Pioglitazone Induces Cardiomyocyte Apoptosis and Inhibits Cardiomyocyte Hypertrophy Via VEGFR-2 Signaling Pathway

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Abstract

Background: Pioglitazone has been widely used as an insulin-sensitizing agent for improving glycemic control in patients with type 2 diabetes mellitus. However, cardiovascular risk and protective effects of pioglitazone remain controversial.

Objectives: In this study, we investigated whether pioglitazone affects cardiomyocyte apoptosis and hypertrophy by regulating the VEGFR-2 signaling pathway.

Methods: Cardiomyocytes were enzymatically isolated from 1- to 3-day-old Sprague-Dawley rat ventricles. Effects of pioglitazone and the VEGFR-2-selective inhibitor apatinib on cardiomyocyte apoptotic rate was determined using flow cytometry, and hypertrophy was evaluated using [3H]-leucine incorporation. The protein expressions of unphosphorylated and phosphorylated VEGFR-2, Akt, P53, and mTOR were determined by Western-Blotting. Analysis of variance (ANOVA) was used to assess the differences between groups.

Results: Pioglitazone and VEGFR-2-selective inhibitor apatinib reduced rat cardiomyocyte viability and cardiomyocyte hypertrophy induced by angiotensin II in vitro. Furthermore, in the same in vitro model, pioglitazone and apatinib significantly increased the expression of Bax and phosphorylated P53 and decreased the expression of phosphorylated VEGFR-2, Akt, and mTOR, which promote cardiomyocyte hypertrophy.

Conclusions: These findings indicate that pioglitazone induces cardiomyocyte apoptosis and inhibits cardiomyocyte hypertrophy by modulating the VEGFR-2 signaling pathway. (Arq Bras Cardiol. 2018; 111(2):162-169)

Keywords: Apoptosis; Myocytes, Cardiac; Cardiomegaly; Heart Failure/physiopathology; Antihypertensive Agents; Thiazolidinediones; Insulin Resistance

Introduction

Heart failure (HF) is the most common consequence of cardiovascular diseases and the leading cause of cardiovascular mortality worldwide.1 Basic pathophysiology of HF is cardiac remodeling, which involves a number of cellular changes including cardiomyocyte hypertrophy, loss of cardiomyocytes due to apoptosis, necrosis, fibroblast proliferation and fibrosis.2 Recent fundamental and clinical studies have demonstrated that diabetes mellitus (DM) drives cardiac remodeling, including myocardial hypertrophy and cardiomyocytes loss, via glucotoxicity and lipotoxicity, eventually resulting in HF.3 Intensive glucose control was shown to reduce the occurrence of major cardiovascular events including HF, but did not improve the overall survival rate in patients with type 2 DM, compared to patients receiving standard therapy.4 Thiazolidinediones, including pioglitazone, have been widely used as peroxisome proliferator-activated receptor (PPAR)-γ agonists and insulin-sensitizing agents for improving glycemic control in patients with type 2 DM. However, cardiovascular risks of pioglitazone remain controversial.

One view is that intensive glycemic control with pioglitazone or rosiglitazone increases the risk of HF (OR ≤ 2.1; 95% CI 1.08–4.08) based on meta-analyses of randomized clinical trials.4-6 Rosiglitazone was more likely to induce HF than pioglitazone.7 Additionally, animal experiments confirmed the increased risk of HF with pioglitazone treatment, as pioglitazone augmented cardiac damage in isoproterenol-induced HF rat model and induced rat ventricular hypertrophy in acute toxicity experiments.8,9 However, another point of view is that pioglitazone use does not significantly increase the risk of myocardial infarction or cardiac death, based on the PROspective pioglitAzone Clinical Trial In macroVascular Events (PROactive) data,10 and that pioglitazone can suppress overload-induced cardiac hypertrophy by inhibiting AKT/GSK3β and MAPK signaling pathways.11

