Down-Regulation of the Expression of Angiotensin II Type 1 Receptor in Neonatal Rat Cardiac Fibroblast by Activation of PPARγ Signal Pathway

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Abstract

Peroxisome proliferator-activated receptor-gamma (PPARγ) is one of the hormone nuclear receptors. Recent data have shown that activation of PPARγ signal pathway has many positive effects on cardiovascular system. The goals of this study were to determine whether PPARγ activator affects cardiac fibrosis and the possible mechanisms. Cardiac fibroblasts (CFs) of SD neonate rats were used in the study. Cells were divided into 4 groups: I – control group; II – pioglitazone group (Piog – PPARγ agonist); III – angiotensin II (Ang II) group; and IV – Piog + Ang II group (Piog plus angiotensin II). mRNA and protein expression of collagen type I, III and angiotensin II type 1 receptor (AT1-R) were tested by reverse transcription - polymerase chain reaction and Western blotting. With the inhibition of actinomycin D, we investigated the impacts of Piog on the stability of AT1-RmRNA. Compared with group I, the mRNA and protein expression of collagen type I, III and AT1-R were up-regulated in group III ($P < 0.05$). However with the effects of Piog in group IV, the expressions mentioned above were attenuated significantly ($P < 0.05$). With the effects of actinomycin D, AT1-RmRNA was reduced at the same degree in control and Piog groups at the same time points. These results indicated that treatment with Piog can attenuate Ang II-induced collagen synthesis in CFs through down-regulation of the AT1-R expression. With the intervention of actinomycin D, we suggested that PPARγ agonist didn't affect the stability of AT1-RmRNA.

Key Words: PPARγ agonist, cardiac fibrosis, angiotensin II type 1 receptor, actinomycin D

Introduction

Peroxisome proliferator-activated receptors gamma (PPARγ) is a member of the nuclear hormone receptor super-family, and it's a ligand-activated transcriptional factor. Activated PPARγ forms heterodimer with retinoid X receptors, binds to specific DNA sequences [PPARγ response element (PPRE)], and activates target genes transcription and expression. The process leads to the production of specific proteins and enzymes and regulates activities of cells, such as preventing growth factor-induced proliferation and migration of cells (8), inhibiting cytokine-mediated cell proliferation (6) and tumor necrosis factor α expression at the transcriptional level (1). PPARγ ligands can improve insulin sensitivity and protect pancreatic β-cell. So its synthetic ligands (thiazolidinediones - TZDs) have been used as an antidiabetic agent. Recent data have shown that PPARγ ligands have many positive effects on cardiovascular system (10), but there are few studies investigating whether PPARγ ligands have impacts on cardiac fibrosis. The goals of this study were to assess whether PPARγ ligands could attenuate angiotensin II (Ang II) – induced fibrosis in cardiac fibroblast and the mechanisms.

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Materials and Methods

Cell Culture and Measurement of Viability

CFs were isolated from the ventricles of 1 to 3 day-old Sprague-Dawley (SD, WeiTongLiHua animal center, Beijing, PRC) rats and maintained as described previously (14). After digesting, the cells were cultured with CFs culture medium [DMEM (Gibco, Invitrogen Corporation,Carlsbad, CA, USA) supplemented with 15% FBS (fetal bovine serum, Gibco, Invitrogen Corporation, Carlsbad, CA, USA)] in 5% CO_2 and 95% humidified air at 37°C. When the cells achieved 80% confluence (after 72 h), trypsin (0.08%) was added for 1 to 2 min to detach the cells, and CFs culture medium was added to discontinue the digestion. After centrifugalization, the cells were seeded in new culture flasks until re-confluence (after 48 h). Passages between 2 and 4 were used for the experiment. For cell viability analysis, CFs cells were seeded at 1×10^4 / well in 96-well plate and cultured in CFs media. After 24 h, the media was discarded and the cells were cultured in media (100 μl - 2.5% FBS + DMEM) with Piog (5, 10, 20, 30 μM, Takeda Pharmaceutical Company Limited, Osaka, Japan) or Ang II (10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} M, Sigma, Saint Louis, MO, USA) for 24 h, respectively. The cells viability was evaluated by a colorimetric MTT {3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenylter-zolium bromide} assay. MTT (100 μl) solution was added to each well (n = 6/group, MTT 1 mg/ml), incubated for 4 h at 37°C. Then the media (containing MTT) was removed and 100 μl dimethyl sulfoxide (DMSO) was added. After slightly shaking, the absorbance at 490 nm of each well was read using a Microplate Reader (ELX808, BioTek Instrument Inc. Winooski, VT, USA).

