Oleuropein Protects Human Retinal Pigment Epithelium Cells from IL-1β –Induced Inflammation by Blocking MAPK/NF- k B Signaling Pathways

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Research Article

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Abstract

Objectives and design

Pro-inflammatory mediators such as interleukin (IL)-1b cause retinal pigment epithelium (RPE) inflammation, which is related to visual deterioration, including age-related macular degeneration and diabetic retinopathy. Oleuropein is a polyphenol compound that shows potent anti-inflammatory, antioxidant, and anti-cancer activities, but its effects on IL-1b–induced inflammation have not been examined in the adult RPE cell line ARPE-19.

Materials/methods

Here, we assessed the ability of oleuropein to attenuate this inflammation in ARPE-19 cells. IL-1β induced secretion of the inflammatory cytokines IL-6, monocyte chemoattractant protein-1 (MCP)-1, and soluble intercellular adhesion molecule (sICAM)-1. As measured by enzyme-linked immunosorbent assay, oleuropein significantly inhibited levels of all three proteins and led to decreased monocyte adhesiveness to ARPE-19 cells. To clarify the underlying anti-inflammatory mechanisms, we used western blots to evaluate the effect of oleuropein on inactivation of the nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways. Result The results showed that oleuropein significantly decreased levels of the inflammatory mediator cyclooxygenase-2 and increased anti-inflammatory protein HO-1 expression. We next examined if the anti-inflammatory activity of oleuropein arises via inactivated NF-κB. We found that suppressing phosphorylation of the JNK1/2 and p38 MAPK signaling pathways inhibited IL-6, MCP-1, and sICAM-1 secretion, implicating these pathways and NF-κB suppression in the effects of oleuropein.

Conclusions

These results indicate that oleuropein shows potential for the prevention and treatment of inflammatory diseases of the retina.

Introduction

Inflammation-induced damage of the retinal pigment epithelium (RPE) has been suggested as a major influence in retinal degenerative diseases (RDs), including age-related macular degeneration (AMD) and diabetic retinopathy (DR). Retinal pericytes are the cell layer surrounding endothelial cells and inflammation stimulus endothelial proliferative and formation of aberrant capillaries. In addition, the RPE is a main source of pro-inflammatory mediators [1–3]. AMD, which is classified into dry (non-neovascular) or wet (neovascular), involves irreversible visual disability [4, 5]. RPE, which lies between the neural retina and the choroid, is a monolayer of pigmented cells and mainly functions as a retinal blood barrier and in photoreception [6]. Major risk factors for AMD are aging, oxidative stress, obesity, and chronic inflammation [7].

Interleukin (IL)-1β is a pro-inflammatory cytokine that initiates and propagates sterile inflammation, which is associated with retinal degenerative diseases [8]. In addition, IL-1β modulates expression of inflammatory mediators of RDs, including the pro-inflammatory cytokine IL-6, monocyte chemoattractant protein (MCP)-1, and soluble (s) intercellular adhesion molecule (ICAM)-1 [9]. IL-6 controls T cell proliferation, and this activation has been associated with inflammatory diseases such as DR [10]. MCP-1 draws monocytes and basophils to the site of inflammation, where macrophages or dendritic cells embed in the tissues, activating the inflammatory reaction and development of related lesions [11]. During inflammation, leukocytes secrete the associated marker and regulator sICAM-1 [12]. Many studies have suggested that these activities are related to the regulation of the nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways [13]. Results suggest that inflammatory mediators and leukocyte attractants would increase retinal inflammation and risk of AMD [14]. Therefore, anti-inflammatory therapies that inhibit these mediators could have potential in preventing progression in AMD [15].

Oleuropein (Fig. 1A) is a major phenolic compound of *Olea europaea L*. or olive leaf extract, and has antioxidant, anti-inflammatory, hepatoprotective, and anti-cancer effects [16, 17]. Because of the potential link between the inflammatory state of the RPE and AMD development [18, 19], here we investigated the anti-inflammatory action of oleuropein in IL-1β–induced adult RPE (ARPE)-19 cells.

Materials And Methods

Materials

Oleuropein (from coumarins, ≥ 98%) was purchased from Sigma-Aldrich (St. Louis, MO, USA) (Fig. 1A), prepared as a 100-mM stock solution in dimethyl sulfoxide (DMSO), and stored at -20°C. The Cell Counting Kit-8 (CCK-8) and 4’,6-diamidino- 2-phenylindole (DAPI) solution were purchased from Sigma-Aldrich. Culture medium was developed to a final concentration ≤ 0.1% in DMSO, as previously described [19]. We purchased IL-1β and enzyme-linked immunosorbent assay (ELISA) kits from R&D Systems (Minneapolis, MN, USA). Antibodies raised against β-actin, cyclooxygenase-2 (COX-2), and heme oxygenase (HO)-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell lines and culture conditions

The ARPE-19 cells were purchased from the Bioresource Collection and Research Center (BCRC, Taiwan). Cells were cultured in DMEM/F-12 medium (Invitrogen-Gibco, Paisley, Scotland) containing heat-inactivated 10% FBS (Invitrogen-Gibco) and penicillin G (100 U/ml), streptomycin (100 μg/ml), and gentamycin (50 ng/ml). Culture took place in a 5% CO_2 humidified atmosphere at 37°C, with subcultures started every 2–3 days. ARPE-19 cells were pretreated or not with oleuropein (3–100 μM) for 1 h, followed by addition of IL-1β (1 μg/ml). ARPE-19 cells were lysed for western blot analysis, and media samples were subjected to ELISA analysis after a 24-h treatment. The THP-1 cells were cultured in RPMI 1640 medium (Gibco) containing 10% FBS in a humidified atmosphere of 5% CO2 at 37°C and subcultured

every 3–4 days. “THP-1 cells is a human monocytic cell line were purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan). THP-1 cells incubated in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum and added 2 mM L-glutamine.