Vascular endothelial growth factor receptors (VEGFR) are considered critical factors for cardiac hypertrophy and HF.
Three different subtypes, VEGFR-1, -2, and -3 have been described. Recent studies showed that VEGFR-1 and VEGFR-2 are essential for regression and induction of cardiomyocyte hypertrophy, respectively, whereas VEGFR-3 was shown to be beneficial for the infarcted myocardium by promoting compensatory cardiomyocyte hypertrophy and improving survival. Additionally, VEGFR-2 is involved in the delayed phase of endothelial cell (pulmonary artery and human aortic endothelial cells) barrier dysfunction caused by high levels of 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphatidylcholine oxidation products, contributes to stress fiber formation, and increases phosphorylation of myosin light chains. Pioglitazone decreased the expression of VEGFR-2 in splanchnic tissues and inhibited neoangiogenesis in a rat model of portal hypertension, indicating a possible direct effect on VEGFR-2 expression. Reverse screening approaches (reverse pharmacophore mapping and reverse docking) have been very important methods to discover new cardiovascular disease-related protein targets for pioglitazone. In this study, we used the PharmMapper for reverse pharmacophore mapping. The structure of pioglitazone in mol2 format was submitted to PharmMapper, obtaining 10 targets and poses, which were sorted by decreasing PharmMapper fit score. The top ten PharmMapper fit scores for potential pioglitazone targets showed that VEGFR-2 was the best-ranked potential target, which will be essential for understanding the interaction between pioglitazone and VEGFR-2.

Given the potential link between pioglitazone and VEGFR-2 and their function in cardiomyocyte hypertrophy and apoptosis in the pathophysiology of HF, we investigated, for the first time, whether pioglitazone affects cardiomyocyte hypertrophy and apoptosis by regulating the VEGFR-2 signaling pathway. Furthermore, it may be expected to explore a promising approach for clarifying the potential mechanism for the effect of pioglitazone on cardiovascular outcomes.

Methods

Ethics Statement

All animal experiments were approved by the Institutional Animal Care and Use Committee of the First Hospital of Nanping City.

Molecule preparation

In order to characterize the binding sites in the predicted protein targets we used Genetic Optimization for Ligand Docking (GOLD) suite v5.3. VEGFR-2-inhibitor complex crystallographic structure (Protein Data Bank ID: 3CP9) was selected as the starting structure for predicting the binding site of pioglitazone at VEGFR-2. GOLD uses a genetic algorithm for docking ligands into the binding site of target proteins, with full conformational flexibility of the ligand and partial receptor flexibility. Ligand binding energy was predicted with the ChemScore scoring function and free energy of binding (ΔG) as implemented in GOLD. Solvent molecules were removed from the crystal structure and protein hydrogen atoms were added. Ligand binding site for docking was defined to include amino acids within 10 Å of the coordinates of the inhibitor in the crystal structure. Top 10 docking poses were obtained by terminating the simulation once root mean square deviation (RMSD) between any five ligand poses was reached < 1 Å. PyMol v.1.3 and LigPlot+ v.1.4 were used to visualize the results.

Isolation and culture of rat neonatal cardiomyocytes

Cardiomyocytes were enzymatically isolated from 1- to 3-day-old Sprague-Dawley rat ventricles as previously described. A total of twelve rats were used to perform twelve independent experiments in the study. Isolated cardiomyocytes were seeded onto cell culture plates precoated with 10 g/ml of fibronectin and cultured in a medium containing DMEMF-12 with HEPES (Invitrogen, Carlsbad, CA, USA), 5% heat-inactivated horse serum, 100 U/ml penicillin, 10 μg/ml streptomycin, 3 mM pyruvic acid, 2 mg/ml bovine serum albumin, 100 g/ml ampicillin, insulin-transferrin-sodium selenite media supplement (Sigma, St. Louis, MO, USA), 5 g/ml linoleic acid, and 100 M ascorbic acid at 37°C in a humidified atmosphere containing 5% CO₂. For all experiments, cells were cultured at 5 × 10⁴ cells/cm² unless otherwise stated.

