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Investigation of the preventive effect of calcium on inflammation-mediated choroidal neovascularization

In Sung Sung[a,1], Seon Young Park[a,1], Keun-Yeong Jeong[b,*,1], Hwan Mook Kim[a,**]

[a] Gachon Institute of Pharmaceutical Science, Gachon University, Incheon, Republic of Korea
[b] MetiMedi Pharmaceuticals Co., Research Center, Incheon, Republic of Korea

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ABSTRACT

Aims: Age-related macular degeneration (AMD) is a leading cause of irreversible blindness in elderly people. The pathogenesis of neovascular AMD is known but is closely related to inflammation and choroidal neovascularization (CNV). The aim of this study was to investigate the anti-inflammatory and anti-angiogenic effects of calcium on neovascular AMD.

Main methods: Human retinal pigment epithelial cells (ARPE-19) were used to identify protein markers of inflammation induced by differentiated macrophages. Choroidal neovascularization (CNV) mouse model was established by rupturing the Bruch's membrane using laser photocoagulation in C57BL/6 mice. Mice were divided into the following groups: untreated control and calcium supplemented. The expression levels of toll-like receptor isotype (TLR) 4, nuclear factor kappa B (NF-κB), hypoxia-inducible factor-1α (Hif-1α), and vascular endothelial growth factor (VEGF) were investigated to check whether calcium supplementation results in suppression of inflammation and has an anti-angiogenic effect. CNV was evaluated by immunofluorescence staining on choroidal flat mounts.

Key finding: The inflammation-induced expression of TLR4, NF-κB, and Hif-1α was decreased in ARPE-19 cells after calcium supplementation. Inhibition of the transcriptional activation of ARPE-19 cells by Hif-1α suppression resulted in decreased VEGF expression. In the laser-induced CNV mouse model, calcium supplementation inhibited inflammatory mediators and neovascularization in the retinal tissue.

Significance: Supplementation with calcium seems to constrain inveterate symptoms of neovascular AMD by inhibiting inflammation and angiogenesis in the laser-induced CNV mouse model.

1. Introduction

Age-related macular degeneration (AMD) is a leading cause of irreversible blindness in people aged 50 years or older [1]. The cause of AMD is not yet known, but genetics, lifestyle (*e.g.*, smoking and high-fat meal) and aging have been associated with AMD pathology [2]. Particularly, aging is the highest risk factor for developing AMD. Recently the prevalence of AMD in the elderly population was calculated to be about 12–30% in Europeans, 10.4% in Hispanics, and 7.4% in Asians [3].

The symptoms of AMD include retinal pigmentary disturbances, focal retinal atrophy, drusen accumulation, and choroidal neovascularization (CNV) [4,5]. Drusen accumulation and CNV are the most significant risk factors for the development of neovascular AMD among all categorized risk factors of AMD and are strongly correlated with advanced age [6]. Despite the well-established relationship between drusen and CNV with regard to the development of neovascular AMD, the underlying mechanism of retinal pigment epithelium (RPE) cell damages is not fully understood.

The pathogenesis of neovascular AMD is known but is closely related to inflammation. Age-related destruction of RPE cells is one of the hallmarks of AMD pathogenesis and it has been suggested to follow

Abbreviations: AMD, age-related macular degeneration; CM, conditioned media; CNV, choroidal neovascularization; TLR, toll-like receptor isotype; NF-κB, nuclear factor kappa B; Hif-1α, hypoxia-inducible factor-1α; VEGF, vascular endothelial growth factor; RPE, retinal pigment epithelium; HUVEC, human umbilical vein endothelial cell; CaLac, lactate calcium salt

* Correspondence to: K.-Y. Jeong, Metimedi Pharmaceuticals Co., Research Center, 263 Central-Ro, Yeonsu-Gu, Incheon 22006, Republic of Korea.
** Correspondence to: H.M. Kim, Gachon Institute of Pharmaceutical Sciences, Gachon University, 191 Hambangmoe-ro, Yeonsu-Gu, Incheon 21936, Republic of Korea.
E-mail addresses: alvirus@naver.com (K.-Y. Jeong), hwanmook@gachon.ac.kr (H.M. Kim).
[1] These authors equally contributed to this work.

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drusen accumulation [7]. Following RPE damage, large numbers of macrophages are recruited *via* chemokine gradients. Furthermore, isolated drusen has been identified to mediate pro-inflammatory responses through the activation of signaling cascades, such as Toll-like receptor isotype (TLR) 4 [7,8].

TLR4 is activated by danger signals in AMD retinas, such as components of drusen, which leads to the activation of the intracellular signaling pathway and increased expression of nuclear factor kappa B (NF-κB) and hypoxia-inducible factor-1α (Hif-1α) [8,9]. This signaling cascade increases the proportion of pro-inflammatory M1 macrophages and cellular stress in RPE. Cellular stress with long-lasting inflammatory responses may result in a vicious cycle of severe RPE damage [7]. Furthermore, Hif-1α increases expression of vascular endothelial growth factor (VEGF), which has been shown to significantly contribute to CNV formation, the major pathological finding in neovascular AMD [10].

Recent clinical studies have reported that dietary and supplementary calcium intake is associated with a lower risk of neovascular AMD [11,12]. Moreover, if we consider the well-established theory that calcium affects anti-inflammatory reactions, it is reasonable to assume that calcium supplementation may lead to the inhibition of neovascular AMD. Therefore, the aim of this study was to investigate the impact of consistent calcium supplementation on inflammatory factors and the CNV, the major pathological findings in neovascular AMD.

