The 15-deoxy-$\delta^{12,14}$-prostaglandin J_2 inhibits LPS-stimulated inflammation via enhancement of the platelet-activating factor acetylhydrolase activity in human retinal pigment epithelial cells

WON-KYO JUNG[1*], CHANG-MIN LEE[2*], DAE-SUNG LEE[3], GIYOUN NA[4], DA-YOUNG LEE[4], INHAK CHOI[4], SAE-GWANG PARK[4], SU-KIL SEO[4], JAE-WOOK YANG[5], JUNG SIK CHOI[6], YOUNG-MIN LEE[6], WON SUN PARK[7] and IL-WHAN CHOI[4]

[1]Department of Biomedical Engineering, Pukyong National University, Busan; [2]Department of Microbiology and Immunology, Medical Research Institute, Pusan National University School of Medicine, Yang-san; [3]POSTECH Ocean Science and Technology Institute, Pohang University of Science and Technology, Pohang; [4]Department of Microbiology, College of Medicine Inje University; Departments of [5]Ophthalmology and [6]Internal Medicine, Busan Paik Hospital, College of Medicine Inje University, Busan; [7]Institute of Medical Sciences, Department of Physiology, Kangwon National University School of Medicine, Chuncheon, Republic of Korea

Received August 30, 2013; Accepted December 11, 2013

DOI: 10.3892/ijmm.2013.1588

Abstract. A well-recognized natural ligand of PPARγ, 15-deoxy-$\delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) possesses immunomodulatory properties. The aim of this study was to elucidate whether 15d-PGJ_2 was able to attenuate lipopolysaccharide (LPS)-induced inflammatory responses in human retinal pigment epithelial (RPE) cells, which are involved in ocular immune responses. In addition, we examined whether the platelet activating factor (PAF) is associated with the anti-inflammatory activity of 15d-PGJ_2. ARPE19 cells treated with varying concentrations of 15d-PGJ_2 and a PAF antagonist (CV3988) were used in this study. The activity of PAF-acetylhydrolase (PAF-AH) was assayed by treatment with 15d-PGJ_2 and CV3988 in the presence of LPS. 15d-PGJ_2 and CV3988 inhibited the LPS-induced mRNA expression and protein production of interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), and intercellular adhesion molecule-1 (ICAM-1) in ARPE19 cells. These effects resulting from 15d-PGJ_2 were not abrogated by the PPARγ antagonist, indicating that the actions were PPARγ-independent. Furthermore, 15d-PGJ_2 and CV3988 enhanced the PAF-AH activity. Additionally, 15d-PGJ_2 inhibited the phosphorylation of the extracellular signal-regulated kinase (ERK) and the activation of nuclear transcription factor-κB (NF-κB). These results demonstrated that 15d-PGJ_2 reduced LPS-stimulated inflammatory responses in ARPE19 cells by enhancing the PAH-AH activity. These results suggest that 15d-PGJ_2 may have potent anti-inflammatory activity against ocular inflammation.

Correspondence to: Professor Il-Whan Choi, Department of Microbiology, College of Medicine Inje University, Bokji-Ro 75, Busanjin-gu, Busan 614-735, Republic of Korea
E-mail: cihima@inje.ac.kr

Professor Won Sun Park, Institute of Medical Sciences, Department of Physiology, Kangwon National University School of Medicine, 1 Kangwondaehak-gil, Chuncheon, Republic of Korea
E-mail: parkws@kangwon.ac.kr

*Contributed equally

Abbreviations: 15d-PGJ_2, 15-deoxy-$\delta^{12,14}$-prostaglandin J_2; PAF, platelet activating factor, PAF-AH, PAF-acetylhydrolase; hRPE, human retinal pigment epithelial; IL-6, interleukin-6, MCP-1, monocyte chemoattractant protein-1; ICAM-1, intercellular adhesion molecule-1; NF-κB, nuclear transcription factor-κB; MAPKs, mitogen-activated protein kinases

Key words: 15-deoxy-$\delta^{12,14}$-prostaglandin J_2, platelet activating factor, PAF-acetylhydrolase, ocular inflammation, human retinal pigment epithelial cells

Introduction

Inflammatory ocular responses are associated with the pathophysiology of several retinal degenerative diseases, including uveitis (1), diabetic retinopathy (2), and age-related macular degeneration (AMD) (3). In the eye, the retinal pigment epithelium (RPE), which is a pigmented layer of the neural retina, provides metabolic support to the photoreceptors that provide visual functionality (4). This epithelium is in the unique position to sense the circulating immune system status and has both macrophage and microglia-like activities in the retina (5,6). The RPE plays a critical role in innate immunity through the expression of pattern recognition receptors (PRRs), such as toll-like receptors (TLR), to detect the unique molecular patterns associated with microbial pathogens prior to evoking RPE cell inflammatory responses via the production of inflammatory mediators (7). If this response is prolonged, then

the subsequent atrophy of RPE and photoreceptors may occur, which is the leading cause of legal blindness.

Activation of an inflammatory response upon encountering a pathogen cause the release of pro-inflammatory mediators. One such pro-inflammatory mediator is the phospholipid platelet-activating factor (PAF, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine), which acts via its G protein-coupled receptor to stimulate numerous complex signaling pathways, producing diverse biological actions (8). Bacterial pathogen lipopolysaccharides (LPS) are known inducers of numerous pro-inflammatory events, including the production of PAF (9). PAF is a lipid molecule involved in inflammatory processes and cell-to-cell communication. PAF is produced by a variety of cells that may be involved in the development of the inflammatory reaction, such as monocytes/macrophages, polymorphonuclear neutrophils (PMN), eosinophils, basophils, and platelets (10-12). PAF is rapidly hydrolyzed into lyso-PAF, which is an inactive phospholipid, by specific enzymes with PAF-AH (13). PAF-AH hydrolyzes the oxidized phospholipids that are structurally similar to PAF and therefore exerts anti-inflammatory activity (14).

Peroxisome proliferator-activated receptors (PPAR) are members of the nuclear receptor superfamily with at least three identified subtypes (PPARα, PPARδ and PPARγ) (15). PPARγ are important in a variety of biological processess, including adipogenesis, glucose metabolism, and inflammation (16). Of the various PPARγ ligands, 15-deoxy-$\delta^{12,14}$-prostaglandin J_2 (15d-PGJ$_2$) has exhibited a potent immuno-modulatory effect on several cell types, including monocytes/macrophages, microglia, astrocytes, neutrophils and lymphocytes (17-19). RPE cells express all three forms of PPARs, although PPARβ is the dominant isoform (20). In particular, PPARγ may be important for processing the lipids generated during the phagocytosis of the outer segments of photoreceptors in RPE cells (20). In earlier studies, 15d-PGJ$_2$ attenuated the degree of inflammation via modulation of the production of cytokines, chemokines, and adhesion molecules (21,22). Therefore, 15d-PGJ$_2$ may be a therapeutic candidate for ocular inflammatory diseases. Human RPE is clinically involved in many ocular inflammatory diseases. In this study, we investigated whether 15d-PGJ$_2$ was capable of attenuating ocular inflammatory responses and elucidate its regulatory molecular mechanisms for the RPE cells that are induced by LPS.

