

Original Article

Squamosamide derivative FLZ inhibits TNF- α -induced ICAM-1 expression via down-regulation of the NF- κ B signaling pathway in ARPE-19 cells

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Abstract: Dysfunction of the retinal pigment epithelium (RPE) resulting from chronic inflammation is implicated in the pathogenesis of age-related macular degeneration (AMD). It has been reported that tumor necrosis factor- α (TNF- α) could induce intercellular adhesion molecule-1 (ICAM-1) expression in RPE cells. FLZ, a novel synthetic squamosamide derivative from a Chinese herb, *Annona glabra*, has displayed significant anti-inflammatory activity. However, the effects of FLZ on TNF- α -induced ICAM-1 expression in RPE cells remain unknown. Therefore, in the present study, we evaluated the effects of FLZ on TNF- α -induced ICAM-1 expression in RPE cells. We found that FLZ prevented TNF- α -induced ICAM-1 expression and the ability of monocytes to adhere to ARPE-19 cells induced by TNF- α . Furthermore, FLZ inhibited TNF- α -induced NF- κ B p65 expression, as well as phosphorylation of I κ B α in ARPE-19 cells. Taken together, these results suggest that FLZ inhibited TNF- α -induced ICAM-1 expression through blocking NF- κ B signaling pathway in ARPE-19 cells. Thus, FLZ could be used for designing novel therapeutic agents against AMD.

Keywords: Squamosamide derivative FLZ, age-related macular degeneration (AMD), retinal pigment epithelium (RPE), tumor necrosis factor- α (TNF- α), intercellular adhesion molecule-1 (ICAM-1)

Introduction

Age-related macular degeneration (AMD) is the leading cause of severe vision loss in the developed world among persons older than 50 years of age [1]. The incidence continues to rise as a result of the expansion in the number of aged people [2]. The exact mechanism of AMD remains yet to be elucidated, but a number of risk factors associated with AMD have been identified. In AMD, pathologic changes in the retinal pigment epithelium (RPE) have been involved in the disease process. Accumulating evidence indicates that cytokines, such as tumor necrosis factor- α (TNF- α) mediate RPE cell dysfunction and contribute to the development of AMD [3-5].

Intercellular adhesion molecule-1 (ICAM-1) is a transmembrane glycoprotein, binds to two integrins of the β 2 subfamily on leukocytes that mediate leukocyte adhesion [6]. Lee et al.

showed that TNF- α could induce ICAM-1 protein and mRNA expression and promoter activity in RPE cells [7]. Thus, inhibition of ICAM-1 expression may lead to therapy for AMD.

FLZ (N-[2-(4-hydroxy-phenyl)-ethyl]-2-(2,5-dimethoxy-phenyl)-3-(3-methoxy-4-hydroxy-phenyl)-acrylamide) is a novel synthetic derivative of squamosamide, which was first isolated from *Annona glabra*. Previous studies have demonstrated that FLZ has protective effects against neuronal damage and neuronal death induced by hydrogen peroxide, glutamate, N-methyl-D-aspartate, hemoglobin and ischemia-reoxygenation [8-10]. In addition, FLZ has strong anti-inflammatory effect. Pang et al. reported that FLZ inhibits lipopolysaccharide-induced inflammatory effects in RAW264.7 macrophages [11]. It has also been shown that FLZ could protect RPE cells from oxidative stress through activation of epidermal growth factor receptor (EGFR)-AKT signaling pathway [12].

However, the effects of FLZ on TNF- α -induced ICAM-1 expression in RPE cells remain unknown. Therefore, in the present study, we evaluated the effects of FLZ on TNF- α -induced ICAM-1 expression in RPE cells.

Materials and methods

Cell culture and reagents

Human RPE cell line (ARPE-19) was purchased from American Type Culture Collection (ATCC, Manassas, VA), and cultured in Dulbecco's modified Eagle's medium (DMEM)/Nutrient Mixture F-12 (DMEM/F12, Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Shanghai, China) and penicillin/streptomycin (1:100, Sigma, St. Louis, MO, USA) in a humidified incubator at 37°C and 5% CO₂. THP-1 human monocytic cells were maintained in RPMI 1640 medium supplemented with 10% FBS, penicillin G (100 U/ml), streptomycin (100 g/ml), and L-glutamine (2 mM) in a humidified incubator at 37°C and 5% CO₂.

