·Basic Research·

Troglitazone induced apoptosis of human pterygium fibroblasts through a mitochondrial -dependent pathway

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Abstract

• AIM: To study the effect of troglitazone on primary culture human pterygium fibroblasts (HPF).

• METHODS: Cell viability loss and apoptosis were quantified by cell counting kit-8, AnnexinV-FITC/PI double staining, caspases activity test and western blotting. Flow cytometry was used to detect mitochondrial membrane potential.

• RESULTS: Peroxisome proliferator-activated receptor γ (PPAR-γ) was positively expressed in pterygium specimens (n=5). Troglitazone showed dose-dependent inhibition of cell survival, induced phospholipids redistribution, activated caspase-3, -9, and altered mitochondrial potential. Western blot assay demonstrated the increase of Bax/Bcl-2 protein ratio.

• CONCLUSION: Troglitazone induced apoptosis of HPF through a mitochondrial-dependent pathway.

• KEYWORDS: pterygium; peroxisome proliferator-activated receptor γ ; troglitazone; apoptosis
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INTRODUCTION

Pterygium is a wing-like growth that invades the cornea, which is characterized by fibrovascular proliferation, collagen elastics degeneration and ocular surface chronic inflammation. Surgical excision is the primary treatment of Pterygium, yet the recurrence rate is high, even using autologous conjunctiva flap or preserved amniotic membrane. Adjuvant therapies such as intraoperative and postoperative use of antimetabolites e.g. mitomycin (MMC) considerably reduced recurrence rate. However, MMC is associated with serious and potentially sight-threatening complications. There is still need for finding therapeutic drugs with low toxicity and enhanced efficacy. Peroxisome proliferator-activated receptor γ (PPAR-γ) belongs to the family of nuclear hormone receptors (NHRs). PPAR-γ is mainly expressed in adipose tissue where it plays a role in lipid and glucose metabolism. Later studies demonstrated that PPAR-γ agonist has potential anti-inflammatory and anti-neoplastic effects as well as inhibition of angiogenesis in devise pathological states [1,2], including arthritis, pulmonary fibrosis, solid cancers and leukemia. The studies of using PPAR-γ in eye diseases are mainly focused on choroidal neovascularization and inflammation pathological ocular conditions such as age-related macular degeneration (AMD), diabetic retinopathy, and autoimmune uveitis [3,4]. The role of PPAR-γ as a potential therapeutic agent for pterygium remains undefined. With the more understanding of molecular events underlying pterygium pathogenesis, multiple factors are believed to be involved [5]. It has been demonstrated that Pterygium was not only a chronic inflammatory reaction associated with free radicals [6], but shared some similarities with neoplastic conditions [7]. Considering the anti-inflammation and anti-cancer roles PPAR-γ played in other diseases, we hypothesized PPAR-γ may have therapeutic potentials in pterygium. Thus, in this study we examined the use of PPAR-γ agonists for possible treatment of pterygium by studying troglitazone's effect on primary cultured human pterygium fibroblasts (HPF).

MATERIALS AND METHODS

Materials Pterygium specimens were obtained from patients undergoing routine pterygium excision at the First Affiliated Hospital of Jinan University, Guangzhou, China.

Informed consent was obtained from each patient. Fresh pterygium specimen was placed in culture plates containing antibiotics phosphate buffer saline PBS buffer. Sterile technique was used to dissect away surrounding normal corneal and conjunctival tissue. The central portion of the pterygium body was used for culture. The specimen was subsequently cut into several 1mm×1mm pieces and placed onto 100-mm tissue culture dishes. Added drops of Fetal Bovine Serum FBS (Sijiqing Science , Hangzhou, China) then incubated the culture dish at 37℃ with 95% humidity and 50mL/L CO_2 for 6 to 8 hours, to help the explants attach to the substratum. DMEM medium (Gibco, CA, USA) enriched with 100mL/L FBS, 100kU/L penicillin, and 0.1g/L streptomycin was added after, and changed medium every 2 days. Cell migration from explants was observed within 6-8 days. Fibroblasts were subcultured to T25 culture flasks with 2.5g/L trypsin and 0.2g/L EDTA (Gibco, CA, USA) in a DMEM medium at 80% to 90% confluence two weeks later, with 1:2 to 1:3 split. Cells were passaged at weekly intervals thereafter. After 3 passages, exponentially growing cells were used throughout the study. Primary cultured pterygium fibroblast cells showed typical spindle cells, and Vimentin positive stained by immunohistochemistry. In initial studies we examined the expression of PPAR-γ in primary Pterygium specimens (n =5, average age is 48.6) by western blotting. All five specimens showed positive expression.