Groups and Treatments

All the cells were grouped as follows. The concentration of Piog and Ang II were mainly based on previous studies (4, 12) and the results of MTT assay. I – control group: Passage cells were cultured only with 2.5% FBS and DMEM for 24 h; II – 2.5% FBS + DMEM + Piog (10 μM) for 24 h; III – A (Ang II A group) 2.5% FBS + DMEM + Ang II (10^{-6} M) for 24 h; III – B (Ang II B group) 2.5% FBS + DMEM + Ang II (10^{-7} M) for 24 h; IV – Piog (10 μM) pretreated the cells for 30 min, then the cells were cultured in Ang II (10^{-7} M) and Piog (10 μM) together for 24 h.

At the end, every group of cells was washed with PBS for 2 times and was digested with 0.25% trypsin for 1 to 2 min. All groups of cells were centrifuged at 1000 rpm for 5 min. Cells sediment was collected and trizol was added to extract total RNA. Total RNA (2 μg) was used to reverse transcribe cDNA at 37°C for 1 h.

Collagen Type I and III (CO I and III) mRNA Expression

CO I and III cDNA were amplified in every cell groups to examine the effects of Ang II or Piog on collagen gene expression in CFs. Internal standard was the oligonucleotide sequence of β-actin (SD rat, a 349 bp fragment). Amplification of CO I (a 282 bp fragment) cDNA used the following primers, forward: 5'-GACCAACAGACTGGCAACCTCAAGAAG-3'; reverse: 5'-GATTGGGATGG—AGGGAGTTTACACGAA-3'. For CO III (a 296 bp fragment), forward: 5'-AGA-GCGGAGAATACTGGGTT-3'; reverse: 5'-GTGGTATGT AAT GTT CTGGGAG-3'.

Angiotensin II Type 1 Receptor (AT1-R) Expression

AT1-RcDNA was amplified in each group to assess the impacts of each stimulant factor on RAS in mRNA level. The forward and reverse primers were 5'-CACCCAATGAAGTCT CGC-3' and 5'-AAGGAAAGGGAACACGAA-3'. It's a 234 bp fragment. The RT-PCR reaction was carried out in a standard buffer with 300 ng of each primer, dNTPs (10 mM) 0.5 μl, and 1 u Taq polymerase for 30 cycles. PCR products were analyzed on a 2% agarose gel.

Western blot was performed as Makino (11) described previously to assess the expression of AT1-R protein in each group. The procedures mainly included: total protein extract, polyacrylamide gel electrophoresis to separate proteins, transfer to membranes, immunoreaction (Antibody: abcam, Cambridge, Cambridge, UK), and visualization.

Effects of Piog on AT1-RmRNA Stability

CFs from 1 to 3 day-old SD rats were divided into 2 groups: A – control group, and B – Piog group. After Piog preincubation for 30 min, actinomycin D (5 μg/ml, Sigma) was added to every group at the same time and the samples were collected at 4 time points: 0 h (immediately after pretreatment, A1 - B1 groups), incubation with actinomycin D – 6 h (A2 - B2 groups), 12 h (A3 - B3 groups), and 24 h (A4 - B4 groups). RT-PCR was used to determine AT1-RmRNA expression in each group at 4 time points.

