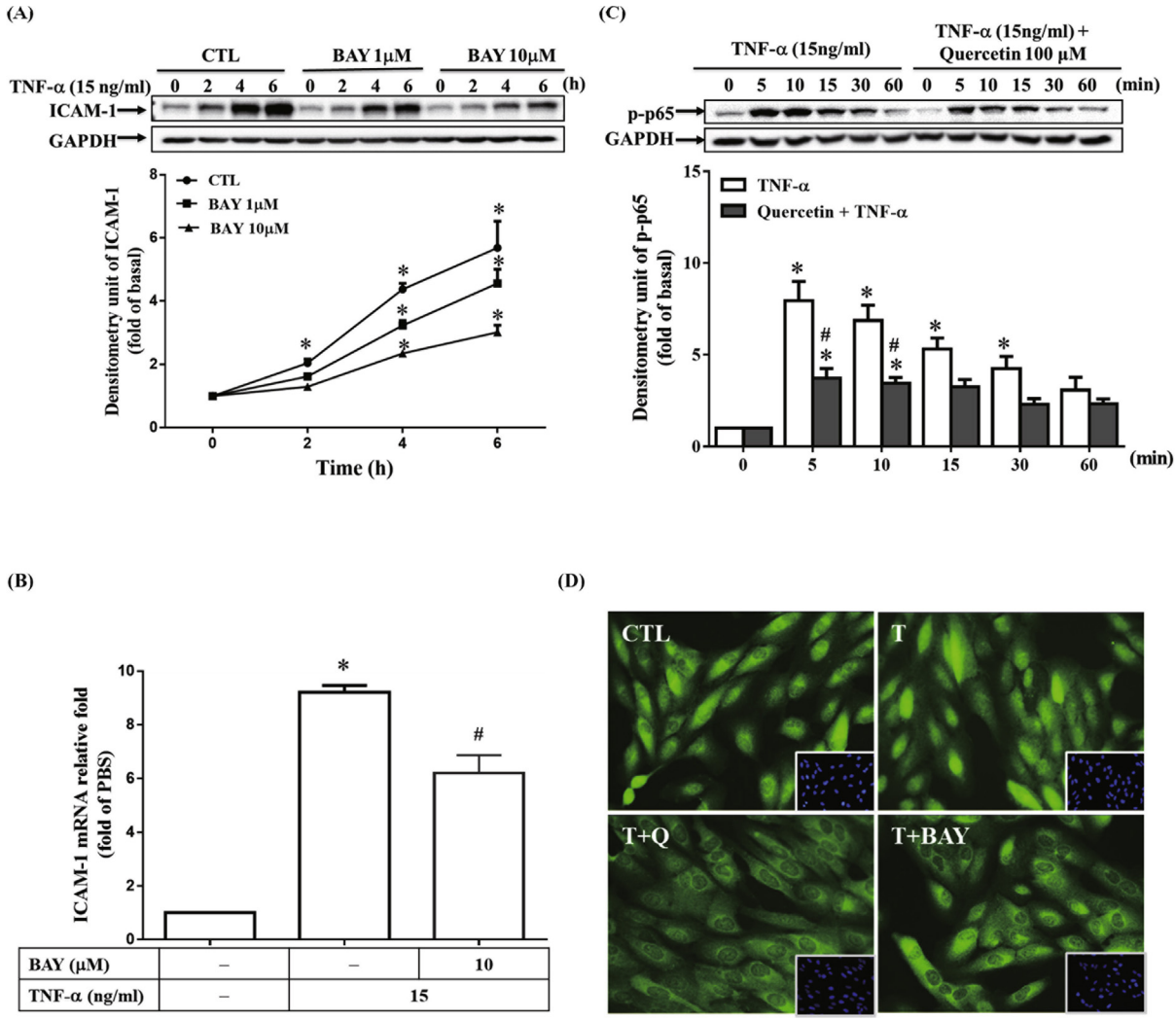

Fig. 5. Quercetin attenuates ICAM-1 expression induced by TNF-α via NF-κB (p65) in ARPE-19 cells. ARPE-19 cells were pretreated with Bay 11-7082 or quercetin for 1 h before stimulation with 15 ng/ml TNF-α for (A, C) the designated time, (B) 4 h, or (D) 5 min. ICAM-1 protein expression and mRNA level were analyzed by (A) Western blotting and (B) real-time PCR, respectively. (C) NF-κB (p65) phosphorylation was analyzed by Western blotting. Data were obtained from at least three independent experiments and are expressed as mean ± SD. $^{*}P < 0.05$ vs. control cells. #P < 0.05 vs. TNF-α-stimulated cells. (D) NF-κB (p65) translocation was determined by immunofluorescence staining. The image is representative of the results of four independent experiments.

in ICAM-1 and MMP-9 expression induced by TNF-α in ARPE-19 cells. As shown in Fig. 5A, the TNF-α-induced ICAM-1 expression was reduced in a concentration- and time-dependent manner in cells pretreated with Bay 11-7082 (an NF-κB inhibitor). ICAM-1 mRNA expression induced by TNF-α was also downregulated by pretreatment with Bay 11-7082 (Fig. 5B). Similar results were also obtained for TNF-α-induced MMP-9 expression (data not shown). These results suggest that NF-κB played an important role in ICAM-1 and MMP-9 expression induced by TNF-α in ARPE-19 cells.