2. Materials and methods

2.1. Cell Culture and reagents

Human retinal pigment epithelium cells (ARPE-19) and human leukemic monocyte (THP-1) were purchased from the American Type Culture Collection (Manassas, VA, USA). ARPE-19 and THP-1 cells were maintained in RPMI 1640 medium (Hyclone Laboratories, Inc., Logan, UT, USA) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT, USA), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Welgene, Daegu, South Korea) and incubated in a humidified atmosphere containing 5% CO_2 at 37 °C. 0.05 mM 2-Mercaptoethanol (Gibco, Grand Island, NY, USA) was additionally supplied to the THP-1 cell line only. Lactate calcium salt (CaLac) was purchased from Sigma-Aldrich (St Louis, MO, USA). CaLac was dissolved in distilled water or dimethyl sulfoxide and kept under 4 °C or −80 °C respectively. Antibody against Hif-1α was purchased from BD Biosciences (Heidelberg, Germany). Antibodies against TLR4, NF-κB, Apolipoprotein E (ApoE), CBP, P300, and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against VEGF was purchased from Abcam (Cambridge, UK).

2.2. Preparation of proinflammatory macrophage

To polarize macrophages toward the pro-inflammatory (M1) phenotypes, THP-1 cells were incubated with 100 ng/ml phorbol 12-myristate 13-acetate (Enzo life sciences, Lörrach, Germany) for 12 h. And then THP-1 cells were treated with 20 ng/ml of interferon-gamma (BPS Bioscience Inc., San Diego, CA, USA) and 1 μg/ml of lipopolysaccharide (Sigma, St. Louis, MO, USA). Polarized M1 macrophages were co-cultured with ARPE-19 cells or cultured in fresh RPMI 1640 media to collect conditioned media (CM), and the CM was kept under −80 °C before use.

2.3. Immunocytochemistry

ARPE-19 cells were fixed on bio-coated coverslips (BD bioscience, NJ, USA) using 4% paraformaldehyde, and incubated for 20 h with the primary antibodies. Specific primary antibodies were as follows: TLR4 (1:500); NF-κB (1:500); Hif-1α (1:500); ApoE (1:500); CBP (1:500); P300 (1:500). Subsequently, coverslips were incubated with an anti-rabbit secondary biotinylated antibody (1:1000, Vector, Burlingame, CA, USA) and visualized with fluorescein-conjugated streptavidin (Vector, Burlingame, CA, USA). The cells were then washed and mounted using the mounting medium (Vector Laboratories, Burlingham, CA, USA) and observed using confocal microscopy (Nikon, Tokyo, Japan) with the oil immersion lens in the magnification range 60 × .

2.4. Western blot

Whole-cell lysates were prepared using lysis buffer (20 mM and pH 7.6 Tris-HCl, 1 mM EDTA, 140 mM NaCl, 1% Nonidet P-40, 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium vanadate). Equivalent amounts of protein (20 μg) were separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane. Nonspecific binding was blocked with 5% nonfat powdered milk. The membrane was incubated with primary antibodies in 5% bovine serum albumin at 4 °C for 16 h. Specific primary antibodies were as follows: TLR4 (1:1000); NF-κB (1:1000); HIF-1α (1:1000); ApoE (1:1000); VEGF (1:500); Actin (1:1000). The membrane was then washed three times with Tris-buffered saline containing 0.1% Tween-20 and incubated with an anti-mouse secondary antibody (1:2000, Sigma-Aldrich, St Louis, MO, USA) for 2 h at room temperature. Immunoblots were developed using Western blotting detection reagents (Abclon, Seoul, Korea) and exposed to X-ray film (Agfa, Leverkusen, Germany) according to the manufacturer's protocol.

2.5. Protein array

ARPE-19 cells were cultured with M1 macrophage CM for 24 h. Cultured media was collected using a centrifugal filter unit (Millipore, Billerica, MA, USA) for VEGF and angiogenin assay. Proteome profiler human protein array kit (R&D Systems, Minneapolis, MN, USA) was used according to the manufacturer's instructions.

2.6. Tube formation assay

Matrigel (BD Bioscience Bedford, MA, USA) was added in a 6-well plate and kept at 37 °C for 30 min. Human umbilical vein endothelial cells (HUVECs; 3×10^4 cells) were suspended in the CM and seeded onto Matrigel. After 12 h, morphological changes were captured using an optical microscope (Leica, Wetzlar, Germany).

2.7. CNV animal model

All experiments were performed under the institutional guidelines established by the Institutional Animal Care and Use Committee at Gachon University (approved number: GIACUC-R2017018). Six-week-old male C57BL/6N mice were purchased from Orient Bio (Seongnam, S. Korea). All animals were maintained in a 12 h light/dark cycle (light on, 08:00) at 22–25 °C with free access to food and water. To induce experimental CNV, laser-induced rupture of Bruch's membrane was performed on eyes under gaseous anesthesia with isoflurane. Laser (wavelength, 532 nm) spots were created by slit lamp (SL120; Carl Zeiss Meditec, Jena, Germany). The laser parameters were as follows: spot diameter, 100 lm; intensity, 200 mW; duration, 0.1 s. 20 mg/kg calcium was subcutaneously injected to the interscapular region for 21 days [13].

