Involvement of Intracellular Calcium Mobilization in *IL-8* Activation in Human Retinal Pigment Epithelial Cells

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Purpose. Calcium signaling is an important intracellular pathway. Increased intracellular calcium is associated with cytokine regulation and inflammatory signals secretion. The purpose of this study is to understand the molecular mechanisms by which calcium signaling controls *IL-8* activation in human RPE cells.

Methods. Fluorescence-based calcium imaging and different mutants of *IL-8* plasmids were used in this study. The *IL-8* promoter activation, gene expression, and secretion were detected by using luciferase reporter assay, quantitative real-time PCR (Q-PCR), and ELISA, respectively. In addition, pharmacological inhibitors and small interfering RNA (siRNA) were applied to clarify the mechanisms of *IL-8* activation.

Results. Our study reported that intracellular calcium mobilization activated *IL-8* gene expression and secretion. Application of pharmacological inhibitor BAY 11-7082, siRNA, and plasmids of the nuclear factor κ light chain enhancer of activated B cells (NF-κB) binding site, we identified that NF-κB is the main transcription factor involved in intracellular calcium mobilization-mediated IL-8 activation in human RPE cells.

Conclusions. Collectively, our findings highlight the important role of intracellular calcium mobilization in the activation of *IL-8*. These findings may be helpful for the clinical applications in the age-related macular degeneration (AMD) prevention and treatment.

Keywords: calcium, IL-8, retinal pigment epithelium

The retinal pigment epithelium (RPE), which is located between the neurosensory retina and choroidal vasculature, is essential for normal visual function by interacting with photoreceptors.[1] In proinflammatory situations, RPE cells become activated and provoke inflammatory processes that contribute to immune-related ocular conditions, such as age-related macular degeneration (AMD).[2]

Several lines of evidence indicated that the proinflammatory cytokine, IL-8, increased in vitreous fluid samples of various vitreoretinal diseases.[3,4] The IL-8 expression also was detected after mechanical injury in the RPE during a wound-healing response.[5] The IL-8 further attracts neutrophils and evokes neutrophil-mediated inflammation leading to tissue destruction.[6,7] The concentration of IL-8 in the aqueous humor was found to be associated with exudative AMD.[8] Single-nucleotide polymorphisms of the *IL-8* gene also were reported to be related to AMD development[9–11] and to bevacizumab treatment responses for exudative AMD.[12]

In the pathogenesis of AMD, lipofuscin granules accumulation is considered to have a significant role.[13] In a further study, the increase in calcium signals was found to trigger lipofuscin accumulation in RPE cells.[14] Previous studies also showed that calcium overload may cause cell impairment and death.[15–17] Since that calcium is important in intracellular signaling, the increase of intracellular calcium concentration can affect cellular functions, such as cell proliferation, secretion of cytokines, and phagocytosis.[18] The calcium signaling pathway was reported to involve in *IL-8* gene expression in neutrophils, osteoblasts, and respiratory epithelial cells.[19–21] Therefore, we hypothesized that intracellular calcium mobilization is involved in *IL-8* activation in RPE cells. Thapsigargin (TG) is used widely to trigger intracellular calcium release and calcium influx.[22,23] Herein, we report that intracellular calcium mobilization and nuclear factor κ light chain enhancer of activated B cells (NF-κB) pathways contributed to *IL-8* activation in RPE cells.