Materials and methods

Cell culture and reagents. Human ARPE19 retinal pigment epithelial cells (RPE) were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were grown to confluency in a standard incubator in Dulbecco's MEM/Nut MIX F-12 medium (Gibco, Paisley, UK) including 10% fetal bovine serum, penicillin/streptomycin (Gibco/BRL, Gaithersburg, MD, USA), and 2 mM glutamine (Life Technologies, UK). LPS was purchased from Sigma-Aldrich (St. Louis, MO, USA). The reverse transcription polymerase chain reaction (RT-PCR) reagents were purchased from Promega (Madison, WI, USA). The light shift chemiluminescent electrophoretic mobility shift assay reagents, nuclear and cytoplasmic extraction reagents, and the enhanced chemiluminescence (ECL)-detecting reagent were purchased from Pierce (Rockford, IL, USA). Rabbit anti-mouse p65 and IκBα antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against ERK, phosphor (p)-ERK 1/2, p38, p-p38, JNK/SAPK, p-JNK/SAPK, and p-IκBα were purchased from Cell Signaling Technology (Beverly, MA, USA).

Determination of cell viability. The cell viability of the ARPE19 cells was determined using the Cell Counting Kit-8 (CCK-8) according to the manufacturer's instructions (Dojindo Laboratories, Kumamoto, Japan). Briefly, the cells were plated onto 96-well plates at a density of 1x10^4 cells/well. Subsequently, 10 μl of CCK-8 reagent was added to each well followed by incubation for 2 h. The amount of CCK-8 reagent that was reduced to yield formazan via cellular dehydrogenase indicated whether the cell was viable. The results were measured by reading the absorbance at 450 nm in a 96-well plate reader (Model EL800, BioTek Instruments, Inc., Winooski, VT, USA). The absorbance reading was subtracted from the background control and reported as the mean of three measurements.

RT-PCR. The ARPE19 cells were plated overnight in 6-well culture plates at a density of 2x10^5 cells/well, and the cells were incubated in a serum-free medium for at least 4 h prior to treatment. Total RNAs were isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The cDNA was generated using ImProm-II Reverse Transcription System (Promega) before being amplified via PCR with specific primers for interleukin-6 (IL-6) (forward, 5'-GAT GGC TGA AAA AGA TGG ATG C-3'; reverse, 5'-TGG TTG GGT CAG GGG TGG TT-3'), monocyte chemoattractant protein-1 (MCP-1) (forward, 5'-AAT GCC CCA GTC ACC TGC TGT TAT-3'; reverse, 5'-GCA ATT TCC CCA AGT CTC TGT ATC-3'), and intercellular adhesion molecule-1 (ICAM-1) (forward, 5'-ACT TTC CCA CTG CCC ATC GG-3'; reverse, 5'-GTG GCT TGT GTG TTC GGT TTC A-3'). After the amplification process, sections of the PCR reactions were subjected to agarose gel electrophoresis.

Enzyme-linked immunosorbent assay (ELISA). Cytokine levels were determined by ELISA. ELISA kits purchased from BioLegend (San Diego, CA, USA) were used to measure IL-6 and MCP-1 levels, and a kit obtained from R&D Systems (Minneapolis, MN, USA) was used to measure ICAM-1 levels. The absorbance at 450 nm was determined using a microplate reader (Model EL800, BioTek Instruments, Inc.).

PAF-acetylhydrolase (PAH-AH) assay. Cytosolic PAF-AH levels were determined with PAF-AH assay kits purchased from Cayman Chemical (Ann Arbor, MI, USA). Briefly, cells (1x10^5 cells/ml, in 60-mm culture dishes) were collected by centrifugation for 10 min at 4˚C followed by sonication of the cell pellet in 1 ml of cold buffer (0.1 M Tris-HCl, pH 7.2). The mixture was centrifuged at 10,000 x g for 15 min at 4˚C. The supernatant was removed and 10 μl of DTNB was added to it, followed by addition of 10 μl of cell lysate, and 5 μl of assay buffer (0.1 M Tris-HCl, pH 7.2) to the wells. The wells were incubated for 30 min at room temperature and 200 μl of substrate solution was added to all of the wells. The 96-well plate was agitated for 30 sec. The absorbance at 412 nm

was determined using a microplate reader (SpetraMax M2, Molecular Devices, Sunnyvale, CA, USA).

Western blot analysis. The ARPE19 cells were plated overnight in a 100-mm culture dish at a density of $1x10^6$ cells/dish. Subsequently, the cells were incubated in serum-free medium for at least 4 h before treatment. The cells were washed three times with PBS buffer before being lysed in a lysis buffer (1% Triton X-100, 1% deoxycholate, 0.1% NaN_3) containing protease inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany). Equal amounts of protein were separated on 10% SDS polyacrylamide mini-gels and transferred to a nitrocellulose transfer membrane (Whatman, Florham Park, NJ, USA). Following incubation with the appropriate primary antibodies, the membranes were incubated for 1 h at room temperature with a secondary antibody conjugated to horseradish peroxidase. After three washes in TBST, the immunoreactive bands were visualized using the ECL detection system (Pierce). In a parallel experiment, a nuclear protein was prepared using nuclear extraction reagents (Pierce) according to the manufacturer's instructions.

Preparation of nuclear extracts and EMSA. Nuclear extracts were prepared with the NE-PER nuclear extraction reagent (Pierce). An oligonucleotide containing the immunoglobulin κ-chain binding site (κB, 5'-CCGGTT AACAGAGGGGGCTTTCCGAG-3') was synthesized as a probe for the gel retardation assay, and the probe was labeled with biotin (Pierce). The binding reactions contained 5 μg of nuclear extract protein, buffer (10 mM Tris, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.05% Nonidet P-40, and 2.5% glycerol), 50 ng of poly-(dI-dC), and a 20 fM solution of biotin-labeled DNA. The reactions were incubated for 20 min at room temperature at a final volume of 20 μl. During the competition reactions, the nuclear extracts were incubated for 15 min at room temperature with a competing cold oligonucleotide (100-fold excess) before the addition of a labeled probe. The reaction mixture was electrophoretically analyzed in a 5% polyacrylamide gel with 0.5X Tris-borate EDTA buffer. The reactions were transferred to nylon membranes and the biotinylated DNA was detected using a LightShift chemiluminescent EMSA kit (Pierce).

Statistical analysis. Statistical analyses were conducted using the Student's t-test. The results are presented as the means ± SD of at least three separate experiments. $P<0.05$ was considered to be statistically significant.

Results

Effects of 15d-PGJ_2 on IL-6, MCP-1, and ICAM-1 production in LPS-stimulated ARPE19. Initially, to exclude the possibility that the inhibition of the production of inflammatory mediators was caused by the cytotoxity of 15d-PGJ_2, we performed CCK-8 assays in ARPE19 cells treated with 15d-PGJ_2 (10-80 μM) (Fig. 1). A decrease in cell survival was detected at concentrations from 40 to 80 μM. The concentrations (10-20 μM) of 15d-PGJ_2 alone did not affect cell viability in this investigation. Therefore, concentrations of 10-20 μM 15d-PGJ_2 were used in subsequent experiments.

