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Phospholipase C-γ as a Potential Therapeutic Target for Graves' Orbitopathy

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Background: Phospholipase C-γ (PLC-γ) plays a crucial role in immune responses and is related to the pathogenesis of various inflammatory disorders. In this study, we investigated the role of PLC-γ and the therapeutic effect of the PLC-specific inhibitor U73122 using orbital fibroblasts from patients with Graves' orbitopathy (GO).

Methods: The expression of phospholipase C gamma 1 (*PLCG1*) and phospholipase C gamma 2 (*PLCG2*) was evaluated using polymerase chain reaction in GO and normal orbital tissues/fibroblasts. The primary cultures of orbital fibroblasts were treated with non-toxic concentrations of U73122 with or without interleukin (IL)-1β to determine its therapeutic efficacy. The proinflammatory cytokine levels and activation of downstream signaling molecules were determined using Western blotting.

Results: *PLCG1* and *PLCG2* mRNA expression was significantly higher in GO orbital tissues than in controls ($P<0.05$). *PLCG1* and *PLCG2* mRNA expression was significantly increased ($P<0.05$) in IL-1β, tumor necrosis factor-α, and a cluster of differentiation 40 ligand-stimulated GO fibroblasts. U73122 significantly inhibited the IL-1β-induced expression of proinflammatory molecules, including IL-6, IL-8, monocyte chemoattractant protein-1, cyclooxygenase-2, and intercellular adhesion molecule-1 (ICAM-1), and phosphorylated protein kinase B (p-Akt) and p38 (p-p38) kinase in GO fibroblasts, whereas it inhibited IL-6, IL-8, and ICAM-1, and p-Akt and c-Jun N-terminal kinase (p-JNK) in normal fibroblasts ($P<0.05$).

Conclusion: PLC-γ-inhibiting U73122 suppressed the production of proinflammatory cytokines and the phosphorylation of Akt and p38 kinase in GO fibroblasts. This study indicates the implications of PLC-γ in GO pathogenesis and its potential as a therapeutic target for GO.

Keywords: Phospholipase C gamma; Graves ophthalmopathy; U73122; Proinflammatory cytokines; Inflammation; Orbital fibroblasts

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INTRODUCTION

Graves' orbitopathy (GO), also known as thyroid eye disease and thyroid-associated ophthalmopathy, is an autoimmune inflammatory disorder of the eye orbit associated with Graves' disease [1]. Although GO pathophysiology is yet to be fully elucidated, the role of thyrotropin receptor (TSHR)- and insulin-like growth factor-1 receptor (IGF-1R)-autoantibody-stimulated orbital fibroblasts in the disease pathogenesis is well-known [2-4]. Current evidence indicates that the inflammatory response is perpetuated and amplified via complex interactions among orbital fibroblasts and infiltrated inflammatory cells [4,5]. During the process, activated orbital fibroblasts can differentiate into adipocytes and produce excessive hyaluronan, a hydrophilic glycosaminoglycan [1,6]. Consequent enlargement of extraocular muscles and an increase in orbital connective tissues can cause various clinical complications, including proptosis, periorbital edema, diplopia, and compressive optic neuropathy [4,6]. To decrease the orbital inflammatory response, which is a major etiology of GO, glucocorticoids have been used as a mainstay treatment [7]; however, concerns regarding systemic side effects and treatment failures indicate the need for developing new effective therapeutic agents [7].

Phospholipase C (PLC) is an enzyme that catalyzes phosphatidylinositol 4,5-bisphosphate hydrolysis into diacylglycerol and inositol triphosphate, which mediates the activation of protein kinase C (PKC) and Ca^{2+} signaling [8]. Mammalian PLC enzymes are categorized into six subtypes (β, γ, δ, ε, ζ, and η) based on their structures [8]. PLC-γ and PLC-β are recognized as primary PLCs that are directly activated by extracellular stimuli, and their strong association with various human diseases has been widely reported [9]. PLC-γ has two isozymes, PLC-γ1 and PLC-γ2, which differ from other PLC subtypes because of their unique γ-specific array (γSA) structure [10]. γSA plays a crucial role in conferring specific functions, such as regulating PLC-γ activity and facilitating interactions with various signaling molecules and receptor tyrosine kinase [10]. PLC-γ1 is widely expressed and regulates cellular growth and differentiation, whereas PLC-γ2 is expressed mainly in immune cells [8]. PLC-γ is a key regulator of mediating immune responses and engages in various signaling pathways, including those associated with T-cell receptors, B-cell receptors, and toll-like receptors (TLRs) [11-13]. Recent studies have revealed that aberrant PLC-γ activation and gene expression induce inflammatory conditions such as autoinflammation and PLC-γ2-associated antibody deficiency and immune dysregulation [14], rheumatoid arthritis [15], viral inflammation [16], and different cancer types [17]. Moreover, animal experiments revealed that gain-of-function mutations in phospholipase C, gamma 2 (*Plcg2*) could induce severe inflammatory reactions and autoimmunity [18,19] and the protective and anti-inflammatory effects of PLC-γ2 deficiency against inflammatory arthritis [20,21].

In the context of its potential role in various inflammatory and immune responses, prospects of PLC-γ as a therapeutic target are currently being explored, and the anti-inflammatory effect of PLC inhibition has been mainly reported in cell experiments [16,22,23]. However, no studies have been conducted to investigate the relationship between PLC-γ and GO. Therefore, in this study, we investigated the role of PLC-γ in the pathogenesis of GO using the PLC-specific inhibitor U73122.