Cell viability assay

We used the CCK-8 kits to assess the effect of oleuropein on cell viability, as described previously (20). Cells were seeded into 96-well plates (10^5 cells/well) and treated with oleuropein (3–100 μM) for 24 h, followed by addition of CCK-8 solution and incubation at 37°C for 2 h. We used a 450-nm microplate reader for counting (Multiskan FC; Thermo, Waltham, MA, USA), and each concentration was evaluated in triplicate. Cell viability was given as percentage cell count relative to count without oleuropein treatment.

ELISA assay

ARPE-19 cells were pretreated or not with oleuropein (3–100 μM) for 1 h in 24-well plates, followed by addition of IL-1β (1 ng/ml) and culture for 24 h. Cell supernatants were assayed using ELISA kits to measure IL-6, MCP-1, and sICAM-1 levels, following the manufacturers’ instructions. We used a microplate reader (Multiskan FC; Thermo) at 450 nm to determine optical density values.

Cell adhesion assay

ARPE-19 cells were pretreated with oleuropein for 1 h and added with 1 ng/mL IL-1β for 24 h. The control groups were incubated with IL-1β alone. ARPE-19 cells were co-cultured with THP-1 cells (10^6 cells/ml), which labeled with calcein-AM for 1 h in a humidified incubator containing 5% CO_2 at 37˚C. Then, using PBS to wash the extent of adhesion of THP-1 cells to ARPE-19 cells. To observe the adhesion of THP-1 cells to ARPE-19 cells under fluorescence microscopy (3 per view; magnification, x200; Olympus, Tokyo, Japan) with excitation and emission wavelengths of 490 and 515 nm, respectively. All experiments were repeated three times.

Preparation of total proteins and Nuclear Proteins

ARPE-19 cells were seeded in 6-well plates pretreated or not with oleuropein (3–100 μM) for 1 h, followed by addition of IL-1β (1 ng/ml) for 24 h to evaluate total protein content or for 30 min to detect protein phosphorylation. A protein lysis buffer (300 μl; Sigma, St. Louis, MO, USA) containing protease inhibitor cocktail and phosphatase inhibitors was used to harvest cells. ARPE-19 cells to investigate the expression of nuclear proteins, cells were treated using the NE-PER® nuclear and cytoplasmic extraction reagent kits (Pierce, Rockford, IL, USA). Total protein and phosphorylated proteins were quantified using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA), as previously described (19).

Western blot analysis

Protein samples were separated on 10% sodium dodecyl sulfate polyacrylamide gels, then transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). Membranes then were incubated

overnight at 4°C with specific primary antibodies against β-actin (Sigma), COX-2, HO-1, P65, phosphorylated (p)P65 (Santa Cruz, CA, USA), and JNK, phosphorylated (p)JNK, p38, pp38, ERK, and pERK (Cell Signaling Technology, Danvers, MA, USA). Then, membranes were washed three times in Tris-buffered saline with Tween 20 buffer and incubated with secondary antibodies for 1 h at room temperature. To detect proteins, we used Luminol/Enhancer solution (Millipore), and we used the BioSpectrum 600 system (UVP, Upland, CA, USA) to detect signals and quantify protein bands.

Immunofluorescence staining

ARPE-19 cells were seeded onto 6-well plates and reached 50–60% confluence. Cells were pretreated or not for 1 h with oleuropein (3, 10, 30, or 30 μM), followed by addition of IL-1β for 15 min. Then cells were suctioned from the medium, washed in phosphate-buffered saline (PBS), fixed in paraformaldehyde 4% (w/v), and incubated overnight at 4°C with anti-NF-κB p65 antibody. Next, the medium was removed, and the cells were washed again in PBS, followed by incubation with secondary antibodies at room temperature for 1 h. After 2–3 more washes in PBS, fluorescent dye (BODIPY493/503 or BODIPY581/591) was added. Following a wash with PBS to remove the dye, the nuclei were stained with DAPI (Sigma). Images were captured using a fluorescence microscope (Olympus, Tokyo, Japan).

Statistical analysis

One-way analysis of variance and post hoc analysis with Dunnett's test were used to analyze data. Data are presented as the mean ± standard deviation (SD) of at least three independent experiments. Differences were considered statistically significant at $P < 0.05$.

Results

No cell viability effect of oleuropein up to 200 μM

Using the CCK-8 assay to detect cytotoxicity in ARPE-19 cells, we tested oleuropein concentrations from 3 to 200 μM. Oleuropein did not significantly affect cell viability compared with the DMSO negative control, suggesting that these concentrations were safe to use (Fig. 1B).

