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Original Article

Effects of PPARγ agonist rosiglitazone on human retinoblastoma cell in *vitro* and in *vivo*

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Abstract: The aim of the study was to evaluate the antitumor effects of the PPARγ agonist rosiglitazone on the human retinoblastoma. The cell biological behavior was detected, specifically, the effects of rosiglitazone on cell viability and apoptosis of the human retinoblastoma Y79 cells were investigated by MTT assay and Hochest 33258 staining and the migration assay showed that rosiglitazone blocked the invasion and migration of the carcinoma cells through the reconstituted extracellular matrix (Matrigel). The effect of rosiglitazone on NF-κB-dependent reporter gene transcription induced by LPS was analyzed by NF-κB-luciferase assay. Then human retinoblastoma Y79 cells were subcutaneously transplanted in BALB/c nude mice, and the animals were treated with rosiglitazone (20 mg/kg, 40 mg/kg, and 80 mg/kg) to verify its anti-tumor effect in vivo. Rosiglitazone suppressed the viability of Y79 cells dose- and time-dependently and induced apoptosis in Y79 cells *in vitro*. Molecular biology analysis found that rosiglitazone could modulate the proliferative and apoptosis related signal, reduce NF-κB-dependent reporter gene transcription induced by LPS. Rosiglitazone markedly reduced the growth of Y79 cells transplanted into the mice without causing significant side effects. Our results suggested that rosiglitazone demonstrated antitumor activity against the human retinoblastoma Y79 cells by inhibiting cell growth, inducing apoptosis and inhibiting metastasis and invasion in vitro and delaying tumor growth in vivo.

Keywords: Rosiglitazone, PPARγ, retinoblastoma, NF-κB, tumor growth

Introduction

Retinoblastoma (RB) is the most common paediatric intraocular malignancy tumor arising in the retina and mainly occurred in infant, the incidence of which is about 1/15,000 to 1/20,000 live births, translating to approximately 9,000 new cases every year worldwide [1-3]. Retinoblastoma was the first disease demonstrating a genetic basis for cancer development caused by RB gene mutations and the loss of the tumor suppressor functions of embryonal nuclear layer cells [4]. Metastasis of Rb, second primary tumors and intracranial neuroblastic malignancy (trilateral Rb) are the three life-threatening problems for Children with Rb [5].

Peroxisome proliferator-activated receptor γ (PPARγ) discovered in 1990 is a subclass of ligand-activated nuclear hormone receptor superfamily, and three related PPAR isotypes have been identified till now including PPARα, PPARβ/δ and PPARγ sharing a high degree of homology but differ in tissue distribution and ligand specificity [6, 7]. The PPARγ agonists were used as the antidiabetic drugs for which could decrease the concentration of serum glucose in diabetes. In recent years, anecdotal evidence suggested that PPARγ and its ligands not only regulated metabolic actions, energy balance, but also demonstrated the potent in the treatment of cancer [8], studies have shown that PPARγ is expressed in various tumor tissues, for example decreased expression of PPARγ has been associated with poor prognosis in lung cancer patients and activating PPARγ by either endogenous or synthetic agonists was found to inhibit growth of human lung cancer cells [9, 10], and combined treatments with PPARγ agonist rosiglitazone and a JNK inhibitor in reducing human colon cancer cell adhesion and migration [11]. However, there are no study reported about the expression of PPARγ and the possible mechanisms in retinoblastoma. In this study, we aimed to evaluate the antitumor

effects of the PPARγ agonist rosiglitazone on the human retinoblastoma and the possible mechanism in vitro.

Materials and methods

Materials

The human retinoblastoma cell lines Y79 (American Type Culture Collection, USA) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, penicillin-streptomycin (100 IU/ml-100 lg/ml), and 10 mM Hepes buffer, and 2.5 μg/mL amphotericin B at 37°C in a humid atmosphere (5% CO_2-95% air). Rosiglitazone was purchased from Sigma-Aldrich, USA.

