Research Article

Pioglitazone Attenuates Vascular Fibrosis in Spontaneously Hypertensive Rats

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1. Introduction

Vascular fibrosis, characterized by excessive deposition of extracellular matrix (ECM) (e.g., collagen and fibronectin), is a major complication of hypertension and diabetes [1]. Connective tissue growth factor (CTGF) is a potent profibrotic factor implicated in pathologic fibrosis processes [2, 3]. In the cardiovascular system, CTGF is overexpressed in atherosclerotic lesions [4] and arteries of hypertensive animals [5]. In vascular smooth muscle cells (VSMCs), CTGF is involved in cell proliferation, migration, and apoptosis [6, 7]. Moreover, angiotensin II (Ang II) increases the production of CTGF and ECM, so CTGF is an intracellular mediator of hypertension-induced vascular fibrosis [8].
**Figure 1:** Systolic blood pressure in WKY and SHR rats after 12 weeks of treatment. WKY, Wistar Kyoto rats; SHR, spontaneously hypertensive rats; PIO, SHRs treated with pioglitazone; HYZ, SHRs treated with hydralazine. Values are mean ± SD. *P < 0.05 versus WKY; #P < 0.05 versus SHR.

**Figure 2:** Masson’s trichrome staining for fibrosis and immunohistochemical staining for collagen III and quantification (n = 6 rats, each group). Scale bar: 50 mm. Data are mean ± SD of 24 measurements in 6 slides. *P < 0.05 versus WKY; #P < 0.05 versus SHR.
Figure 3: Effect of pioglitazone on collagen I, III and fibronectin mRNA expression. Data are relative to GAPDH expression and are mean ± SD of 3 experiments. *P < 0.05 versus WKY; #P < 0.05 versus SHR.

In this study, we observed the effects of pioglitazone on vascular fibrosis, and the expression of transforming growth factor β (TGF-β) and CTGF in spontaneously hypertensive rats (SHRs) to ascertain whether administering pioglitazone could attenuate vascular fibrosis with hypertension and to understand the underlying mechanisms.

2. Materials and Methods

Our study was approved by the Institutional Animal Care Committee of Xian Jiaotong University and was conducted in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (Publication no. 85-23, revised 1996).

The experiments involved 8-week-old male SHRs and age-matched male Wistar Kyoto rats (provided by the Shanghai SLAC Laboratory Animal Technique Corp.).

2.1. Reagents. Pioglitazone was from Takeda Pharmaceuticals (Japan). Polyclonal anti-rat CTGF and TGF-β antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibody against PPAR-γ was from Upstate Inc. (Chicago, IL, USA). DAB Horseradish Peroxidase Color Development Kit was from Beyotime (Suzhou, China), TRIzol and SuperScript III Platinum SYBR-Green Two-Step qRT-PCR kits were from Invitrogen (Carlsbad, CA, USA). DNA-free kit was from Ambion (Austin, TX, USA). Agarose gels were from Spanish Biochemicals (Pronadisa, Madrid). Reagents for enhanced chemiluminescence were from Pierce Corp. (Rockford, IL, USA).

2.2. Protocol. Normal WKY rats were the normal control group (n = 8). SHRs were randomly divided into 3 groups (n = 8 each) for treatment by oral gavage for 12 weeks: pioglitazone, 10 mg/kg/day [15]; hydralazine, 25 mg/kg/day; saline control. All rats were housed under similar conditions with a 12 hr light/dark cycle at 21 ± 1°C and humidity 55 ± 5%. Rats had free access to an ordinary diet and water.

Systolic blood pressure (SBP) was measured biweekly in conscious rats by tail-cuff plethysmography. Body weight was recorded every week throughout the study period.

At 12 weeks, rats were killed by intra-aortic administration of 10% potassium chloride and an excess amount of pentobarbital. One portion of the aorta was dissected and cleaned of fat and then frozen in liquid nitrogen for RNA extraction, and another portion was fixed in 4% formaldehyde solution, embedded in paraffin, and cut into sections, 4-5 μm each, and, underwent trichromic Masson's trichrome and immunohistochemistry staining.

2.3. Real-Time RT-PCR. Total RNA was extracted by the use of TRIzol reagent, and DNA was removed by the use of the DNA-free kit. Real-time qRT-PCR with SYBR involved the use of the SuperScript III Platinum Two-Step qRT-PCR Kit on an ABI PRISM 7000 sequence detection PCR system (Applied Biosystems, Foster City, CA, USA).
2.4. Western Blot Analysis. Protein samples (20 μg) were resolved on 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes in a semidry system (Bio-Rad, Hercules, CA), and incubated with antibodies against CTGF (1:500), TGF-β (1:500), and β-actin (1:2000). Signals were revealed by chemiluminescence and visualized by exposure to X-ray films. Optical density was quantified with use of the Gel Doc 2000 system (Bio-Rad).

2.5. Immunohistochemistry. Paraffin-embedded rat thoracic aorta sections were incubated with primary antibodies against PPAR-γ (1:300) and collagen type III (Col III) (1:250) overnight at 4 °C and then biotinylated and affinity-purified IgG (Zymed, USA) secondary antibody for 1 hr at 37 °C. A streptavidin-enzyme conjugate was sequentially added for 20 min, and samples were incubated with substrate 3′,3′-diaminobenzidine (DAB), and then they underwent haematoxylin counterstaining. Negative control had no primary antibody. Quantitative analyses were using Qwin 550 quantitative image analysis system (Leica, German) by measuring the gray scale.

