Experimental Eye Research 113 (2013) 1–8

Contents lists available at SciVerse ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

Pirfenidone attenuates IL-1β-induced COX-2 and PGE_2 production in orbital fibroblasts through suppression of NF-κB activity☆

Youn-Hee Choi [a], Keum Ok Back [b], Hee Ja Kim [a], Sang Yeul Lee [c], Koung Hoon Kook [b,*]

[a] Department of Physiology, Tissue Injury Defense Research Center, Ewha Womans University School of Medicine, Seoul, Republic of Korea
[b] Department of Ophthalmology, Ajou University School of Medicine, World cup-ro 164, Woncheon-dong, Yeongtong-gu, Suwon 443-721, Republic of Korea
[c] Department of Ophthalmology, Yonsei University College of Medicine, Seoul, Republic of Korea

ARTICLE INFO

Article history:
Received 21 December 2012
Accepted in revised form 1 May 2013
Available online 9 May 2013

Keywords:
thyroid-associated ophthalmopathy
orbital fibroblast
pirfenidone
COX-2
PGE_2
IL-1β
NF-κB

ABSTRACT

The aim of this study was to determine the effect of pirfenidone on interleukin (IL)-1β-induced cyclooxygenase (COX)-2 and prostaglandin (PG)E_2 expression in orbital fibroblasts from patients with thyroid-associated ophthalmopathy (TAO). Primary cultures of orbital fibroblasts from patients with TAO ($n = 4$) and non-TAO subjects ($n = 4$) were prepared. The level of PGE_2 in orbital fibroblasts treated with IL-1β in the presence or absence of pirfenidone was measured using an enzyme-linked immunosorbent assay. The effect of pirfenidone on IL-1β-induced COX-2 expression in orbital fibroblasts from patients with TAO was evaluated by reverse transcription-polymerase chain reaction (PCR) and quantitative real-time PCR analyses, and verified by Western blot. Activation of nuclear factor-κB (NF-κB) was evaluated by immunoblotting for inhibitor of κB (IκB)α and phosphorylated IκBα, and DNA-binding activity of p50/p65 NF-κB was analyzed by electrophoretic mobility shift assay. In addition, IL-1 receptor type 1 (IL-1R1) expression was assessed by RT-PCR in IL-1β-treated cells with or without pirfenidone. Pirfenidone significantly attenuated IL-1β-induced PGE_2 release in both TAO and non-TAO cells. IL-1β-induced COX-2 mRNA and protein expression decreased significantly following co-treatment with pirfenidone. IL-1β-induced IκBα phosphorylation and degradation decreased in the presence of pirfenidone and led to decreased nuclear translocation and DNA binding of the active NF-κB complex. In our system, neither IL-1β nor pirfenidone co-treatment influenced IL-1R1 expression. Our results suggest that pirfenidone attenuates the IL-1β-induced PGE_2/COX-2 production in TAO orbital fibroblasts, which is related with suppression of the NF-κB activation.

1. Introduction

Thyroid-associated ophthalmopathy (TAO) occasionally accompanies thyroid disease such as Graves' disease. Almost half of patients with Graves' disease report symptoms of TAO including a dry ocular sensation, photophobia, excessive tearing, double vision, and a pressure sensation behind the eyes (Bahn, 2010). Upper lid retraction is one of the common clinical features, and 3–5% of patients suffer from severe ophthalmopathy, including intense pain, inflammation, and sight-threatening corneal ulceration or compressive optic neuropathy (Garrity and Bahn, 2006).