FLZ (purity = 99.6%) was supplied by Professor Ping Xie (Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing, China).

Cell viability assay

Cell viability was assessed in 96-well dishes using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, 1×10⁴ cells/well were pretreated with different concentrations (0, 10, 25 and 50 µg/ml) of FLZ for 24 h, then, 20 µL of 5 mg/mL MTT was added to each well and incubated at 37°C for 4 h. The medium was then gently aspirated and 150 µL of dimethyl sulfoxide (DMSO) was added to each well to solubilize the formazan crystals. The spectrophotometric absorbance was measured at 570 nm on a multifunctional microplate reader (Tecan, Durham, NC, USA).

RNA extraction and quantitative reverse transcription polymerase chain reaction (RT-qPCR)

ARPE-19 cells were pretreated with FLZ for 12 h, and then stimulated with TNF- α (10 ng/ml) for 6 h. Total RNA was extracted from the treated cells (TRIzol, Invitrogen-Gibco, Carlsbad, USA), according to the manufacturer's protocol. Oligo-dT first strand cDNA was synthesized from total RNA using First-Strand cDNA Syn-

thesis Kit (Invitrogen, Grand Island, NY, USA). Real-time PCR was performed using an Applied Biosystems StepOne™ real-time PCR system (Applied Biosystems, Bedford, MA, USA). The specific primer sequences are shown as follow: for ICAM-1, 5'-CCGGAAGGTGTATGAACTG-3' (sense), 5'-CAGTTCATACACCTTCCGG-3' (antisense); and for GAPDH, 5'-AGAAGGCTGGGGC-TCATTG-3' (sense), 5'-AGGGGCCATCCACAGTCTTC-3' (antisense). Quantitative real-time PCR data were calculated by the 2- $\Delta\Delta$ CT method [13].

Western blot

ARPE-19 cells were pretreated with FLZ for 12 h, and then stimulated with TNF- α (10 ng/ml) for 6 h. Then ARPE-19 cells were washed twice with ice-cold PBS, lysed on ice with buffer (RIPA; 50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate) containing a protease and phosphatase inhibitor cocktail (Sigma Aldrich). Cell lysates were centrifuged at 13,000× g for 15 min, and protein concentrations of the cell supernatants were measured by BCA Protein Assay kit. The sample (10 µg protein) was loaded in SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Whatman Schleicher & Schuell, Middlesex, UK). The membranes were blocked in a Tris-buffered saline with 0.05% Tween-20 (TBST) solution containing 5% nonfat dried milk for 2 h and then incubated overnight at 4°C with specific antibodies against ICAM-1, p-IkBa, IkBa, p65, Lamin B1 and β -actin (Invitrogen, Carlsbad, CA, USA). After washing and blocking, the membranes were further incubated with horseradish peroxidase-linked secondary antibodies (1:10,000) at room temperature for 1 h. After washing with TBST buffer, the protein bands were detected using enhanced chemiluminescence (ECL) reagent and visualized and photographed using a LAS 3000 chemiluminescence system (Fujifilm, Tokyo, Japan).

Cell adhesion assay

ARPE-19 cells were pretreated with FLZ for 12 h, and then stimulated with TNF- α (10 ng/ml) for 6 h, and then adhesion assays were performed as described previously [14]. In brief, THP-1 cells were labeled with a fluorescent dye, 10 µM calcein-AM, at 37°C for 1 h in RPMI-1640 medium (Gibco BRL, Grand Island, NY). The labeled THP-1 cells were co-cultured with

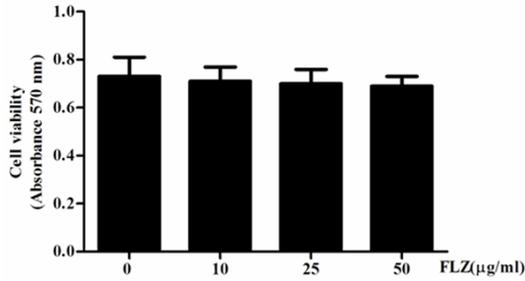


Figure 1. Effect of FLZ on ARPE-19 cell viability. ARPE-19 cells were treated with various concentrations (0, 10, 25 and 50 µg/ml) of FLZ for 24 h. The cytotoxicity was then measured by MTT assay. All experiments were repeated at least three times. Data are means ± SD.