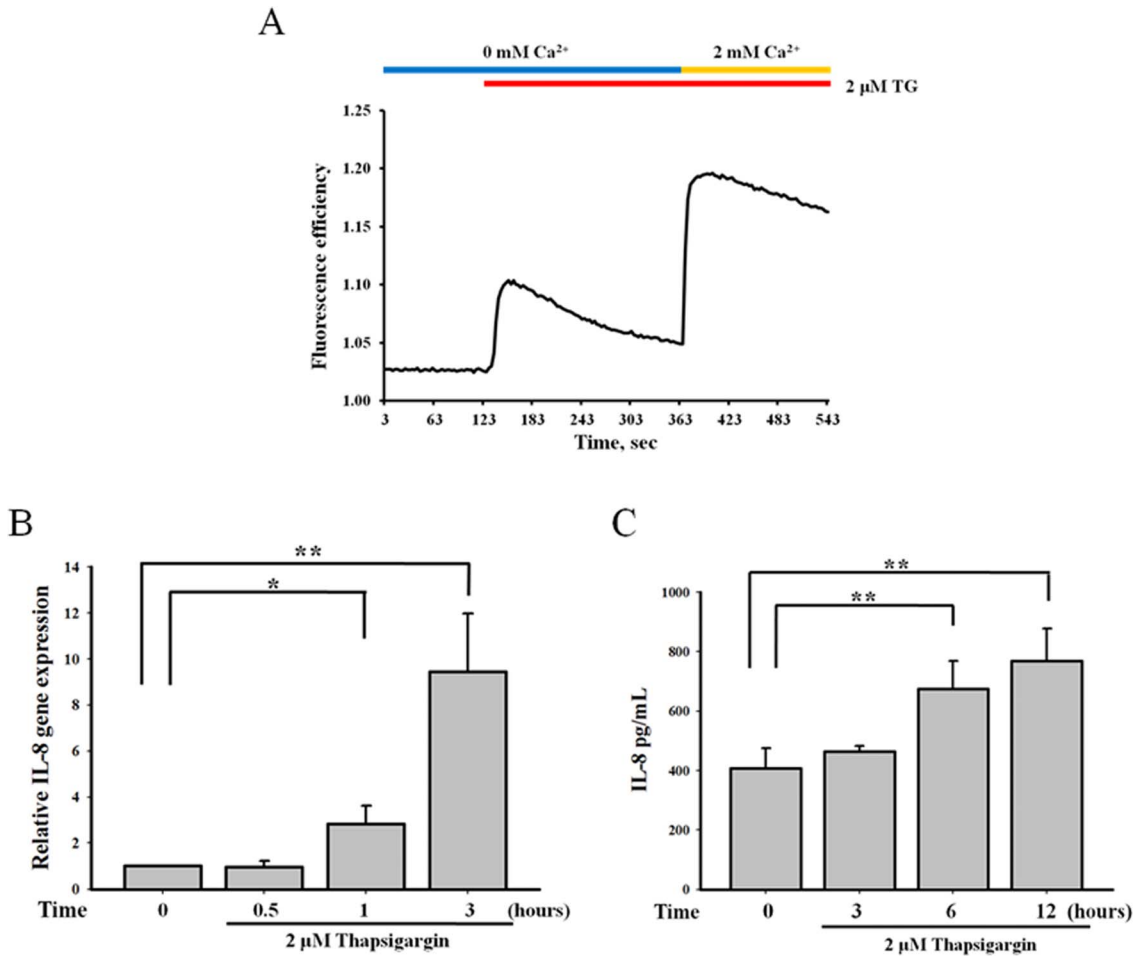

Figure 1. Effects of TG on intracellular calcium mobilization, *IL-8* gene expression, and IL-8 secretion. (**A**) Intracellular calcium signals to TG in the absence and presence of external 2 mM calcium. (**B**) Time course of *IL-8* gene expression following stimulation with TG. (**C**) The 2 μM TG induced a time-dependent increase in IL-8 secretion.

Methods

Cell Culture

The human retinal epithelial (ARPE-19) cells were acquired from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in a Dulbecco's modified Eagle's medium (DMEM):F-12 nutrient mixture (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS; Invitrogen), and 1% penicillin/streptomycin mixture (Invitrogen) at 37°C in a humidified atmosphere with 5% CO_2. The medium was replaced with DMEM:F-12 containing 0.5% FBS 3 hours before the beginning of treatment.

Determination of Calcium Concentrations

After ARPE-19 cells were seeded onto glass coverslips for 24 hours, the attached cells were loaded with 1 μM Fluo-4 (Invitrogen) at 37°C for 20 minutes in the dark. Cells then were washed in a standard external solution: 130 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 2 mM $MgSO_4$, 5.5 mM D-glucose, and 20 mM HEPES at pH 7.4. Changes in the fluorescence intensity of Fluo-4 in loaded cells were detected by time-lapse video-microscopy (IX70; Olympus, Tokyo, Japan) and analyzed by the cell $^{\Lambda}$R system (Olympus).

RNA Extraction and RT-PCR

Total RNA was extracted from ARPE-19 cells using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. The RNA samples of 1 μg were used to synthesize complementary DNA (cDNA) by RT using an RT kit (Invitrogen). Incubation conditions included 10 minutes at 25°C, 120 minutes at 37°C, and 5 minutes at 85°C. Except the time determination tests, samples of RNA experiments were collected after 3 hours of TG treatment.

Quantitative Real-Time PCR (Q-PCR)

After obtaining cDNA, *IL-8* gene expression was quantified by real-time PCR SYBR green assays. Sequences of the primers used were: IL-8 forward primer: CAG AGA CAG CAG AGC ACA C and IL-8 reverse primer: AGT TCT TTA GCA CTC CTT GGC, and β-actin forward primer: ATC TCC TTC TGC ATC CTG TCG GCA AT and β-actin reverse primer: CAT GGA GTC CTG GCA TCC ACG AAA C. The SYBR Green PCR master mix reagent (Applied Biosystems, Carlsbad, CA, USA) was used to amplify cDNA, and products were detected with a 7500 Real-Time PCR System (Applied Biosystems).