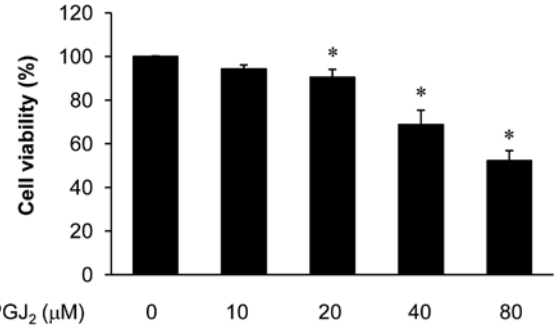

Figure 1. Effects of 15-deoxy-$\delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) on the viability of ARPE19 cells. The cells were treated with various concentrations (10-80 μM) of 15d-PGJ_2 for 24 h. Cell viability was measured via CCK-8 assays, and the results are expressed as the percentage of surviving cells over control cells (no addition of 15d-PGJ_2). Each value is reported as the mean ± SD and is representative of results obtained from three independent experiments. *$P<0.05$ compared with non-treated cells.

The effect of 15d-PGJ_2 on IL-6, MCP-1, and ICAM-1 production in LPS-stimulated ARPE19 cells was examined. The ARPE19 cells were incubated with 15d-PGJ_2 (10 or 20 μM) in the presence of LPS (10 μg/ml) for 24 h, and the mediator levels in the culture media were measured via ELISA. As shown in Fig. 2A, the IL-6, MCP-1, and ICAM-1 levels were increased in the culture media from the LPS-stimulated ARPE19. These increases were significantly decreased in a concentration-dependent manner by treatment with 15d-PGJ_2. In a parallel experiment, RT-PCR was performed to determine whether 15d-PGJ_2 inhibits the expression of these mediators at the transcriptional level. As shown in Fig. 2B, the treatment of ARPE19 cells with different concentrations of 15d-PGJ_2 2 h prior to LPS treatment caused a dose-dependent decrease in the IL-6, MCP-1, and ICAM-1 mRNA. These results suggested that 15d-PGJ_2 acts primarily by preventing the expression of IL-6, MCP-1, and ICAM-1 at the transcriptional level. Therefore, the results indicated that 15d-PGJ_2 inhibited the expression of these mediators, which are involved in the inflammatory process. We further explored the mechanism of inhibitory action for 15d-PGJ_2.

Relationship between 15d-PGJ_2 levels and PAF on IL-6, MCP-1, and ICAM-1 expression. The effect of CV-3988 on IL-6, MCP-1, and ICAM-1 production in LPS-stimulated ARPE19 cells was examined. The PAF released in response to LPS is a major contributor to the pathological events associated with numerous pro-inflammatory events. PAF may be a major mediator of retinal inflammation. Rosenbaum *et al* reported that intravitreal injection of PAF induces retinitis in experimental animals (23). To validate the above information, we examined the relationship between 15d-PGJ_2 levels and the repression of PAF. In order to exclude the possibility that the inhibition of inflammatory mediators production was caused by the cytotoxity of CV-3988, we initially performed CCK-8 assays in ARPE19 cells treated with CV-3988 (0.1-80 μM) (Fig. 3). A decrease in cell survival was detected at 80 μM. The concentrations (10-40 μM) CV-3988 alone did not affect cell viability in this investigation. Therefore, concentrations of 10-40 μM CV-3988 were used in the subsequent experiments.

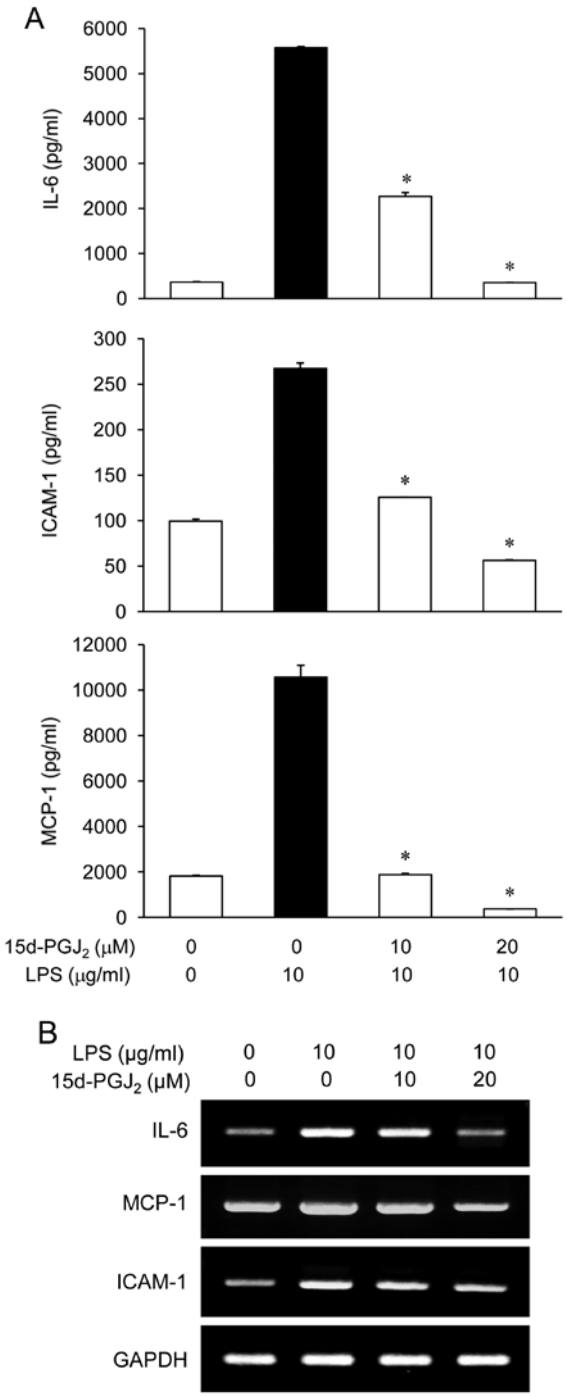

Figure 2. Inhibition of interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), and intercellular adhesion molecule-1 (ICAM-1) expression by 15-deoxy-$\delta^{12,14}$-prostaglandin J_2 (15d-PGJ$_2$) in lipopolysaccharide (LPS)-stimulated ARPE19 cells. The ARPE19 cells were pre-treated with 15d-PGJ$_2$ (10 and 20 μM) for 2 h before a 24-h incubation with LPS (10 μg/ml). (A) The IL-6, ICAM-1, and MCP-1 concentrations were measured in culture media using a commercial ELISA kit. (B) After 16 h of LPS treatment, the total RNA was prepared for the RT-PCR analysis of IL-6, ICAM-1, and MCP-1 gene expression in the LPS-stimulated ARPE19 cells. Each value reported is the mean ± SD representative of the results obtained from four independent experiments. *P<0.05 indicates a significant difference from the value obtained for cells treated with LPS in the absence of 15d-PGJ$_2$.

