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Peroxisome proliferator-activated receptor-γ agonist pioglitazone suppresses experimental autoimmune uveitis

Yoko Okunuki*, Yoshihiko Usui, Hayate Nakagawa, Kazuki Tajima, Ryusaku Matsuda, Shunichiro Ueda, Takaaki Hattori, Takeshi Kezuka, Hiroshi Goto

Department of Ophthalmology, Tokyo Medical University, 6-7-1 Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan

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ABSTRACT

Peroxisome proliferator-activated receptor (PPAR)-γ agonists are clinically used as anti-diabetes agents. Recent research has discovered that an anti-inflammatory effect of PPAR agonist may have the potential to treat autoimmune disease. In the present study, we investigated the anti-inflammatory effects of PPAR-γ agonist, pioglitazone, on murine model of endogenous uveitis. Experimental autoimmune uveoretinitis (EAU) was induced by immunizing C57BL/6 mice with human interphotoreceptor retinoid binding protein-derived peptide (1–20). Pioglitazone or vehicle was injected intravenously from day −1 (whole phase treatment) or day 8 (effector phase study) until day 20. Severity of EAU was assessed clinically and pathologically on day 21. Immunological status was assessed by measuring intraocular inflammatory factors, and activation and regulatory markers of $CD4^+$ T cells in draining lymph nodes (LNs). Treatment with pioglitazone suppressed both whole-phase and effector-phase of EAU. In effector-phase treatment, intraocular concentrations of TNF-α and IL-6 were significantly suppressed, and $CD4^+Foxp3^+$ regulatory T cells and $CD4^+CD62L^{high}$ naïve T cells increased in draining LNs, although there were no differences in $CD4^+CD44^{high}$ effector T cells and IL-17 producing $CD4^+$ T cells between pioglitazone- and vehicle-treated mice. Administration of pioglitazone before and after the onset of EAU significantly reduced disease severity. The present results suggest that pioglitazone may be a novel therapeutic agent for endogenous uveitis.

1. Introduction

Endogenous uveitis, represented by Behcet's disease, sarcoidosis, and Vogt-Koyanagi-Harada disease, is a sight-threatening ocular inflammatory disease. Although progress has been made in the treatment of this important disease, decisively effective therapies have not yet been established. While systemic and local corticosteroids or immunomodulatory agents are the major agents used, recently infliximab, an anti-tumor necrosis factor (TNF)-α antibody, has been shown to be effective for severe Behcet's disease with uveitis. However, serious side effects are not negligible (Evereklioglu, 2005; Okada, 2005; Accorinti et al., 2007). Patients who cannot take medications because of adverse effects or patients who are not responsive to the existing medications experience unavoidable vision loss. Thus, it is necessary to find new medications that are effective for endogenous uveitis but have less serious adverse effects than existing medications. With this aim, we previously focused on angiotensin II type 1 receptor blockers (ARBs), which are clinically used as anti-hypertensive agents, but their anti-inflammatory effects have also been investigated in various diseases. We reported the suppressive effect of telmisartan, an ARB, on experimental autoimmune uveitis (EAU) which is an animal model of endogenous uveitis (Okunuki et al., 2009). In addition to ARB activity, telmisartan is also a partial agonist of peroxisome proliferator-activated receptor (PPAR)-γ. This receptor is the target of the insulin sensitizing thiazolidinediones (TZDs), a class of drugs used in the treatment of type 2 diabetes mellitus. The PPARs belong to the nuclear hormone receptor superfamily and have different subtypes; α, β/δ and γ, coded by three separate genes that have been identified in rodents and humans (Lemberger et al., 1996). Several PPAR-γ agonists such as pioglitazone and rosiglitazone are clinically used in the treatment of diabetes mellitus.

Besides their original indication as anti-diabetic agents, multiple functions of PPAR-γ agonists, such as anti-inflammatory, anti-tumor, and anti-angiogenic effects, as well as neuroprotection have been investigated recently. As an example of anti-inflammatory function, PPAR-γ has been shown to be important for the differentiation of monocytes into macrophages and to act as a negative regulator of

* Corresponding author. Tel.: +81 3 3342 6111x5880; fax: +81 3 3346 9170.
E-mail address: yoko-okunuki@nifty.com (Y. Okunuki).

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macrophage activation (Ricote et al., 1998a, 1998b; Daynes and Jones, 2002). Also, activated T cells express PPAR-γ and treatment with PPAR-γ agonists inhibits antigen-specific T cell proliferation and promotes apoptosis of activated T cell, suggesting a role of PPAR-γ in the regulation of T cell-mediated immune and inflammatory responses (Clark et al., 2000; Harris and Phipps, 2001). Interestingly, recent studies also have shown the importance of PPAR-γ in the regulation of immune and inflammatory responses in animal models. For example, PPAR-γ agonists prevent rheumatoid arthritis (Kawahito et al., 2000), atherosclerosis (Neve et al., 2000; Chen et al., 2001), colitis (Su et al., 1999), and psoriasis (Pershadsingh et al., 1998) in animal models, suggesting their potential use for the treatment of human inflammatory diseases.

Accumulating data indicate the promising effect of the anti-inflammatory effect of PPAR-γ agonists in autoimmune disease. However, whether PPAR-γ agonists are effective in treating endogenous uveitis is unknown. In this study, we investigated the effect of pioglitazone, a PPAR-γ agonist, on EAU.