Although the pathogenesis of TAO is not completely understood, it is widely accepted that this disease is induced by an autoimmune response resulting in active inflammation of orbital fat and extraocular muscles and not merely by the metabolic perturbations associated with thyroid hormone overproduction (Kazim et al., 2002; Prummel et al., 1990). Cyclooxygenase (COX)-2 is an enzyme that catalyzes the production of prostaglandin (PG)E_2 which modulates the inflammatory process (O'Banion, 1999). Orbital fibroblasts from patients with TAO produce high levels of PGE_2 associated with coordinate induction of COX-2 when treated with interleukin (IL)-1β (Han et al., 2002), and the induction of COX-2 is currently believed to be critical to the inflammatory response in patients with TAO. In various cells, IL-1β acts as a proinflammatory cytokine inducing the expression of canonical IL-1β target genes such as *IL-6*, *IL-8* and *COX-2*. In this process, the ligand binding of IL-1β to the cell surface IL-1 receptor (IL-1R) leads

Abbreviations: COX, cyclooxygenase; IL, interleukin; NF, nuclear factor; PG, prostaglandin; TAO, thyroid-associated ophthalmopathy.

☆ This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2011-0013586).

* Corresponding author. Tel.: +82 31 219 5260; fax: +82 31 219 5259.
E-mail addresses: drkook@ajou.ac.kr, drkook71@hotmail.com (K.H. Kook).

0014-4835/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.exer.2013.05.001

to a complex sequence of combinatorial phosphorylation and ubiquitination events resulting in the activation of intracellular signaling pathways such as nuclear factor kappa B (NF-κB) and p38 mitogen-activated protein kinase (MAPK) pathways (Weber et al., 2010). Recently, it is reported that IL-1β-induced COX-2 gene expression in orbital fibroblasts from TAO patients is mediated by an NF-κB dependent pathway (Yoon et al., 2011). Therefore, the effective inhibition of this pathogenic process could be one of the therapeutic targets in TAO.

Pirfenidone (5-methyl-1-phenyl-2-[1H]-pyridone) is a small molecule that exhibits novel anti-fibrotic effects not only in several experimental disease models of pulmonary fibrosis (Card, 2003; Kakugawa, 2004; Oku, 2008) but also in clinical trials of patients with idiopathic pulmonary fibrosis (Azuma et al., 2011; Taniguchi et al., 2011). Pirfenidone inhibits proliferation, migration, and collagen contraction in human Tenon's fibroblasts (Lin et al., 2009), and improves trabeculectomy bleb survival when rabbits are treated postoperatively with 0.5% eye drops (Zhong et al., 2011). We reported previously that pirfenidone attenuates IL-1β-induced tissue inhibitor of metalloproteinase (TIMP)-1 level without significant toxicity at the concentrations used, indicating the anti-fibrotic effect of this agent in TAO (Kim et al., 2010).

In this study, we demonstrate that pirfenidone effectively decreased the levels of PGE_2 and COX-2 induced by IL-1β in orbital fibroblasts from patients with TAO and that this inhibitory effect of pirfenidone was, at least in part, mediated by the suppression of NF-κB activity. This is the first study to evaluate the anti-inflammatory effects of pirfenidone in orbital fibroblasts in the context of TAO.

2. Material and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, and trypsin/ethylenediaminetetraacetic acid (EDTA) were purchased from HyClone (North Logan, UT). Bovine serum albumin (BSA) and pirfenidone were obtained from Sigma–Aldrich (St. Louis, MO). Recombinant human IL-1β was purchased from Peprotech (Rocky Hill, NJ). Trizol® was obtained from Invitrogen (Carlsbad, CA). The nuclear extraction kit was obtained from Cayman Chemical Co. (Ann Arbor, MI). Anti-human COX-2 antibody was purchased from Abcam (Cambridge, UK). Antibodies to NF-κB (p65, p50), inhibitor of κB (IκB)α, phosphorylated (p)-IκBα, and actin were purchased from Cell Signaling Technology (Beverly, MA). The PGE_2 enzyme-linked immunosorbent assay (ELISA) kit was obtained from R&D Systems (Minneapolis, MN).