Methods

Cell proliferation assay The effect of troglitazone (Sigma-Aldrich, Saint Louis, MO) on the proliferation of human pterygium HPF was assessed utilizing commercially available cell counting kit-8 (Dojindo Molecular Technologies, Japan). HPF were seeded in 96-well microplates at 5×10^3 cells/well, allowed to attach, and then incubated overnight in DMEM nutrient media containing 100mL/L FBS. According to the CCK-8 protocol, briefly, HPF were treated with troglitazone for 12 and 24 hours. Then CCK-8 solutions with tetrazolium salt WST-8 were added and incubated at 37℃ for an additional 2 hours. WST-8 is bio-reduced by cellular dehydrogenases to an orange formazan product in culture medium. The amount of formazan, which is directly proportional to the number of living cells, was evaluated by measurement of the optical density at 450nm in the microplate spectrophotometer (Safire2, Tecan, Switzerland).

HPF apoptosis detection Cells were placed in 6-well plates at the density 2×10^5 cells/well and cultured in DMEM supplemented with 100mL/L FBS. After 24 hours, troglitazone of various concentrations 20 to 80μmol/L was added to the culture medium and cells were incubated for an additional 12 hours. For apoptosis detection, cells were harvested using trypsin-EDTA treatment and washed with DMEM containing 10% FBS, and stained with AnnexinV FITC/PI Kit (Nanjing Keygen Biotech, China) for fluorescence activated cell sorter analysis using a Flow Cytometer (Becton Dickinson, CA, USA).

Caspase assays HPF were placed in 6-well plates at 2×10^5 cells per well and treated similarly. The activity of caspase-3, caspase-9 was assayed using caspase colorimetric assay Kits (abcam, MA, USA). According to the manufacturer's instructions, cells were harvested and washed with cold PBS, resuspended in lysis buffer and left on ice for 15 minutes. The lysate was centrifuged at 16000g at 4℃ for 15 minutes. Activities of caspase-3, and -9 were measured using substrate peptides Ac-DEVD-pNA, Ac-LEHD-pNA respectively. The release of p-nitroanilide (pNA) was quantified with a microplate spectrophotometer (Safire2, TECAN, Switzerland) at an absorbance of 405nm.

Mitochondrial membrane potential detection Cells were placed in 6-well plates at the density 2×105 cells/well and cultured in DMEM supplemented with 100mL/L FBS. After 24 hours, HPF cells were treated with Troglitazone for 12 hours. Cells were labeled with JC-1 reagent (JC-1 Mitochondrial Membrane Potential Detection Kit, beyotime China) for 15 minutes. After washing, cells were analyzed on a FACS flow cytometer. (Becton Dickinson, CA, USA).

Protein analysis Total cell protein was isolated with RIPA lysis buffer (10mmol/L Tris [pH 7.4], 150mmol/L NaCl, 5g/L NP-40, 1g/L SDS, 1g/L deoxycholate, 1mmol/L PMSF). Protein concentrations were measured using the BCA protein assay kit (Pierce, Rockford, IL). Proteins were separated on 100g/L SDS-polyacrylamide gels, and transferred onto PVDF transfer membrane (Millipore, Bedford, MA).After blocking in 50mL/L nonfat milk for 2 hours at room temperature, Filters were incubated overnight at 4℃ with primary antibodies anti- PPAR-γ (Santa Cruz, CA, USA), bax and Bcl-2 (Bioworld, OH, USA), and actin (Sigma-Aldrich, Saint Louis, MO). Then washed membranes with ice-cold PBS-T before probing with a peroxidase(HRP)-conjugated secondary antibody, anti-rabbit IgG (KPL, Gaithersburg, Maryland) for 1 hour at room temperature and developed using SuperSignal West Pico kit (Pierce Biotechnology, Rockford, IL,USA).

Statistical Analysis All the experiments were replicated at least three times. Data were presented as mean ±standard deviation and analyzed using Student's t-test and one way ANOVA. P <0.05 was considered statistically significant.

RESULTS

HPF Proliferation After treated with different dose of troglitazone (5, 10, 20, 40, 60, 80 μmol/L) by cell counting kit-8, the application of troglitazone induced a dose-dependent viability loss in HPF. The IC50 value for growth inhibition for 12 hours was 47.8μmol/L. Therefore, 40μmol/L troglitazone was used in subsequent experiments.