ARPE-19 cells for 1 h. Non-adherent THP-1 cells were removed and plates were washed twice with PBS. Then monocyte-RPE adhesion was observed under a Nikon E600 fluorescent microscope. The number of fluorescence-labeled THP-1 was determined from five randomly selected high-power fields (200×). Experiments were performed in triplicate and repeated at least three times.

Statistical analysis

Data were presented as means ± SD from three or more independent experiments. Statistical analysis involved use of the Student's *t* test for the comparison of 2 groups or 1-way ANOVA for multiple comparisons. A value of *P*<0.05 was considered statistically significant.

Results

Cytotoxicity of FLZ to ARPE-19 cells

To investigate the effect of FLZ on ARPE-19 cells viability, the cells were treated with various concentrations of FLZ (0, 10, 25 or 50 µg/ml). As indicated in **Figure 1**, the viabilities of ARPE19 cells were unaffected by FLZ. These results showed that FLZ did not have cytotoxic effects on ARPE19 cells at the concentration range of 10-50 µg/ml.

Effects of FLZ on ICAM-1 expression in TNF-α-stimulated ARPE-19 cells

In order to determine the effect of FLZ on ICAM-1 expression in ARPE-19 cells, we treated ARPE-19 cells with TNF-α in the presence or absence of FLZ. As indicated in **Figure 2**, TNF-α treat-

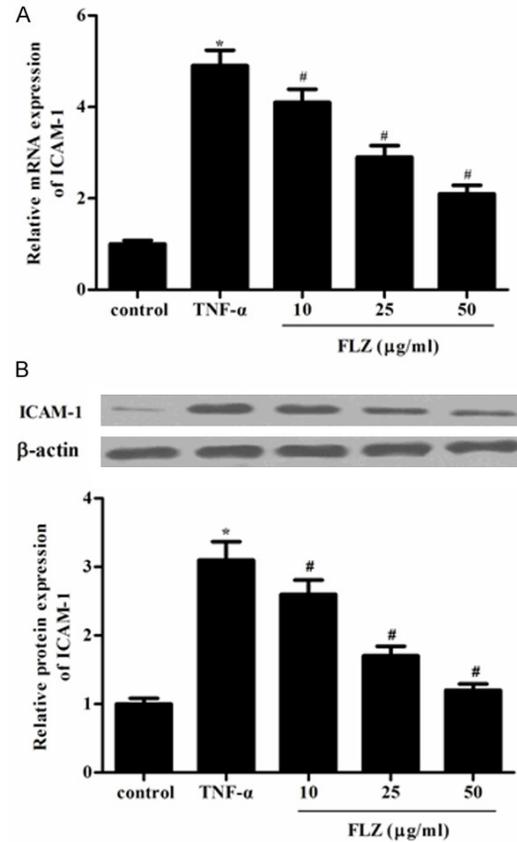


Figure 2. Effect of FLZ on adhesion molecule expression in TNF-α-stimulated ARPE-19 cells. ARPE-19 cells were preincubated with various concentrations (0, 10, 25 and 50 µg/ml) of FLZ for 12 h and stimulated with TNF-α (10 ng/ml) for 6 h. A. ICAM-1 mRNA expression was determined by qRT-PCR. B. ICAM-1 protein expression was determined by Western blot. All experiments were repeated at least three times. Data are means ± SD. **P*<0.05 vs. control group, #*P*<0.05 vs. TNF-α group.

ment obviously increased the mRNA of ICAM-1 expression in ARPE-19 cells, while, FLZ prevented TNF-α-induced ICAM-1 mRNA expression (**Figure 2A**). Consistent with RT-PCR analysis, FLZ also prevented TNF-α-induced ICAM-1 protein expression (**Figure 2B**). These results suggest that FLZ inhibited TNF-α-induced ICAM-1 expression in ARPE-19 cells.