METHODS

Reagents

The reagents used in the study were obtained from the following sources: U73122 (catalog number 1268, Tocris Bioscience, Minneapolis, MN, USA); Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM/F12, 1:1), penicillin, and streptomycin (Welgene, Gyeongsan, Korea); fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA); 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (catalog number 475989, Sigma-Aldrich, St. Louis, MO, USA); recombinant human interleukin (IL)-1β (catalog number 201-LB) and tumor necrosis factor (TNF)-α (catalog number 210-TA, R&D systems, Minneapolis, MN, USA); recombinant human cluster of differentiation 40 ligand (CD40L) (catalog number ALX-850-064-KI01, Enzo Life Sciences, Farmingdale, NY, USA); antibodies for phosphorylated protein kinase B (p-Akt) (catalog number 9271), total Akt (t-Akt) (catalog number 9272), phosphorylated extracellular signal-regulated kinase (p-ERK) (catalog number 9101), t-ERK (catalog number 9102), p-p38 mitogen-activated protein kinase (MAPK) (catalog number 9211), t-p38 (catalog number 9212), phosphorylated c-Jun N-terminal kinase (p-JNK) (catalog number 9251), t-JNK (catalog number 9252), IL-6 (catalog number 12153), IL-8 (catalog number 94407), monocyte chemoattractant protein-1 (MCP-1, also known as C–C motif chemokine ligand 2 [CCL2]) (catalog number 2027), cyclooxygenase-2 (COX-2) (catalog number 4842), and intercellular adhesion molecule-1 (ICAM-1) (catalog number 4915, Cell Signaling Technology, Danvers, MA, USA); and anti-β-actin antibody (catalog number sc-47778, Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Subjects and cell culture

The orbital adipose/connective tissue specimens were harvested from orbital decompression performed on 15 patients with GO having proptosis (12 females and three males; age, 24 to 62 years). During the surgery, all patients were in a stable euthyroid state and did not receive any steroid or radiation therapy for ≤3 months before surgery. The control tissue samples were obtained from 14 healthy control subjects who underwent blepharoplasty, all without thyroid disease and GO (10 females and four males; age, 24 to 69 years). Written informed consent was obtained from all the participants of the study. The protocol of this study, including specimen acquisition, was approved by the Institutional Review Board of Soonchunhyang Hospital, Soonchunhyang University College of Medicine (2020-04-006) and Severance Hospital, Yonsei University College of Medicine (4-2022-0272). The work was done in accordance with the ethical principles for medical research involving human subjects outlined in the Declaration of Helsinki. The detailed clinical characteristics of all the participants are presented in Supplemental Table S1.

Orbital fibroblasts were cultured as described in a previous study [24]. Briefly, the tissue explants were minced and placed directly in DMEM/F12 (1:1 ratio) containing 20% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. After cell growth was confirmed, the cultured monolayers were passaged serially with the trypsin/ethylenediaminetetraacetic acid solution. The cells were cultured in a humidified 5% CO_2 incubator at 37°C. The cell strains used in this study were stored in liquid N_2 until utilized, and cells from the third–seventh passages were used for experiments.

Cell viability assay

To determine the non-toxic concentration of U73122 for treating the cells, orbital fibroblasts from patients with GO were seeded into 24-well culture plates (1×10^5 cells/well) and treated with different concentrations of U73122 (control, 0.1, 0.3, 0.5, 1, and 5 μM) for 24 and 48 hours. Thereafter, the treated cells were washed and incubated with the MTT solution (5 mg/mL) for 3 hours at 37°C, then solubilized in ice-cold isopropanol. The absorbance of the dye was measured using a microplate reader (VersaMax, Molecular Devices, Sunnyvale, CA, USA) at 560 nm with background subtraction at 630 nm, based on which the corresponding cell viability was assessed.

Reverse transcription-quantitative polymerase chain reaction

To assess phospholipase C gamma 1 (*PLCG1*) and phospholipase C gamma 2 (*PLCG2*) mRNA levels in each orbital sample, Precellys 24 tissue homogenizer (catalog number P000669-PR 240-A, Bertin Instruments, Montigny-le-Bretonneux, France) and Precellys lysing kit (catalog number KT03961-007.2, Bertin Instruments) were used for homogenizing the tissue samples. The total RNA was extracted using TRIzol (catalog number 15596026, Invitrogen, Carlsbad, CA, USA), and complementary DNA (cDNA) was synthesized from 1 μg of the isolated RNA according to the manufacturer's protocol (catalog number 74104, Qiagen, Valencia, CA, USA). The cDNA was amplified with SYBR Green polymerase chain reaction (PCR) Master Mix (catalog number RR420A, Takara Bio, Kusatsu, Japan) in a QuantStudio 3 real-time PCR thermocycler (catalog number A28136, Applied Biosystems). The PCR conditions were set as follows: 50°C for 2 minutes and 95°C for 10 minutes; followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute; followed by 95°C for 15 seconds, 60°C for 1 minute, and 95°C for 15 seconds. All PCRs were performed in triplicate, and the primer sequences used are listed in Table 1. The glyceraldehyde-3-phosphate dehydrogenase level was used to normalize the results, and the $2^{-\Delta\Delta Cq}$ method was used to present the results as the relative fold changes in the threshold cycle value [25].