ARPE-19 cells were incubated with CV-3988 (10, 20 or 40 μM) in the presence of LPS (10 μg/ml) for 24 h, and the mediator levels in the culture media were measured via ELISA. As shown in Fig. 4A, the IL-6, MCP-1, and ICAM-1 levels were

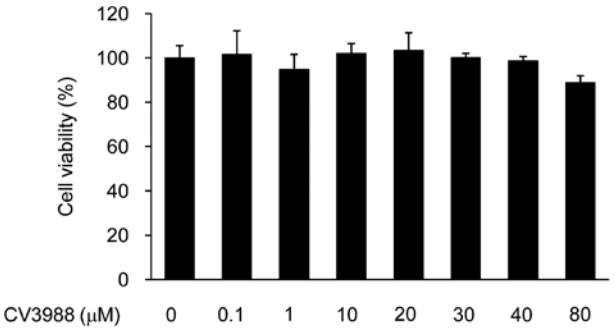

Figure 3. Effects of CV-3988 (PAF receptor antagonist) on the viability of ARPE19 cells. The cells were treated with various concentrations (0.1-80 μM) of CV-3988 for 24 h. Cell viability was measured via CCK-8 assays, and the results are expressed as the percentage of surviving cells over control cells (no addition of CV-3988). Each value reported is the mean ± SD representative of the results obtained from three independent experiments.

increased in the culture media of LPS-stimulated ARPE19 cells, and these increases were significantly decreased in a concentration-dependent manner by treatment with CV-3988.

Bulger *et al* demonstrated that treatment of alveolar macrophages with PAF-acetylhydrolase (PAF-AH) *in vitro* caused significant inhibition of the cytokine response to the endotoxin (24). To determine whether 15d-PGJ$_2$ and CV-3988 were capable of upregulating PAF-AH activity in ARPE19 cells, the cells were treated with 15d-PGJ$_2$ (20 μM) and CV-3988 (40 μM) in the presence of LPS (10 μg/ml) for 24 h, and then PAF-AH activity in the cytosols was measured. As shown in Fig. 4B, the PAF-AH activity decreased in response to LPS treatment in the cultured ARPE19 cells, and these decreases were reversed by the administration of 15d-PGJ$_2$ and CV-3988. The results suggested that the anti-inflammatory effects of 15d-PGJ$_2$ are generated via enhancement of PAH-AH activity.

Effects of 15d-PGJ$_2$ on the activation of NF-κB in LPS-stimulated ARPE19 cells. Since 15d-PGJ$_2$ inhibited the expression of the inflammatory mediators in ARPE19 cells, we examined the mechanism of inhibitory action for 15d-PGJ$_2$. Activation of nuclear transcription factor-κB (NF-κB) is necessary to induce the IL-6, MCP-1, and ICAM-1 genes. Therefore, using western blot and EMSA, we investigated whether 15d-PGJ$_2$ acts on NF-κB activity. The effect of 15d-PGJ$_2$ on LPS-induced IκBα phosphorylation and degradation was also examined. The imunoblotting results shown in Fig. 5A reveal that the LPS-induced IκBα phosphorylation/degradation were inhibited after 1 h of exposure to 15d-PGJ$_2$. Additionally, 15d-PGJ$_2$ inhibited the LPS exposure-induced translocation of the NF-κB p65 subunit from the cytosol to the nucleus. To further characterize the mechanism, the effect of 15d-PGJ$_2$ on the DNA-binding activity of NF-κB was determined by EMSA (Fig. 5B). LPS treatment caused a significant increase in the DNA-binding activity of NF-κB. By contrast, treatment with 15d-PGJ$_2$ markedly reduced the LPS-induced DNA-binding activity of NF-κB. Additionally, pretreatment with a PAF antagonist significantly abrogated the LPS-induced NF-κB activation. When combined, these results suggested that 15d-PGJ$_2$ may inhibit NF-κB activation in ARPE19 cells by suppressing the IκBα phosphorylation/degradation, as well as the binding of NF-κB.

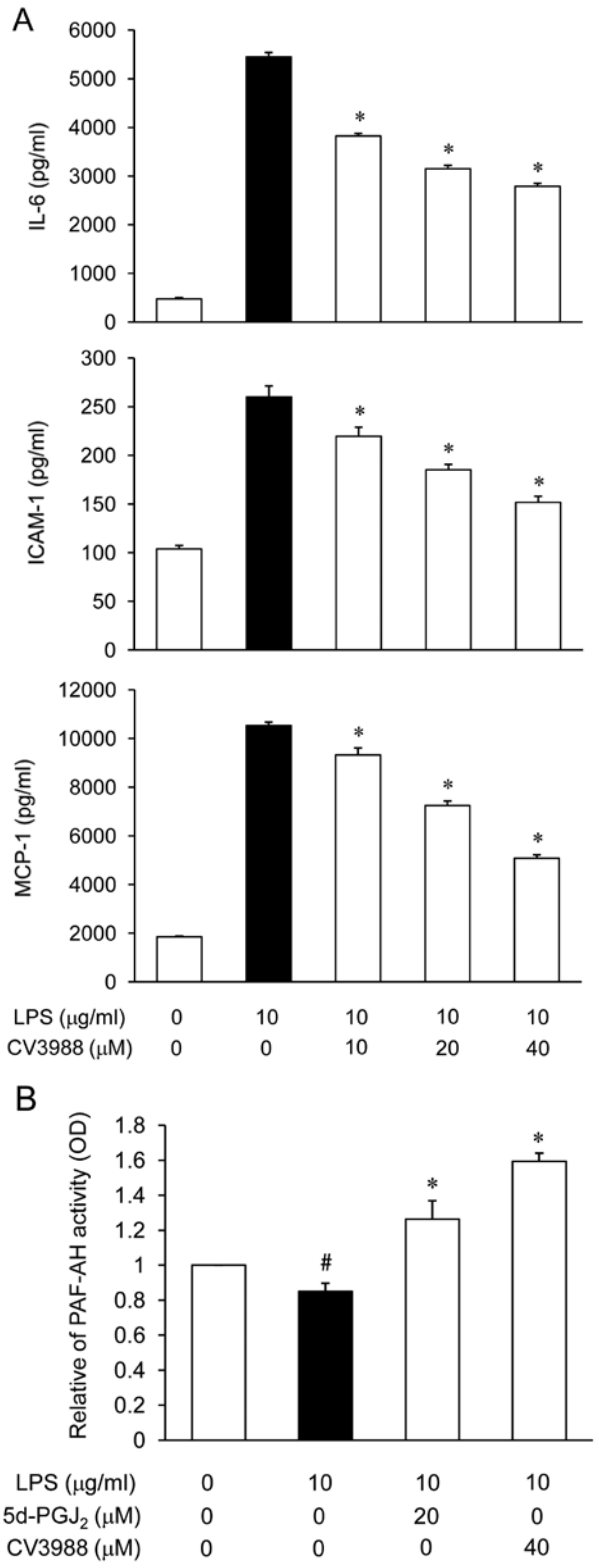

Figure 4. Inhibition of interleukin-6 (IL-6), intercellular adhesion molecule-1 (ICAM-1), and monocyte chemoattractant protein-1 (MCP-1) production by CV3988 in lipopolysaccharide (LPS)-stimulated ARPE19 cells. The ARPE19 cells were pre-treated with CV3988 (10, 20, and 40 μM) for 2 h before a 24-h incubation with LPS (10 μg/ml). (A) The IL-6, ICAM-1, and MCP-1 concentrations were measured in culture media using a commercial ELISA kit. (B) After LPS treatment for 24 h, the cytosolic fraction was prepared for PAF-AH activity in LPS-stimulated ARPE19 cells. Each value reported is the mean ± SD representative of results obtained from four independent experiments. $^{\#}$P<0.05 indicates a significant difference from the value obtained for non-treated cells. *P<0.05 indicates a significant difference from the value obtained for cells treated with LPS in the absence of 15-deoxy-$\delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) or CV3988.