Effects of FLZ on cell adhesion assays in vitro

Since FLZ inhibited TNF-α-induced ICAM-1 expression in ARPE-19 cells, we asked whether it also has an effect on monocyte adhesion (THP-1 cells) to ARPE-19 cells. To explore the effect of FLZ on TNF-α-induced monocyte adhesion (THP-1 cells) to ARPE-19 cells, we

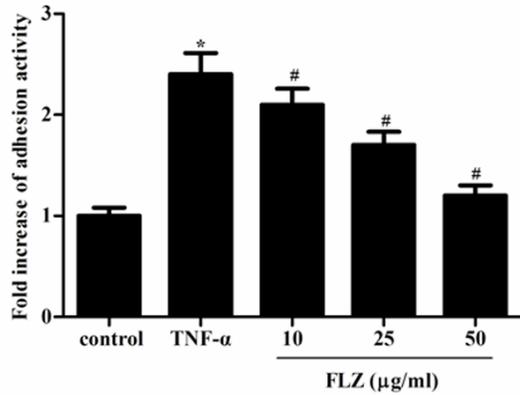


Figure 3. Effects of FLZ on cell adhesion assays *in vitro*. ARPE-19 cells were preincubated with various concentrations (0, 10, 25 and 50 μg/ml) of FLZ for 12 h and subsequently stimulated with TNF-α for 6 h. Fluorescein-labeled THP-1 cells were added to cytokine treated or untreated monolayers of ARPE-19 cells. The numbers of fluorescein-labeled THP-1 cells were determined. FLZ inhibited TNF-α-increased the ability of monocytes to adhere to ARPE-19 cells. All experiments were repeated at least three times. Data are means ± SD. *P<0.05 vs. control group, #P<0.05 vs. TNF-α group.

adopted a cell adhesion assay. As indicated in **Figure 3**, TNF-α treatment significantly increased the ability of monocytes to adhere to ARPE-19 cells, while FLZ reversed this effect, exhibiting a dose-dependent manner.

Effects of FLZ on TNF-α-induced NF-κB activation

NF-κB signaling pathway plays a critical role in the development of AMD [15, 16], and transcription factors have been known to be important mediators of adhesion molecule expression [17, 18]. Therefore, we investigated whether FLZ could inhibit NF-κB pathway in ARPE-19 cells. As indicated in **Figure 4**, TNF-α treatment significantly increased the expression of NF-κB p65. However, FLZ decreased the expression level of NF-κB p65 induced by TNF-α. In addition, FLZ also inhibited TNF-α-induced phosphorylation of IκBα. These results suggest that FLZ inhibits TNF-α-induced NF-κB activation in ARPE-19 cells.

Discussion

Monocyte-RPE cells adhesion is an early step in inflammatory disorders such as AMD [19]. Therefore, reducing monocyte adhesion to RPE cells is a promising pharmacological target for