Statistical Analysis

All values were expressed as means ± SD. Variance analysis (one-way ANOVA), S-N-K test, paired *t*-test, and two-sample *t*-test for independent sample were used to determine statistical differences among groups. All values were analyzed using SPSS11.5 software. Statistical differences were considered significant at a value of $P < 0.05$.

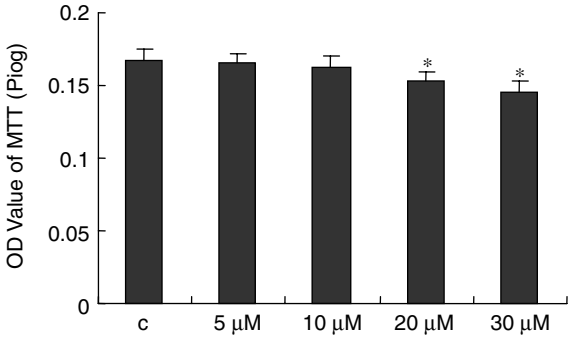

Fig. 1. OD value of MTT in each Piog group. The OD value of 20 μM and 30 μM groups had significant difference ($P < 0.05$) compared with control group. Piog (5 μM and 10 μM group) had less effect of CFs viability.

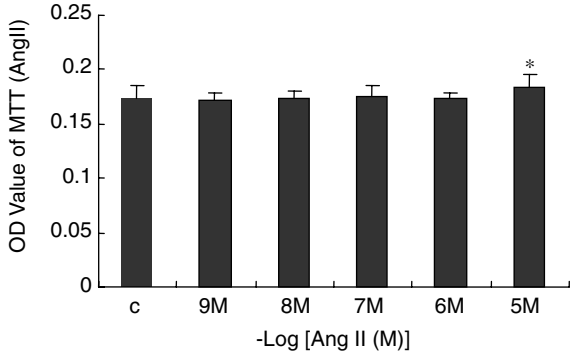

Fig. 2. OD value of MTT in each Ang II group. Treatment with Ang II (10^{-5} M) led to significant cell proliferation ($P < 0.05$). Exposure to Ang II (10^{-9} ~ 10^{-6} M group) didn't affect cell viability significantly.

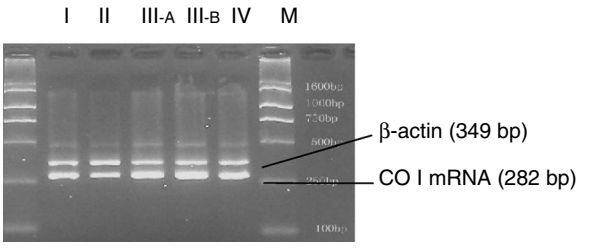

Fig. 3. Expression of CO I mRNA in each group. The expression of CO I mRNA was reduced in group II and increased in group III significantly ($P < 0.05$). Compared with group III - B, the expression in IV was also reduced significantly ($P < 0.05$).

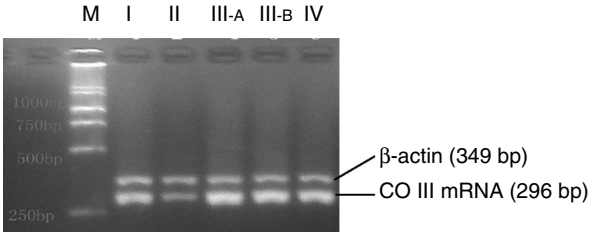

Fig. 4. Expression of CO III mRNA in each group. The expression of CO III mRNA was reduced in group II and increased in group III significantly ($P < 0.05$), but there is no significant difference between III - A and III - B ($P > 0.05$). Compared with group III - B, the expression in IV was also reduced significantly ($P < 0.05$).