Western blotting assay

For the Western blotting assay, orbital fibroblasts were washed with phosphate-buffered saline to remove any attached reagents and lysed in lysis buffer as described previously [24]. The cell lysates were centrifuged for 15 minutes at 14,000 rpm and the pellets were discarded. The protein concentrations in the supernatant fractions were quantified using the bicinchoninic acid assay, and the extracted proteins were denatured and resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Subsequently, the electrophoresed proteins were transferred onto nitrocellulose membranes (Millipore Corporation, Billeri-

Table 1. Primer Sequences Used in This Study

Gene name	Sequences (5′-3′)	Type
PLCG1	AGT TTG TGG TGG ACA ATG GAC T	Forward
	ATA CAC CAC GAA GCG CAG AA	Reverse
PLCG2	GCG TCT ACC CAA AGG GAC AA	Forward
	GCC GTC TGG AAA TTG AGT GC	Reverse
GAPDH	ATG GGG AAG GTG AAG GTC G	Forward
	GGG GTC ATT GAT GGC AAC AAT A	Reverse

PLCG1, phospholipase C gamma 1; *PLCG2*, phospholipase C gamma 2; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

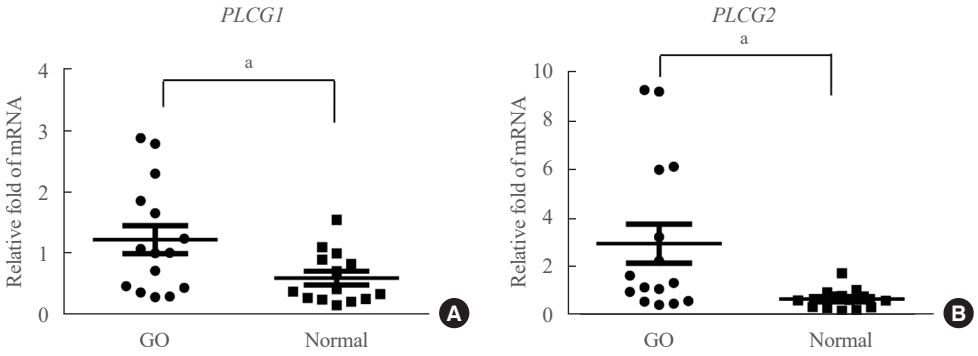

Fig. 1. The mRNA expression of (A) phospholipase C gamma 1 (*PLCG1*) and (B) phospholipase C gamma 2 (*PLCG2*) was higher in Graves' orbitopathy (GO) orbital tissues than in normal tissues. The transcript levels of *PLCG1* and *PLCG2* were evaluated through real-time polymerase chain reaction using orbital tissues obtained from subjects with GO ($n=15$) and healthy controls ($n=14$). Each dot corresponds to data obtained from one subject. Results are presented as mean±standard deviation. [a]$P<0.05$.

ca, MA, USA), treated with primary antibodies (1:1,000 dilution), and incubated overnight at 4°C. The membranes were blocked with 5% skim milk and incubated with horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature; the immunoreactive bands were visualized using chemiluminescence (Thermo Fisher Scientific). The relative protein amount in each band was quantified through densitometry using the ImageJ software version 1.54 (National Institutes of Health, Bethesda, MD, USA) and normalized to that of β-actin in the same sample.

Statistical analysis

At least three strains from three different individuals were used in the experiments and each experiment was performed in triplicates. The Mann-Whitney *U* test and Kruskal-Wallis test were performed to analyze the nonparametric data. *Post hoc* analysis was performed using Dunn's multiple comparison tests for pairwise comparisons between conditions. The Kolmogorov-Smirnov test was used to analyze the data that were not normally distributed. The experimental results in this study are expressed as mean±standard deviation. For statistical analyses, the SPSS version 22 (IBM, Armonk, NY, USA) was used, and a $P<0.05$ indicated statistical significance.

RESULTS

PLCG1 and *PLCG2* mRNA expression in GO and normal orbital tissues

The transcript levels of *PLCG1* and *PLCG2* were quantitatively compared using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) in orbital tissues from patients with GO ($n=15$; age, 39.13±10.88 years) and healthy control ($n=14$; age, 44±13.70 years) subjects. The analysis revealed significantly higher expression of both *PLCG1* and *PLCG2* in GO tissues than in normal orbital tissues (Fig. 1). The mean expression of *PLCG1* in GO orbital tissues was 2.1-fold higher than that in normal tissues, whereas the mean expression of *PLCG2* was 4.2-fold higher than that in normal tissues.

IL-1β, TNF-α, and CD40L upregulated *PLCG1* and *PLCG2* mRNA expression in GO fibroblasts

To investigate the effect of external stimuli on *PLCG1* and *PLCG2* mRNA expression, the transcript levels of *PLCG1* and *PLCG2* in GO fibroblasts were evaluated after treatment with various stimulants, including IL-1β (10 ng/mL), TNF-α (10 ng/mL), and CD40L (1 μg/mL). Quantitative analysis using RT-qPCR was performed at 1, 3, 6, 16, and 24 hours after treatment. The results indicated a significant increase in the expression of *PLCG1* and *PLCG2* following treatment with IL-1β, TNF-α, and CD40L compared with that the untreated controls (Fig. 2). Specifically, *PLCG1* mRNA expression exhibited the highest values after 3 hours of IL-1β treatment, 16 hours of TNF-α treatment, and 24 hours of CD40L treatment, with approximate fold increases of 6.4, 4.5, and 2.7, respectively, compared with the corresponding control groups. Contrastingly, *PLCG2* mRNA expression exhibited the highest increase after 1 hour of IL-1β treatment, 6 hours of TNF-α treatment, and 24 hours of CD40L treatment, with approximate fold increases of 2.9, 16, and 44, respectively, compared with the corresponding control groups. Unlike treatments with IL-1β and TNF-α, CD40L treatment exhibited a gradual increase in the expression of *PLCG1* and *PLCG2* throughout the experimental period. The increase was especially prominent