MTT assay

Cells seeded in 96-well plates (5×10^3 per well) were incubated with increasing concentrations of rosiglitazone (0.04 μM, 0.2 μM, 1 μM, 5 μM, 25 μM, and 125 μM) for 24, 48 and 72 h, respectively. The controls were treated with an equal volume of the drug's vehicle DMSO. The cell growth inhibition was evaluated by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma, USA) assay as described elsewhere [12].

Hoechst 33258 staining

Y79 cells at the logarithmic-growth phase were seeded into 96-well plates (1×10^4/well). The cells were cultured in normal medium (control group) or with increasing concentrations of rosiglitazone (5 μM and 25 μM) for 24 h. Then, the cells were fixed with 3.7% paraformaldehyde for 30 min at room temperature, then washed and stained with Hoechst 33258 (Sigma-Aldrich) for 30 min at 37°C. Cells were observed under a Nikon 80i fluorescence microscope equipped with a UV filter (Nikon Corporation, Tokyo, Japan).

Cell migration assay

3×10^5 cells/ml of Y79 cells treated with different concentrations of rosiglitazone (5 μM and 25 μM) were prepared to perform the migration and invasion assays by using QCM™ Laminin Migration Assay (24-well, colorimetric, Millipore, ECM220) according to the manufacturer' protocols, respectively. Finally, absorbance was measured at 57 nm for migration or at 560 nm for invasion.

Western blot analysis

Y79 cells (3×10^5) seeded in 6-well plates were treated with various concentrations of rosiglitazone for 72 h. The cells were harvested for cell lysates, tumor tissues from mice model were also prepared. Samples of 30 μg of protein per lane were fractionated by 10% SDS-PAGE. The proteins were electro-transferred onto PVDF membranes and then protein levels were detected using dilutions of the primary antibodies. The primary antibodies were washed in 0.05% Tween-20/PBS and then incubated with horseradish peroxidase-conjugated secondary antibody. The bound antibodies were visualized using an enhanced chemiluminescence reagent (Millipore, USA) and quantified by densitometry using ChemiDoc XRS+ image analyzer (Bio-Rad, USA). Densitometric analyses of bands were adjusted with β-actin as loading control. Triplicate experiments with triplicate samples were performed.

Luciferase reporter gene assay

The effect of rosiglitazone on NF-κB-dependent reporter gene transcription induced by LPS was analyzed by NF-κB-luciferase assay. Briefly, Y79 cells (5×10^5 cells/well) were plated in 6-well plates and transiently transfected by the liposome 2000 method with pNF-κB-luc plasmid reporter gene (0.5 μg, Beyotime) and β-galactosidase (90 ng). Twenty-four hours after transfection, cells were treated with rosiglitazone (5 μM and 25 μM) and 10 μg/mL LPS for another 20 h. Cells were harvested for measuring β-galactosidase activity and luciferase activity. Relative luciferase activity was normalized to β-galactosidase value to correct transfection efficacy. Triplicate experiments with triplicate samples were performed.

In vivo inhibition of tumor growth

The in vivo efficacy of rosiglitazone was evaluated in an Y79 xenograft mice model. Balb/c athymic (nu+/nu+) female nude mice (4-6 weeks) were purchased from Beijing Colab Bio-Technology Ltd., China. The animals were housed under pathogen-free conditions. Y79 cells (1×10^7) were suspended in 100 μl of Matrigel (Collaborative Biomedical) and injected subcutaneously into the left anterior flank of nude mice. After 7 days, when tumor volume had reached approximately 0.1 cm^3, then different concentration of rosiglitazone was administered via oral gavage. Administration of