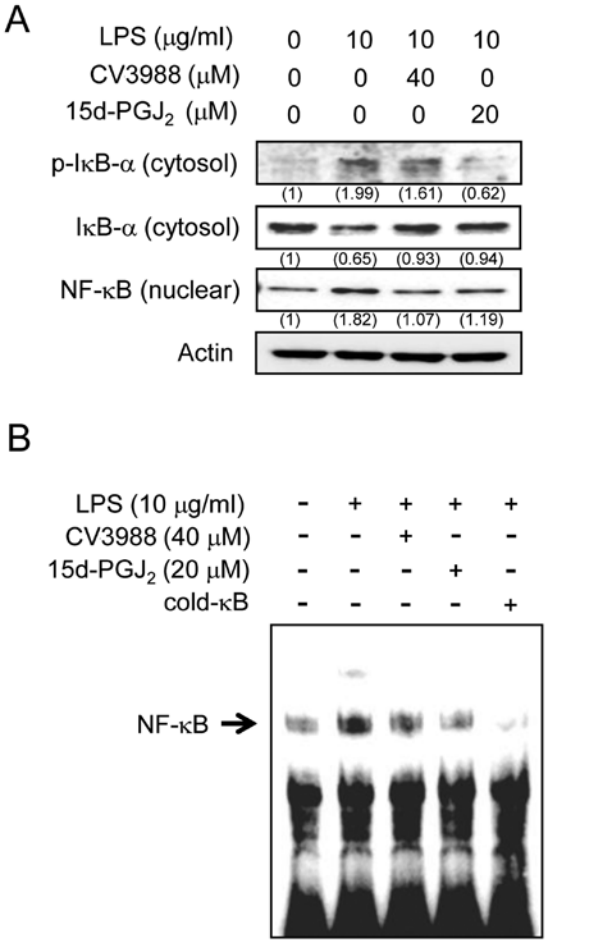

Figure 5. Effects of 15-deoxy-$\delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) and CV3988 on the nuclear transcription factor-κB (NF-κB) activity in lipopolysaccharide (LPS)-stimulated ARPE19 cells. (A) The p65 subunit of NF-κB in the nuclear protein extracts and the levels of p-IκBα and IκBα in the cytosolic protein were determined using western blot analysis.The ARPE19 cells were treated with LPS (10 μg/ml) for 1 h and the p65, p-IκBα, and IκBα proteins were detected using specific antibodies. (A) The nuclear extracts (5 μg) were prepared and analyzed for the DNA binding activity for NF-κB using an electrophoretic mobility shift assay. The ARPE19 cells were pretreated with the indicated concentrations of 15d-PGJ_2 or CV3988 for 2 h prior to stimulation with LPS (10 μg/ml) for another hour. The results are the mean representative of three independent experiments.

Effect of 15d-PGJ_2 on the phosphorylation of MAP kinases in LPS-stimulated ARPE19. Experiments were designed to elucidate the signaling cascades that regulate the expression of the inflammatory mediators in ARPE19 cells in responding to LPS stimulation. MAP kinases are important for the expression of IL-6, MCP-1 and ICAM-1. MAP kinases therefore act as a specific target for inflammatory responses. To investigate whether the inhibition of inflammation by 15d-PGJ_2 is regulated by the MAP kinase pathway, we examined the effect of 15d-PGJ_2 on LPS-induced phosphorylation of ERK, JNK, and p38 kinase in ARPE19 cells using western blot analysis. We first demonstrated that ERK, JNK, and p38 kinase were phosphorylated following ARPE19 cell stimulation with LPS, and then examined the effect of 15d-PGJ_2 on the LPS-induced activation of MAP kinases. As shown in Fig. 6, 15d-PGJ_2 (20 μM) markedly inhibited ERK activation, while the phosphorylation of JNK and the p38 kinase were not affected. The total amount of ERK was not affected by treatment with LPS or 15d-PGJ_2

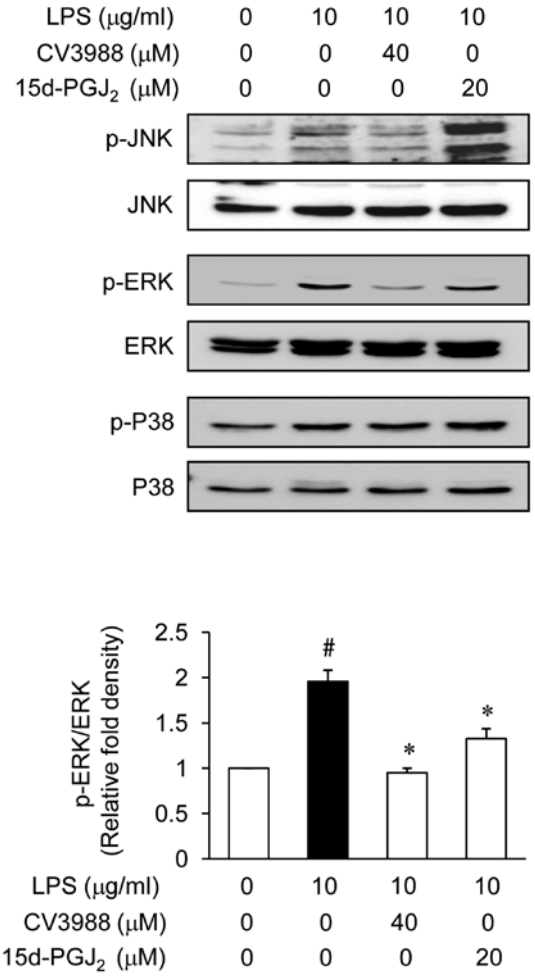

Figure 6. Effects of 15-deoxy-$\delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) and CV3988 on lipopolysaccharide (LPS)-stimulated phosphorylation of mitogen-activated protein kinase (MAPK) in ARPE19 cells. The ARPE19 cells were treated with the vehicle or the indicated concentrations of 15d-PGJ_2 and CV3988 for 2 h prior to incubation with LPS (10 μg/ml) for 0.5 h. The cell extracts were then prepared and subjected to a western blot with antibodies specific for the phosphorylated forms of JNK, ERK, and p38. $^{\#}$P<0.05 indicates a significant difference from the value obtained for non-treated cells. *P<0.05 indicates a significant difference from the value obtained for cells treated with LPS in the absence of 15d-PGJ_2 or CV3988.