Results

Cells Viability

Figures 1 and 2 showed the effects of Piog and Ang II on the viability of CFs. The OD values of each Piog group (from 5 μM to 30 μM) were 0.165 ± 0.007, 0.163 ± 0.008, 0.153 ± 0.006, and 0.145 ± 0.009, respectively. Of all the Piog groups, the OD value of 20 μM and 30 μM group had significant differences ($t = 3.145$, $P < 0.05$ and $t = 4.216$, $P < 0.05$) with that of the control group (OD 0.167 ± 0.008). So the exposure to 20 μM and 30 μM Piog led to a loss of cell viability.

The OD values of each Ang II group (from 10^{-9} M to 10^{-5} M) were 0.172 ± 0.007, 0.173 ± 0.008, 0.176 ± 0.010, 0.174 ± 0.005, and 0.184 ± 0.011, respectively. Of all the Ang II groups, the OD value of 10^{-5} M group had significant differences ($t = 2.473$, $P < 0.05$, n = 6) with that of the control group (OD 0.173 ± 0.012). So, treatment with Ang II (10^{-5} M) increased absorbance on 490 nm, which means there may be significant cell proliferation.

Based on the results and previous studies [4, 12], Piog (10 μM) and Ang II (10^{-6} M, 10^{-7} M) were selected to continue the study.

CO I and III mRNA Expressions

Compared with group I, CO I and CO III mRNA expressions were reduced in group II, and enhanced in group III. The differences were significant ($P < 0.05$). Compared with group III – B, CO I and CO III, mRNA expressions were depressed in group IV by Piog and the differences were significant ($P < 0.05$). The *F value* of all groups in CO I and CO III were $F(20, 4) = 6.83$, and $F(20, 4) = 7.01$, respectively. (Fig. 3, Fig. 4 and Table 1)

Compared with group III – B (Ang II 10^{-7} M), CO I and CO III mRNA expressions tended to be higher in group III – A (Ang II 10^{-6} M), but the differences were insignificant ($P > 0.05$).

Expression of AT1-RmRNA and Protein

Compared with group I, AT1-RmRNA and protein expressions were attenuated in group II and

Table 1. Expression of CO I mRNA and CO III mRNA in each group (means ± SD)

	I	II	III - A	III - B	IV
CO I/β-actin	0.421 ± 0.03	0.247 ± 0.05*	1.410 ± 0.100*	1.242 ± 0.080#	1.004 ± 0.100**
q value	--	*3.006*	*6.964*	*2.183*	*3.265*
CO III/β-actin	0.476 ± 0.04	0.279 ± 0.05*	1.593 ± 0.130*	1.404 ± 0.050#	1.135 ± 0.130**
q value	--	*3.132*	*5.481*	*1.974*	*3.126*

*The differences were significant comparing with group I ($P < 0.05$).
#There were no significant differences between III - A and III - B ($P > 0.05$).
**The differences were significant comparing with group III - B ($P < 0.05$).

Table 2. Expression of AT1-RmRNA and protein in each group (n = 5)

	I	II	III - B	IV
AT1-RmRNA/β-actin	0.245 ± 0.015	0.026 ± 0.010*	0.539 ± 0.017*	0.408 ± 0.021**
q value	--	*3.283*	*4.421*	*3.026*
AT1-R Protein	0.916 ± 0.163	0.702 ± 0.179*	2.257 ± 0.223*	1.673 ± 0.204**
q value	--	*3.011*	*6.208*	*3.164*

*The differences were significant comparing with group I ($P < 0.05$).
**The differences were significant comparing with group III - B (Ang II 10^{-7} M) ($P < 0.05$).