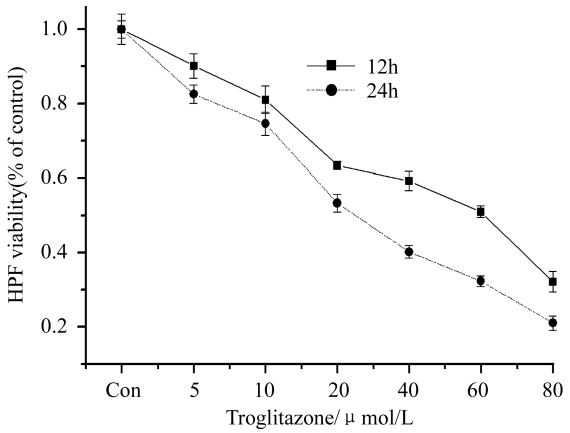

Figure 1 Cell viability loss induced by troglitazone

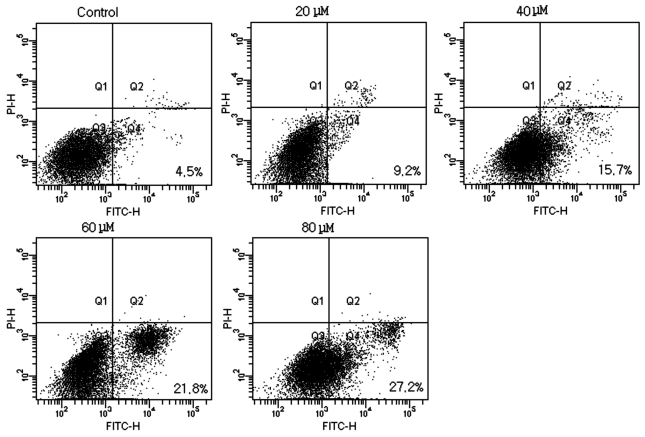

Figure 2 Troglitazone induced apoptosis of HPF

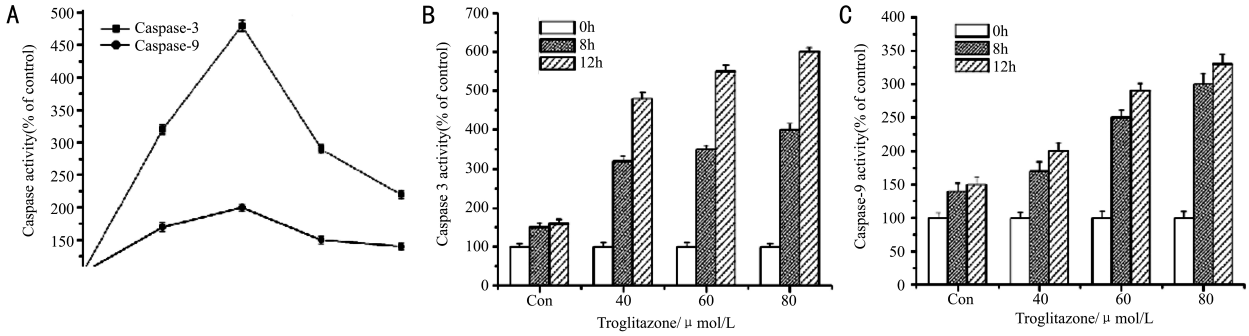

Figure 3 Activity of caspase-3, -9 of troglitazone treated HPF (P <0.05)

Cell viability was measured by cell counting kit 8 assay under exposure to various concentrations of Troglitazone for 12 hours and 24 hours in cultured human pterygium fibroblasts (Figure 1).

HPF Apoptosis To investigate whether cell death induced by troglitazone occurs through apoptosis, we performed annexin V-FITC/PI double staining by flow cytometry. Apoptotic cells are stained positively for Annexin V-FITC that bind to phosphatidylserine (PS), but are negative for staining with propidium iodide (PI). Apoptotic induction was observed in cells treated with 20μmol/L. Troglitazone for 12 h. As shown in the Figure 2, Q4, the lower right quadrant represented the early apoptosis of cells. The percentages of apoptotic cells were 4.5%, 9.2%, 15.7%, 21.8%, and 27.2% corresponding to 0, 20, 40, 60, and 80μ mol/L, respectively (Figure 2).

After treated with 40μmol/L Troglitazone for various time points, our results showed an obvious increase in caspase-3 as well as caspase-9. Both Caspase-3 and 9 reached maximal levels by 12 hours, which was 4.8-fold and 2-fold greater than that of control respectively and decreased thereafter (Figure 3A). Then we measured the caspases activity with different dose of troglitazone. As shown in Figure 3B, 3C, exposure to troglitazone (0, 40, 60, and 80μmol/L) for 12 hours resulted in a significantly effect on the caspases activity of HPF in a dose-dependent manner.

When cells were treated with troglitazone, as in Figure 4A, a remarkable increase of green fluorescence was observed. The mean green and red fluorescence intensity ratio (530nm/590nm) in the mitochondria leaped (Figure 4B), eliciting the depolarization of the mitochondrial potential.

Bax/Bcl -2 ratio We found over-expression of Bax and down-regulation of Bcl-2 after treated with 40μmol/L troglitazone for 12 hours. Thus the Bax/Bcl-2 ratio increased significantly and lead to cells apoptosis (Figure 5).