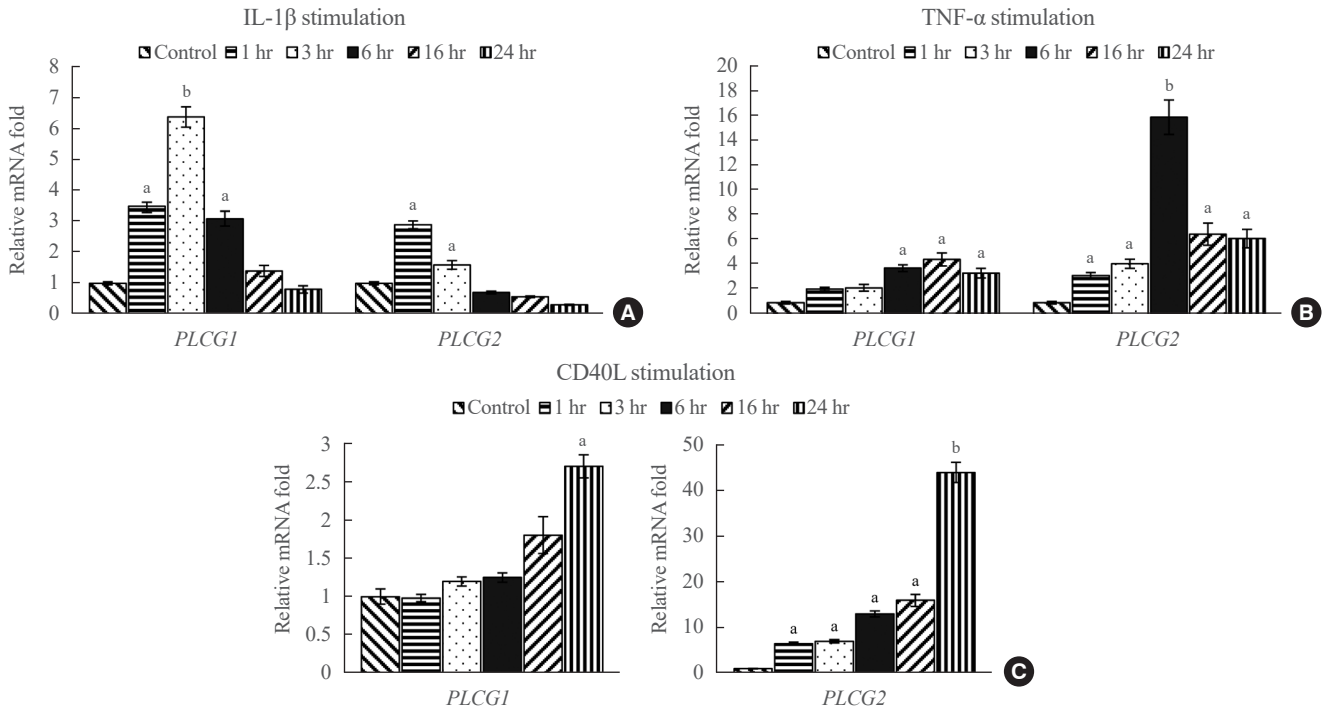

Fig. 2. External stimulation upregulated phospholipase C gamma 1 (*PLCG1*) and phospholipase C gamma 2 (*PLCG2*) mRNA expression in orbital fibroblasts obtained from patients with Graves' orbitopathy ($n=3$). *PLCG1* and *PLCG2* transcript levels were measured through real-time polymerase chain reaction for increasing length of time (0 to 24 hours). (A) Orbital fibroblasts were treated with interleukin (IL)-1β (10 ng/mL). *PLCG1* and *PLCG2* mRNA expression peaked at 3 and 1 hour of treatment, respectively. (B) Orbital fibroblasts were treated with tumor necrosis factor (TNF)-α (10 ng/mL). *PLCG1* and *PLCG2* mRNA expression peaked at 16 and 6 hours of treatment, respectively. (C) After stimulation of cluster of differentiation 40 ligand (CD40L) (1 μg/mL), *PLCG1* and *PLCG2* mRNA expression gradually increased and was highest at 24 hours of treatment. Results are presented as mean±standard deviation. $^{a}P<0.05$; $^{b}P<0.01$, compared with the control group.

in *PLCG2* mRNA expression, exhibiting an increase of more than 40-fold compared with the corresponding control at 24 hours of treatment ($P<0.01$).

Effect of U73122 on cell viability

We determined the non-toxic concentrations of U73122 on orbital fibroblasts using the MTT assay. Orbital fibroblasts from GO were treated with U73122 (0.1 to 5 μM) to determine its effect on viability for 24 and 48 hours. Cell viability was expressed as a percentage relative to the respective control. Results obtained from treatment with 1 μM or lower concentrations of U73122 indicated that cell viability did not significantly decrease regardless of the time (Supplemental Fig. S1). Based on these results, the maximal non-toxic concentration of U73122 in orbital fibroblasts was considered at 1 μM for 48 hours, and this concentration was used for subsequent experiments.