The effect of oleuropein on expression of HO-1, IL-6, MCP-,1 and COX-2

ARPE-19 cells stimulated by IL-1β for 24 h showed a significant increase in the inflammatory mediators IL-6, MCP-1, and COX-2 compared to control (Fig. 2A–D). Treatment with oleuropein significantly inhibited IL-6, MCP-1, and COX-2 expression compared with cells treated with IL-1β alone. In addition, oleuropein was associated with increased levels of the anti-inflammatory HO-1 protein compared with cells treated with IL-1β alone (Fig. 2E, F). Based on these results, pretreatment with oleuropein reduced IL-1β–induced cytokine and COX-2 production and increased HO-1 expression in ARPE-19 cells.

Oleuropein inhibition of sICAM-1 and attenuation of THP-1 cell adherence to IL-1β–stimulated ARPE-19 cells

IL-1β treatment significantly stimulated sICAM-1 levels and THP-1 cell adhesion to IL-1β–stimulated ARPE-19 cells compared to control (Fig. 3A–C). Exposure of the cells to oleuropein concentrations ≥ 3 μM significantly inhibited the IL-1β–induced release of sICAM-1 compared to IL-1β treatment alone (Fig. 3A). In addition, oleuropein concentrations ≥ 10 μM strongly attenuated THP-1 cell adhesion to IL-1β–stimulated ARPE-19 cells compared to IL-1β treatment alone (Fig. 3B, C).

Oleuropein inhibition of phosphorylated JNK and p38 MAPK in activated ARPE-19 cells

IL-1β strongly stimulated phosphorylation of JNK and p38, and ERK1/2 compared to control (Fig. 4A–F). Conversely, oleuropein at ≥10 μM and ≥30 μM significantly inhibited JNK1/2 and p38 phosphorylation, respectively (Fig. 4A–D). However, oleuropein did not significantly affect ERK1/2 phosphorylation compared with IL-1β alone (Fig. 4E, F). These results suggest that oleuropein might act to suppress IL-1β–induced inflammation via blocking the JNK1/2 and p38 MAPK pathways.

Oleuropein suppression of IL-1β–induced NF-κB activation in ARPE-19 cells

We wanted to explore whether the effects of oleuropein on HO-1, COX-2, and the cytokines sICAM-1, IL-6, and MCP-1 arise in part via suppression of IL-1β–induced NF-κB activation. We found that IL-1β caused a significant increase in NF-κB p65 translocation into the nucleus, which oleuropein ≥ 30 μM suppressed. Under oleuropein treatment, the p65 subunit was retained in the cytoplasm in IL-β–activated ARPE-19 cells (Fig. 5A). These results indicate that the anti-inflammatory effect of oleuropein is associated with suppression of IL-1β–induced NF-κB p65 translocation into the nucleus of ARPE-19 cells.

Discussion

Inflammation is thought to cause RPE damage related to vision loss and RDs such as dry AMD and DR (20,21). Therefore, ophthalmologic agents are needed that can attenuate RPE inflammation to protect against, treat, or slow progression of RDs. Current candidates include oleuropein, lutein, β-carotene, zeaxanthin, and vitamins C and E. Oleuropein is reported to have powerful anti-inflammatory properties *in vivo* and *in vitro* (22,23), but research into its effects in ARPE-19 cells has so far been limited (24). In line with earlier work, our current results indicate that polyphenols such as oleuropein may be candidates for supplementation or focused dietary intake in the treatment or prevention RDs, especially AMD (25,26). We found that oleuropein, which did not substantially affect ARPE-19 cell viability up to 200 μM (Fig. 1B), is effective *in vitro* in reducing IL-1β–induced inflammation in ARPE-19 cells.

In this study, oleuropein inhibited levels of IL-6, MCP-1, and sICAM-1, which normally increase in response to IL-1β stimulation (Figs. 2A, B, and 3A). It also was associated with decreased levels of the inflammatory mediator COX-2, increased expression of the anti-inflammatory protein HO-1 (Fig. 2C, D and 2E, F), and attenuation of monocyte adhesion to IL-1β–stimulated ARPE-19 cells (Fig. 3B, C). Moreover, we found that the MAPK and NF-κB signaling pathways regulate the inflammatory response activated by IL-1β in ARPE-19 cells. Oleuropein inhibited the JNK1/2 and pP38 MAPK pathways but had no effect on the ERK1/2 MAPK pathway (Fig. 4). Finally, oleuropein down-regulated NF-κB p65 translocation from the

cytoplasm to the nucleus, with the p65 subunit being retained in the cytoplasm in oleuropein-pretreated IL-β–activated cells (Fig. 5). Taken together, these results show a pattern of inactivated NF-κB and down-regulated phosphorylation of JNK1/2 and p38 MAPKs following oleuropein pretreatment in IL-1β–induced ARPE-19 cells.