2.8. H&E and immunofluorescence

Three weeks after laser injury, mice were anesthetized and perfused with PBS. The eyes were enucleated and fixed in 4% paraformaldehyde for 48 h. Fixed eyes were embedded in paraffin and sectioned at 5 mm. Slides were incubated at 55 °C for 2 h, and subsequently deparaffinized in xylene and rehydrated in a graded ethanol series. H&E staining was

performed following this step. For immunofluorescence staining, endogenous peroxidase activity was blocked by 3% H_2O_2 containing distilled water for 30 min. Subsequently, slides were incubated with primary antibodies under 4 °C. The primary antibodies were as follows: TLR4 (1:300); NF-κB (1:300); Hif-1α (1:300). After 24 h incubation, the slides were incubated room temperature 1 h 30 min with anti-mouse secondary antibody (1:2000, Vector, Burlingame, CA, USA) and visualized with streptavidin conjugated to Fluorescein (Vector, Burlingame, CA, USA). Fluorescence images were obtained by confocal laser scanning microscopy (Nikon, Tokyo, Japan) with the oil immersion lens in the magnification range 20 × .

2.9. Fluorescein angiography

Three weeks after laser injury, mice were anesthetized and perfused with PBS containing 25 mg FITC-dextran (Sigma-Aldrich, St Louis, MO, USA). Then, eyes were enucleated and fixed in 4% paraformaldehyde for 48 h under dark and 4 °C. After hemisecting the eye, with total removal of the lens, vitreous body, and retina, RPE-choroid-sclera eyecups were prepared and mounted on a slide glass by mounting solution (Thermo science, Cheshire, UK). Fluorescence images were obtained by confocal laser scanning microscopy (Nikon, Tokyo, Japan) with the oil immersion lens in the magnification range 60 × .

2.10. Statistical analysis

All data are presented as the mean ± standard deviation (SD). Statistical significance was analyzed by the Student's *t*-test or One-way ANOVA, depending on the normality of the data. A difference of $p < 0.05$ was considered to be statistically significant. All statistical analyses were carried out using Sigma Stat (ver. 3.5, Systat Software Inc., Chicago, IL, USA).

3. Results

3.1. Induction of inflammatory factors in human retinal pigment epithelium cells by polarized M1 macrophages

Pro-inflammatory M1 macrophages are recruited to lesions to mediate inflammatory responses in pathological conditions of the eye. To induce a pathological condition similar to inflammatory-mediated neovascular AMD, human retinal pigment epithelium cells (ARPE-19) were co-cultured with M1 macrophages and the expression of inflammatory and angiogenic factors was examined (Fig. 1). Under this co-culture condition, ARPE-19 cells became elongated, and impurities were observed around them as compared to ARPE-19 cells cultured alone (Fig. 1A). The expression of inflammatory factors was also increased in ARPE-19 cells co-cultured with M1 macrophages (Fig. 1B–G). Cytochemical staining indicated increased TLR4 expression in ARPE-19 cells co-cultured with M1 macrophages (Fig. 1B). Quantitation analysis indicated that this increase was statistically significant (Fig. 1C). NF-κB and Hif-1α expression levels were also increased in ARPE-19 co-cultured with M1 macrophages (Fig. 1D, F). Quantitative analysis indicated that this increase was statistically significantly (Fig. 1E, G). VEGF and angiogenin expression levels were also significantly increased and followed the expression pattern of the inflammatory factors (Fig. 1H). The expression levels of ApoE, which is a prominent component of drusen, were qualitatively and quantitatively increased in the co-cultures (Fig. 1I, J).

3.2. Inhibition of the expressing inflammatory factors in human retinal pigment epithelium cells via consistent calcium supplementation

To investigate the effect of calcium on the expression of inflammatory factors, ARPE-19 cells co-cultured with M1 macrophages were treated with calcium and its effect on the expression levels of TLR4, NF-κB, Hif-1α, and ApoE was examined (Fig. 2). Cytochemical staining showed that TLR4 expression was decreased in the calcium-treated ARPE-19 cells as compared to untreated cells (Fig. 2A), which was also confirmed by quantitative analysis (Fig. 2B). It was confirmed that the expression levels of NF-κB and Hif-1α were decreased by calcium supplementation in ARPE-19 cells co-cultured with M1 macrophages (Fig. 2C, E). It was also confirmed by quantitative analysis and the results indicated that the effect of calcium supplementation on the expression levels of NF-κB and Hif-1α statistically significant (Fig. 2D, F). The results of cytochemical staining indicated that ApoE, a drusen marker, expression was also decreased in the calcium-treated ARPE-19 cells as compared to untreated cells (Fig. 2G). Quantitative analysis indicated that the expression level of ApoE was significantly decreased by calcium supplementation in ARPE-19 cells co-cultured with M1 macrophages (Fig. 2H).

3.3. Inhibition of the transcriptional activation in human retinal pigment epithelium cells upon consistent calcium supplementation

Activation of signaling cascades by inflammatory factors results in increased Hif-1α expression, which acting as a transcription factor leads to an increase in the expression of angiogenic factors. ARPE-19 cells co-cultured with M1 macrophages were treated with calcium to investigate its effects on Hif-1α-mediated transcriptional activity and expression of angiogenic factors (Fig. 3). Following calcium supplementation, expression of CBP or P300, which act as transcription factors with Hif-1α, was decreased compared to the untreated cells, despite the fact that ARPE-19 cells were co-cultured with M1 macrophages (Fig. 3A, C). Quantitative analysis indicated that the relative intensity of CBP, P300, or Hif-1α was significantly decreased by calcium supplementation in ARPE-19 cells co-cultured with M1 macrophages (Fig. 3B, D). As the transcriptional activity of CBP and P300, including Hif-1α, was inhibited by calcium supplementation, the expression levels of VEGF protein were also significantly decreased compared to the untreated cells (Fig. 3E). Moreover, tube formation was also inhibited by conditioned media supplemented with calcium (Fig. 3F). Quantitative analysis indicated that the rate of tube formation was significantly decreased to about 24.9 ± 4.98% by the conditioned media supplemented with calcium (Fig. 3F).