U73122 inhibits *PLCG1* and *PLCG2* expression

The effect of U73122 on *PLCG1* and *PLCG2* mRNA expression was assayed through RT-qPCR. GO fibroblasts were treated with 1 μM of U73122 for 3, 24, and 48 hours. The results indicated that U73122 significantly decreased the transcript levels of *PLCG1* and *PLCG2* in GO fibroblasts regardless of time (Supplemental Fig. S2). The average transcriptional inhibitory effect of U73122 on the *PLCG1* gene was highest at 48 hours after treatment, whereas it was highest at 24 hours after treatment for *PLCG2*.

U73122 inhibits IL-1β-induced expression of proinflammatory molecules

Western blot analysis was performed to investigate the effect of U73122 on the protein expression of IL-1β-induced proinflammatory molecules (IL-6, IL-8, MCP-1, COX-2, and ICAM-1) in orbital fibroblasts. GO and normal orbital fibroblasts were treated with U73122 (1 μM) with or without IL-1β stimulation (10 ng/mL) for 48 hours (Fig. 3). The results indicated an increase in proinflammatory molecules in both GO and normal fibroblasts after 48 hours of IL-1β treatment. Notably, IL-6 expres-

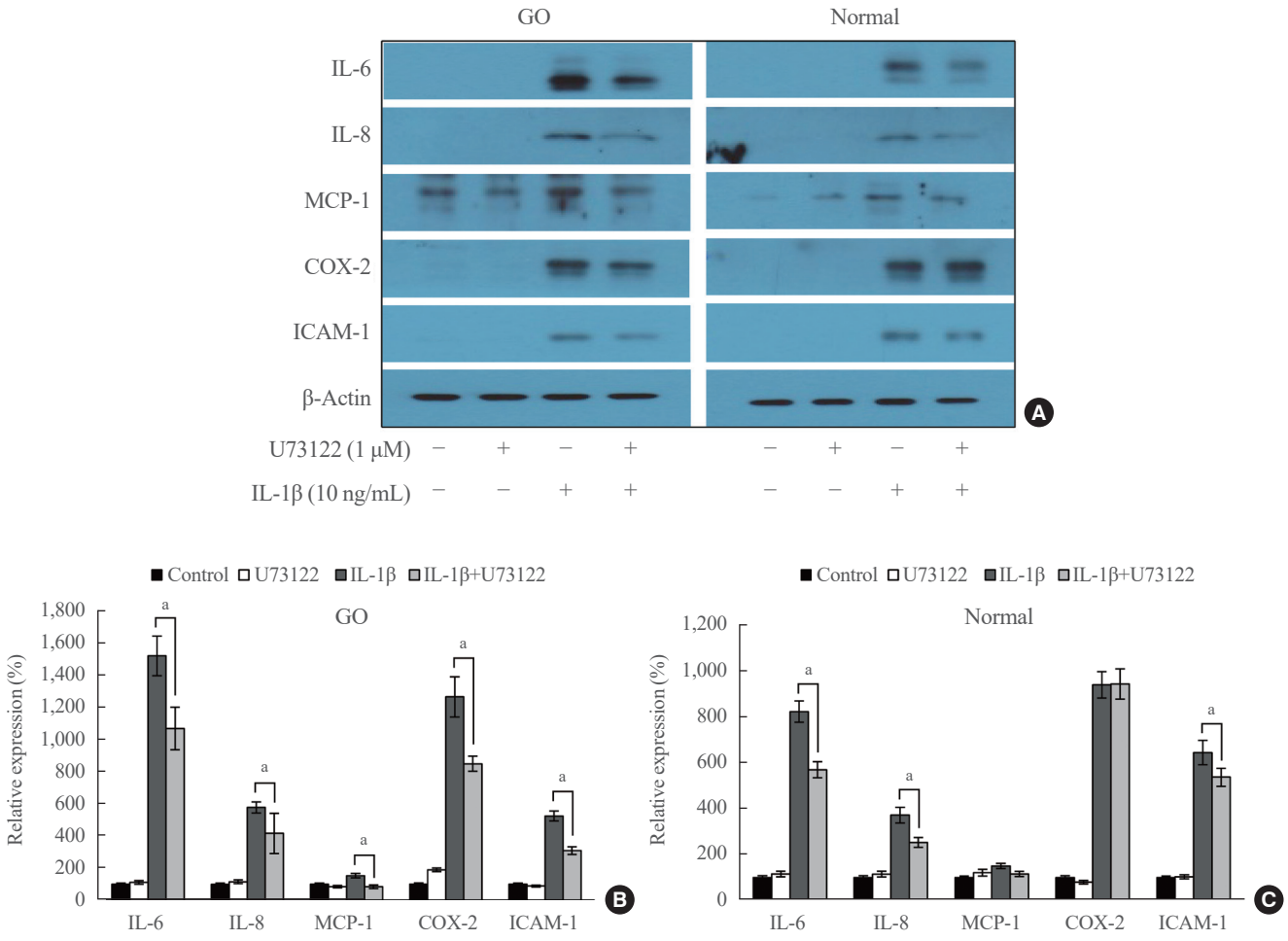

Fig. 3. U73122 inhibits interleukin (IL)-1β-induced expression of proinflammatory molecules in orbital fibroblasts. Fibroblasts obtained from patients with Graves' orbitopathy (GO) (n=3) and healthy controls (n=3) were treated with 1 μM U73122 with or without IL-1β (10 ng/mL) for 48 hours. (A) Western blot analyses were performed to analyze the expression of proinflammatory molecules IL-6, IL-8, monocyte chemoattractant protein-1 (MCP-1), cyclooxygenase-2 (COX-2), and intercellular adhesion molecule-1 (ICAM-1). (B) U73122 treatment significantly decreased the expression of all the IL-1β-induced proinflammatory molecules in GO fibroblasts. (C) In normal orbital fibroblasts, IL-6, IL-8, and ICAM-1 expression induced by IL-1β were significantly decreased. Results are presented as the mean relative expression ratio±standard deviation and are normalized to the level of β-actin in the same sample. [a]P<0.05.

sion in GO cells exhibited the highest increase, with a 15.2-fold increase by IL-1β induction, followed by a 12.6-fold increase in COX-2 expression. The expression of all IL-1β-induced molecules, including IL-6 and COX-2, significantly decreased in GO fibroblasts treated with U73122. In normal cells, IL-1β induced the highest increase in COX-2 expression by 9.4-fold, followed by an 8.3-fold increase in IL-6. However, the IL-1β-induced expression of MCP-1 and COX-2 was not significantly attenuated by U73122 in normal orbital fibroblasts, whereas the IL-1β-induced expression of IL-6, IL-8, and ICAM-1 was significantly diminished.

Effect of U73122 on the phosphorylation of signaling molecules

To investigate the signaling pathways affected by U73122, Western blotting analysis was performed on many downstream signaling kinases in GO and normal orbital fibroblasts. GO and normal orbital fibroblasts were treated with U73122 (1 μM) with or without IL-1β (10 ng/mL) for 1 hour (Fig. 4). Following treatment with IL-1β, the phosphorylated forms of signaling molecules involved in the inflammatory response, including Akt and MAPKs (ERK, p38, and JNK), were increased in both GO and normal orbital fibroblasts. When U73122 was treated for 1 hour, the expression of p-Akt induced by IL-1β significantly decreased