Earlier reports have indicated that the pro-inflammatory cytokine IL-1β initiates and propagates inflammation in RDs, including AMD and DR (8, 27). Inflammation in ARPE-19 cells can induce expression of other chemokines and cytokines, including IL-6, MCP-1, and sICAM-1 (17,19, 28). MCP-1 is primarily produced by Müller cells in RDs, and it recruits macrophages to the site of damage (29, 30). In the presence of IL-1β, mesenchymal stem cells simultaneously secrete a significant amount of IL-6, leading to production of this cytokine in the inflammatory environment of the diseased retina (31). Studies also have shown that ICAM-1 is involved in leukocyte–endothelial interactions and that cell migration may contribute to the development of choroidal neovascularization. In addition, sICAM-1 appears to be more closely related to neovascular AMD (32–34). The activities of these inflammation-related molecules are associated with the NF-κB and MAPK signaling pathways (12), implicated here in the mechanism of action of oleuropein.

When RPE cells were active by IL-1β will release cytokines and chemokines to trigger inflammatory responses in the inflammation-relative eye diseases progressive. RPE cells are the important role, of which AMD. We firstly observed oleuropein anti-inflammatory in IL-1β-induced ARPE-19 cells. In ophthalmology, oleuropein has not been used to treat inflammation-relative eye diseases. However, quercetin is also a phenolic compound has recently been used to treat dry eye, corneal inflammation and corneal neovascularization (9). This studies provide a basal theoretical for in vivo study, even clinical application of oleuropein in the prevention and treatment of retinal inflammatory diseases in future. On this regards it could be useful develop a biodegradable deliver system to inject oleuropein, avoiding a multiple treatment (35).

In addition, oleuropein is a natural compound from *Olea europaea L.* Previous studies found that 400 μM oleuropein did not significantly affect cell viability in 3T3-L1 adiocytes and 300 μM oleuropein inhibited Sterol regulatory element-binding transcription factor 1 (SREBP1c) and fatty acid synthase (FAS) gene expressions for suppressed lipogenesis in 3T3-L1 adipocytes (36).Others studies indicated that 400 μM oleuropein could activated the phosphorylation AMPK and AKT for promoted insulin sensitivity in C2C12 muscle cells (37). And Colorectal cancer mice treated with100 mg/kg oleuropein, the result demonstrated that could improve clinical symptoms of colonic tumors by ameliorating inflammatory responses (38). Therefore, we suggest that the experimental treatment of ARPE-19 cells with a maximum dose of 100 μM oleuropein has anti-inflammatory effects that should have physiologically usable dosage significance in ARPE-19 cells. Moreover, ARPE-19 cells have phenotype characteristic of native RPE. The ARPE-19 RNA-Seq data set compared with primary human fetal RPE, native RPE revealed the similar expression ratio among all the models and native tissue (39). The results of this experiment, we hope to development of an eye drop form to treat in diabetic mice-induced eye disease and can be used in clinical practice in the future.

Conclusions

These results indicate that oleuropein attenuates IL-1β–induced IL-6, MCP-1, and sICAM-1 secretion and leads to increased levels of anti-inflammatory HO-1 by repressing activation of the NF-κB and JNK1/2–pP38 MAPK pathways. Oleuropein thus shows potential for anti-inflammation in IL-1β-induced ARPE-19 cells. In further, we will confirm that oleuropein inhibit inflammatory effects in RDs in vivo study.

Declarations

COMPLIANCE WITH ETHICAL STANDARDS

Ethics Approval and Consent to Participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors have no conflicts of interest to declare.

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Authors' contributions

Designed and performed the experiments: SJW, WCH, and CHH.; Analysis and interpretation of data: MLH. and YRZ; Drafting the manuscript: SJW and SH. All authors have read and agreed to the published version of the manuscript.