2.2. Cell culture

Human orbital fibroblasts were cultured from orbital fatty connective tissue obtained as surgical waste during decompression surgery for patients with TAO or during orbital surgery for non-TAO patients to resolve non-inflammatory conditions as described previously (Kim et al., 2010). These activities were undertaken after informed consent was obtained from the donors according to protocols approved by the Institutional Review Board of Ajou University Hospital and following the tenets of the Declaration of Helsinki. Four different orbital fibroblast culture strains were obtained from each of four patients with TAO and four non-TAO subjects. All patients with TAO had experienced at least 6 months of inactive disease status with a euthyroid condition prior to the decompression surgery. Patient characteristics are listed in Table 1. Cell cultures were maintained in a humidified 5% CO_2 incubator at 37 °C in DMEM containing 10% FBS and antibiotics. Once a fibroblast monolayer was obtained, cultures were serially passaged after gentle treatment with trypsin/EDTA. Liquid nitrogen was used for long-term storage of some cultures. The medium was changed every 3 days, and cells at passages 3–8 were used for experiments.

Table 1
Characteristics of patients with thyroid-associated ophthalmopathy (TAO) and subjects without inflammatory disease from whom the fibroblast strains were obtained.

	Patients with TAO ($n = 4$)	Non-TAO subjects ($n = 4$)
Mean age, *y* (range)	46 (39–57)	51 (40–65)
Sex (m/f)	2/2	2/2
Smoking (yes)	1	1
Graves' disease	4	0
Radioactive iodine therapy	0	–
Surgery	1	–
Methimazole	3	–
Treatment TAO	4	0/4
Surgery	4	–
Prednisone	1	–
Radiation	0	–
Euthyroid	4	4
TSH-receptor antibodies	4	0
Clinical activity score (range)	1 (1–2)	–

TSH, thyroid-stimulating hormone.

2.3. ELISA

Orbital fibroblasts were seeded in six-well cell culture plates and co-treated with 10 ng/ml IL-1β and 10 mM pirfenidone in DMEM containing 1% FBS for 48 h. Supernatants from the cell cultures were collected, and PGE_2 concentrations were determined using a competitive binding PGE_2 ELISA kit according to the manufacturer's instructions. Briefly, after addition of 150 μl of standards and samples to the corresponding wells, 200 and 150 μl of diluent was added to the blank and zero PGE_2 control wells, respectively. A 50 μl aliquot of the primary antibody solution was added to all wells except the blank, and the plate was covered and incubated for 1 h at room temperature on a horizontal orbital microplate shaker set at 500 ± 50 rpm. A 50-μl aliquot of PGE_2 conjugate solution was added to each well and incubated for 2 h at room temperature on the shaker. After washing, 200 μl of substrate solution was added to each well, and the plate was incubated in the dark at room temperature for 30 min. Finally, absorbance was spectrophotometrically measured at 450 nm using a microplate reader (Molecular Devices, Sunnyvale, CA). The PGE_2 concentration in each sample was determined by reference to a standard curve generated using known amounts of PGE_2.

2.4. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Orbital fibroblasts (5×10^5) were seeded in six-well cell-culture plates and treated with 10 ng/ml IL-1β in the presence or absence of pirfenidone. Total RNA was extracted using TRIzol reagent at each designated time point, and cDNA was generated from RNA (1 μg) using the SuperScript® First-Strand Synthesis System (Invitrogen). PCR was performed using 1 μl cDNA, 0.25 mM dNTP, 1 U *Taq* DNA polymerase, and 10 pmole of the primer pair in a thermal cycler. PCR cycles were composed of one cycle of 95 °C for 5 min, 30 cycles of 45 s at 94 °C, 45 s at 55 °C, and 60 s at 72 °C. The reaction was terminated at 72 °C for 5 min and quenched at 4 °C. Band densities were quantified using the Image J software (National Institutes of Health, Bethesda, MD). The measured intensities were corrected to the β-actin level in each lane and subjected to statistical analyses. Primer sequences are listed in Table 2.