To elucidate whether quercetin attenuated ICAM-1 and MMP-9 expression induced by TNF-α in ARPE-19 cells through the inhibition of NF-κB, the cells were pretreated with quercetin before TNF-α incubation. The results are displayed in Fig. 5C; NF-κB (p65) phosphorylation was significantly reduced by quercetin in a time-dependent manner. Next, we used immunofluorescence staining to assess the effect of quercetin on NF-κB translocation from the cytoplasm into the nucleus. Both quercetin and Bay 11-7082 reduced NF-κB translocation induced by TNF-α (Fig. 5D). These results suggest that quercetin attenuated TNF-α-induced ICAM-1 and MMP-9 expression via NF-κB (p65) in ARPE-19 cells.

3.6. Quercetin attenuated TNF-α-induced NF-κB (p65) phosphorylation, translocation, and promoter activity via the PKCδ–JNK1/2 pathway in ARPE-19 cells

We next investigated whether MEK1/2–ERK1/2, PKCδ, and JNK1/2 are involved in NF-κB (p65) phosphorylation induced by TNF-α in ARPE-19 cells. As shown in Fig. 6A and B, TNF-α-induced p65 phosphorylation was markedly reduced in cells pretreated with SP600125 and rottlerin, but not with U0126. Stimulation by TNF-α in ARPE-19 cells resulted in translocation of NF-κB into the nucleus. Therefore, we examined further whether MAPK activation leads to NF-κB translocation and then induces ICAM-1 and MMP-9 expression in ARPE-19 cells. First, cells were stimulated with TNF-α (15 ng/ml) for 5, 10 and 15 min. TNF-α induced NF-κB translocation within 5 min and the maximal response was achieved within 15 min, as shown by immunofluorescence staining (Fig. 6C). These effects were attenuated by rottlerin or SP600125, as shown in Fig. 6D, but not by U0126 (data not shown).