Cell proliferation assay

Effects of pioglitazone and the VEGFR-2-selective inhibitor apatinib (Apxebio Technology LLC, Houston, TX, USA) on cardiomyocyte proliferation were measured by counting crystal violet-stained cells 24 h after treatment using an automated cell counter (BioRad). Briefly, cardiomyocytes (5 × 10⁴ cells/well) were seeded in 96-well plates and cultured in standard medium for 24 h. After 24 h serum starvation, cardiomyocytes were treated with 0.1 μM angiotensin (Ang) II for 24 h. Pioglitazone (0, 10 or 20μM) and apatinib (2 μM) was added to the culture medium 2 h prior to Ang II administration. For crystal violet staining, the cells were washed twice with 1× phosphate-buffered saline, fixed with 20% methanol for 30 min, and stained with 0.2% crystal violet solution for 14 min. Stained cells were washed with water until a clear background was visible. Crystal violet dye was extracted using 1% SDS and the cells were counted using an automated cell counter.

Detection of apoptosis by flow cytometry

Cardiomyocyte apoptotic rate was determined using flow cytometry with the annexin V-FITC (AV)/propidium iodide (PI) dual staining. Briefly, after treatment, the cells (1-5 × 10⁴/ml) were collected, washed twice with phosphate-buffered saline, and resuspended in 500 μl of binding buffer. Next, the cells were incubated with 10 μl AV and 5 μl PI in the dark at room temperature for 15 min. The apoptotic cells were identified by FCM within 30 min.

Cardiomyocyte hypertrophy

Cardiomyocytes were seeded at a density of 5 × 10⁴ cells/well in 96-well plates and hypertrophy was evaluated using [³H]-leucine incorporation, as previously described. Briefly, 2 h after treatment with pioglitazone (0-20 μmol/l) and apatinib (2 μM), 0.1 μM Ang II was used to stimulate cardiomyocyte hypertrophy and 1 μCi [³H]-leucine was simultaneously added.
to each well. After stimulation with Ang II for 60 h, cells were harvested by precipitation with 10% trichloroacetic acid on ice for 30 min before being solubilized with 1 mol/l NaOH overnight at 4°C. The samples were neutralized with 1 mol/l HCl and [3H] levels were determined in scintillation fluid using a β counter to assess [3H]-leucine incorporation.

**Western Blot Analysis**

Trypsinized cells were lysed in radioimmunoprecipitation assay buffer, homogenized on ice, and centrifuged. Supernatants were resolved via 10% SDS-PAGE and transferred to PVDF membranes (Millipore). The membranes were blocked with Tris-buffered saline (TBS) containing 5% non-fat milk and incubated with the following primary antibodies at 4°C overnight: VEGFR-2 (ab39256; 1:1000; Abcam), phospho-VEGFR-2 (Tyr1175) (#2478; 1:1000; Cell Signaling Technology), Akt (#9272; 1:1000; Cell Signaling Technology), phospho-Akt (Thr308) (#9275; 1:1000; Cell Signaling Technology), P53 (#9282; 1:1000; Cell Signaling Technology), phospho-P53 (Ser15) (#9284; 1:1000; Cell Signaling Technology), Bax (#14796; 1:1000; Cell Signaling Technology), rabbit anti-phosphorylated-mTOR (#2972; 1:1000; Cell Signaling Technology), rabbit anti-phosphorylated-mTOR (Ser2448) (#5536; 1:1000; Cell Signaling Technology), and GAPDH (#2118, 1:1000; Cell Signaling Technology). On the following day, the membranes were washed three times with tris-buffered saline with tween (TBST) for 5 min at room temperature and subsequently incubated with anti-rabbit IgG secondary antibodies, for 1 h at room temperature. Following incubation, the membranes were washed with TBST and exposed to an X-ray film. Band intensities on the film were analyzed by densitometry, and the results were normalized to β-actin. GAPDH was used as the protein loading control.

**Statistical analysis**

Statistical analysis was performed using the SPSS 13.0 software package. Kolmogorov Smirnov test was used to verify the normality of data distribution and Levene test was used to inspect the homogeneity of variance. All data satisfied normal distribution and homogeneity of variance were presented as mean ± SD. One-way ANOVA was used for comparisons between multiple groups, whereas post-hoc Bonferroni test was used for pairwise comparisons. P < 0.05 indicated statistical significance.