3.4. Suppression of the expression of inflammatory and angiogenic factors in laser-induced CNV model via consistent calcium injections

To further investigate the inhibitory effect of calcium supplementation on inflammatory reactions and angiogenesis *in vivo*, a laser-induced CNV mouse model was established, and calcium was administered daily for 21 days (Fig. 4). The results of fluorescence staining indicated that the inflammatory factors were widely expressed from the retina to the sclera of the laser-induced CNV animal model (Fig. 4A, B and C). In the group treated with calcium after CNV induction (CNV + Calcium), the expression of inflammatory factors, TLR4, NF-κB, and Hif-1α, was decreased (Fig. 4A, B and C). Quantitative analysis of these results clearly indicated that the decrease in the expression levels of TLR4, NF-κB, and Hif-1α was statistically significant (Fig. 4A, B and C). As a consequence, vascularization was inhibited by calcium administration even although blood vessels had formed in the choroid layer under the retina in the CNV-induced tissue (Fig. 4D). An analysis of choroidal flat mounts by fluorescein angiography revealed that calcium administration suppressed laser-induced CNV development (Fig. 4E). Quantitative analysis indicated that the relative intensity of fluorescein angiography was significantly decreased by calcium supplementation in the choroid layer (Fig. 4F).

4. Discussion

In the present study, we investigated whether consistent calcium

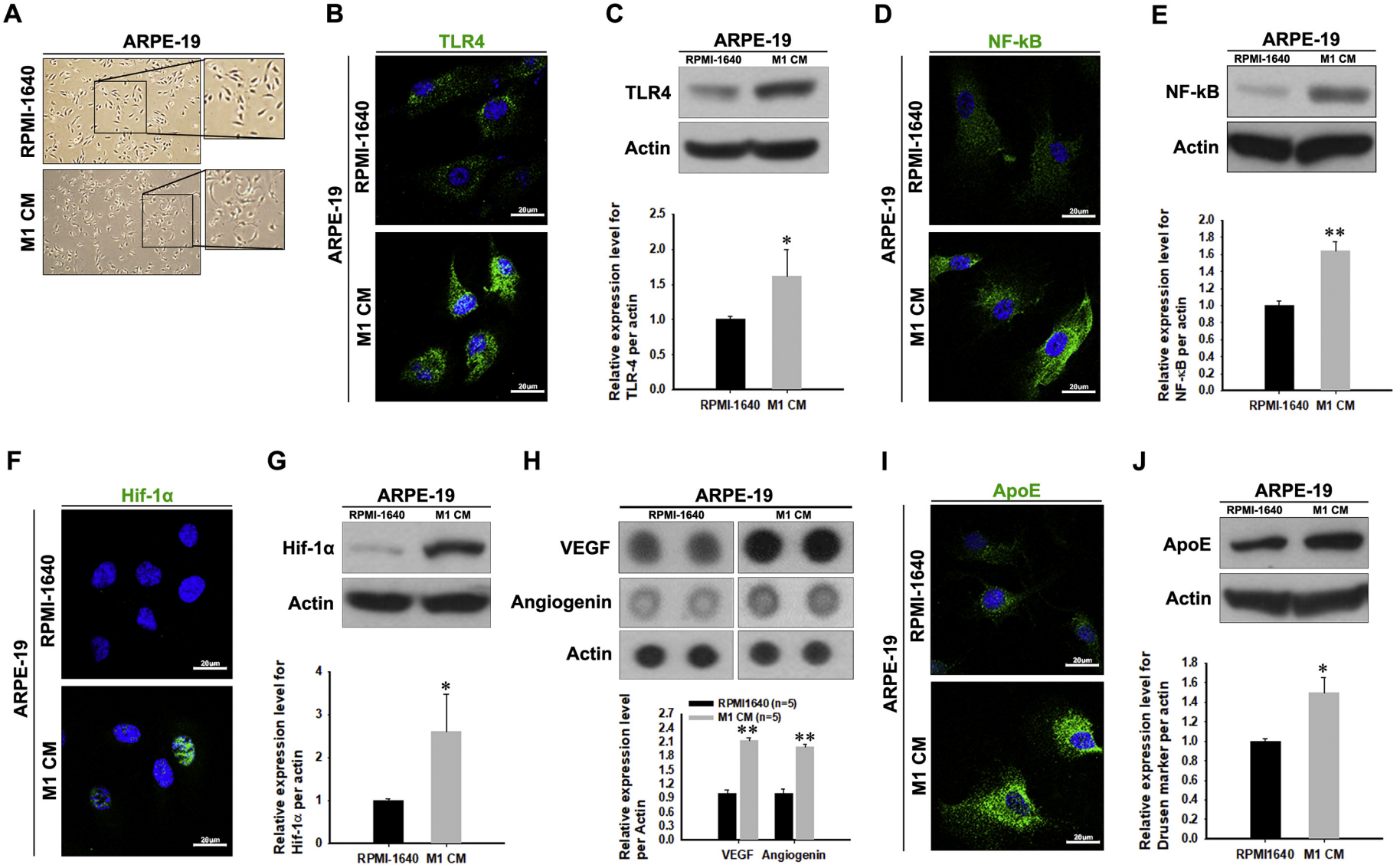

Fig. 1. Induction of inflammatory and angiogenic factors in human retinal pigment epithelium cells (ARPE-19) co-cultured with M1 macrophages. (A) Observation of morphological changes in ARPE-19 cells co-cultured with M1 macrophages. (B) Increased expression of TLR4 in ARPE-19 cells co-cultured with M1 macrophages. Scale bar, 20 μm. (C) Qualitative and quantitative analysis of TLR4 expression in ARPE-19 cells co-cultured with M1 macrophages. (D) Increased expression of NF-κB in ARPE-19 cells co-cultured with M1 macrophages. Scale bar, 20 μm. (E) Qualitative and quantitative analysis of NF-κB expression in ARPE-19 cells co-cultured with M1 macrophages. (F) Increased expression of Hif-1α in ARPE-19 cells co-cultured with M1 macrophages. Scale bar, 20 μm. (G) Qualitative and quantitative analysis of Hif-1α protein expression in ARPE-19 cells co-cultured with M1 macrophages. (H) Quantitative analysis of VEGF and angiogenin protein expression in ARPE-19 cells co-cultured with M1 macrophages. (I) Increased expression of ApoE in ARPE-19 cells co-cultured with M1 macrophages. Scale bar, 20 μm. (J) Qualitative and quantitative analysis of ApoE protein expression in ARPE-19 cells co-cultured with M1 macrophages. The experiments were done in quintuplicate. $^{*}p < 0.05$ and $^{**}p < 0.001$ *vs* RPMI-1640 group. Results are the mean ± SD.