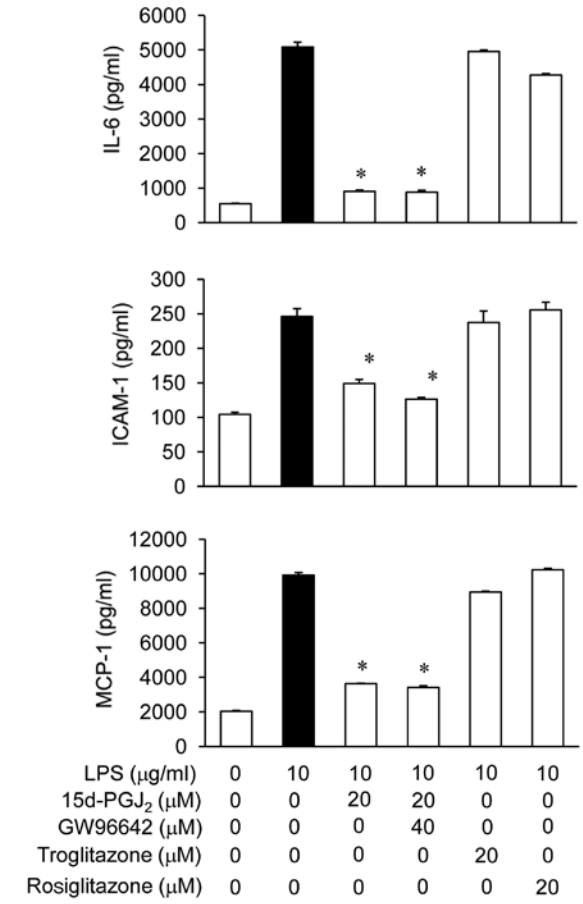

Figure 7. Effects of 15-deoxy-$\delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) on lipopolysaccharide (LPS)-stimulated interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), and intercellular adhesion molecule-1 (ICAM-1) production in ARPE19 cells via PPARγ-independent mechanisms. The ARPE19 cells were pretreated with PPARγ agonists, such as troglitazone, rosiglitazone, or 15d-PGJ_2, at the indicated concentrations for 2 h, followed by stimulation with lipopolysaccharide (LPS) (10 μg/ml) for 24 h. In addition, the cells were pretreated with a specific PPARγ antagonist, GW9662, at 40 μM for 0.5 h followed by treatment with 15d-PGJ_2 for 2 h; subsequently, they were stimulated with LPS at the indicated concentrations. Twenty-four hours later, the cultured supernatants were isolated and analyzed for IL-6, MCP-1, and ICAM-1 levels using commercial ELISA kits. *P<0.05 indicates a significant difference from the value obtained for cells treated with only LPS. The experiments are reported as the mean representative of three independent experiments.

treatment. These results suggested that the ERK pathways are relevant during the LPS-mediated expression of IL-6, MCP-1, and ICAM-1.

15d-PGJ_2 inhibition of the LPS-stimulated IL-6, MCP-1, and ICAM-1 production via PPARγ-independent pathways. To verify whether the action of 15d-PGJ_2 was PPARγ-dependent or -independent, we examined the effects of GW9662, which is a potent, irreversible and selective PPARγ antagonist, on the expression of IL-6, MCP-1, and ICAM-1. Fig. 7 shows that, in the presence of LPS, GW9662 did not reverse the inhibitory effect of 15d-PGJ_2 on the expression of IL-6, MCP-1, and ICAM-1. Additionally, we compared the inhibitory effects of different PPARγ agonists on the LPS-stimulated production of IL-6, MCP-1, and ICAM-1. 15d-PGJ_2 inhibited the LPS-stimulated production of IL-6, MCP-1, and ICAM-1, but other PPARγ agonists, such as troglitazone and rosiglitazone, did not inhibit the LPS-stimulated production of IL-6, MCP-1, and ICAM-1 in ARPE19 cells. These results supported the hypothesis that 15d-PGJ_2 regulates the LPS-stimulated production of IL-6, MCP-1, and ICAM-1 via PPARγ-independent mechanisms.

Discussion

The present study was undertaken to elucidate the anti-inflammatory effects and mechanisms of 15d-PGJ_2 on the production of inflammatory mediators in RPE cells stimulated with LPS. In particular, we investigated whether the anti-inflammatory effects of 15d-PGJ_2 were associated with PAF activation. The bacterial LPS activates the RPE cells to produce and release potent inflammatory mediators, including IL-6, MCP-1, and ICAM-1. The results suggest that 15d-PGJ_2 is an effective inhibitor of the LPS-induced inflammatory mediators through the blockade of the NF-κB and mitogen-activated protein kinase (MAPK) pathways via PAF release in the retinal pigment epithelium cell line, ARPE19. The inhibitory effect of

15d-PGJ_2 on the inflammatory mediator expression suggests efficacy, which is responsible for its anti-inflammatory action, as well as its potential use as a therapeutic agent for treating LPS-stimulated ocular diseases.

Bacterial LPS can elicit acute ocular inflammation in animals and lead to uveitis, various degrees of degeneration of the retina, and loss of vision (1,25). The RPE is a major component of the blood retinal barrier and controls the nutrient flow to the photoreceptors. It has been reported that the bacterial endotoxin activates the RPE to enhance the expression of various inflammatory mediators, such as IL-6, MCP-1, and ICAM-1 (26,27). IL-6 is an important mediator of the acute-phase response and possesses biological activities that support host immune reactions. The pro-inflammatory cytokine IL-6 is an important mediator of inflammation and has chemotactic activity for neutrophils and macrophages, in addition to activating T lymphocytes, stimulating the secretion of immunoglobulin, and triggering the release of acute phase proteins (28,29). The local production of IL-6 by resident cells and infiltrating inflammatory cells has been detected during a variety of inflammatory ocular conditions (30,31). MCP-1 is overexpressed in human eyes during acute anterior uveitis and is known to have strong chemotactic activity for monocytes/macrophages (32). Retinal detachment-induced photoreceptor apoptosis has been associated with MCP-1 (33). ICAM-1 expression is upregulated in the iris and ciliary bodies following LPS application (34). Leukocyte adhesion to the vessel walls is an important process during inflammation. When leukocytes are recruited to inflammatory sites, the adhesion molecules play essential roles during the first step of inflammation. Therefore, the downregulation of IL-6-, MCP-1-, and ICAM-1-mediated migration and adhesion of leukocytes may lead to the suppression of ocular inflammation. In the present study, the application of 15d-PGJ_2 reduced the retinal expression of IL-6, ICAM-1, and MCP-1 (Fig. 2), suggesting that the anti-inflammatory effects of 15d-PGJ_2 on the eye resulted from inhibition of the inflammation-related molecules.