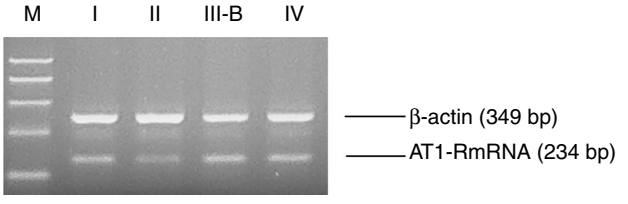

Fig. 5. Expression of AT1-RmRNA in each group. Compared with group III - B, the expression of AT1-RmRNA was significantly reduced in group IV ($P < 0.05$). Piog down regulated the expression of AT1-R in mRNA level.

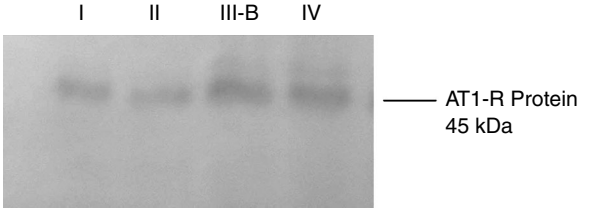

Fig. 6. Expression of AT1-R protein in each group (result of western blotting). Compared with group III - B, the expression of AT1-R protein was significantly reduced in group IV ($P < 0.05$). Piog down regulated the expression of AT1-R in protein level.

increased in III – B ($P < 0.05$). Compared with group III – B (Ang II 10^{-7} M), AT1-RmRNA and protein expressions were reduced significantly ($P < 0.05$) in group IV (Ang II 10^{-7} M + Piog 10 μM). The *F value* of all groups in expression of mRNA and protein were F(16, 3) = 8.37 and F(16, 3) = 7.41, respectively. (Fig. 5, Fig. 6 and Table 2)

In group III – A (Ang II 10^{-6} M), CO I and CO III mRNA expressions were higher than they were in III – B (Ang II 10^{-7} M), but the difference is not significant. So, one commonly used concentration (Ang II 10^{-7} M) was used to continue the study.

Assessment of AT1-RmRNA Stability

As time went by, AT1-RmRNA expression reduced both in control groups and Piog groups at 4 time points. But at each time point, AT1-RmRNA expression in the two groups doesn't have statistical difference ($P > 0.05$). It suggests that when Actinomycin D inhibits mRNA expression in transcription level, the decreases of AT1-RmRNA expression were the same in both groups. So Piog has no effects on stability of existed AT1-RmRNA. (Fig. 7, Fig. 8 and Table 3)

Discussion

PPARγ is an isoforms of PPARs family, and is involved in fatty acid metabolism, adipocyte differentiation, and inhibition of macrophage activation. As shown in recent data, activation of PPARγ signal pathway can block atherosclerosis procession, decrease blood pressure (3), inhibit left ventricular hypertrophy, and improve cardiac function after myocardial infarction (9). At the same time, PPARγ signal pathway has

Table 3. Expression of AT1-RmRNA in each group at 4 time points (n = 5)

	0 h	6 h	12 h	24 h
Control (A1 – 4 group)	0.443 ± 0.081	0.236 ± 0.047	0.142 ± 0.051	0.067 ± 0.033
Piog (B1 – 4 group)	0.421 ± 0.079	0.224 ± 0.055	0.129 ± 0.035	0.058 ± 0.029
t value	*0.611*	*0.579*	*0.962*	*1.016*

AT1-RmRNA expression in each group at the same time points had no statistical differences ($P > 0.05$).

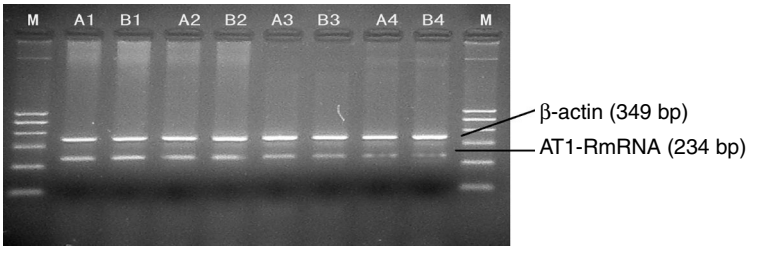

Fig. 7. With the effect of Actinomycin D, AT1-RmRNA expressed in each group at 4 time points. As time went by, AT1-RmRNA expression reduced step by step. However, at each time point, the reduction of AT1-RmRNA expression in both groups had no significant difference ($P > 0.05$).