DISCUSSION

To confirm the apoptosis underlying Troglitazone treated HPFs, we assay the activity of caspase-3 caspase-9 using caspase colorimetric assay Kits. Caspase-3 and -9 are important in both intrinsic and extrinsic pathway of apoptosis. Caspase activity has been found in many models of apoptosis. Caspase-3 plays an important role as an executor of cell death, while, caspase-9 is an initiator caspase and has linked to the mitochondrial cell death pathway.

Several reports have indicated a possible role for in Troglitazone-induced apoptosis. Mitochondrial damage is often associated with loss of mitochondrial membrane potential which has been shown to be a hallmark of early apoptosis [8]. To determine the exact contribution of mitochondrial alteration of potential, we therefore probed HPFs with JC-1. In live cells, JC-1 exhibits potential-dependent accumulation in mitochondria forming J-aggregates. These aggregates can be detected within the

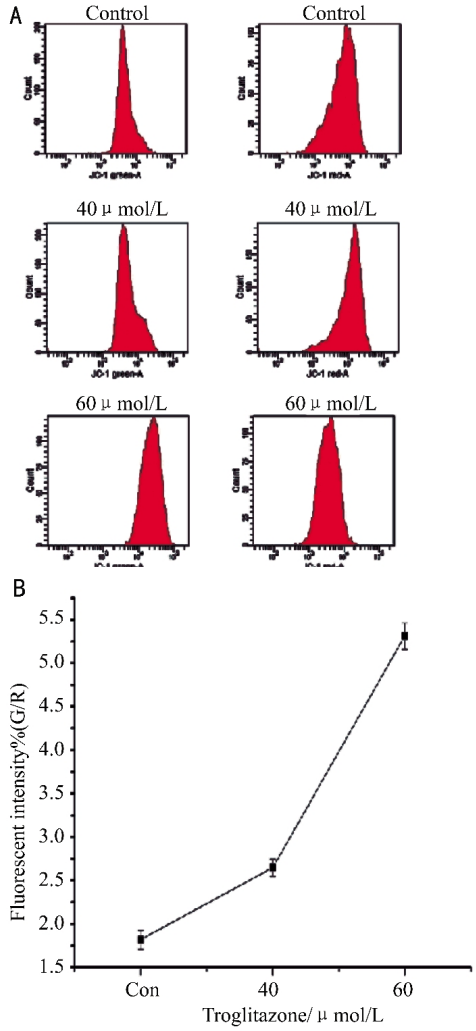

Figure 4 Loss of mitochondrial membrane potential A: FACS flow cytometry of JC-1 staining; B: The mean green and red fluorescence ratio

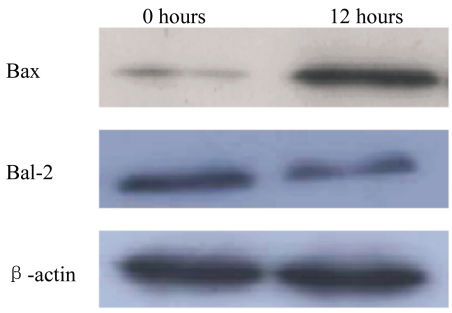

Figure 5 Bax and Bcl-2 expression

red fluorescence spectrum (-590nm), in contrast to the green fluorescence (-530nm) emitted by JC-1 monomers. An increase in green fluorescence indicates depolarization of the mitochondrial membrane potential.

The mitochondrial apoptotic signaling pathway involves activation of the pro-apoptotic Bcl-2 family member---Bax, which induce permeabilization of the mitochondrial outer membrane. The relative levels of Bax (pro-apoptotic) and Bcl-2 (anti-apoptotic) in mitochondria determine the fate of the cell.

In present study, we examined the potential use of PPAR-γ agonists in pterygium treatment and the mechanisms underlying for the fist time. We elucidated troglitazone induced apoptotic cell death in human pterygium fibroblasts HPF through a mitochondrial-dependent pathway. As the complexity of its pathogenesis, pterygium has no established animal model. Our study based on cultured pterygium fibroblasts could explain just part of the story.

There is a growing body of literature demonstrating that PPAR-γ may be involved in various mechanisms associated with Pterygium. The function of PPAR-γ in adipogenesis is well established. Recent study showed fibroblast isolated from human pterygium exhibit altered lipid metabolism characteristics [9]. Altered MMP expression of pterygium limbal basal epithelial cells enables them to invade Bowman's layer. Saika *et al* [10] showed PPAR-γ suppressed upregulation of inflammation growth factors and matrix metalloproteinase (MMPs) in mice cornea after alkali burn. Pterygium is in a disrupted redox state [11]. It has been found PPAR-γ ligands protect a variety of cell types, including retinal cells, from oxidative stress injury *in vitro*. All of these indicated that PPAR-γ may be a promising multi-target treatment of pterygium to replace or supplement surgical intervention, and is worth further investigations.

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