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Not applicable

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Figures

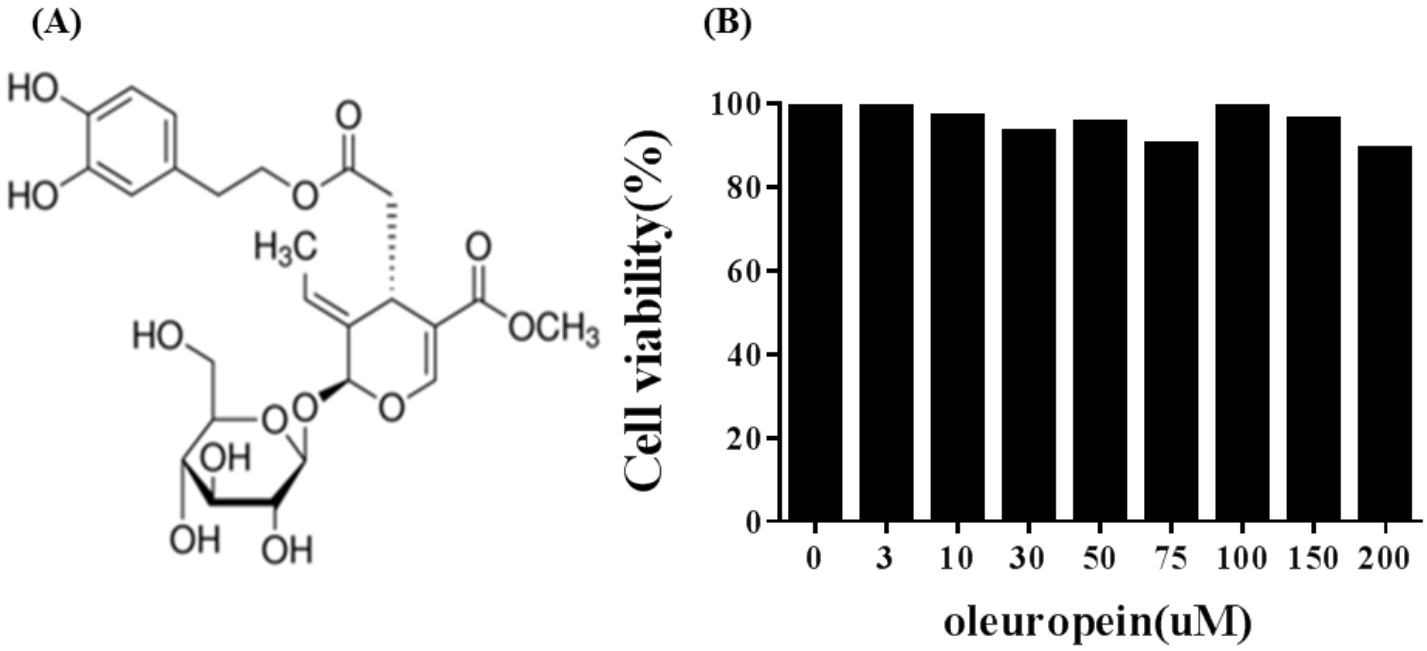

Figure 1

Cell viability effects of oleuropein (Ole). (A) Structure of oleuropein. (B) Cytotoxicity of oleuropein in ARPE-19 cells.