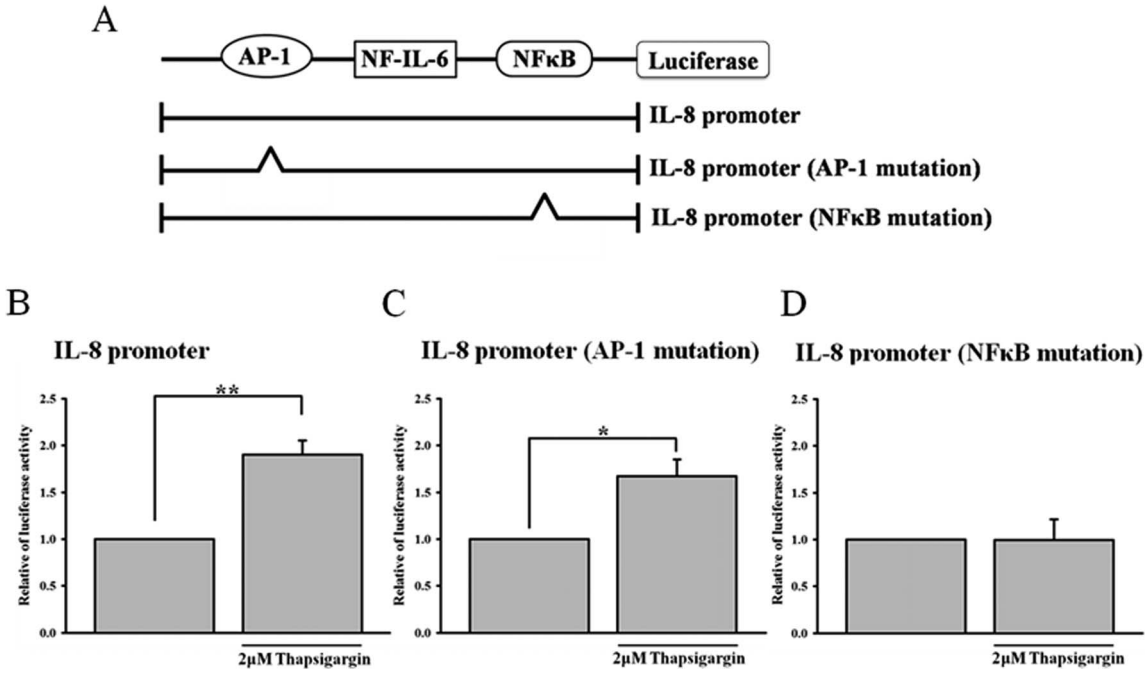

Figure 2. Determination of TG-responsive regions in the *IL-8* gene promoter. (**A**) The *IL-8* gene promoter area contains AP-1–, NF-IL-6–, and NF-κB–binding regions. The ARPE-19 cells were transiently transfected with different mutants of *IL-8* promoter plasmids, including wild-type, an AP-1 mutation, and an NF-κB mutation. (**B**) Activation of the wild-type *IL-8* promoter revealed an almost 2-fold luciferase activity following stimulation with 2 μM TG (**$P < 0.01$). (**C**) Mutation of the AP-1 site in *IL-8* promoter revealed a 1.7-fold luciferase activity following stimulation with 2 μM TG (*$P < 0.05$). (**D**) Mutation of the NF-κB site in *IL-8* promoter abolished TG-induced activity.

ELISA

After various treatments, IL-8 protein secretion in the supernatant was measured using an ELISA kit (PeproTech, Rocky Hill, NJ, USA) with specific antibodies against human IL-8 according to the manufacturer's protocol. Plates were read at 405 nm with a microplate spectrophotometer. Except the time determination tests, samples for ELISA experiments were collected after 6 hours of TG treatment.

Transfection and Luciferase Reporter Assay

After seeding of ARPE-19 cells for 24 hours, luciferase reporter plasmids with the *IL-8* promoter (gifts from Marc Hershenson, University of Michigan, Ann Arbor, MI, USA) or with multimers of NF-κB (a kind gift from Wan-Wan Lin, National Taiwan University, Taipei, Taiwan) were transfected into cells using Opti-MEM medium (Invitrogen) containing Lipofectamine 2000 (Invitrogen). The *IL-8* promoter contained binding sites for activator protein (AP)-1, nuclear factor-interleukin-6 (NF-IL-6)/nuclear factor of activated T cells, and NF-κB.[24–26] The luciferase reporter *IL-8* plasmids provided by Hershenson were with −162/+44 fragment of the wild-type human *IL-8* promoter (−162/+44 hIL-8/Luc), and of mutations at AP-1– or NF-κB–binding site (ΔAP-1 −162/+44 hIL-8/Luc or ΔNF-κB −162/+44 hIL-8/Luc).[27–30] Cells were cultured for an additional 24 hours before 3 hours of low-serum medium and further treatment. Following TG stimulation for 3 hours with conditioned pretreatment for 30 minutes, cells were lysed with cell lysis buffer (Promega, Madison, WI, USA), and cell lysates were assayed with a luciferase assay kit (Promega) according to the manufacturer's protocol.

Transfection of Small Interfering RNA (siRNA)

After seeding of ARPE-19 cells for 24 hours, cells were transiently transfected with control siRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA), NF-κB p65 siRNA (Santa Cruz Biotechnology) in Opti-MEM medium containing Lipofectamine 2000 for 24 hours. The luciferase reporter plasmids were further transfected on the following day as the same methods mentioned above for another 24 hours. Next, the cells were treated according to individual experiments before final harvest.

Protein Extraction and Western Blotting

Whole-cell lysates (30 μg) were isolated in radioimmunoprecipitation assay (RIPA) buffer with a protease inhibitor. Proteins were analyzed by SDS-PAGE on a 10% gel and transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 5% nonfat dry milk for 1 hour at room temperature. Blots were probed overnight at 4°C with diluted primary antibodies specific for the individual target protein. The antibody against NF-κB p65 (Cell Signaling, Beverly, MA, USA) was diluted 1:1000. The antibody against β-actin (Sigma-Aldrich Corp., St. Louis, MO, USA) was diluted 1:20,000. Membranes were washed three times with 0.1% Tween 20 in 1× PBS (PBST) and incubated with a 1:4000 to 1:10^4 dilution of peroxidase-linked anti-rabbit or anti-mouse IgG secondary antibodies (Millipore) for 1 hour at room temperature. Last, protein bands were visualized using an enhanced chemiluminescence (ECL)–plus Western blotting detection system (Millipore).

Statistical Analysis

Statistical analyses were performed using the Statistical Package for Social Science program (SPSS for Windows, vers. 13.0; SPSS, Chicago, IL, USA). Student's *t*-test and a 1-way ANOVA were used to compare differences between groups. *P* values of less than 0.05 were considered statistically significant.