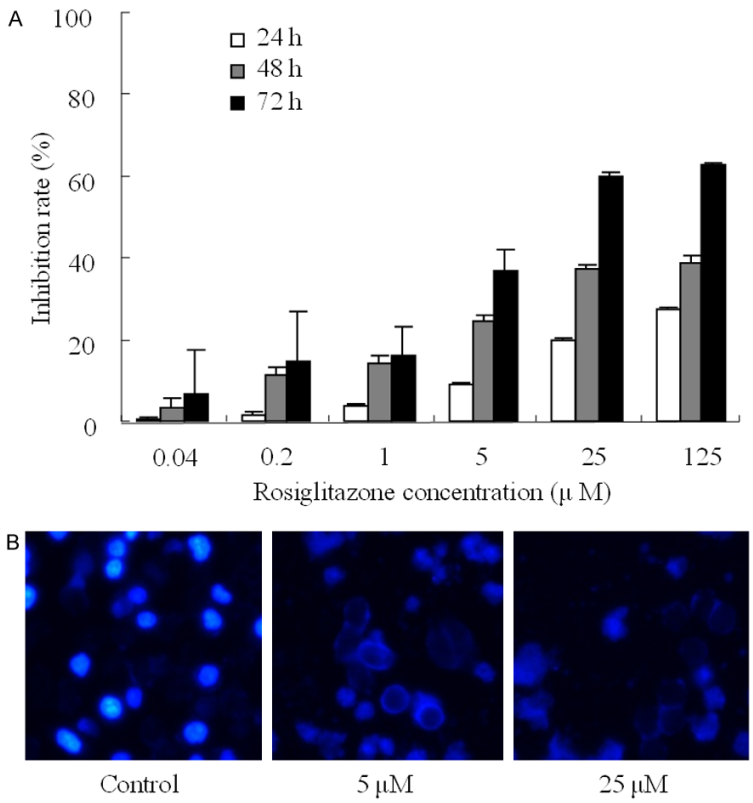

Figure 1. Effects of rosiglitazone on human retinoblastoma Y79 cells proliferation and apoptosis. A. Growth inhibition of Y79 cells exposed to different concentrations of rosiglitazone (bars indicate means ± S.D, n=3); B. Morphological changes of Y79 cells in nuclear chromatin treated by rosiglitazone.

rosiglitazone (20 mg/kg, 40 mg/kg, and 80 mg/kg) was performed daily for three consecutive weeks. Tumor growth inhibition rates were defined as a ratio to the control tumor weight. The research protocol was approved and in accordance with the institutional guidelines of the Animal Care and Use Committee at Kunming Medical University.

Statistical analysis

Data was described as the mean ± S.D. and analyzed by one-way ANOVA. A p value <0.05 was considered statistically significant. Statistical analysis was performed using the SPSS/Win13.0 software (SPSS, Inc., Chicago, IL).

Results

Detection of retinoblastoma cell growth and cell apoptosis

Y79 cells were exposed to different concentrations of rosiglitazone and then subjected to the MTT assay. Rosiglitazone significantly inhibited Y79 cell proliferation in a dose- and time-dependent pattern. As shown in **Figure 1A**, in the range of 0.04-125 μM of rosiglitazone, the rates of inhibition varied from 1.7% (0.04 μM-24 h exposure) to a maximum of 63.2% after 72 h of exposure (the statistical significance was not observed in low concentrations and short time of exposure, 0.04 μM-0.2 μM, 24 h exposure, P>0.05 *vs.* vehicle control; other treated group P<0.05 *vs.* vehicle control).

Alterations of cellular morphology were assessed using Hoechst 33258 staining in order to characterize the effects of rosiglitazone on cell apoptosis (**Figure 1B**). The nuclei of the Y79 cells in control group revealed big and round nuclei with smooth nuclear membrane, there were few cells with nuclear chromatin condensation and fragmentation as well as cell shrinkage and the formation of apoptotic bodies observed. While in the rosiglitazone treated groups, cells appeared fragmented, indicating that rosiglitazone induced apoptosis affected the morphology of the cells.

Cell Invasion and migration

The effect of rosiglitazone on invasion and migration of Y79 cells was examined using QCM™ Laminin Migration Assay. As shown in **Figure 2**, the activity of invasion and migration of Y79 cells was significantly decreased by treatment of different concentrations of rosiglitazone. The results showed that 5 μM and 25 μM rosiglitazone could inhibit cell invasion and migration by 29.3% and 54.1% (P<0.05 and P<0.01 *vs.* untreated controls respectively). These suggested that rosiglitazone suppressed the metastasis and invasion of Y79 cells.