2.6. Statistical Analysis. Results are expressed as mean ± SD. Statistical significance of groups was assessed by one-way ANOVA, followed by post hoc Duncan multiple comparisons, with the use of SPSS v13.0 (SPSS Inc., Chicago, IL). A P < 0.05 was considered statistically significant.

3. Results

3.1. SBP in Rats. At baseline and after treatment, SBP was higher in the SHR treatment group than in WKY rats (P < 0.05) (Figure 1). After treatment, SBP was significantly lower with pioglitazone and hydralazine treatment than in the SHR alone group (P < 0.05); the groups did not differ in body weight or heart rate at baseline or after treatment (data not shown).

3.2. Pioglitazone Attenuated ECM Expression in Thoracic Aorta. We then evaluated whether pioglitazone attenuated vascular fibrosis in SHRs. SHRs showed significant ECM deposition in thoracic aortas on Masson’s trichrome and immunohistochemical staining of Col III (Figure 2). Real-time RT-PCR revealed increased fibronectin (FN), collagen I (Col I), and Col III mRNA expression in the aortic tissue of SHRs, which was significantly inhibited by pioglitazone treatment (Figure 3). These results indicated that pioglitazone but not hydralazine attenuated ECM deposition as compared with SHRs alone.

3.3. Vascular PPAR-γ Expression in Thoracic Aorta. Pioglitazone treatment increased PPAR-γ protein expression as seen on immunohistochemistry (Figure 4) and was mainly located in nuclei of VSMCs, which indicates that PPAR-γ activation by pioglitazone may be involved in the suppression of ECM expression in vivo. Hydralazine had little effect on PPAR-γ expression.

3.4. Effect of Pioglitazone on CTGF and TGF-β Expression in Thoracic Aorta. Real-time RT-PCR revealed increased CTGF
mRNA expression in the aortic tissue of SHRs, which was significantly inhibited by pioglitazone treatment. Pioglitazone also attenuated CTGF protein expression (Figure 5(a)) but had no effect on TGF-β mRNA or protein expression in rat thoracic aortas (Figure 5(b)).

4. Discussion

Hypertension causes structural changes in the arteries (vascular remodeling) that involve alterations in cell growth, VSMC hypertrophy, and accumulation of ECM [19, 20]. Our data demonstrate that the PPAR-γ ligand pioglitazone can attenuate ECM production in the SHR aorta. Importantly, these effects are mediated in part by PPAR-γ activation likely through a TGF-β-independent pathway to inhibit CTGF expression. These findings provide novel evidence for the beneficial vascular effect of pioglitazone.

We previously showed that another PPAR-γ ligand, rosiglitazone, inhibited vascular fibrosis in Ang-II-infused rats [16]. In this study, we used SHRs, a typical rat model of hypertension, as a hypertension and vascular fibrosis model [10]. As expected, we found hypertension and vascular fibrosis in SHRs. Both pioglitazone and hydralazine treatment significantly lowered SBP, but only pioglitazone attenuated ECM production in the rat aorta. Pioglitazone
has antihypertensive effects on other hypertensive models and hypertensive patients [21–24]. The novel finding that this agent reduced vascular fibrosis in SHRs may be explained by a direct action of pioglitazone on the vessel wall. BP lowering does not appear to play a role, because hydralazine decreased BP without any effect on vascular fibrosis and CTGF or TGF-β expression. These in vivo data confirm and extend results from our previous in vitro studies in which PPAR-γ ligands were shown to down-regulate angiotensin-induced ECM production in VSMCs [16].

CTGF has been postulated to be involved in conditions involving overgrowth of connective tissue cells (e.g., systemic sclerosis, cancer, fibrotic conditions, and atherosclerosis). Our previous study confirmed that PPAR-γ ligand inhibited CTGF overexpression in response to Ang II in cultured VSMCs [16]. In the present study, treatment with pioglitazone for 12 weeks in SHRs significantly reduced vascular CTGF expression and markedly alleviated fibrosis infiltration. Thus, inhibiting vascular CTGF expression to inhibit vascular fibrosis may be a mechanism of the beneficial effects of pioglitazone on vascular fibrosis in SHRs.

In vascular fibrosis, TGF-β participates in regulating CTGF and ECM production [25]; importantly, the CTGF promoter has a TGF-β binding element [26], so we tested whether reduction of CTGF by pioglitazone was due to downregulated TGF-β expression. TGF-β expression in SHR aortas was not affected by pioglitazone, so pioglitazone attenuated CTGF expression by a TGF-β-independent mechanism. Some recent studies support our results. Pioglitazone inhibited CTGF and ECM production through the mTOR-4EBP1 pathway in cultured VSMCs [16]. In the present study, treatment with pioglitazone for 12 weeks in SHRs significantly reduced vascular CTGF expression and markedly alleviated fibrosis infiltration. Thus, inhibiting vascular CTGF expression to inhibit vascular fibrosis may be a mechanism of the beneficial effects of pioglitazone on vascular fibrosis in SHRs.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

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