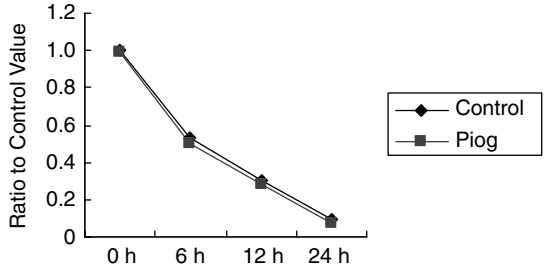

Fig. 8. The baseline was set to 1 at 0 h. AT1-RmRNA expression in each group at the same time points had no statistical differences ($P > 0.05$).

close relation with the generation and development of organ fibrosis. In previous studies, it can exert the effects of anti-inflammation, inhibition of NF-κB, oxidative stress, and inhibition of the expression of prefibrosis factors. It was found to block the progress of fibrosis in liver, kidney, lung, and pancreas (2, 13, and 16).

Cardiac fibrosis is characterized by the proliferation of CFs and abundant accumulation of matrix proteins (80% CO I and III) in the extracellular space. It may occur in hypertension, diabetes and ischemia heart disease, and is closely associated with diastolic dysfunction, heart failure and arrhythmia. Chen *et al.* (4) reported that pioglitazone can mediate oxidative stress and release transcription factor NF-κB, and it attenuates hydroxyproline synthesis and fibrosis in CFs. Diep *et al.* (5) proved that PPARγ agonist reduced inflammation, the expression of adhesion molecule and profibrosis factors, and it attenuated the accumulation of Collagen. In our studies, we found pioglitazone repressed Ang II-mediated Collagen mRNA expression and fibrosis in CFs.

It has been proved that Ang II can lead to cardiac fibrosis, and PPARγ agonist blocks the effects (4, 5, and 12). But how dose it work? In this study, we demonstrated PPARγ agonist reduced AT1-R both in mRNA and protein level in cultured CFs. Most of the cardiovascular effects of Ang II are mediated by AT1-R. Our data suggest that Ang II incubation up-regulated the expression of CO I and III mRNA and AT1-R in a model of dose-dependence. However, there is no statistical difference of expression in the concentration of Ang II 10^{-6} M and 10^{-7} M. Compared to treatment with Ang II alone, Ang II and Piog co-incubation significantly reduced the expression of AT1-R and Collagen mRNA. So, suppression of AT1-R by PPARγ agonist attenuated the effects of RAS in cardiac fibrosis.

Actinomycin D (ActD) is a cell cycle non-specific medicine and a conventional inhibitor of gene transcription. It was always used as a molecular tool to block target mRNA transcription in previous studies (7, 15), to assess whether stimulant factors affected target mRNA expression on transcription level. The molecular mechanism involved is that it can insert into DNA double strands, bind to guanine group, repress the activity of DNA-dependent RNA polymerase and block the mRNA synthesis. In this study, ActD was added to both control groups and Piog pretreated groups. As time went by, AT1-RmRNA expression reduced step by step, but at the same time point the expression didn't have statistical

significance in the two groups. In our study, we had demonstrated that Piog alone significantly reduced AT1-RmRNA expression in CFs when compared with control group (shown in Figure 5). However, when ActD was added and it inhibits mRNA in transcription level, AT1-RmRNA expression didn't show statistical significance in the two groups on the same time point. It suggests that Piog didn't affect the breakdown of AT1-RmRNA at co-incubation circumstance and it did not affect the stability of existed AT1-RmRNA.

Acknowledgments

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