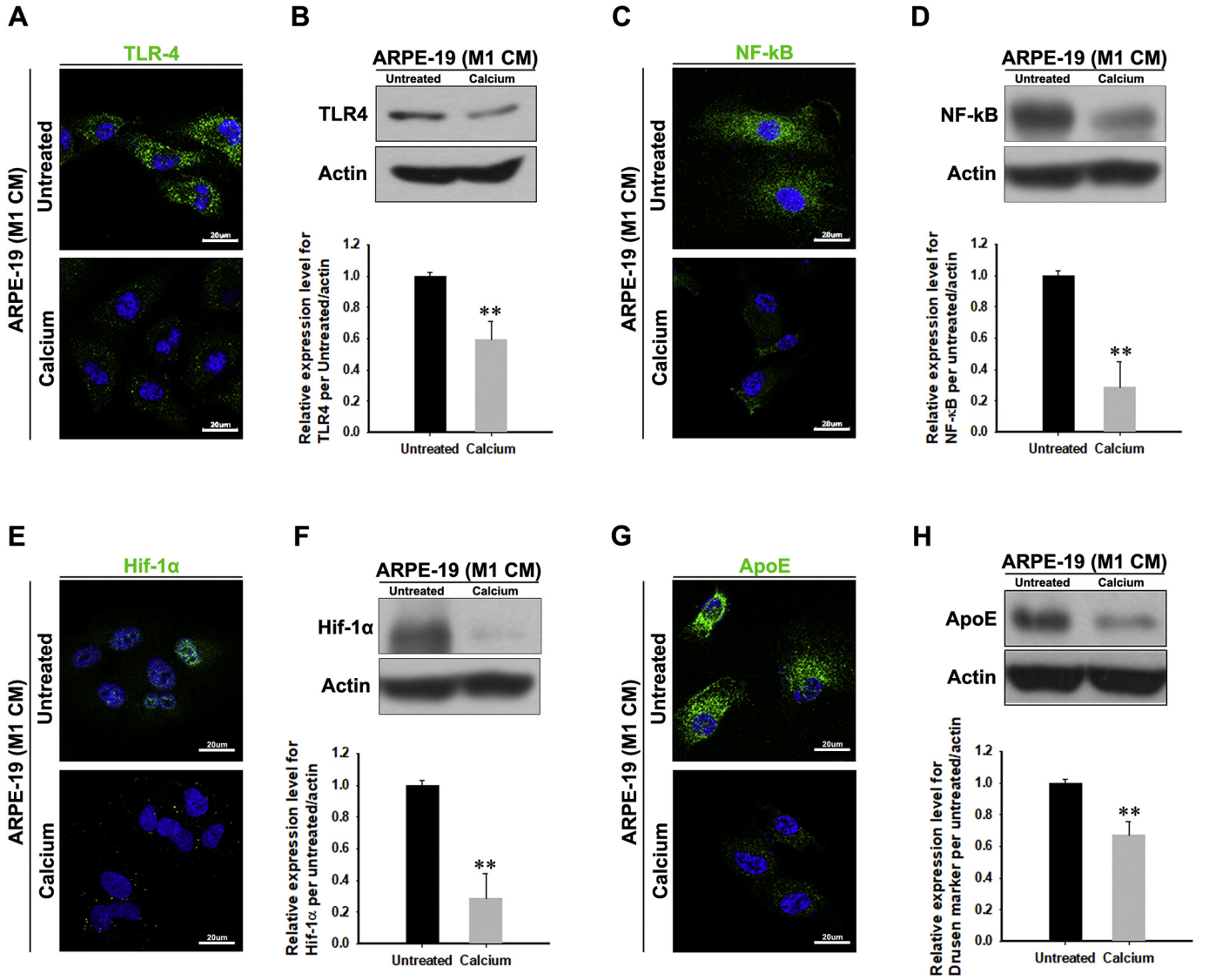

Fig. 2. The anti-inflammatory effect of calcium supplementation in human retinal pigment epithelium cells (ARPE-19) co-cultured with M1 macrophages. (A) Reduction of TLR4 expression by calcium supplementation in ARPE-19 cells cultured with M1 macrophages. Scale bar, 20 μm. (B) Qualitative and quantitative analysis of changes in TLR4 protein expression by calcium supplementation. (C) Reduction of NF-κB expression by calcium supplementation in ARPE-19 cells cultured with M1 macrophages. Scale bar, 20 μm. (D) Qualitative and quantitative analysis of changes in NF-κB protein expression by calcium supplementation. (E) Reduction of Hif-1α expression by calcium supplementation in ARPE-19 cells cultured with M1 macrophages. Scale bar, 20 μm. (F) Qualitative and quantitative analysis of changes in Hif-1α protein expression by calcium supplementation. The experiments were done in quintuplicate. (G) Reduction of ApoE expression by calcium supplementation in ARPE-19 cells cultured with M1 macrophages. Scale bar, 20 μm. (H) Qualitative and quantitative analysis of changes in ApoE expression by calcium supplementation. The experiments were done in quintuplicate. $^{**}p < 0.001$ *vs* Untreated group. Results are the mean ± SD.

supplementation could inhibit expression angiogenic factors following inhibition of the inflammatory reaction in ARPE-19 cells co-cultured with M1 macrophages and whether calcium administration could inhibit laser-induced CNV in an experimental CNV mouse model. The *in vitro* studies showed that calcium supplementation inhibited the expression of inflammatory and angiogenic factors, and that consistent calcium administration in laser-induced CNV animal model suppressed inflammatory responses and choroidal neovascularization.

AMD has been recognized not only as an exudative vascular event but also as a chronic inflammatory disease of the retina and choroid [7,8]. THP-1 can be recruited from blood vessels to sites of tissue damage and inflammation where they differentiate in proinflammatory M1 macrophages in the presence of lipopolysaccharides and interferon-gamma [14]. In addition, an increase in the expression of TLR4 and NF-κB, which are formed by the activation of M1 macrophages, is a critical factor involved in the stimulation of angiogenic factors in human ARPE-19 cells [15]. Therefore, we simulated this similar pathogenic environment of neovascular AMD by co-culturing differentiated M1 macrophages and ARPE-19 cells.

It is well-established that chronic insults related to aging alter the immune system so that it no longer resembles the immune system of young individuals, and the macrophage polarization may be shifted toward M1 [16]. Referring to the more specific role of macrophages, the proportion of M1 macrophages was upregulated in the early stages of AMD, while the proportion of M2-macrophages was slightly upregulated in the middle stage and remained stable until the late stage [16,17]. Therefore, even though M1-macrophages express factors related to a variety of inflammatory responses, the co-culture model is a limited experimental condition that does not reflect the full pathophysiological mechanism of chronic neovascular AMD. Nevertheless, M1 macrophages are significantly involved in the deterioration of tissue damage due to aging, and the mechanism by which they contribute to