Molecular analysis by Western blotting

Protein levels were examined by Western blotting assay. First we measured several molecu-

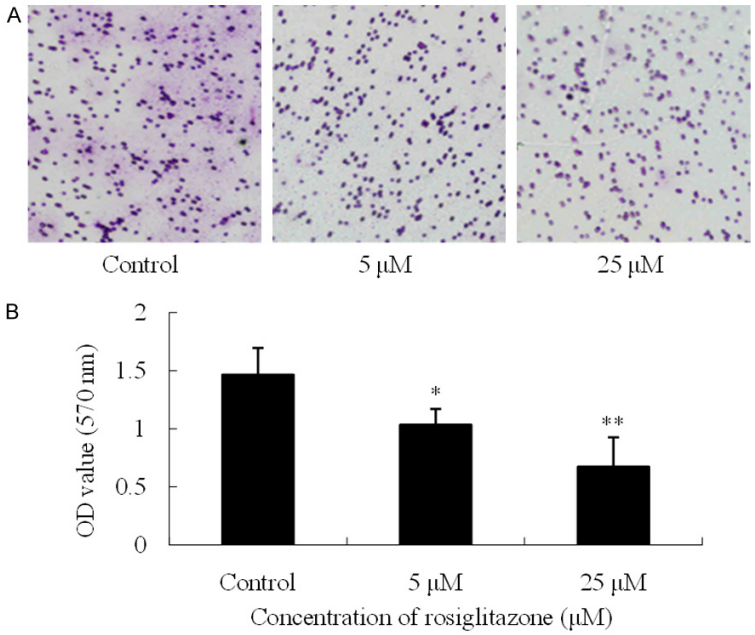

Figure 2. Rosiglitazone suppressed the of Y79 cells *in vitro*. 3×10^5 Y79 cells were treated with different concentrations of rosiglitazone for 24 h, respectively, were used to perform the migration assay. A. The light microscopy view of the cells at a magnification of ×200; B. Histogram showed the values of absorbance at 570 nm for migration. The bars indicate mean ± S.D. (n=3). *P<0.05; **P<0.01 vs. untreated controls.

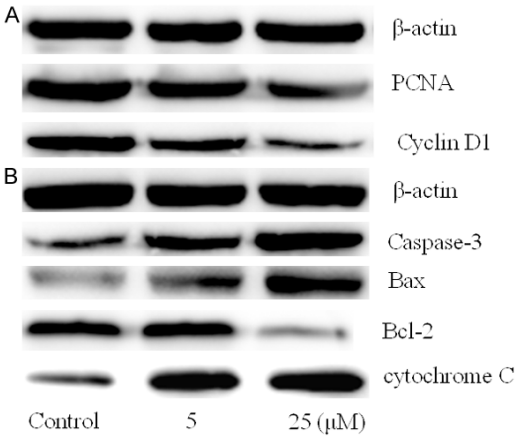

Figure 3. Regulation effect of rosiglitazone on proliferation and apoptosis related molecular. A. Rosiglitazone modulated the expression of PCNA and cyclin D1; B. Rosiglitazone affected the levels of apoptosis proteins.

lar related to cell proliferation and apoptosis. Our results showed that rosiglitazone could downregulate expression of PCNA and cyclin D1 in vitro, and rosiglitazone also affect levels of apoptosis proteins including cytochrome C, caspase-3 and Bax/Bcl-2 (**Figure 3**). As shown in **Figure 3**, the inhibition rate were 18.6% and 41.3% at 5 μM and 25 μM of rosiglitazone for PCNA, 30.5% and 56.2% for cyclin D1 (P<0.05 *vs.* control group). As for the apoptosis related proteins, the expression of caspase-3 was upregulated to 1.7-fold and 2.3-fold of the control group at 5 μM and 25 μM of rosiglitazone, 2.5-fold and 1.9-fold of control for cytochrome C, and for Bax: Bcl-2, the ratio was elevated to 2.4-fold and 5.3-fold at 5 μM and 25 μM of rosiglitazone respectively (P<0.05 *vs.* control group).