2. Materials and methods

2.1. Mice, reagents and monoclonal antibodies

Seven- to 9-week-old female C57BL/6 mice were obtained from Japan CLEA (Shizuoka, Japan). The mice were treated according to the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. High pressure liquid chromatography-purified human interphotoreceptor retinoid binding protein peptide 1–20 (hIRBP-p) was purchased from Operon (Tokyo, Japan). Complete Freund's Adjuvant (CFA) and *Mycobacterium tuberculosis* H37Ra were purchased from Difco (Detroit, MI). Purified *Bordetella pertussis* toxin (PTX), Phorbol 12-myristate 13-acetate (PMA) and ionomycin were purchased from Sigma–Aldrich (St. Louis, MO). Pioglitazone was a gift from Takeda (Osaka, Japan). Fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4 mAb, phycoerythrin (PE)-conjugated anti-CD44 mAb, PE-conjugated anti-Foxp3 mAb, PE-conjugated anti-mouse CD62L mAb, and PE-conjugated anti-mouse IL-17 mAb were purchased from eBiosicience (San Diego, CA). Purified anti-mouse CD16/32 (2.4G2; FcBlock) was purchased from BD Pharmingen.

2.2. Induction and scoring of EAU and pioglitazone treatment

Human IRBP-p (200 μg) was emulsified in CFA (1:1 w/v) containing 5 mg/ml *M. tuberculosis* H37Ra. On day 0, 200 μl of the emulsion was injected subcutaneously in the neck. Concurrent with immunization, 1 μg of PTX was injected intraperitoneally (i.p.). Indicated doses of pioglitazone in 50 μl of dimethyl sulfoxide (DMSO) or vehicle only were injected i.p. once daily from day −1 to the day before sacrifice as whole-phase treatment, or from day 8 to the day before sacrifice as effector-phase treatment. Pioglitazone- or vehicle-treated mice were assessed clinically and histologically for the severity of EAU on day 21 after immunization. Clinical score was graded on a scale between 0 and 4 in half-point increments as described previously (Thurau et al., 1997). For histological assessment, enucleated eyes were fixed in 4% paraformaldehyde. The severity of EAU in each eye was scored on a scale of 0–4 in half-point increments, according to a semiquantitative system described previously (Chan et al., 1990).

2.3. Flow cytometric analysis of lymph nodes

Cervical and axillary lymph nodes (LNs) were harvested from EAU and control mice. After counting cell number of LNs in each mouse, the cells were first preincubated with unlabeled anti-CD16/32 mAb to avoid nonspecific binding of mAb to FcγR. Then the cells were incubated with FITC-conjugated anti-CD4 and PE-conjugated anti-CD44 mAb, PE-conjugated anti-CD62L, or PE-conjugated anti-Foxp3 mAb (Usui et al., 2006). After washing with PBS, the stained cells (live-gated based on forward and side scatter profiles and propidium iodide exclusion) were analyzed on a flow cytometer (FACSCalibur; BD Bioscience, Franklin, Lakes, NJ). The data were processed using the accompanying software (CellQuest; BD Bioscience), and expressed as mean fluorescence intensity (MFI) or percent. For intracellular cytokine staining of IL-17, purified T cells, stimulated with PMA/ionomycin for 6 h in the presence of GolgiPlug (Becton Dickinson), were stained with FITC anti-CD4 antibody. Then, intracellular staining with PE-conjugated anti-IL-17 mAb was performed using Fix buffer and permeabilization buffer (eBioscience), according to the manufacturer's instructions. Analysis was performed using FACSAccuri (BD Bioscience).

2.4. Measurement of intraocular inflammatory cytokines and chemokines using flow cytometer

We prepared intraocular protein extracts as follow. On day 21 after immunization, both eyes were enucleated. Eyes from each mouse were homogenized in a 1.5 ml tube using micropestles (Eppendorf, Hamburg, Germany) after adding 100 μl of PBS. The tubes were centrifuged at 15,000 rpm for 15 min, and the supernatants were stored at −80 °C until use. We measured the intraocular concentrations of IFN-γ, IL-6, IL-9, IL-10, IL-12p70, IL-17, IL-21, tumor necrosis factor (TNF)-α, monocyte chemotactic protein-1 (MCP-1), and monokine induced by interferon-γ (Mig) using Cytometric Beads Array Flex Set (BD Bioscience) and FACSCalibur according to the manufacture's protocol. The minimal detection levels were 10 pg/μl. Fifty μl of undiluted protein extract from each sample was used.

2.5. In vitro cell proliferative activity

Draining LN cells were isolated and pooled on day 21 and cultured in triplicate in 96-well flat-bottomed microculture plates at a density of 6×10^5 cells/well in the presence or absence of indicated dose of hIRBP-p. To assess proliferative responses, the cultures were pulsed with [3H]dThd (0.5 μCi/well) for the last 8 h of a 72-h culture and were harvested onto glass filters with an automated cell harvester. Radioactivity was measured by liquid scintillation spectrometry (Tomtec, Orange, CT), and expressed as counts per minute.

2.6. Statistical analysis

The significance of differences between means was determined using the Mann–Whitney *U* test or student *t*-test. *P* values less than 0.05 were considered significant. All analyses were performed using JMP statistical analysis software, version 10 (SAS Institute, Cary, North Carolina, USA).

3. Results

3.1. Whole-phase pioglitazone treatment suppressed EAU

We first studied the effect of whole-phase treatment with pioglitazone on EAU. Clinical severity of pioglitazone- or vehicle-treated EAU mice was evaluated on day 21 after immunization. Clinical EAU score was significantly suppressed in 25 mg/kg pioglitazone-treated mice compared with vehicle-treated mice (mean; 0.69 ± 0.61 versus 1.63 ± 0.83; $p = 0.034$) (Fig. 1A), although