Next, a luciferase gene activity assay was used to confirm whether quercetin can attenuate NF-κB promoter activity and, if so, whether this occurs through the signaling pathways mentioned above. The RelA/p65–Luc reporter gene was transfected into ARPE-19 cells, which were then stimulated with TNF-α for 0.5, 1, 2, 4 and 6 h. As shown in Fig. 6E,

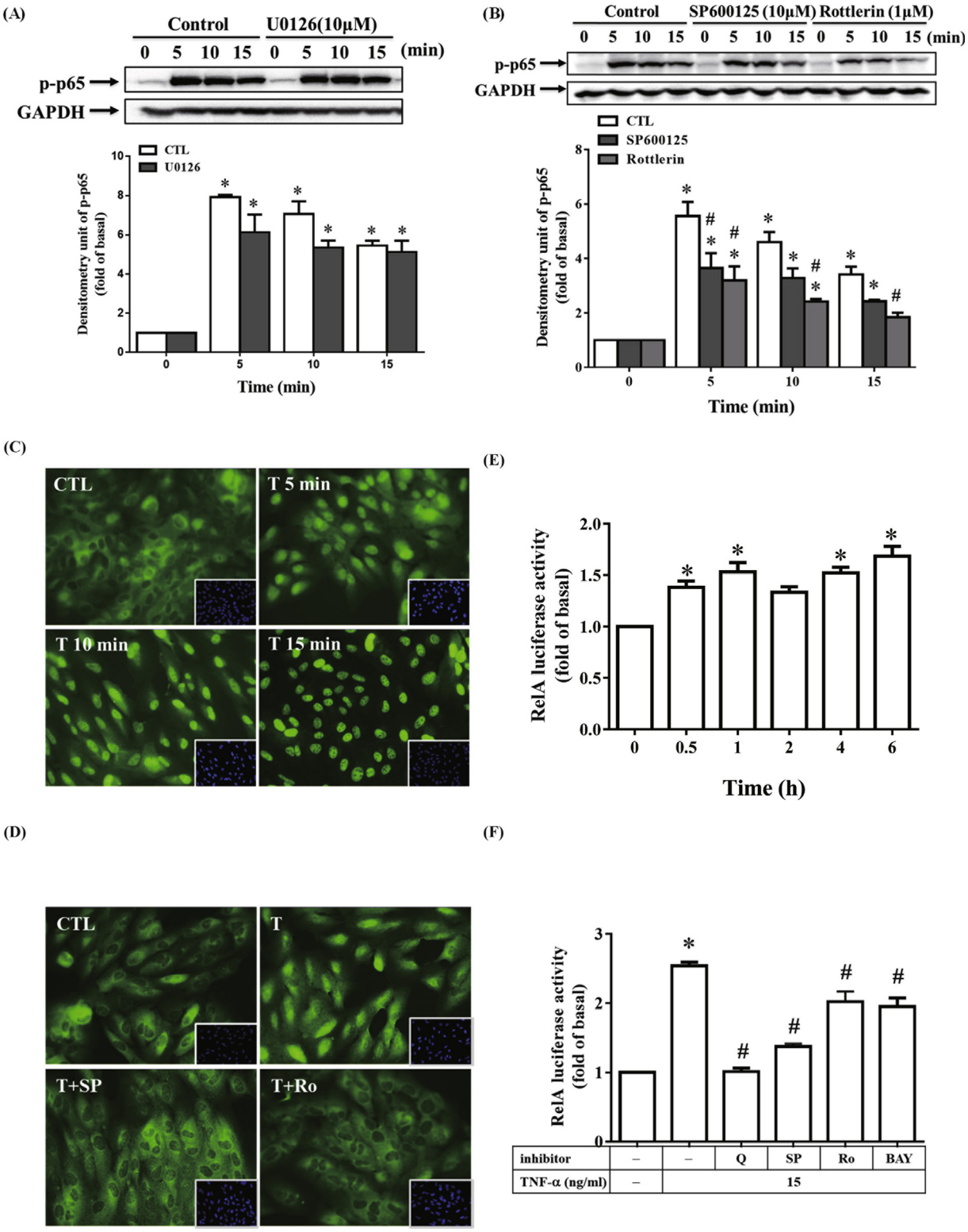

Fig. 6. Quercetin attenuates TNF-α-induced NF-κB (p65) phosphorylation, translocation, and promoter activity via the PKCδ–JNK1/2 pathway in ARPE-19 cells. ARPE-19 cells were pretreated with (A) U0126, (B) SP600125, or rottlerin for 1 h before stimulation with 15 ng/ml TNF-α for 5, 10 and 15 min. NF-κB (p65) phosphorylation was analyzed by Western blotting. (C, D) ARPE-19 cells were treated with 15 ng/ml TNF-α for 5, 10 and 15 min or with SP600125 (10 μM) or rottlerin (1 μM) for 1 h before stimulation with 15 ng/ml TNF-α for 5 min. NF-κB (p65) translocation was determined by immunofluorescence staining. The image is representative of the results of four independent experiments. (E, F) To determine luciferase activity, ARPE-19 cells were first transiently transfected with RelA/p65–Luc and β-galactosidase plasmids. The cells were then treated with 15 ng/ml TNF-α for 0.5, 1, 2, 4 and 6 h. or pretreated with quercetin (100 μM), SP600125 (10 μM), rottlerin (1 μM), or Bay 11-7082 (10 μM) for 1 h and then exposed to TNF-α for 6 h. Data were obtained from at least three independent experiments and are expressed as mean ± SD. $^{*}P < 0.05$ vs. control cells. $^{\#}P < 0.05$ vs. TNF-α-stimulated cells.