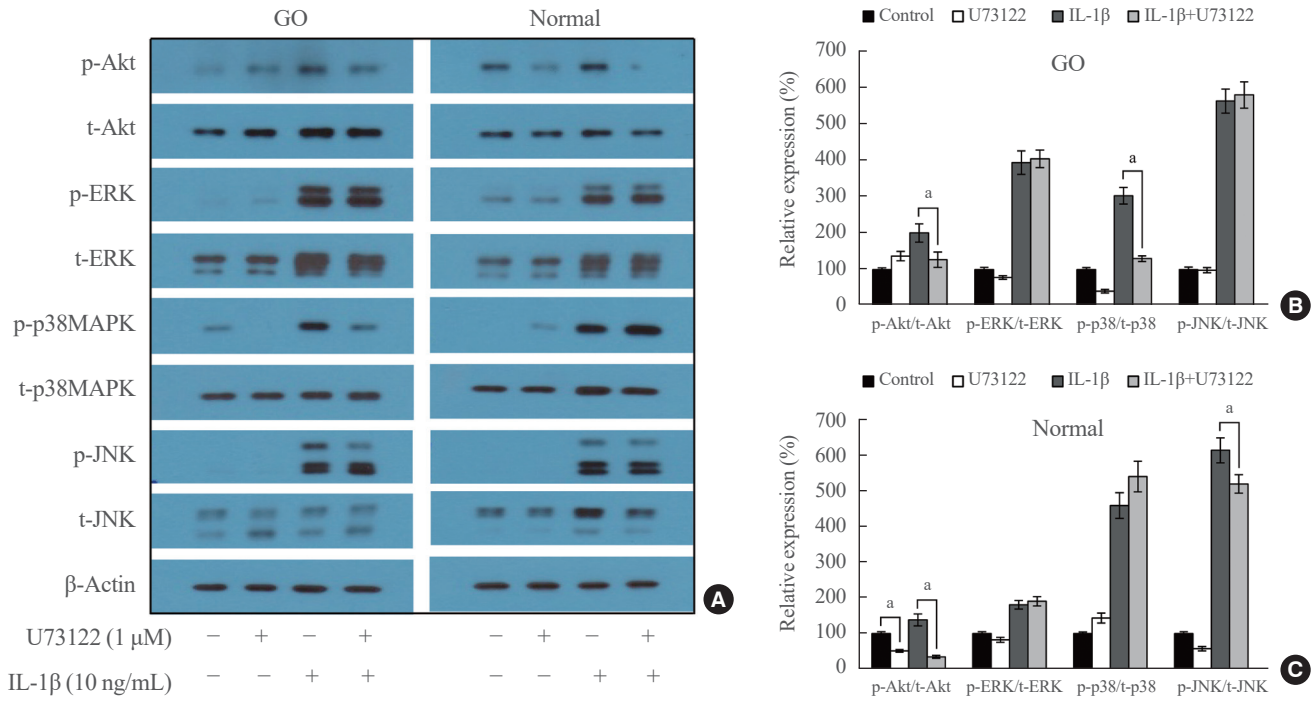

Fig. 4. U73122 inhibits interleukin (IL)-1β-induced expression of downstream signaling molecules in orbital fibroblasts. Fibroblasts obtained from patients with Graves' orbitopathy (GO) (n=3) and healthy controls (n=3) were treated with 1 μM U73122 with or without IL-1β (10 ng/mL) for 1 hour. (A) Western blot analyses were performed to investigate phosphorylated protein kinase B (p-Akt), phosphorylated extracellular signal-regulated kinase (p-ERK), p-p38, and phosphorylated c-Jun N-terminal kinase (p-JNK) protein expression. (B) U73122 treatment significantly decreased IL-1β-induced expression of p-Akt and p-p38 in GO fibroblasts, whereas (C) IL-1β-induced expression of p-Akt and p-JNK was significantly decreased in normal orbital fibroblasts. Results are presented as the mean relative expression ratio of each protein (phosphorylated form/total form)±standard deviation, normalized to the level of β-actin in the same sample. MAPK, mitogen-activated protein kinase. [a]P<0.05.

in GO and normal cells. Moreover, U73122 significantly suppressed IL-1β-induced p-p38 expression in GO fibroblasts and significantly inhibited IL-1β-induced p-JNK expression in normal fibroblasts. However, no significant inhibitory effect of U73122 was observed on other signaling molecules.

DISCUSSION

Based on the pathogenesis of GO, we investigated the roles of PLC-γ in orbital tissues and the molecular pathways and interactions involved using primary cultures of orbital fibroblasts. *PLCG1* and *PLCG2* were overexpressed in GO tissues compared with normal orbital tissues. The production of proinflammatory cytokines, which is closely associated with the development of GO, was upregulated after IL-1β treatment in GO fibroblasts but was significantly suppressed after treatment with U73122, a well-known PLC inhibitor. To the best of our knowledge, this is the first study to show that PLC-γ mediates the inflammatory reactions in GO fibroblasts.