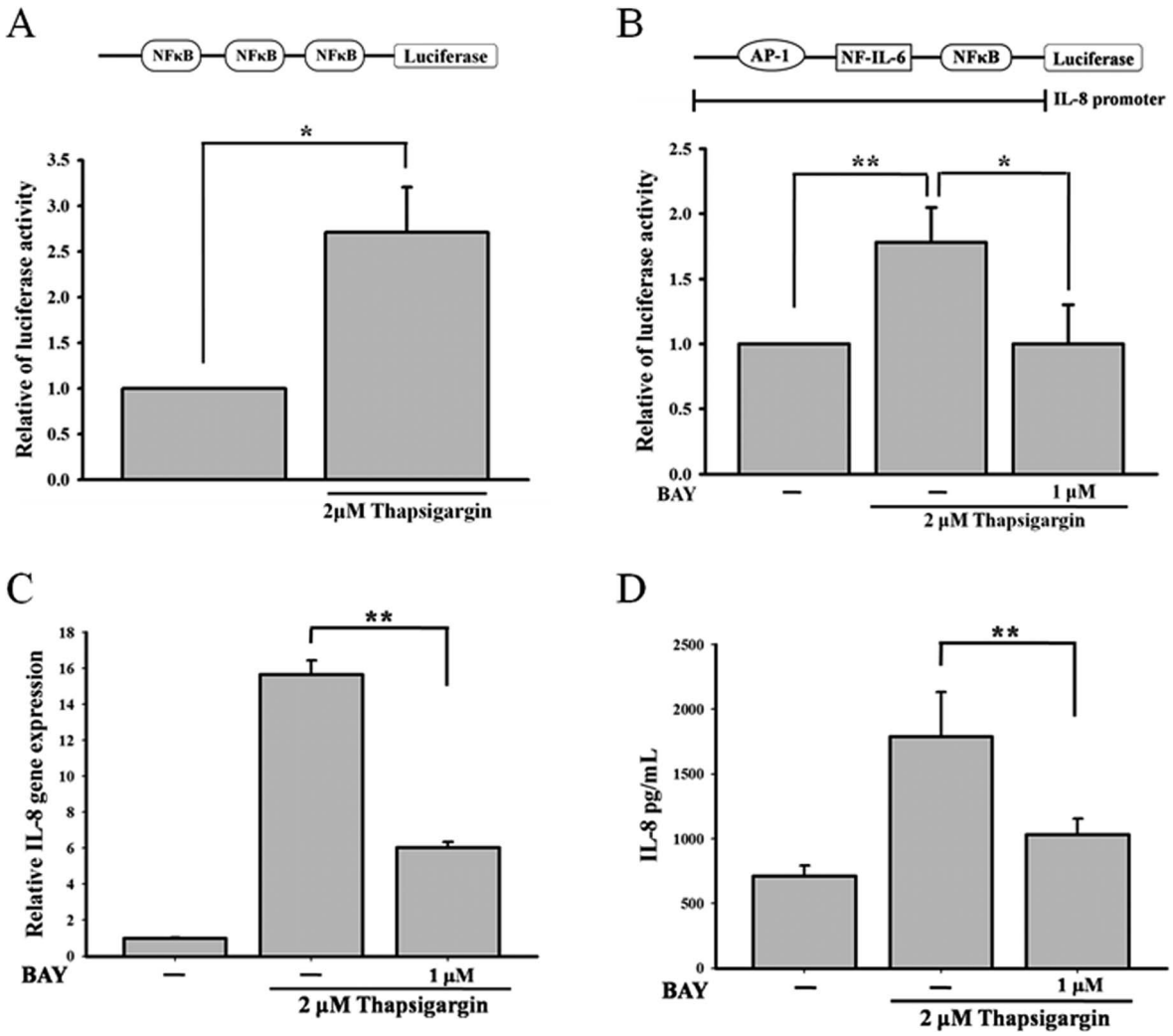

Figure 3. Nuclear factor-κB is an important transcription factor involved in TG-induced IL-8 expression. (**A**) The luciferase reporter gene with three copies of NF-κB binding site was transfected into cells. The NF-κB multimer was activated following stimulation with 2 μM TG. (**B–D**) The IκB kinase inhibitor, BAY 11-7082, significantly suppressed TG-induced IL-8 luciferase activity, gene expression, and protein secretion.

Results

TG-Induced Calcium Signals Activated *IL-8* Expression in ARPE-19 Cells

As shown in Figure 1A, cells were stimulated with 2 μM TG in a calcium-free solution, and a short-term calcium release was detected. With the add-back protocol of adding 2 mM calcium solution, calcium influx signals were detected. Application of 2 μM TG to ARPE-19 cells stimulated *IL-8* gene expression in a time-dependent manner (Fig. 1B). The *IL-8* gene expression was not obviously detected until 1 hour and was significantly increased by 3 hours. Stimulation with 2 μM TG also resulted in a time-dependent increase in IL-8 secretion (Fig. 1C). The results indicated a strong correlation between TG-induced calcium signals and *IL-8* activation in ARPE-19 cells.

Identification of TG-Responsive Regions in the Promoter Area of the *IL-8* Gene

To clarify the mechanisms of how the TG-induced calcium signals regulate *IL-8* promoter activation, various mutants of *IL-8* promoter plasmids were used. As shown in Figure 2A, the −162/+44 fragment of human *IL-8* promoter contained AP-1–, NF-IL-6/NFAT–, and NF-κB binding–sites. Plasmids with site-directed mutations in *IL-8* promoter at AP-1 and NF-κB binding sites also were used. The 2 μM TG stimulation for 3 hours triggered a significant induction of *IL-8* promoter activity (Fig. 2B). Expression of the mutant *IL-8* promoter activity with AP-1 site mutation was slightly reduced after 2 μM TG stimulation (Fig. 2C). Importantly, expression of the mutant *IL-8* promoter with NF-κB site mutation was significantly decreased to the level of the control (Fig. 2D), indicating that NF-κB is the most important binding site responsible for TG-induced *IL-8* transcription.