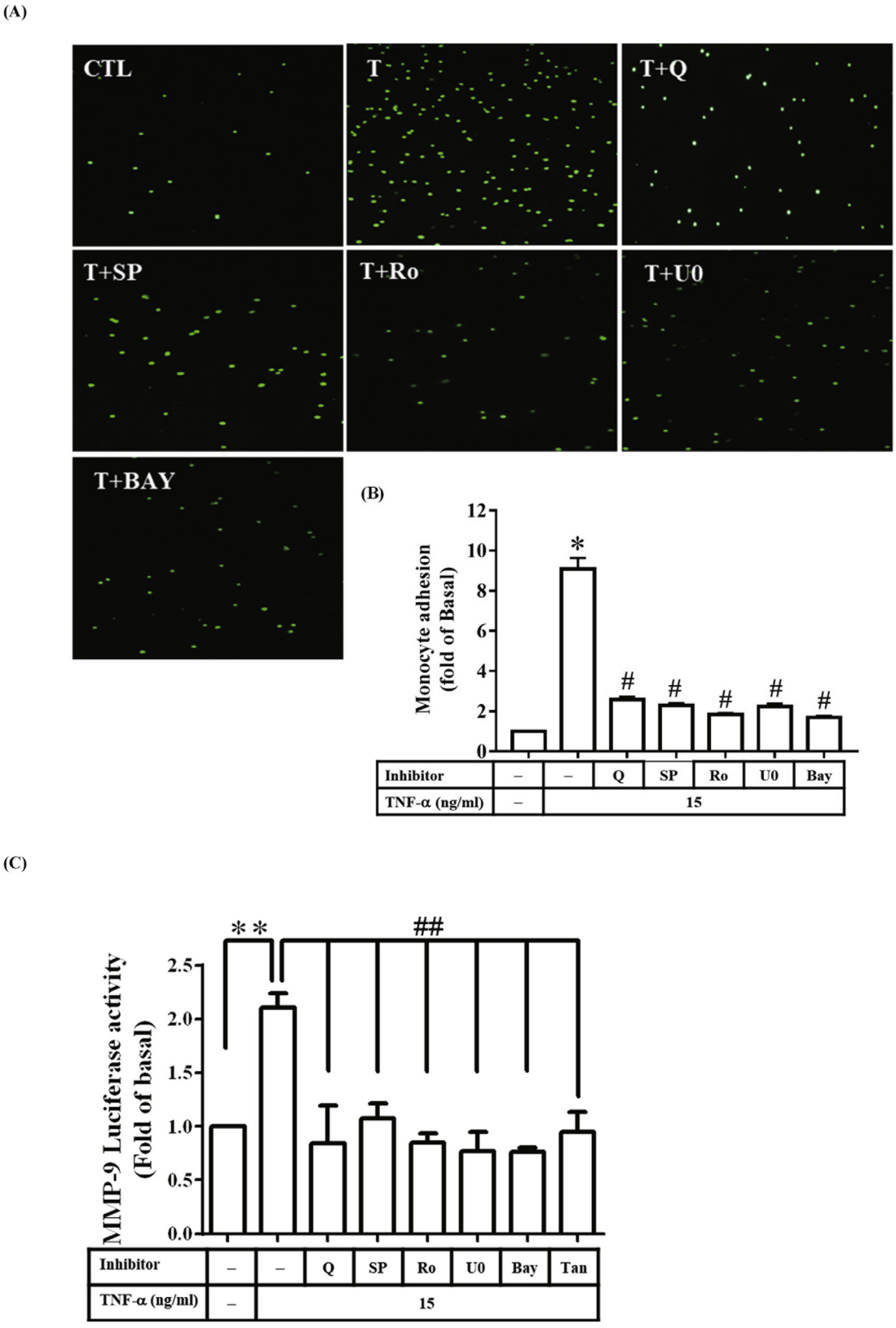

Fig. 7. Quercetin inhibition of TNF-α-induced monocyte adhesion and MMP-9 promoter activity involves PKCδ, ERK1/2, JNK1/2, c-Jun, and NF-κB (p65) in ARPE-19 cells. ARPE-19 cells were preincubated with quercetin (100 μM), SP600125 (10 μM), rottlerin (1 μM), U0126 (10 μM), Bay 11-7082 (10 μM), or tanshinone IIA (0.1 μM) for 1 h and then stimulated with 15 ng/ml TNF-α for 6 h. (A, B) THP-1 monocyte adhesion assay was used to determine the physiological functional of ICAM-1, which was quantified using Image J software. (C) To determine MMP-9 promoter activity, cells were transfected with MMP-9–Luc and β-galactosidase plasmids. The cells were pretreated with quercetin or inhibitors for 1 h and then exposed to TNF-α for 1 h. The luciferase activity was determined. Data were obtained from at least three independent experiments and are expressed as mean ± SD. $^{*}P < 0.05$ vs. control cells. #$P < 0.05$ vs. TNF-α-stimulated cells.

RelA/p65–Luc activity was significantly stimulated by TNF-α at 0.5 h and this stimulation peaked at 6 h. This activity was attenuated by pretreatment with quercetin and the inhibitors rottlerin, SP600125, or Bay 11-7082 (Fig. 6F).

Taken together, these observations suggest that ICAM-1 and MMP-9 expression was regulated through the activation of a PKCδ–JNK1/2–NF-κB-dependent pathway in ARPE-19 cells.

3.7. Quercetin attenuated TNF-α-induced THP-1 cell adherence and MMP-9 promoter activity via the PKCδ–JNK1/2–c-Jun or NF-κB and MEK1/2–ERK1/2 pathways in ARPE-19 cells

Administration of inhibitors of ICAM-1 or MMPs is an attractive potential therapy for diabetic retinopathy and is being investigated in several experiments [23–25]. We investigated whether quercetin can attenuate ICAM-1-dependent monocyte adhesion to ARPE-19 cells stimulated by TNF-α. As shown in Fig. 7A, TNF-α significantly increased THP-1 cell adhesion to ARPE-19 cells, and this effect was attenuated by quercetin.