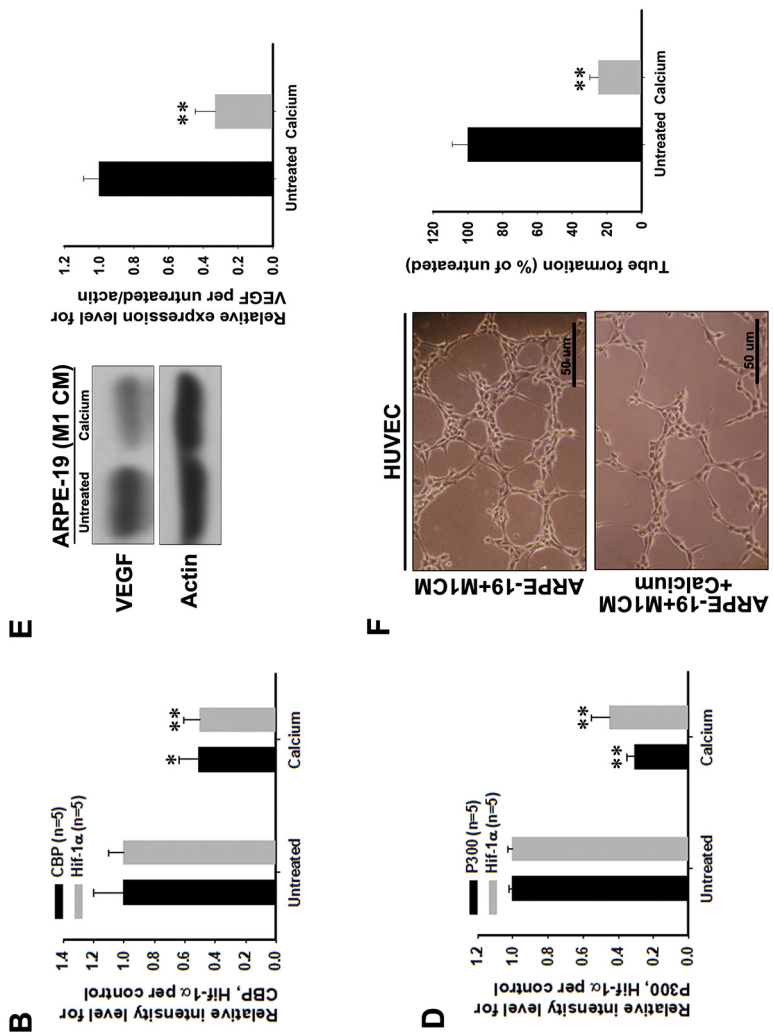

Fig. 3. Calcium-mediated inhibition of transcriptional activation and human umbilical vein endothelial cell (HUVEC) tube formation. (A) Decreased co-expression of Hif-1α and CBP by calcium supplementation in ARPE-19 cells cultured with M1 macrophages. Scale bar, 20 μm. (B) Quantitative analysis of CBP and Hif-1α by calcium supplementation in ARPE-19 cells cultured with M1 macrophages. (C) Decreased co-expression of Hif-1α and P300 by calcium supplementation in ARPE-19 cells cultured with M1 macrophages. Scale bar, 20 μm. (D) Quantitative analysis of CBP and Hif-1α by calcium supplementation in ARPE-19 cells cultured with M1 macrophages. (E) Qualitative and quantitative analysis of changes in VEGF expression by calcium supplementation. (F) Representative pictures of HUVECs plated on Matrigel. Scale bar, 50 μm. Quantitative analysis for the tube-like structures. All experiments were done in quintuplicate. $^{*}p < 0.05$ and $^{**}p < 0.001$ *vs* Untreated or ARPE-19 + M1CM group. Results are the mean ± SD.