NF-κB is an Important Transcription Factor in TG-Mediated *IL-8* Expression

To further confirm the role of NF-κB in the regulatory pathways for *IL-8*, the plasmids with three copies of NF-κB binding sites were transfected into ARPE-19 cells. Activity of the NF-κB multimer was induced greater than 2.5-fold ($^*P < 0.05$) by 2 μM TG (Fig. 3A). We then applied the IκB kinase inhibitor, BAY

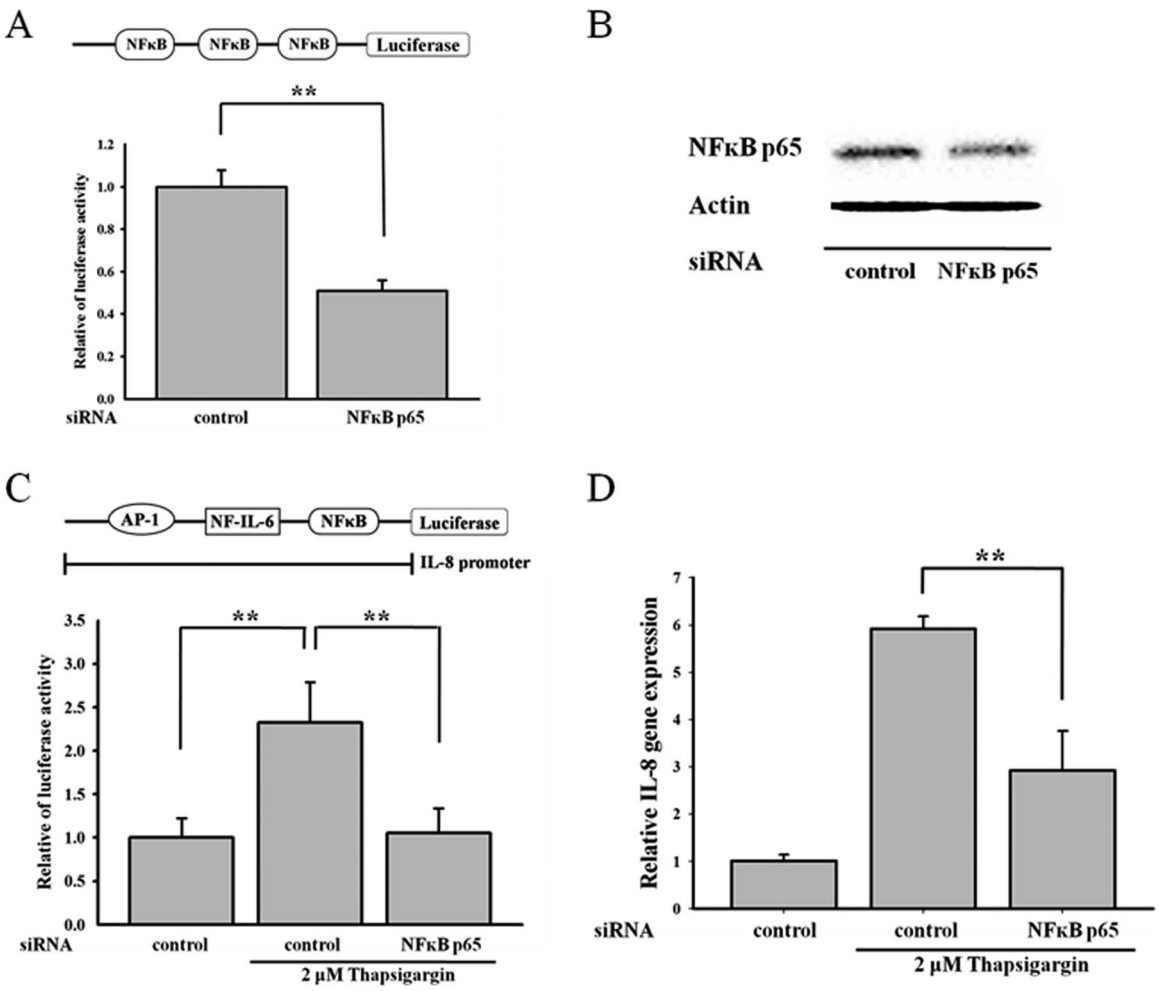

FIGURE 4. Knockdown of NF-κB p65 impairs TG-induced *IL-8* transcription and gene expression. (**A**) The ARPE-19 cells were transfected with siRNA of NF-κB p65. The NF-κB multimer activity was reduced by 50% (**$P < 0.01$). (**B**) The expression of NF-κB p65 protein following transfection with siRNA of NF-κB p65 was obtained by Western blot analysis. (**C–D**) Knockdown of NF-κB p65 suppressed the TG-induced activity of *IL-8* promoter and gene expression.

11-7082, to further verify the functional role of NF-κB. As shown in Figure 3B, pretreatment with 1 μM BAY 11-7082 for 30 minutes significantly suppressed TG-induced *IL-8* promoter activation. In addition, 1 μM BAY 11-7082 inhibited 2 μM TG-induced *IL-8* gene expression (**$P < 0.01$, Fig. 3C) and IL-8 secretion (**$P < 0.01$; Fig. 3D) in ARPE-19 cells.