We next investigated whether PKCδ, JNK1/2, ERK1/2, and NF-κB are involved in the inhibition by quercetin of THP-1 cell adhesion to ARPE-19 cells induced by TNF-α. The inhibitors of kinases, including rottlerin, SP600125, U0126 or Bay 11-7082 greatly attenuated TNF-α-induced THP-1 cell adhesion to ARPE-19 cells (Fig. 7B). This phenomenon was also observed for TNF-α-regulated MMP-9 promoter activity (Fig. 7C). These results suggest that ICAM-1 and MMP-9 expression induced by TNF-α was regulated through the PKCδ, JNK1/2, ERK1/2, c-Jun, and/or NF-κB (p65) pathways, and that this effect contributed to retinal inflammatory responses.

4. Discussion

Quercetin, a polyphenolic flavonoid, was used in the present study because of its anti-inflammatory effects. Quercetin has been reported to significantly inhibit ICAM-1 expression in response to inflammatory stimuli in the A549 pulmonary epithelial cell line [36], human endothelial cells [37], and after acute lung injury in rats with sepsis [38]. A previous study has shown that quercetin downregulates MMP-9 expression and prevents ethanol-induced gastric inflammation [39]. Quercetin also has inhibitory effects on MMP-9 expression and the production of proinflammatory cytokines, which attenuates LPS-induced lung inflammation [40]. We observed that the expression of ICAM-1 and MMP-9, and THP-1 cell adherence were upregulated in TNF-α-stimulated ARPE-19 cells. We investigated further whether quercetin could inhibit the responses induced by TNF-α. The results indicated that quercetin significantly reduced the expression of ICAM-1 and MMP-9, and THP-1 cell adherence, which suggests that quercetin may be effective in preventing the progression of inflammation.

PKC is involved in various pathways that regulate cell functions and proliferation [41]. In this study, we found that quercetin decreased ICAM-1 and MMP-9 expression induced by TNF-α through the PKCδ–JNK1/2–c-Jun pathway. We also used the inhibitors SP600125 or rottlerin to investigate the relationships between PKCδ, JNK1/2, and c-Jun in TNF-α-stimulated ARPE-19 cells. Pretreatment with SP600125 or rottlerin significantly inhibited JNK1/2 and c-Jun phosphorylation, which indicated that TNF-α evoked ICAM-1 and MMP-9 expression through the PKCδ–JNK1/2–c-Jun pathway in ARPE-19 cells; this is consistent with the findings of a previous study [14].

Several researchers have provided evidence that the MAPK family participates in various pathophysiological processes in the retina and cornea [42–45]. Three prominent groups of MAPKs have been identified in mammals—ERK1/2, p38 MAPK, and JNK1/2—and play essential roles in cell proliferation and differentiation, and inflammation [46]. The expression of cytokines and ICAM-1 have been shown to be regulated through the MAPK and NF-κB pathways [35]. IL-1β has been reported to stimulate ICAM-1 expression via differential activation of PI3K–Akt and MEK–ERK1/2 in the pulpal inflammatory processes [47]. Glucose or mannitol induce ICAM-1 expression through the ERK1/2, p38, and JNK1/2 cascades in glomerular endothelial cells [48]. TNF-α induces the expression of proinflammatory cytokines/chemokines and ICAM-1 via activation of NF-κB, ERK1/2, and p38 MAPK in HaCaT cells [49].

In human RPECs, TNF-α induces ICAM-1 expression via ERK1/2 and JNK1/2 [14]. This evidence suggests that ICAM-1 expression induced by inflammatory stimuli is strongly associated with MAPKs in various cell types. Our findings suggest that quercetin inhibited TNF-α-induced ERK1/2 phosphorylation. The increase in TNF-α-induced ERK1/2 phosphorylation was markedly suppressed by pretreatment with U0126 but not with rottlerin. In addition, U0126 pretreatment suppressed the increase in c-Jun phosphorylation induced by TNF-α. Taken together, our findings suggest that TNF-α induced the expression of ICAM-1 and MMP-9 through both the PKCδ–JNK1/2–c-Jun and MEK1/2–ERK1/2–c-Jun signaling pathways in ARPE-19 cells.