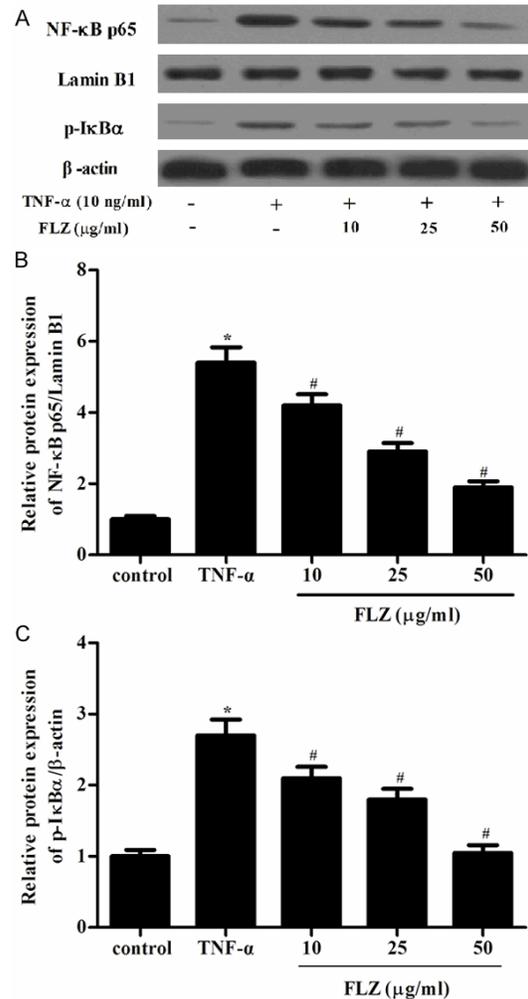


Figure 4. Effects of FLZ on NF-κB signaling pathway in TNF-α-stimulated ARPE-19 cells. A. ARPE-19 cells were preincubated with or without various concentrations of FLZ for 12 h, then treated with TNF-α for 30 min. Then, lysates of cells were blotted with an antibody against the p65 subunit of NF-κB (nucleus) and an anti-phospho-IκBα antibody (cytoplasm). Lamin A and β-actin were used as loading controls for nuclear and cytosolic protein fractions, respectively. B and C. results were quantified with densitometry. All experiments were repeated at least three times. Data are means ± SD. *P<0.05 vs. control group, #P<0.05 vs. TNF-α group.

the prevention of AMD. Thus, in the present study, the effect of FLZ on the TNF-α-mediated adhesion of monocytes to RPE cells was investigated. In this study, we found that FLZ prevented TNF-α-induced ICAM-1 expression and the ability of monocytes to adhere to ARPE-19 cells induced by TNF-α. Furthermore, FLZ inhibited TNF-α-induced NF-κB p65 expression, as well as phosphorylation of IκBα in ARPE-19 cells.

Previous studies have demonstrated that the expression of ICAM-1 was increased in eyes with diabetic retinopathy [20] and in subfoveal choroidal neovascular membranes (CNVMs) surgically excised from patients with AMD [21]. These results suggest that ICAM-1 may play a critical role in the pathogenesis of AMD. TNF is a multifunctional cytokine shown to be involved in inflammation through expression of various inflammatory molecules [22-24]. It has been reported that expression of adhesion molecules on endothelial cells (ECs) is induced by stimulation with inflammatory cytokines, including TNF- α , IL-1 β , IL-4, and IL-13 [25]. In addition to ECs, several studies have demonstrated that RPE cells also express ICAM-1 in AMD [26], TNF- α increased the expression of the inflammatory protein ICAM-1 and increased monocyte-RPE adhesion [27]. It is well known that interactions between *monocytes* and *RPE cells* can occur via ICAM-1, which can be antagonized by the inhibition of ICAM-1 [28]. To confirm this hypothesis, we investigated the effects of FLZ on the monocyte THP-1 adherence to TNF- α -stimulated *ARPE-19 cells*; we observed FLZ prevented *the ability of monocytes to adhere to ARPE-19 cells induced by TNF- α* . These results suggest that FLZ inhibits ICAM-1 expression which could decrease the adhesion of monocytes in TNF- α -stimulated ARPE-19 cells.

NF- κ B has been considered a prototypical pro-inflammatory signaling pathway, and the role of NF- κ B in the expression of other proinflammatory genes including cytokines, chemokines, and adhesion molecules [29-31]. It has been well established that the NF- κ B pathway is activated by proinflammatory cytokines such as TNF- α and IL-1 β . In the resting state, NF- κ B resides in the cytoplasm as a heterotrimer consisting of p50, p65, and the inhibitory subunit I κ B. When the pathway is activated, the I κ B protein is phosphorylated and subsequently degraded [32]. Previous study showed that FLZ suppressed LPS-induced increase in the DNA binding activity of NF- κ B and activator protein 1 (AP-1), the nuclear translocation of NF- κ B p65, the degradation of I κ B α and the phosphorylation of I κ B α in RAW264.7 macrophages [11]. In line with above results, in the present study, we found that FLZ inhibited TNF- α -induced NF- κ B p65 expression, as well as phosphorylation of I κ B α in ARPE-19 cells. This finding suggests that the inhibitory effects of FLZ on the expres-

sion of ICAM-1 may be associated with the suppression of expression of NF- κ B in ARPE-19 cells.

In conclusion, to our knowledge, this is the first time to find out that FLZ inhibited monocyte-RPE cells adhesion and ICAM-1 expression through blocking NF- κ B signaling pathway in TNF- α -stimulated ARPE-19 cells. Thus, FLZ could be used for designing novel therapeutic agents against AMD.

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Disclosure of conflict of interest

None.

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