KnockDown of NF-κB p65 Decreased TG-Induced *IL-8* Expression in ARPE-19 Cells

To confirm the involvement of NF-κB in TG-induced *IL-8* activation, siRNA of NF-κB p65 was transfected into ARPE-19 cells. As shown in Figures 4A and 4B, luciferase activity of the NF-κB multimer and NF-κB protein was reduced by transfection of NF-κB p65 siRNA. In addition, knockdown of NF-κB p65 was further shown to significantly suppress the TG-induced *IL-8* promoter activation (**$P < 0.01$, Fig. 4C) and the TG-induced *IL-8* gene expression (**$P < 0.01$, Fig. 4D).

Intracellular Calcium Mobilization Is Involved in TG-Mediated *IL-8* Gene Expression

Since TG induces calcium release from the endoplasmic reticulum (ER) and subsequent calcium influx, we explored the source of calcium for *IL-8* gene expression. After chelating intracellular calcium with BAPTA-AM, TG-induced *IL-8* gene expression was significantly suppressed (**$P < 0.01$, Fig. 5A). However, chelation of extracellular calcium by ethylene glycol tetraacetic acid (EGTA) did not influence TG-induced *IL-8* gene expression (Fig. 5B). Application of neither SKF96365, an store-operated calcium (SOC) channel inhibitor, nor nifedipine, an L-type calcium channel inhibitor, suppressed TG-induced *IL-8* gene expression (Figs. 5C, 5D). These results indicated that TG-evoked *IL-8* gene expression was mediated by TG-induced intracellular calcium mobilization.

Intracellular Calcium Mobilization Mediated TG-Induced IL-8 Secretion and NF-κB Activation in ARPE-19 Cells

We further applied BAPTA-AM to confirm the role of calcium mobilization in NF-κB activation and IL-8 secretion. As shown in Figures 6A and 6B, 5 μM BAPTA-AM suppressed TG-induced NF-κB activation (*$P < 0.05$) and IL-8 secretion (**$P < 0.01$). Application of 1 mM EGTA chelating extracellular calcium was failed to inhibit TG-induced NF-κB multimer activity (Fig. 6C). Moreover, to ascertain the source of calcium for *IL-8* activation, further experiments were performed in the calcium-free buffer.

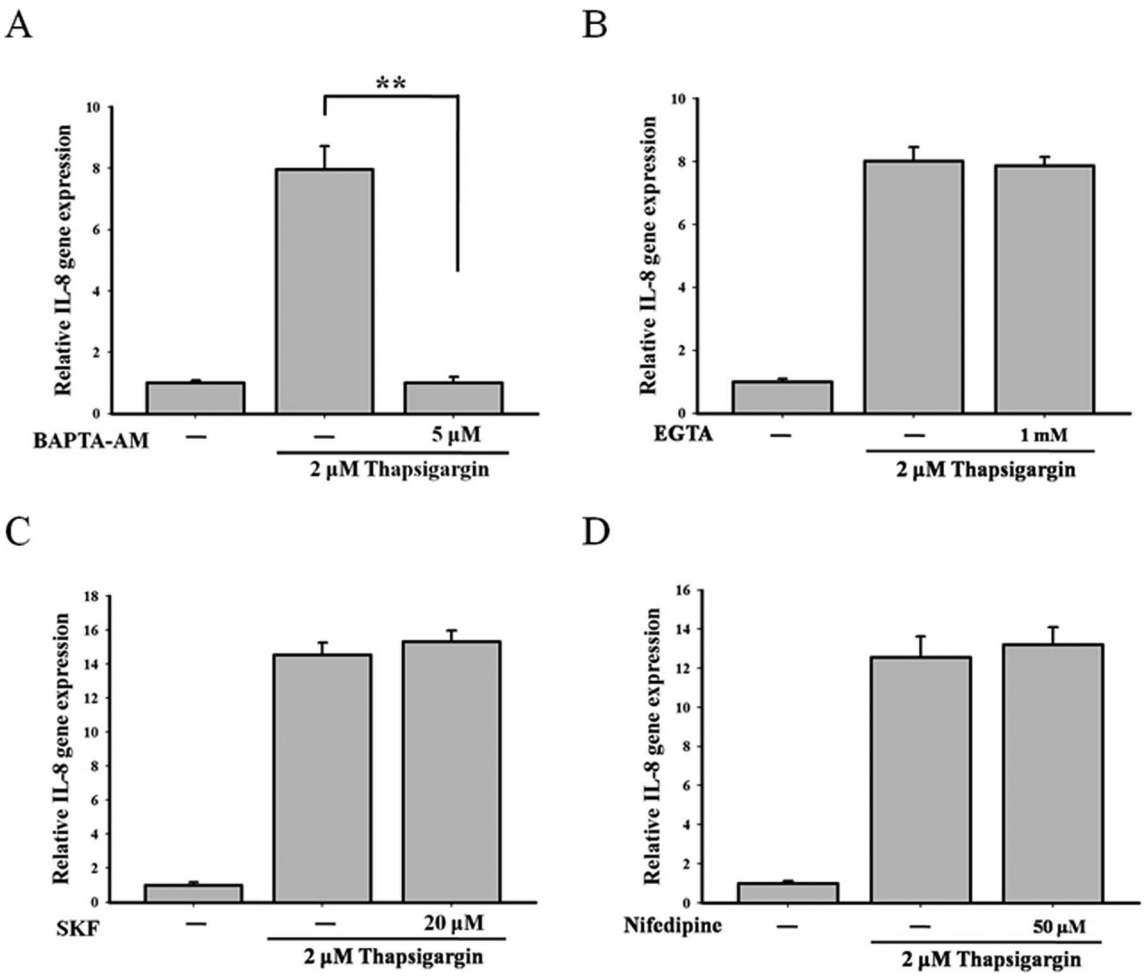

Figure 5. Determination of calcium source for TG-induced *IL-8* gene expression. (**A**) In the presence of BAPTA-AM, TG fails to induce *IL-8* gene expression. (**B**) Expression level of *IL-8* gene did not change following stimulation with TG in the presence of EGTA. (**C**) The store-operated calcium channel inhibitor, SKF96365 did not block TG-induced *IL-8* gene expression. (**D**) Nifedipine did not reduce TG-induced *IL-8* gene expression.