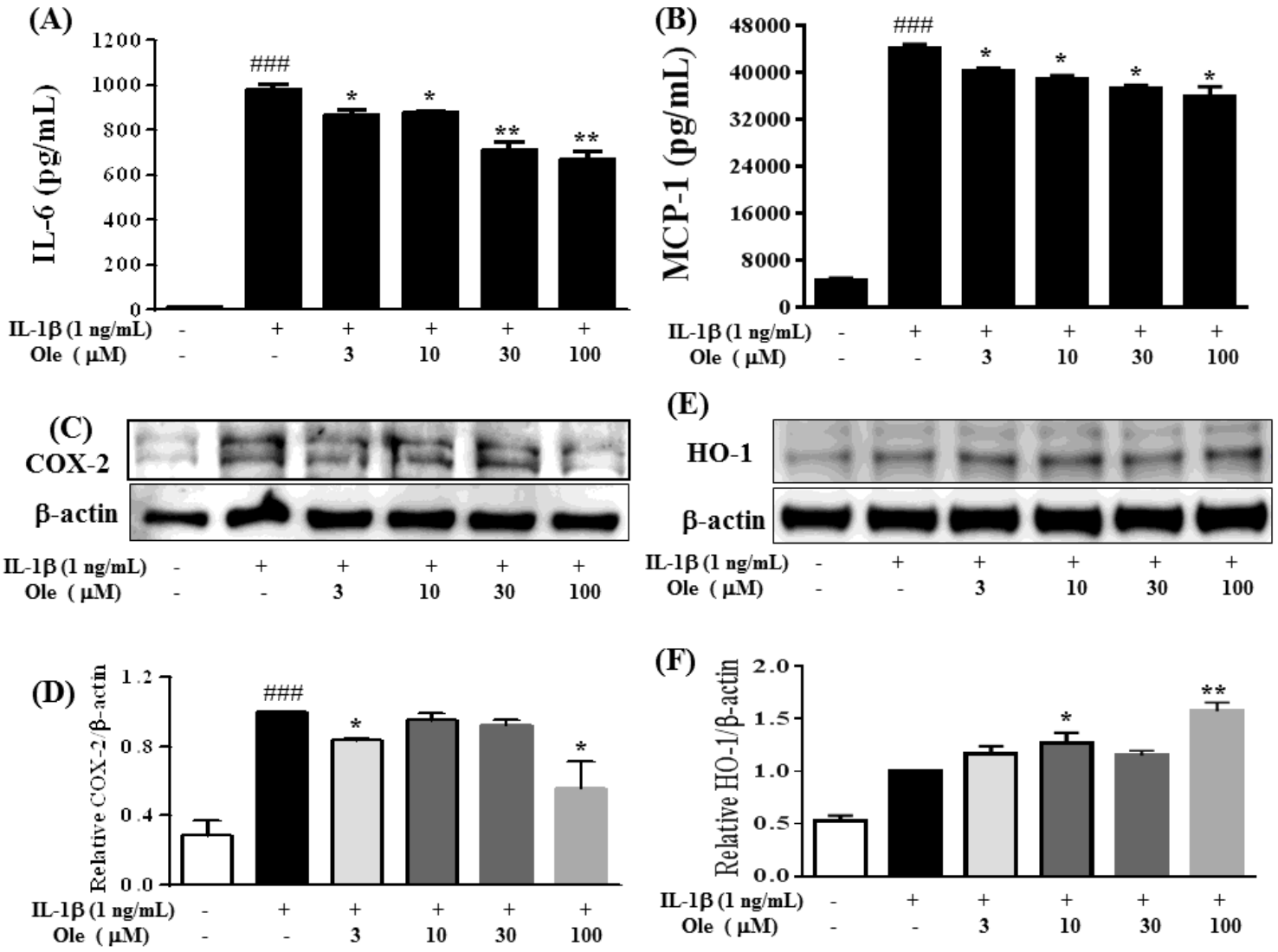

Figure 2

Oleuropein (Ole) inhibited the inflammatory mediators IL-6, MCP-1, and COX-2 and increased HO-1 protein expression. (A, B) Oleuropein inhibited IL-1β–induced cytokine and chemokine levels: IL-6 (A) and MCP-1 (B). (C, D) Oleuropein decreased COX-2 protein expression. (E-F) Oleuropein increased HO-1 protein expression. ARPE-19 cells (105 cells/well) were pretreated with the indicated concentrations of oleuropein (3–100 μM) for 1 h and then stimulated with IL-1β (1 ng/mL) for 24 h. The presented data are mean ± SD. *P<0.05 and **P<0.01, comparisons to cells treated with IL-1β only.

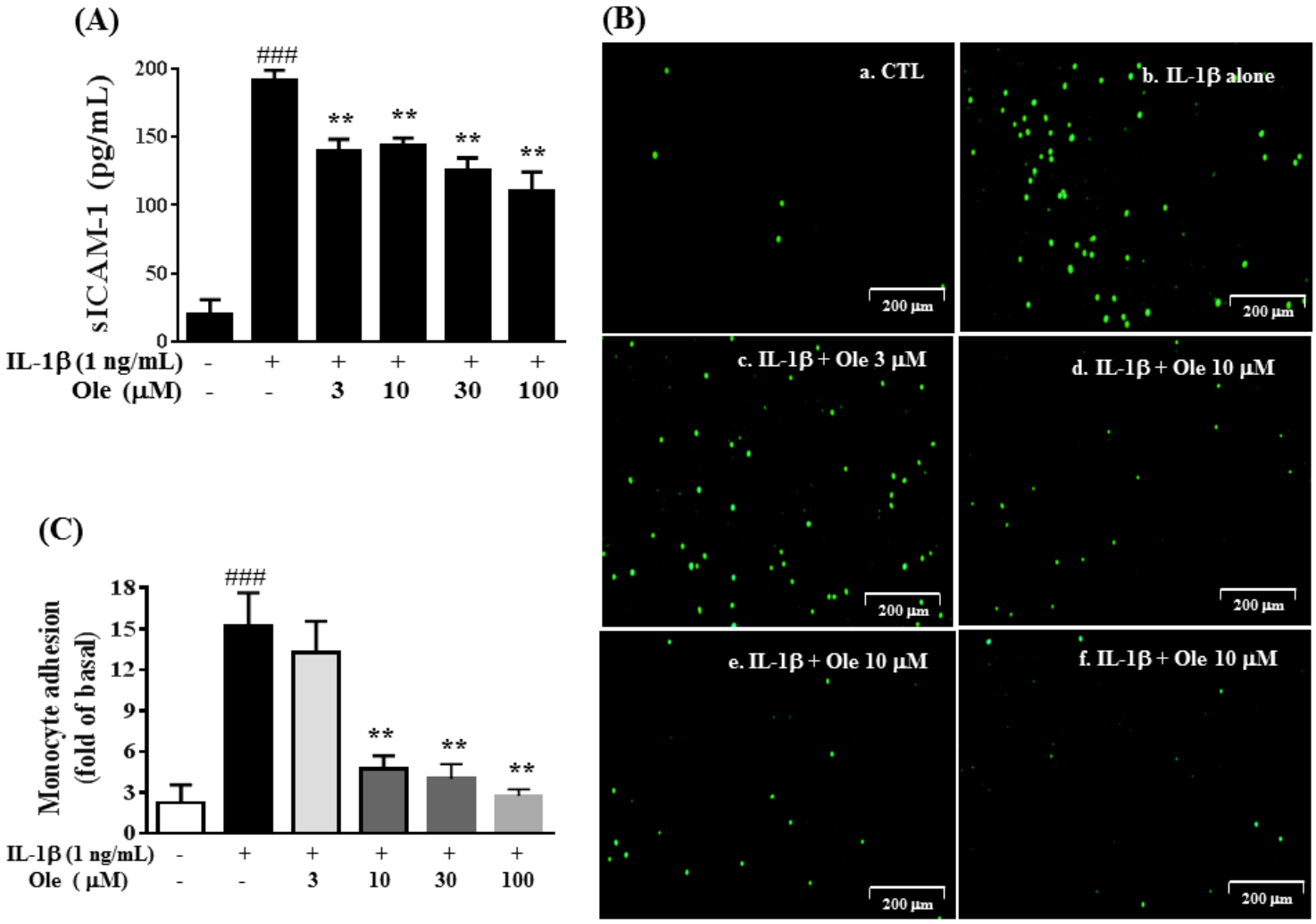

Figure 3

Oleuropein (Ole) inhibited sICAM-1levels and decreased THP-1 cell adherence to IL-1β–stimulated ARPE-19 cells. (A) Oleuropein inhibited IL-1β-induced sICAM-1 levels. (B) Oleuropein decreased monocyte adhesiveness in IL-1β–induced ARPE-19 cells. THP-1 cells were incubated with: (B-a) Untreated ARPE-19 cells; (B-b) IL-1β-treated ARPE-19 cells; (B-c)ARPE-19 cells treated with 3 μM Ole; (B-d) ARPE-19 cells treated with 10 μM Ole; (B-e) ARPE-19 cells treated with 30 μM Ole; and (B-f) ARPE-19 cells treated with 100 μM Ole, respectively. Scale bar=200 μm. (C) A fluorescence plate reader was used to quantify calcein AM fluorescent intensity. Data are presented as mean ± SD. *P<0.05 and **P<0.01 for comparisons to cells treated with IL-1β only.