**Results**

**VEGFR-2 was the optimal potential target for pioglitazone**

Top ten PharmMapper fit scores of potential pioglitazone targets shown VEGFR-2 was the best-ranked potential target. To understand the interaction between pioglitazone and VEGFR-2 and assess ligand binding energy, we performed a docking study using GOLD. Optimal binding conformation of the pioglitazone-VEGFR-2 complex is presented in Figure 1A and 1B. ChemScore score and binding energy of pioglitazone-VEGFR-2 complex were comparable to the VEGFR-2-inhibitor complex crystallographic structure. Pioglitazone was predicted to form van der Waals interactions with Val363, Leu428, Cys454, Leu444, Leu310, Phe456, Gly387, Phe383, Val364, and Ile453 and bind to Cys384 and Asp455 with hydrogen bonds. Predicted hydrogen bonds are shown in Fig. 1B. Western blot was conducted to examine the effect of pioglitazone on expression of VEGFR-2 and phospho-VEGFR-2 in rat neonatal cardiomyocytes under hypertrophic stimuli. Pioglitazone treatment decreased VEGFR-2 phosphorylation in rat neonatal cardiomyocytes in a dose-dependent manner (Figure 1C).

**Pioglitazone promoted cardiomyocyte apoptosis and inhibited cardiomyocyte hypertrophy induced by Ang II**

To validate the effects of pioglitazone, cardiomyocyte viability and Ang II-induced cardiomyocyte hypertrophy were evaluated. Crystal violet staining, a quick and versatile assay for screening cell viability under diverse stimulation conditions, was used to analyze cell viability. Pioglitazone inhibited cardiomyocyte viability in a dose-dependent manner (Figure 2A and 2B, p < 0.01), with effective concentrations ranging from 0 to 20 μmol/l. Apatinib also inhibited cardiomyocyte viability (Figure 2A and 2B, p < 0.01). Cardiomyocyte apoptotic rate was determined using FCM with AV/PI staining. Apoptotic rates in pioglitazone 20 μM, pioglitazone 10 μM, and apatinib 2 μM groups were significantly higher compared to control (Figure 2C and 2D, p < 0.01), with apoptotic rate in the pioglitazone 10 μM group significantly lower than rates in pioglitazone 20 μM and apatinib 2 μM groups (Figure 2C and 2D, p < 0.01). Ang II-induced [3H]-leucine incorporation was significantly decreased after pioglitazone or apatinib treatment, indicating that both inhibited cardiomyocyte hypertrophy.

**Pioglitazone inhibited cardiomyocyte hypertrophy and promoted cardiomyocyte apoptosis by suppressing VEGFR-2 signaling**

Potential mechanisms of pioglitazone-induced inhibition of cardiomyocyte hypertrophy and promotion of cardiomyocyte apoptosis were assessed in vitro. Compared to cardiomyocytes under control conditions, pioglitazone significantly increased the expression of Bax and phospho-P53 and decreased the expression of phospho-VEGFR-2 in neonatal rat cardiomyocytes under hypertrophic stimuli (Figure 1C, Figure 3).

To further investigate whether pioglitazone targets VEGFR-2, effects of pioglitazone on the VEGFR-2 expression and VEGFR-2-regulated intracellular signaling were determined in neonatal rat cardiomyocytes, under hypertrophy induced by Ang II. Compared to untreated hypertrophic cardiomyocytes, pioglitazone significantly decreased the expression of phospho-VEGFR-2, phospho-Akt, and phospho-mTOR, which may contribute to cardiomyocyte hypertrophy. Likewise, apatinib significantly decreased the expression of VEGFR-2, phospho-VEGFR-2, phospho-Akt, and phospho-mTOR (Figure 1C, Figure 3).