As shown in Figure 6D, 2 μM TG stimulation can induce *IL-8* gene expression in the calcium-free condition ($^{**}P < 0.01$). These data indicated that TG-induced NF-κB and *IL-8* activation is via intracellular calcium mobilization.

Discussion

In this study, we demonstrated the effects of intracellular calcium mobilization in the activation of *IL-8* gene in human RPE. Calcium signaling in RPE cells is involved in the interaction between RPE and photoreceptors,[31] and influences RPE cell proliferation, migration, and survival.[16,32] Previous studies have indicated that arachidonic acid-mediated intracellular calcium release is essential for *c-jun* gene expression.[33] Moreover, *IL-8* gene activation and secretion were regulated by calcium mobilization from intracellular stores in human colonic epithelial cells and human airway epithelial cells.[34,35] Consistent with previous reports, our study indicated that intracellular calcium mobilization stimulated IL-8 production via NF-κB pathways in ARPE-19 cells (Fig. 7).

A strong correlation was obtained between elevated levels of IL-8 concentrations in aqueous humor and exudative AMD patients.[8] In addition, genetic association studies between polymorphism of *IL-8* gene and AMD were reported.[9,12] Patients with *IL-8* +781 T allele not only had a higher risk of wet AMD, but also correlated with higher vitreous levels of IL-8.[10] Although the role of IL-8 in pathogenesis of AMD has not yet clearly understood, various molecules and mechanisms have been reported in cell-based studies to link AMD and IL-8. Oxidative stress,[36–38] complement pathways,[39] and senescence,[40] were considered as important factors in *IL-8* activation and AMD development. Clinically, AMD presents with pigmentary abnormalities attributing to RPE degeneration. The RPE associated with drusen in AMD has been histologically evident and regarded as pathological risk for AMD.[41] In this study, ARPE-19 cell line was used since it is known to have structural and functional characteristics of RPE cells.[42] This cell line has been used widely as RPE representative in numerous in vitro studies.

In aged cells, altered functions of ryanodine receptors (RYRs), inositol-1,4,5-trisphosphate receptors (IP_3Rs), and sarcoplasmic/endoplasmic reticulum calcium-ATPase (SERCA) pumps caused disturbance of subtle calcium homeostasis. In addition, the accumulation of oxidized proteins or ER stress, which might lead to disease.[43,44] Previous studies showed the increase of intracellular calcium and lipofuscin in the aging brain.[43] Interestingly, the pathogenesis of AMD, similar to brain aging, also shows an increase of intracellular calcium and