Effect of rosiglitazone on NF-κB-mediated transcriptional activity

After examine the expression levels of the above molecular, we studied rosiglitazone effect on NF-κB-mediated transcriptional activity using Y79 cells, wherein 5 μM and 25 μM of rosiglitazone reduced luciferase activity by 27.5% and 43.1% (P<0.05) compared with control cells (**Figure 4**). These results provide additional insight into suppression of tumor growth by rosiglitazone.

In vivo inhibition of tumor growth by rosiglitazone

The anticancer activity of rosiglitazone was evaluated in a Y79 xenografts mouse model after 3 weeks of treatments. The inhibition of cancer growth effects of rosiglitazone was defined as a ratio to the tumor weight of animals treated with control vehicle. Our results showed that 20 mg/kg, 40 mg/kg and 80 mg/kg of rosiglitazone delayed the growth of Y79 xenografts by 32.1%, 56.7% and 69.5%, respectively (**Figure 5A**, P<0.05 *vs.* control group).

And the body weight of animals in each group were also recorded during the experiment period, the data showed that continuous use of low dose of rosiglitazone (20 mg/kg) did not significantly influence the body weight of the animals (P>0.05 *vs.* control group), and in 40 mg/kg

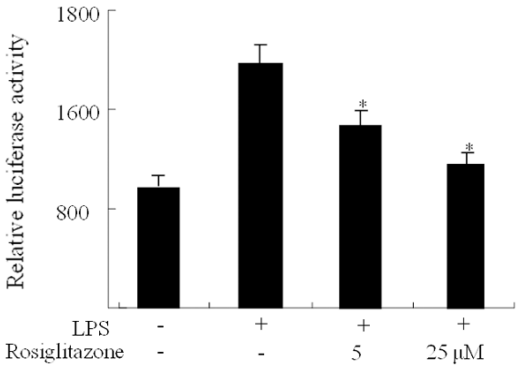

Figure 4. Effect of rosiglitazone on NF-κB-dependent reporter gene transcription ability. The bars indicate means ± S.D. *$P<0.05$ *vs.* vehicle control group.

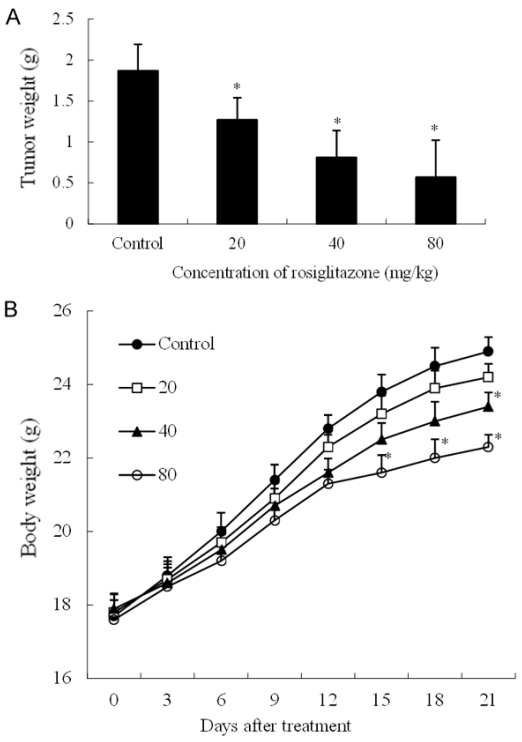

Figure 5. Rosiglitazone inhibited growth of H460 xenografts bearing in mice. The mice were injected intravenously with rosiglitazone every day for three weeks. A. Tumor weight measurement was made after mice were sacrificed; B. Body weights were measured every three days. The bars indicate means ± S.D. *P<0.05 *vs.* vehicle control group.

rosiglitazone group, only in the last day (3 weeks after continuous use of 40 mg/kg rosiglitazone) of the experiment we detected a statistically significant difference compared with control group, while in the high dose group (80 mg/kg), the body weight measured was lower than control group since 15 days after rosiglitazone administration (**Figure 5B**, $P<0.05$ *vs.* control group).