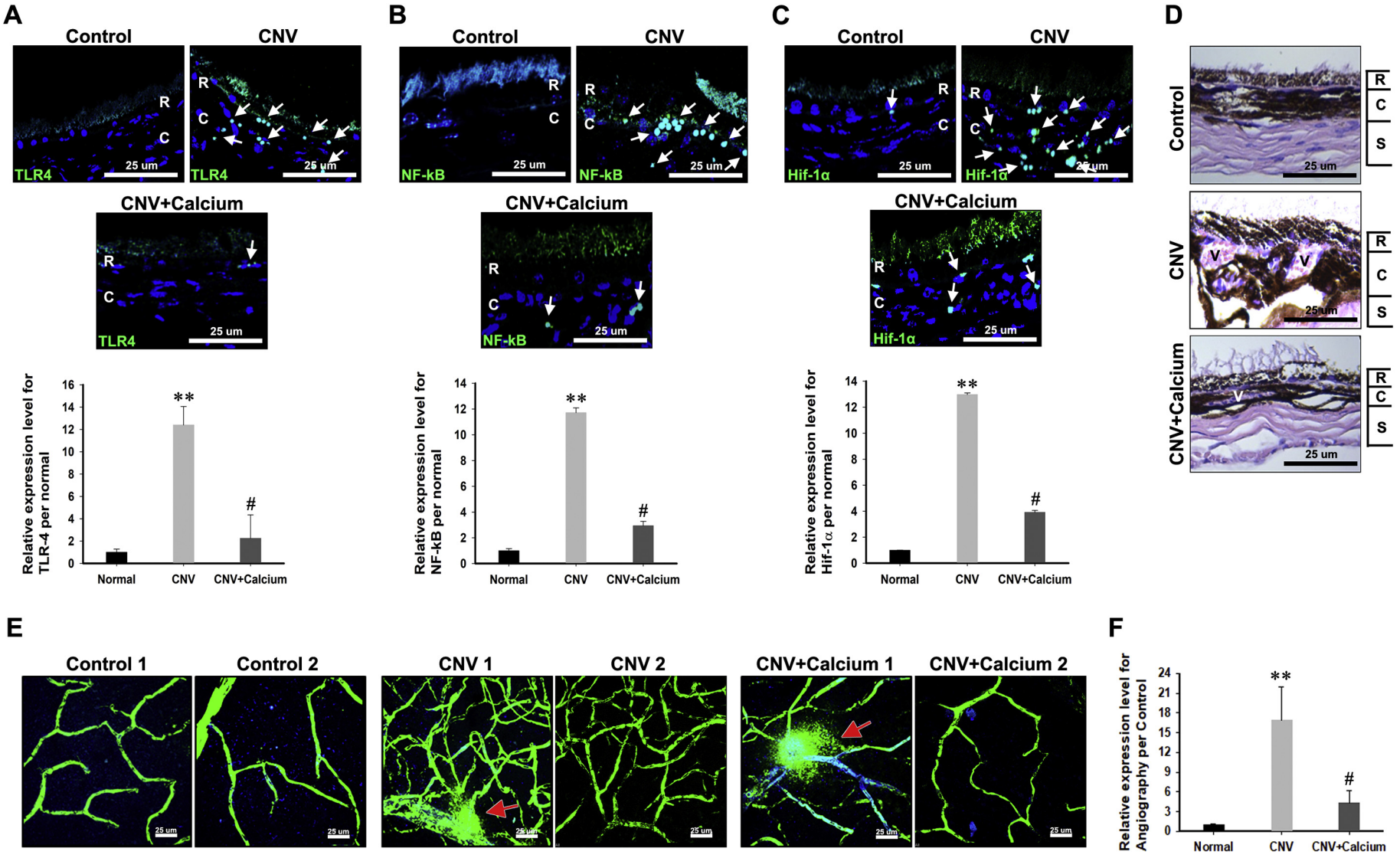

Fig. 4. Calcium-mediated anti-inflammatory and anti-angiogenic effects in laser-induced choroidal neovascularization (CNV) animal models. (A) Qualitative and quantitative analysis of TLR4 protein expression in retinal pigment epithelium-choroid–sclera eyecups of laser-induced CNV mouse. (B) Qualitative and quantitative analysis of NF-κB protein expression in retinal pigment epithelium-choroid–sclera eyecups of laser-induced CNV mouse. (C) Qualitative and quantitative analysis of Hif-1α protein expression in retinal pigment epithelium-choroid–sclera eyecups of laser-induced CNV mouse. (D) Hematoxylin and eosin staining for confirming calcium-mediated inhibition of CNV. R, retina; C, choroid; S, sclera; V, retinal blood vessel. (E) Fluorescence angiography for confirming calcium-mediated inhibition of CNV. (F) Quantitative analysis for relative expression for angiography. The experiments were done in quintuplicate. $^{**}p < 0.001$ *vs* Normal group; $^{\#}p < 0.001$ *vs* CNV group. Results are the mean ± SD. Scale bar, 25 μm.

CNV is well-established [15–17]. The study design to investigate the effect of the inhibition of inflammatory responses of polarized M1 macrophages in human ARPE-19 cells is undoubtedly valid.

A variety of proinflammatory cytokines and chemokines such as interleukin (IL)-1, inducible nitric oxide synthase, tumor necrosis factor-α, IL-6, C-X-C motif chemokine (CXCL) 10, and CXCL11 can be released by M1 macrophages [18]. Under pathological conditions such as bacterial infection, lipopolysaccharide is released from damaged bacteria stimulates a variety of immune responses through activation of the TLR4 signaling pathway [19,20]. TLRs also detect IL-1 receptor members respond to the IL-1 cytokine. It induces initiation of the intracellular signaling cascade through adaptor proteins, MyD88 [19].

While lipopolysaccharides can directly act on TLR4 to mediate an inflammatory signal transduction pathway, co-culture with polarized M1-macrophages has been applied to maximize the inflammatory response to human ARPE-19 cells by various cytokines or chemokines [8,19]. Activation of TLR4 and IL-1 receptor signaling cascades results in the activation of mitogen-activated protein kinases and the IκB kinase complex, which initiate an NF-κB-dependent transcriptional response of pro-inflammatory genes [19,21].

Hif-1α binds to the VEGF promoter, where they form a molecular complex with the transcription coactivators CBP and p300 [22]. Thus, while Hif-1α is required for maximal transcription of VEGF, our results indicated that consistent calcium supplementation inhibited Hif-1α expression followed by a decrease in the molecular complex with transcriptional coactivators such as CBP and p300. VEGF has an important role during developmental, physiological, and pathological neovascularization [10]. The currently used regimen for the treatment of neovascular AMD includes inhibition of VEGF through intravitreal injections with the repositioned anticancer drug Avastin® (bevacizumab) [23]. Thus, the expression of VEGF in neovascular AMD should be considered an important target. It is well known that TLRs are involved in the expression of Hif-1α through NF-κB and that Hif-1α increases TLR4 expression through direct promoter binding [9,19,21]. Thus, signaling through TLR4, NF-κB, and Hif-1α is an important pathway that forms a positive feedback loop involved in the inflammatory response and the progression of neovascular AMD.