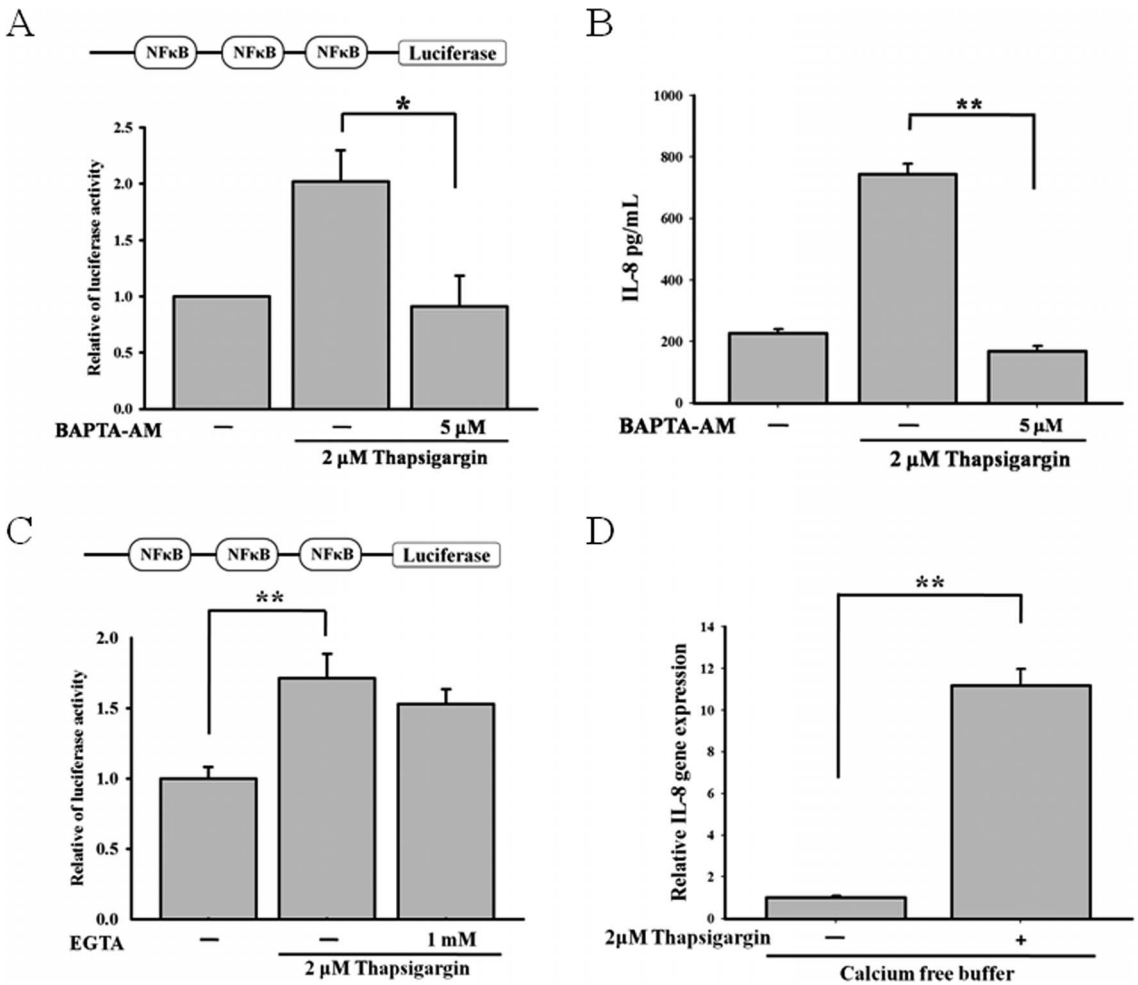

Figure 6. Involvement of intracellular calcium signaling in *IL-8* expression and NF-κB activation. (**A**) The TG-induced NF-κB multimer luciferase activity is suppressed in the presence of 5 μM BAPTA-AM (**B**) The 5 μM BAPTA-AM suppressed TG-induced IL-8 secretion (**$P < 0.01$). (**C**) In the presence of 1 mM EGTA, NF-κB multimer activity did not change following stimulation with TG. (**D**) The 2 μM TG induced *IL-8* gene expression in the calcium-free condition (**$P < 0.01$).

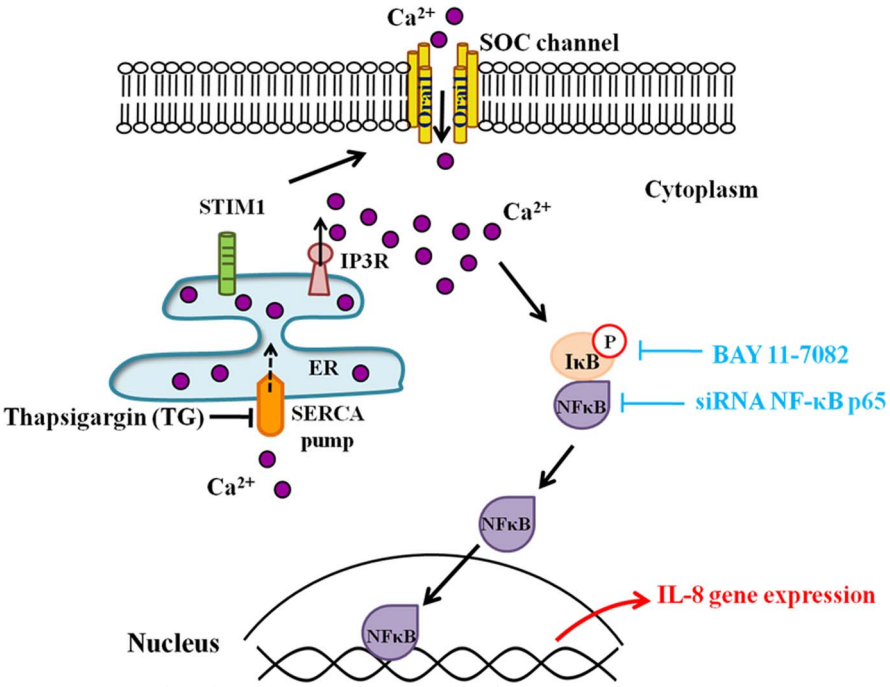

Figure 7. Schematic representation of TG-induced intracellular calcium mobilization and *IL-8* gene expression in ARPE-19 cells. Intracellular calcium mobilization activates NF-κB transcription factor, which, in turn, triggers *IL-8* gene expression. The IκB kinase inhibitor, BAY 11-7082 and siRNA of NF-κB inhibited calcium-mediated *IL-8* gene expression.

lipofuscin, which is the product of RPE cells phagocytizing wastes of photoreceptor outer segments.[14,45] In this study, TG was used to mimic the aging-mediated intracellular calcium change in RPE cells. Our results elucidated the involvement of intracellular calcium mobilization in activation of NF-κB as well as *IL-8*. In addition, oxidative stress has been considered as an important factor in the pathogenesis of AMD.[46,47] Hydrogen peroxide (H_2O_2) is thought to lead to oxidative stress. Also, H_2O_2 was reported to regulate intracellular calcium signals and *IL-8* expression.[36,48,49] Consistent with this, our study also indicated that intracellular calcium mobilization was involved in H_2O_2-mediated IL-8 production in ARPE-19 cells (Supplementary Fig. S1). These findings support a functional role of intracellular calcium signals in *IL-8* gene regulation.

In conclusion, our results demonstrated that intracellular calcium mobilization is able to trigger the activation of NF-κB and IL-8. The IL-8 is a proinflammatory cytokine and has been linked to AMD. As a result, intracellular calcium signaling and NF-κB may become rational targets for treatment of AMD.

Acknowledgments

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