In a previous report, when LPS was injected intracamerally, PAF was detected in the aqueous humor and found to enhance the intraocular inflammation (35). In this study, we found that LPS induces the mRNA expression of the PAF receptor in ARPE19 cells. In addition, the LPS-induced expression of the PAF receptor mRNA was completely inhibited by the PAF receptor antagonist, CV3988. Therefore, we hypothesized that the anti-inflammatory effects of 15d-PGJ_2 may be mediated through inhibition of the PAF secretion. To prove this hypothesis, we investigated the relationship of 15d-PGJ_2 and PAF with the expression of inflammatory mediators (IL-6, MCP-1, and ICAM-1) in LPS-stimulated ARPE19 cells. We initially examined whether CV3988 reduced the expression of these inflammatory mediators in the presence of LPS in the ARPE19 cells. As demonstrated in Fig. 4A, the expression levels of the inflammatory LPS-induced mediators were significantly suppressed when the cells were pretreated with 20 and 40 μM CV3988 for 2 h prior to LPS stimulation for 24 h. To elucidate whether 15d-PGJ_2 is related to the PAF response, we examined PAF-acetylhydrolase (PAH-AH) activity in the presence of LPS. In a previous study, the PAF-AH protein was upregulated by 15d-PGJ_2 (13). This previous finding, in accordance with our data, indicated that the decreased activities of PAH-AH, which were stimulated by LPS, were significantly enhanced when the cells were pretreated with 20 μM 15d-PGJ_2 and 40 μM CV3988 for 2 h prior to LPS stimulation for 24 h (Fig. 4). This suggest that 15d-PGJ_2 induces PAF-AH, leading to the prevention of inflammatory reactions in RPE cells, considering that PAF-AH rapidly hydrolyzed PAF to form lyso-PAF.

Bacterial LPS stimulate the transcription of several genes involved in inflammatory and immune responses, including the NF-κB and MAPK pathways. Previously, NF-κB and MAPK signaling pathways were demonstrated to be involved in the anti-inflammatory responses of various primary human ocular cells (36). To determine the mechanisms by which 15d-PGJ_2 inhibited the production of inflammatory mediators, we examined its effects on LPS-induced NF-κB activation (Fig. 5). NF-κB is a pleiotropic regulator of various genes involved in cell responses to infection and participates in inflammatory responses, leading to organ dysfunction and death in patients with sepsis (37). In addition, PAF is a potent inducer of NF-κB activity. IκBα is one of the inhibitor proteins that binds to NF-κB. Degradation of IκBα after its phosphorylation is required to translocate NF-κB to the nucleus. 15d-PGJ_2 represses the NF-κB transcriptional activity (38). Our findings show that 20 μM 15d-PGJ_2 significantly inhibits the LPS-stimulated IκBα phosphorylation/degradation and nuclear translocation of p65, as well as the DNA binding activity of NF-κB in ARPE19 cells. Therefore, inhibiting the NF-κB signaling pathways in RPE cells with 15d-PGJ_2 may cause the downregulation of inflammatory mediators, generating an anti-inflammatory effect. The involvement of various intracellular signaling pathways, such as MAPKs, is needed to induce and maintain the inflammatory process. Activation of these kinases leads to the nuclear translocation of the NF-κB. Previous studies have revealed that MAPKs have a significant role in the regulation of IL-6, MCP-1, and ICAM-1 production in LPS-stimulated ocular cells (36). Therefore, experiments were performed to determine whether the 15d-PGJ_2 tightly regulates the expression of MAPKs to induce anti-inflammatory effects in LPS-stimulated ARPE19 cells. In the present study, 15d-PGJ_2 inhibits the activation of ERK, which is induced by LPS stimulation in ARPE19 cells (Fig. 6). Therefore, the inhibition of ERK and the NF-κB signaling pathways in ARPE19 cells by 15d-PGJ_2 may cause the downregulation of inflammatory mediators, resulting in an anti-inflammatory effect.

Previous studies have indicated that 15d-PGJ_2 exerts its anti-inflammatory action via a PPARγ-dependent (39) or -independent (40) mechanism. To verify the PPARγ-dependence or -independence of 15d-PGJ_2, we examined the effects of PPARγ antagonists and agonist on the production of IL-6, MCP-1, and ICAM-1 (Fig. 7). In this study, we investigated the mechanism of action for 15d-PGJ_2 with respect to the LPS response. GW9662, which is a selective PPAR antagonist, was not able to efficiently reduce the LPS response. The inhibition of the expression of LPS-stimulated inflammatory mediators by 15d-PGJ_2 was not reversed by GW9662. Additionally, troglitazone and rosiglitazone, which are synthetic PPARγ agonists, were administered at concentrations that induced PPARγ activity, but did not affect the inhibitory effect on the expression of inflamma-

tory mediators. Taken together, these results suggest that the higher potency and efficiency of 15d-PGJ$_2$ for attenuating the expression of inflammation-related molecules (IL-6, MCP-1, and ICAM-1) triggered by LPS are mainly achieved through PPARγ-independent mechanisms.

In conclusion, the results of the present study have demonstrated that 15d-PGJ$_2$ significantly suppresses the LPS-induced expression of pro-inflammatory mediators. Specifically, 15d-PGJ$_2$ significantly inhibited the release of IL-6, MCP-1, and ICAM-1 by inhibiting NF-κB and ERK activation, as well as through a PPARγ-independent pathway in the RPE cells. Additionally, the anti-inflammatory properties of 15d-PGJ$_2$ are mediated by the enhancement of PAF-AH activity. Therefore, our results suggest that 15d-PGJ$_2$ is considered for use as a treatment for ocular inflammatory disorders. Future studies are to address this aspect in ocular inflammation models performed *in vivo*.

Acknowledgements

This study was supported by a grant of the Korea Healthcare Technology R&D Project, Ministry of Health & Welfare Affairs, Republic of Korea (A120006).