**Discussion**

In the present study, we demonstrated that pioglitazone reduced cardiomyocyte viability and hypertrophy induced by Ang II in vitro. We further show that pioglitazone increased the expression of Bax and phosphorylated P53, and decreased the...
expression of phosphorylated VEGFR-2, Akt, and mTOR in vitro. These findings suggest that pioglitazone induces cardiomyocyte apoptosis and inhibits cardiomyocyte hypertrophy through effects on the VEGFR-2 signaling pathway.

Pioglitazone has been widely used to improve glycemic control in patients with type 2 DM. Additionally, the PROactive trial showed that pioglitazone reduced the main secondary composite outcome of cardiovascular death/myocardial infarction/stroke vs. placebo by 43 % in the trial population. These findings indicate that pioglitazone improves vascular function in diabetic patients and non-diabetic patients with insulin resistance and suggesting a possible beneficial effect of pioglitazone treatment on cardiovascular prognosis. Furthermore, a meta-analysis showed that supplementing insulin treatment with pioglitazone in type 2 DM patients with poorly controlled glucose levels could help decrease glucose levels and reduce the daily insulin dose without increasing the risks of myocardial infarction, HF, cardiac death and all-cause death, but at the cost of increasing total cholesterol levels and risks of hypoglycemia and edema.

Given available evidence, pioglitazone treatment appears advantageous in patients with HF. However, pioglitazone was also reported to increase the risk of hospitalization for HF over a 30-day period, even though patients at high risk of HF were unlikely to be prescribed the drug. Furthermore, clinical studies indicated that low dose pioglitazone treatment does not reduce the rate of in-stent restenosis, neointima volume nor atheroma volume in DM patients who have undergone percutaneous coronary intervention with drug-eluting stents. Beyond different methodologies applied in the discussed studies, reasons for contradictory reports on effects of pioglitazone on HF remain unclear. Investigating cardiovascular targets of pioglitazone is a promising approach for clarifying the effect of the drug on cardiovascular outcomes.

Effects of pioglitazone on the cardiovascular system have been previously reported. Pioglitazone attenuated monocrotaline-induced rat right ventricular hypertrophy and fibrosis and decreased cardiomyocyte size. Pioglitazone (2.5 mg/kg) ameliorated systolic and diastolic cardiac dysfunction in a rat model of Ang II-induced...
**Figure 2** – Pioglitazone and apatinib induced apoptosis and inhibited hypertrophy of rat neonatal cardiomyocytes (n = 12 in each group). A, Crystal violet staining of rat neonatal cardiomyocytes in response to various concentrations of pioglitazone or apatinib. Both inhibited the viability of rat neonatal cardiomyocytes. B, Cell proliferation was determined using the automated cell counter. C and D, Pioglitazone and apatinib induced neonatal rat cardiomyocytes apoptosis, which was detected by flow cytometry with the annexin V (AV) / propidium iodide (PI) dual staining. Pioglitazone (10, 20μM) and Apatinib (2μM) increased the apoptosis of the cardiomyocytes compared with the control group. E, Angiotensin II-induced [3H]-leucine incorporation following various concentrations of Pioglitazone or Apatinib. All data represent the means ± SD Statistical comparison with control group: *p < 0.01 compared with controls; #p < 0.01 compared with pioglitazone 10 μM group, calculated by one-way ANOVA followed by the post hoc Bonferroni test for pairwise comparisons.