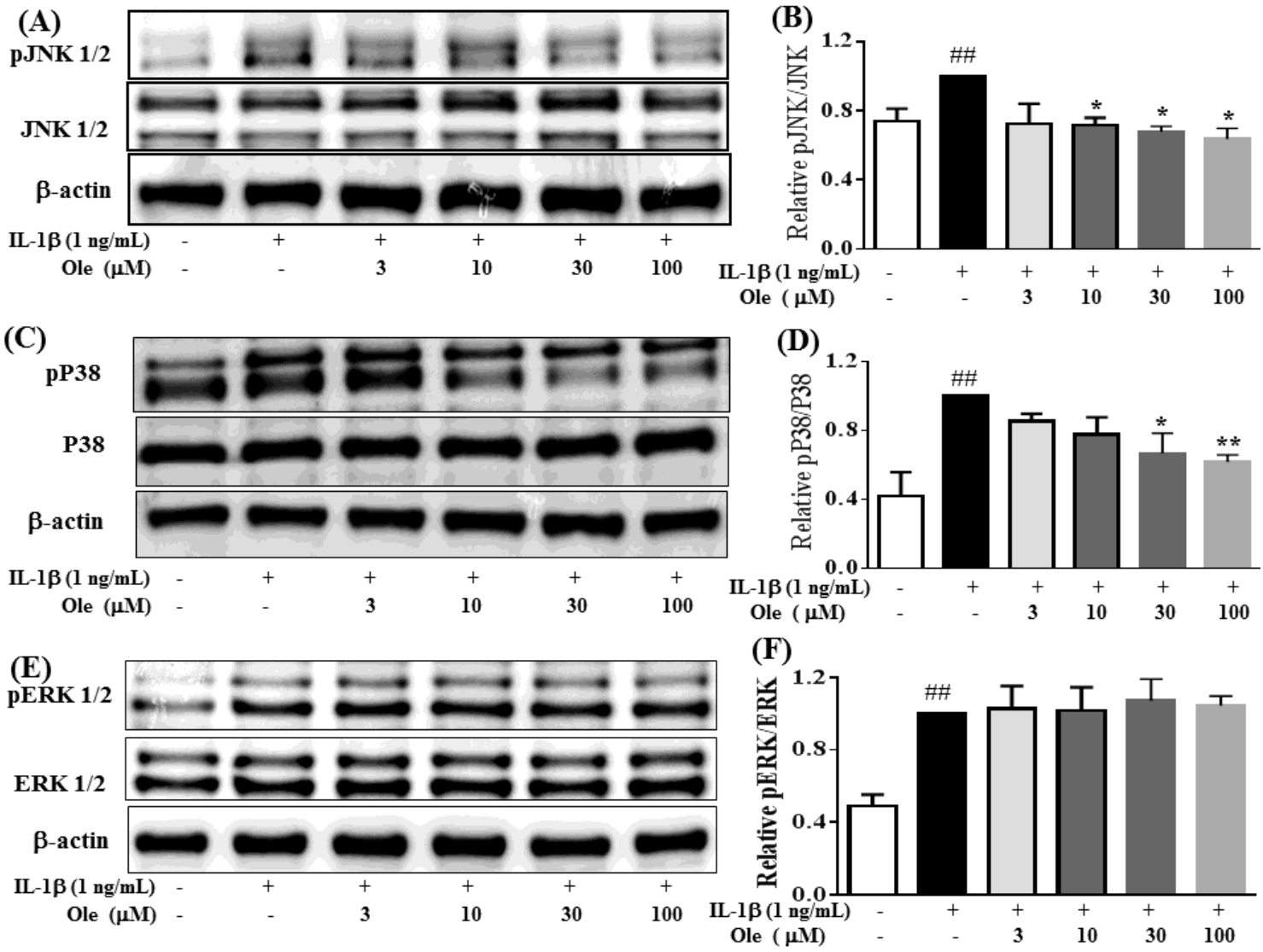

Figure 4

Oleuropein (Ole) inhibited JNK1/2 and p38 phosphorylation in IL-1β–induced ARPE-19 cells. (A, B) Oleuropein inactivated the pJNK1/2 pathway. (C, D) Oleuropein inactivated the p38 pathway. (E, F) Oleuropein had no effect on the pERK1/2 pathway. ARPE-19 cells (106 cells/mL) were incubated in the absence or presence of oleuropein 3–100 μM for 1 h and then exposed to IL-1β (1 ng/mL) for 24 h (total proteins) or 30 min (phosphorylated proteins). Protein levels were determined by western blot. Results are mean ±SD. Statistical significance: *P<0.05 and **P<0.01, comparisons to cells treated with IL-1β only.