PLC-γ has been implicated in various human diseases and modulates the immune system by activating the downstream of various cell surface receptors [10,17,26]. The T-cell receptor complex recruits IL-2-inducible T-cell kinase (ITK) to activate PLC-γ1 [27]; similarly, the B-cell receptor recruits kinases, such as spleen tyrosine kinase (Syk) and Bruton's tyrosine kinase (BTK), which activate PLC-γ2 [26]. The activation of PLC-γ ultimately leads to downstream signaling that activates transcription factors, including the nuclear factor of activated T-cells (NFAT), activator protein-1 (AP-1), and nuclear factor κB (NF-κB) [28,29]. NF-κB, a ubiquitous transcription factor, plays a fundamental role in mediating inflammatory and immune responses, including CD40-mediated interactions between orbital fibroblasts and T-cells [30,31]. CD40 is a transmembrane protein that is overexpressed in GO fibroblasts, and the CD40–CD40L interaction plays a crucial role in perpetuating orbital inflammation [32]. In this study, we found that *PLCG1* and

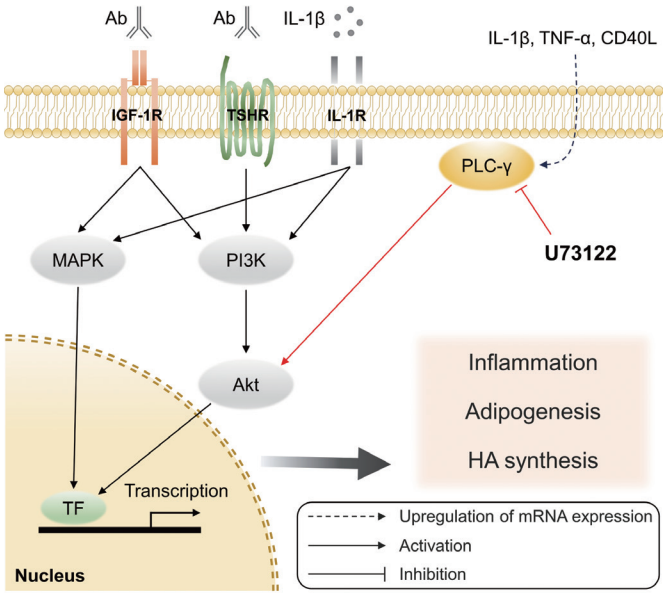

Fig. 5. Schematic diagram of the relationships among U73122, phospholipase C (PLC)-γ, and downstream signaling pathways of insulin-like growth factor-1 receptor (IGF-1R), thyrotropin receptor (TSHR), and interleukin-1 receptor (IL-1R) in orbital fibroblasts from patients with Graves' orbitopathy. Ab, autoantibody; TNF-α, tumor necrosis factor-α; CD40L, cluster of differentiation 40 ligand; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; Akt, protein kinase B; TF, transcription factors; HA, hyaluronan.

PLCG2 mRNA expression was higher in GO tissues than in control, and their expression was significantly increased by stimulation with IL-1β, TNF-α, and CD40L in GO fibroblasts. Notably, *PLCG2* mRNA expression increased more than 40-fold with 1 μg/mL of CD40L, suggesting that PLC-γ may play a crucial role in orbital inflammation.