CaLac is a food supplement that has been generally recognized as safe by the U.S. Food and Drug Administration. Due to its neutral chemical structure, CaLac diffuses easily into cells without the need of a specific ion channel [24]. Since this study was performed on normal cells, CaLac was chosen since it is not toxic and can easily supplement cells with calcium. To select the optimal dose for the present study, the expression level of TLR4 in ARPE-19 was investigated at a dose range 0.1–2.5 mM (Supplementary Fig. 1). The expression level of TLR4 was reduced at 1 and 2.5 mM CaLac. Since 2.5 mM CaLac did not cause toxicity to ARPE-19 cells (Supplementary Fig. 2), 2.5 mM was selected for this study. Our results indicated that co-culture of human ARPE-19 with M1-macrophages clearly reflected the characteristics of proinflammatory conditions with increased expression of TLR4, NF-κB, Hif-1α, and VEGF. The inflammatory response was inhibited by consistent supplementation of CaLac without any effect on the viability of human ARPE-19 cells (Supplementary Fig. 1).

Although various studies have proposed and confirmed that calcium supplementation has an anti-inflammatory effect including reduction of NF-κB, the anti-inflammatory effect of calcium is controversial. However, there is a growing number of clinical reports investigating the relationship between long-term calcium intake and AMD progression [11,12]. Therefore, we tried to put more emphasis on the theory of anti-inflammatory response among various controversial theories of calcium supplementation, one more reason is that our previous studies also have provided the clue that consistent calcium supplementation reduced the inflammatory factors in cancer cells when cancer cells become malignant and VEGF-mediated angiogenesis is induced [24]. This mechanism of changing the tumor microenvironment with inflammatory responses is very similar to the mechanism of the CNV, the main feature of neovascular AMD.

5. Conclusion

These results support the idea that consistent calcium supplementation contributes to a reduced risk of neovascular AMD. Therefore, calcium can be combined with existing compounds, such as AVASTIN, for the treatment of neovascular AMD. However, we clearly recognize that to establish the potential of clinical applications, the mechanism of CNV suppression through calcium should be clarified and further studies are necessary to determine the optimal pathways and concentrations in humans.

Declaration of competing interest

No potential conflicts of interest were disclosed.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.116727.

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