NF-κB plays an essential role in regulation of inflammatory responses. The stimulation of proinflammatory cytokines such as TNF-α can increase NF-κB activity after phosphorylation and degradation of IκBα. Active NF-κB then translocates to the nucleus to regulate the expression of genes for proinflammatory factors including cytokines, chemokines, and adhesion molecules [50–52]. We observed that pretreatment with Bay 11-7082 attenuated ICAM-1 and MMP-9 expression induced by TNF-α in ARPE-19 cells. A previous study has reported that quercetin prevented esophageal mucosal injury caused by chronic mixed reflux esophagitis in rats via suppressing the NF-κB signaling pathway [53].

Quercetin also downregulates the activation of NF-κB and p38 MAPK, and inhibits the expression of inflammatory cytokines in the HMC-1 human mast cell line [54]. Our study found that quercetin reduced TNF-α-induced NF-κB phosphorylation and translocation, and RelA/p65–Luc activity, which suggests that quercetin decreased TNF-α-induced ICAM-1 and MMP-9 expression by inhibiting NF-κB activation. Given the previously reported implications that MAPKs are involved in regulating NF-κB transcriptional activity [55,56], we investigated further whether ERK1/2, PKCδ, and JNK1/2 participate in TNF-α-induced NF-κB transcriptional activity in ARPE-19 cells. We confirmed that TNF-α-induced NF-κB (p65) phosphorylation and translocation, and RelA/p65–Luc activity were mediated via the PKCδ–JNK1/2 but not the ERK1/2 pathway. These findings suggest that quercetin attenuated ICAM-1 and MMP-9 expression induced by TNF-α partially by suppressing the PKCδ–JNK1/2–NF-κB pathway.

5. Conclusion

Our results point to a possible mechanism for the effects of quercetin in the treatment of human retinal inflammatory diseases (Fig. 8) Quercetin seems to inhibit monocyte adherence ability, which then suppresses the expression of ICAM-1 and MMP-9. In our study, quercetin suppressed ICAM-1 and MMP-9 expression by inhibiting the phosphorylation of PKCδ, JNK1/2, ERK1/2, c-Jun, and NF-κB. Our findings suggest that quercetin may provide a novel and effective therapeutic strategy for the prevention or treatment of retinal inflammatory diseases.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgements

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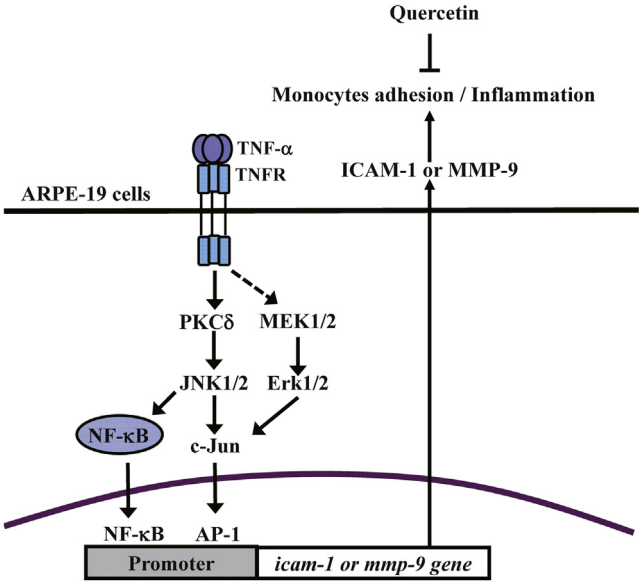

Fig. 8. Schematic diagram of the signaling pathways for attenuation by quercetin of TNF-α-induced inflammation via downregulation of ICAM-1 and MMP-9 expression in ARPE-19 cells. Quercetin attenuates TNF-α-induced ICAM-1 and MMP-9 expression in ARPE-19 cells via the MEK1/2–ERK1/2 and PKCδ–JNK1/2–c-Jun or NF-κB pathways.

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