The phosphatidylinositol 3-kinase (PI3K) signaling, which is essential for the development, activation, and differentiation of T- and B-cells [33], plays a crucial role in the pathogenesis of GO [34,35]. The kinase Akt is a key PI3K effector, and the PI3K-Akt signaling pathway activated in GO fibroblasts by stimulation of TSHR and IGF-1R is associated with adipogenesis and hyaluronan production [34-36]. Our previous study showed that the production of proinflammatory cytokines and adipogenesis induced by treating IL-1β in GO fibroblasts was inhibited by the specific PI3Kδ inhibitor, idelalisib [37]. Furthermore, studies have suggested that PLC-γ downstream signaling pathway interacts with the PI3K-Akt pathway in several cell types [38,39]. Using U73122 in Balb 3T3 fibroblasts, Amin et al. [40] showed that the PLC-PKC signaling cascade involving PLC-γ was required for IL-1β-induced Akt activation. Similarly, we showed that the phosphorylation of Akt and MAPK was increased in both GO and control fibroblasts after IL-1β treatment, and inhibiting PLC-γ via U73122 treatment significantly suppressed the expression of p-Akt and p-p38 in GO fibroblasts. This suggests that PLC-γ may play an important role in activating the PI3K-Akt pathway, which is triggered by surface receptors on orbital fibroblasts, including the TSHR and IGF-1R (Fig. 5).

Previous studies have suggested that PLC-γ plays an important role in the innate immune system. Triggering receptor expressed on myeloid cells 1 (TREM1) is a receptor expressed on neutrophils and monocytes/macrophages, and its downstream signaling increases inflammation [41]; PLC-γ-induced calcium mobilization and activation of transcription factors are involved in this pathway [41]. Further, Aki et al. [13] showed that PLC-γ2 is required to induce normal production of TNF-α and IL-6 when stimulated with peptidoglycan (a ligand for TLR2) and lipopolysaccharide (LPS) (a ligand for TLR4) using bone marrow-derived macrophages. In the context of GO, the levels of macrophage infiltration in the orbital adipose tissue were significantly higher in patients with GO than in normal controls, which exhibited a significant positive correlation with the *CCL2* mRNA expression [42]. Moreover, our previous study revealed that *TLR2* and *TLR4* expression was significantly higher in GO tissues than in controls and TLR2 blockade with antibodies significantly decreased the release of proinflammatory cytokines [43]. These findings indicate that PLC-γ activation may increase the inflammatory response in GO via the innate and adaptive immune system.

The aminosteroid U73122 is the most widely used PLC inhibitor, and several previous studies have used U73122 as a PLC-γ inhibitor [16,39,40,44]. In this study, we showed that U73122 significantly inhibited the mRNA expression of *PLCG1* and *PLCG2* in GO fibroblasts. Although the underlying precise mechanism is yet to be elucidated, these findings are consistent with a previous study that indicated that U73122 affects both PLC activity and gene expression [45]. The blockade of PLC and Ca^{2+} accumulation by U73122 inhibits LPS-induced mRNA expression of proinflammatory cytokines and NF-κB nuclear translocation in human gingival fibroblasts [23]. An *in vivo* study showed that LPS-induced cardiac *Tnf* expression and myocardial dysfunction were significantly suppressed in heterozygous phospholipase C, gamma 1 (*Plcg1*) knockout mice and U73122-treated wild-type mice [46]. These results indicate that the treatment with U73122 leads to the blockade of inflammation both *in vitro* and *in vivo*. Our study also showed that U73122 treatment on GO fibroblasts significantly suppressed the IL-1β-induced ex-

pression of proinflammatory molecules, suggesting that the inhibition of PLC-γ can potentially exhibit a therapeutic effect by modulating the inflammatory response in GO.

However, there are some limitations in the study. The absence of reported therapeutic applications or clinical trials involving U73122 in human clinical studies leads to insufficient pharmacological information. Moreover, U73122 may not be highly specific for PLC because it can inhibit Ca^{2+} pumps [47], potassium channels [48], and enzymes, including 5-lipoxygenase [49], in various cells. Based on the evidence that U73122 is less selective for PLC inhibition, the results using U73122 to analyze the roles and the molecular mechanisms of PLC-γ should be carefully interpreted. Inhibitors that can reduce off-target effects and directly modulate PLC-γ enzymes should be developed. Additionally, in this study using orbital fibroblasts, the interactions with T-cells, B-cells, and innate immune cells related to GO pathogenesis were not sufficiently considered. While orbital fibroblasts may not completely encompass the inflammatory response seen in patients with GO, we did simulate the environment using IL-1β, a proinflammatory cytokine that is highly associated with GO pathogenesis [24,50].

To conclude, in this study, we showed that U73122, which is used to inhibit PLC-γ, exhibits anti-inflammatory effects on orbital fibroblasts in patients with GO. We postulate that the inhibition of PLC-γ can be a potential candidate for GO therapy. Further *in vivo* studies using animal models and clinical trials are required to elucidate the specific mechanism of targeting PLC-γ in the pathogenic mechanism of GO.

CONFLICTS OF INTEREST

Don O. Kikkawa is a former consultant of Horizon Therapeutics. Remaining authors have no potential conflict of interest to declare.

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AUTHOR CONTRIBUTIONS

Conception or design: S.Y.J., J.S.Y. Acquisition, analysis, or interpretation of data: T.H.R., M.K.C., J.S.K., J.S.Y. Drafting the work or revising: T.H.R., D.O.K. Final approval of the manuscript: T.H.R., M.K.C., J.S.K., D.O.K., S.Y.J., J.S.Y.

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