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**Original Article**

Furthermore, pioglitazone protected from Ang II-induced cardiomyocyte hypertrophy by inhibiting AKT/GSK3β and MAPK signaling pathways. However, pioglitazone (40 mg/kg) was observed to induce cardiac hypertrophy with increase in plasma volume, without compromising it effects on the metabolic switch in the heart and whole-body insulin sensitivity. These contradicting findings may be caused by differences in administered doses of pioglitazone, as treatment with pioglitazone at supratherapeutic doses was shown to induce cardiotoxicity. Chemical proteomics-based analysis of off-target binding profiles for pioglitazone suggested potential sources contributing to efficacy and cardiotoxicity: perturbations in mitochondrial function, cardiac ion channels, and disruption of the cardiac sympathetic signaling. In the present study, pioglitazone induced cardiomyocyte apoptosis and inhibits cardiomyocyte hypertrophy. We inferred that these findings indicate that pioglitazone treatment appears disadvantageous in patients with HF. It was reported increased number of apoptotic cells in the heart of spontaneously hypertensive rats, suggesting that apoptosis might be a mechanism involved in the reduction of myocyte mass that accompanies the transition from stable compensation to HF in this model. Furthermore, cardiac myocyte apoptosis is a more critical determinant during the transition from compensatory cardiac hypertrophy to HF. However, available studies on the mechanisms underlying possible cardiovascular risk effects of pioglitazone on cardiovascular risk factors have been conducted in vitro conditions and therefore, prospective cohort studies are needed to confirm these effects.

In this study, reverse screening approaches (reverse docking and reverse pharmacophore mapping) were used to predict potential cardiovascular disease-related protein targets of pioglitazone. Pioglitazone was shown to bind strongly to VEGFR-2, suggesting that cardiovascular effects of pioglitazone may be related to the regulation of angiogenesis, neointima formation, and atherosclerosis associated with VEGFR-2-participating pathways. VEGFR-2 is a tyrosine kinase receptor that dimerizes upon ligand binding and is activated by trans-phosphorylation. VEGFR-2 activation stimulates downstream signaling, including activation of the c-Raf/MEK/ERK and PI3K/Akt pathways leading to increased cell proliferation, migration, and survival. VEGFR-2 is a critical factor in hypertrophic growth of cardiomyocytes. We found that pioglitazone directly targeted VEGFR-2 and inhibited phospho-VEGFR-2 expression, suggesting that pioglitazone induces cardiomyocyte apoptosis and inhibits cardiomyocyte hypertrophy in neonatal rats by inhibiting VEGFR-2 signaling. Downstream PI3K/Akt signaling pathway...
Figure 3 – Pioglitazone and Apatinib regulate VEGFR-2 signaling in neonatal rat cardiomyocytes under hypertrophy induced by Angiotensin II (n = 12 in each group). A and B, Representative western blotting trace of phospho-VEGFR-2, VEGFR-2, phospho-mTOR, mTOR, phospho-Akt, Akt, Bax, phospho-P53 and P53 protein levels in rat neonatal cardiomyocytes under hypertrophic stimuli, and treated with pioglitazone (20 μM) or apatinib (2 μM) for 24 hours, and intensity of the above bands in A normalized to GAPDH. All data represent the means ± SD. *p < 0.01 compared with controls; #p < 0.01 compared with pioglitazone 20 μM group, calculated by one-way ANOVA followed by the post hoc Bonferroni test for pairwise comparisons.

also participates in survival and hypertrophy of these cells by inhibiting P53-dependent pathways and activating mTOR-dependent pathways, respectively. In this study, pioglitazone and VEGFR-2 inhibitor apatinib increased phospho-P53 and Bax expression in cardiomyocytes and decreased phospho-Akt and phospho-mTOR expression in hypertrophic cardiomyocytes, indicating the connection between pioglitazone and the VEGFR-2 signaling pathway.

Conclusion

In conclusion, these findings indicate that pioglitazone induces apoptosis and inhibits hypotrophy of cardiomyocytes in part by acting on the VEGFR-2 signaling pathway. These findings contribute to understanding cardiovascular risks of pioglitazone.

Author contributions

Conception and design of the research: Zhong W, Chen L; Acquisition of data: Zhong W, Jin W, Wu Y, Luo S, Liang M; Analysis and interpretation of the data and statistical analysis: Zhong W, Jin W, Xu S, Wu Y, Luo S, Liang M; Obtaining financing: Zhong W; Writing of the manuscript: Zhong W, Jin W, Liang M; Critical revision of the manuscript for intellectual content: Zhong W, Xu S, Liang M.

Potential Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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Study Association

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Ethics approval and consent to participate

This study was approved by the Ethics Committee on Animal Experiments of the First Hospital of Nanping City under the protocol number NP01371.
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