Discussion

PPARγ is a nuclear receptor with multiple biologic effects belonging to ligand-activated nuclear hormone receptor superfamily, and there has been evidence demonstrating that PPARγ activation by the hiazolidinedione group of drugs (TZDs) inhibits cell growth and induces cell apoptosis in liver cancer, breast cancer, colon cancer and other cancer types [13-18]. Rosiglitazone, as a PPARγ synthetic ligand, has been shown to enhance the antitumor activity of some chemotherapeautic drugs by the modulation of cancer cell agents [19-21]. However, the effects of rosiglitazone on human retinoblastoma cells have been poorly understood until now.

Tumor cells are characterized by uncontrolled cell proliferation without a balanced extent of apoptosis, and apoptosis is an important evolutionary conserved mechanism and plays a key role in organic evolution, the maintenance of the internal environment and the development of tumors defined as programmed cell death to balance cell proliferation essential for maintenance of tissue homeostasis [22, 23]. There are many molecular closely related with cell proliferation and cell cycle. Proliferating cell nuclear antigen (PCNA), which is also known as cyclin, has been found in the nuclei of yeast, plant and animal cells that undergo cell division, suggesting a function participating in DNA metabolic processes and cell cycle regulation during the S phase of the cell cycle [24, 25]. The cell-cycle control protein cyclin D1 is an important regulator of the G1 to S phase transition in normal cells, which also plays as a protooncogene that is activated or overexpressed in many types of human cancers, including human B cell tumors, lymphoid, breast, esophageal, lung, and bladder tumors [26-31].

Our preliminary experiment results have showed that the Y79 cells revealed a relatively high expression of proliferation markers including PCNA and cyclin D1 (data not shown). In this study, rosiglitazone was found to reduce the expressions PCNA and cyclin D1 in Y79 cells in vitro, implying that rosiglitazone might had a

strong ability in regulating Y79 cell proliferation in vitro.

One of the other important aspects in cancer progression is the acquisition of invasive behavior [32]. As per International Retinoblastoma Staging Working Group (IRSWG) most of the RB tumors are found to have massive choroidal, optic nerve, and anterior segment invasion [33, 34]. In this study, we evaluated the efficacy of rosiglitazone on the migration ability of Y79 cells in vitro using a migration assay. And the results proved that rosiglitazone could significantly suppress the metastasis and invasion of Y79 cells

Furthermore, enormous studies suggest that chronic inflammation plays an important role in tumorigenesis [35-37]. The NF-κB family of transcription factors regulates multiple biological functions. NF-κB can function as an oncogene through its ability to stimulate cell proliferation and survival, serve a major role in the inflammatory and innate immune responses by stimulating expression of cytokines, cytokine receptors, and histocompatibility genes [38, 39]. NF-κB is constitutively activated in several human cancers [40, 41]. It has been proved that the active form of NF-κB could activate cyclin D1 which has the NF-κB binding site in its transcription promoter [42]. In this study, we examined the effect of rosiglitazone on NF-κB-dependent reporter gene transcription induced by LPS by NF-κB-luciferase assay. The results indicated that the transcriptional activity of NF-κB was obstructed by treatment of rosiglitazone.

There are other anti-diabetic drugs revealed anti-cancer effects such as metformin, ciglitazone, and troglitazone, however, not all of the drugs could inhibit in vivo tumor growth, metformin was reported to fail to suppress growth of xenografted tumors of Y79 human retinoblastoma cells in nu/nu mice, even when treated with a maximally tolerated dose level achieved in human patients [43, 44]. In this study we found that the growth of the retinoblastoma Y79 cells transplanted in nude mice was extensively suppressed after three weeks of rosiglitazone administration. These results suggesting that rosiglitazone may be useful for tumor suppression when used in vivo.

In summary, this study demonstrated that administration of rosiglitazone leads to the inhibition of cell proliferation, induction of apoptosis, modulation of related molecular expression in vitro in human retinoblastoma cell lines Y79, and significantly delayed the tumor growth in vivo, and moreover rosiglitazone did not show apparent toxicity to animals during the long-term treatment. These results suggested that rosiglitazone could be a potential chemotherapy regimen for human retinoblastoma.

Disclosure of conflict of interest

None.

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