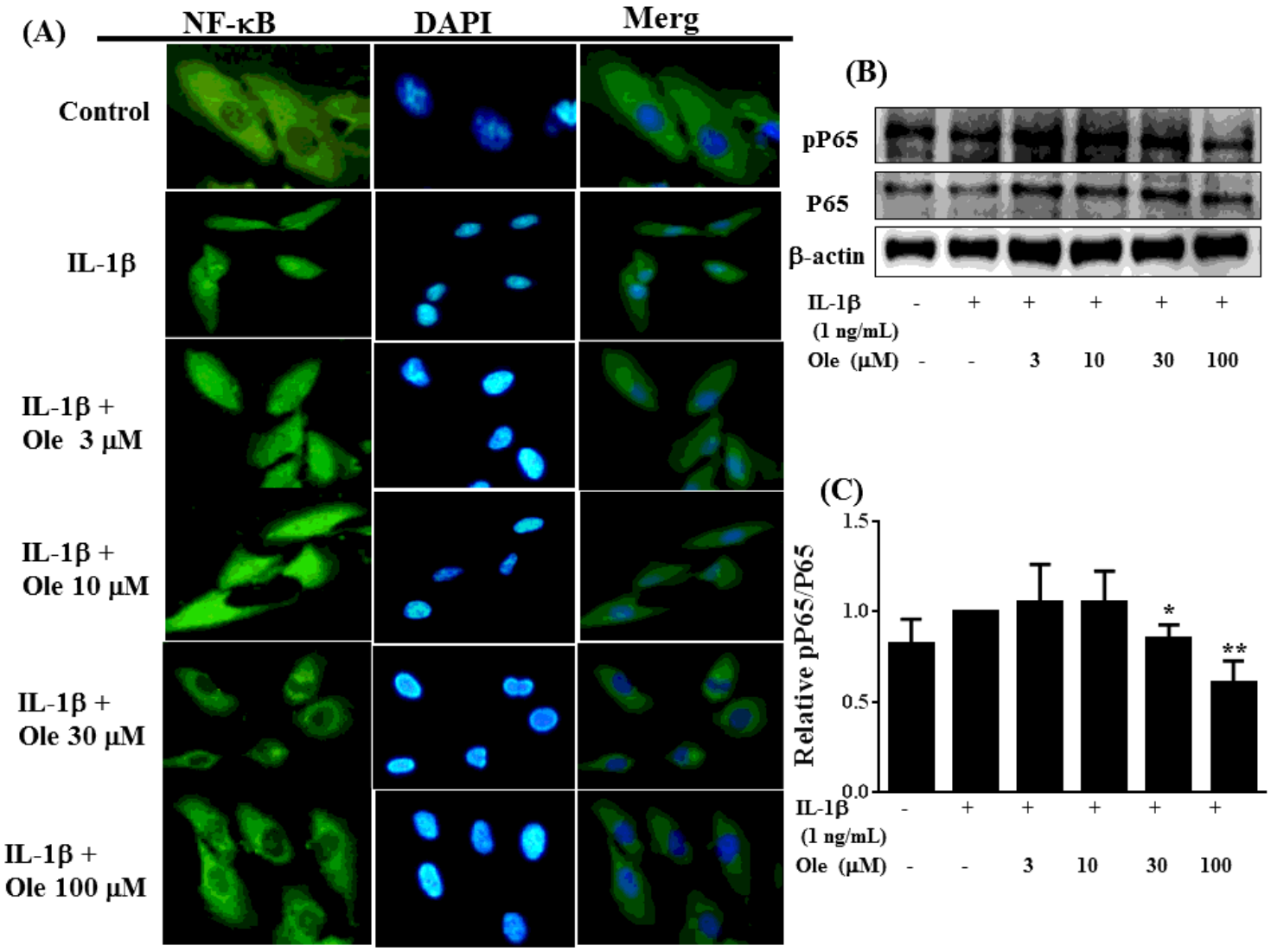

Figure 5

Oleuropein suppression of IL-1β–induced NF-κB activation in ARPE-19 cells. (A) Oleuropein (Ole) inhibited NF-κB p65 translocation from the cytoplasm into the nucleus in IL-1β–stimulated ARPE-19 cells, as observed by DAPI staining. Cells were pretreated with different doses of oleuropein and then incubated with IL-1β (1 ng/mL) for 15 min. NF-κB p65 translocation was evaluated by immunofluorescence staining. (200x magnification). Green: location of the p65 subunit. Blue: DAPI for nuclear staining. (B, C) Oleuropein inactivated the p65 pathway. ARPE-19 cells (106 cells/mL) were incubated in the absence or presence of oleuropein 3–100 μM for 1 h and then exposed to IL-1 (1 ng/mL) for 24 h (total proteins) or 30 min (phosphorylated proteins). Protein levels were determined by western blot. Results are mean ±SD. Statistical significance: *P<0.05 and **P<0.01, comparisons to cells treated with IL-1β only.