References

1. Shen DF, Chang MA, Matteson DM, Buggage R, Kozhich AT and Chan CC: Biphasic ocular inflammatory response to endotoxin-induced uveitis in mouse. Arch Ophthalmol 118: 521-527, 2000.
2. Joussen AM, Poulaki V, Le M, *et al*: A central role for inflammation in the pathogenesis of diabetic retinopathy. FASEB J 18: 1450-1452, 2004.
3. Rodrigues EB: Inflammation in dry age-related macular degeneration. Ophthalmologica 221: 143-152, 2007.
4. Strauss O: The retinal pigment epithelium in visual function. Physiol Rev 85: 845-881, 2005.
5. Kumaki N, Anderson DM, Cosman D and Kumaki S: Expression of interleukin-15 and its receptor by human fetal retinal pigment epithelial cells. Curr Eye Res 15: 876-882, 1996.
6. McLaren MJ: Kinetics of rod outer segment phagocytosis by cultured retinal pigment epithelial cells. Relationship to cell morphology. Invest Ophthalmol Vis Sci 37: 1213-1224, 1996.
7. Yu FS and Hazlett LD: Toll-like receptors and the eye. Invest Ophthalmol Vis Sci 47: 1255-1263, 2006.
8. Ishii S and Shimizu T: Platelet-activating factor (PAF) receptor and genetically engineered PAF receptor mutant mice. Prog Lipid Res 39: 41-82, 2000.
9. Han SJ, Choi JH, Ko HM, *et al*: Glucocorticoids prevent NF-kappaB activation by inhibiting the early release of platelet-activating factor in response to lipopolysaccharide. Eur J Immunol 29: 1334-1341, 1999.
10. Camussi G, Tetta C and Baglioni C: The role of platelet activating factor in inflammation. Clin Immun Immunopathol 57: 331-338, 1990.
11. Lotner GZ, Lynch JM, Betz SJ and Henson PM: Human neutrophil-derived platelet activating factor. J Immunol 124: 676-684, 1980.
12. Triggiani M, Schleimer RP, Warner JA and Chilton FH: Differential synthesis of 1-acyl-2-acetyl-sn-glycero-3-phosphocholine and platelet-activating factor by human inflammatory cells. J Immunol 147: 660-666, 1991.
13. Sumita C, Maeda M, Fujio Y, *et al*: Pioglitazone induces plasma platelet activating factor-acetylhydrolase and inhibits platelet activating factor-mediated cytoskeletal reorganization in macrophage. Biochim Biophys Acta 1673: 115-121, 2004.
14. Tjoelker LW, Wilder C, Eberhardt C, *et al*: Anti-inflammatory properties of a platelet-activating factor acetylhydrolase. Nature 374: 549-553, 1995.
15. Tontonoz P, Nagy L, Alvarez JG, Thomazy VA and Evans RM: PPARγ promotes monocyte/macrophage differentiation and uptake of oxidized LDL. Cell 93: 241-252, 1998.
16. Mueller E, Sarraf P, Tontonoz P, *et al*: Terminal differentiation of human breast cancer through PPARγ. Mol Cell 1: 465-470, 1998.
17. Buckingham RE: Thiazolidinediones: pleiotropic drugs with potent anti-inflammatory properties for tissue protection. Hepatol Res 33: 167-170, 2005.
18. Consoli A and Devangelio E: Thiazolidinediones and inflammation. Lupus 14: 794-797, 2005.
19. Drew PD and Chavis JA: The cyclopentone prostaglandin 15-deoxy-$\delta^{12,14}$ prostaglandin J$_2$ represses nitric oxide, TNF-, and IL-12 production by microglial cells. J Neuroimmunol 115: 28-35, 2001.
20. Garg TK and Chang JY: Oxidative stress causes ERK phosphorylation and cell death in cultured retinal pigment epithelium: prevention of cell death by AG126 and 15-deoxy-delta^{12,14}-PGJ$_2$. BMC Ophthalmol 3: 5, 2003.
21. Surh YJ, Na HK, Park JM, *et al*: 15-Deoxy-$\Delta^{12,14}$-prostaglandin J$_2$, an electrophilic lipid mediator of anti-inflammatory and pro-resolving signaling. Biochem Pharmacol 82: 1335-1351, 2011.
22. Abdelrahman M, Sivarajah A and Thiemermann C: Beneficial effects of PPAR-gamma ligands in ischemia-reperfusion injury, inflammation and shock. Cardiovasc Res 65: 772-781, 2005.
23. Rosenbaum JT, Angell E, Wilson D, Broquet C, Boney RS and Braquet P: Intravitreally injected platelet activating factor induces retinitis in experimental animals. Curr Eye Res 18: 342-348, 1999.
24. Bulger EM, Arbabi S, Garcia I and Maier RV: The macrophage response to endotoxin requires platelet activating factor. Shock 17: 173-179, 2002.
25. Cousin SW, Guss RB, Howes EL Jr and Rosenbaum JT: Endotoxin-induced uveitis in the rat: observations on alters vascular permeability, clinical findings and histology. Exp Eye Res 35: 665-676, 1984.
26. Suzuki M, Noda K, Kubota S, *et al*: Eicosapentaenoic acid suppresses ocular inflammation in endotoxin-induced uveitis. Mol Vis 16: 1382-1388, 2010.
27. Leung KW, Barnstable CJ and Tombran-Tink J: Bacterial endotoxin activates retinal pigment epithelial cells and induces their degeneration through IL-6 and IL-8 autocrine signaling. Mol Immunol 46: 1374-1386, 2009.
28. Planck SR, Dang TT, Graves D, Tara D, Ansel JC and Rosenbaum JT: Retinal pigment epithelial cells secrete interleukin-6 in response to interleukin-1. Invest Ophthalmol Vis Sci 33: 78-82, 1992.
29. Feghali CA and Wright TM: Cytokines in acute and chronic inflammation. Front Biosci 2: d12-d26, 1997.
30. Biswas PS, Banerjee K, Kinchington PR and Rouse BT: Involvement of IL-6 in the paracrine production of VEGF in ocular HSV-1 infection. Exp Eye Res 82: 46-54, 2006.
31. De Vos AF, Hoekzema R and Kijlstra A: Cytokines and uveitis, a review. Curr Eye Res 11: 581-597, 1992.
32. Verma MJ, Lloyd A, Rager H, *et al*: Chemokines in acute anterior uveitis. Curr Eye Res 16: 1202-1208, 1997.
33. Nakazawa T, Hisatomi T, Nakazawa C, *et al*: Monocyte chemoattractant protein 1 mediates retinal detachment-induced photoreceptor apoptosis. Proc Natl Acad Sci USA 104: 2425-2430, 2007.
34. Kanagawa T, Matsuda S, Mikawa Y, *et al*: Role of ICAM-1 and LFA-1 in endotoxin-induced uveitis in mice. Jpn J Ophthalmol 40: 174-180, 1996.
35. Tsuji F and Shirasawa E: The role of platelet-activating factor in cell infiltration in endotoxin-induced uveitis in guinea pigs. Curr Eye Res 17: 501-505, 1998.
36. Zhang JZ, Cavet ME, VanderMeid KR, Salvador-Silva M, López FJ and Ward KW: BOL-303242-X, a novel selective glucocorticoid receptor agonist, with full anti-inflammatory properties in human ocular cells. Mol Vis 15: 2606-2616, 2009.
37. Rodrigues CE, Sanches TR, Volpini RA, *et al*: Effects of continuous erythropoietin receptor activator in sepsis-induced acute kidney injury and multi-organ dysfunction. PLoS One 7: e29893, 2012.
38. Straus DS, Pascual G, Li M, *et al*: 15-deoxy-delta^{12,14}-prostaglandin J$_2$ inhibits multiple steps in the NF-kappa B signaling pathway. Proc Natl Acad Sci USA 97: 4844-4849, 2000.
39. Inoue H, Tanabe T and Umeson K: Feedback control of cyclooxygenase-2 expression through PPARgamma. J Biol Chem 275: 28028-28032, 2000.
40. Thieringer R, Fenyk-Melody JE, Le Grand CB, *et al*: Activation of peroxisome proliferator-activated receptor gamma does not inhibit IL-6 or TNF-alpha responses of macrophages to lipopolysaccharide in vitro or in vivo. J Immunol 164: 1046-1054, 2000.