Imatinib Attenuates Myocardial Fibrosis in Association with Inhibition of the PDGFRα Activity

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Abstract

Background: Imatinib is a tyrosine kinase receptor inhibitor that has been confirmed to exert inhibitory effect on the platelet derived growth factor PDGF receptor (PDGFRα and PDGFRβ) activity.

Objective: To investigate the protective effect of imatinib on the myocardial fibrosis in deoxycorticosterone-acetate (DOCA)/salt induced hypertensive rats.

Methods: Sixty male uninephrectomized Sprague-Dawley rats were assigned to three groups: control rats (CON group); deoxycorticosterone group (DOCA group); deoxycorticosterone and imatinib group (DOCA+IMA group). Systolic blood pressure (SBP) was measured biweekly. The apical portion of the left ventricle was studied. Sirius-Red staining, Hematoxylin-Eosin staining, immunohistochemistry and Western blot assay were employed.

Results: SBP in the DOCA group and DOCA+IMA group was higher than that in the CON group on day 14 and 28. Animals in the DOCA group showed severe interstitial and perivascular fibrosis on day 28, and the expressions of PI, PIII, tenascin-C and fibronectin were significantly higher than those in the DOCA+IMA group and CON group. When compared with the CON group, myocardial tissue inflammatory response and monocyte/macrophage infiltration of different degrees were observed in the DOCA group and DOCA+IMA group. Protein expressions of PDGF-A, PDGF-C and PDGFRα were significantly higher in the DOCA and DOCA+IMA groups than those in the CON group, but the p-PDGFRα protein expression in the DOCA+IMA group was lower than that in the DOCA group.

Conclusion: Imatinib can exert inhibitory effects on myocardial fibrosis in DOCA/salt induced hypertensive rats, which may be attributed to the inhibition of PDGFR-α activity. (Arq Bras Cardiol 2012;99(6):1082-1091)

Keywords: Receptors, Eph Family; Receptor, Platelet-Derived Growth Factor Alpha; Endomyocardial Fibrosis; Desoxycorticosterone.

Introduction

Myocardial fibrosis is a common clinical problem, can be found in several heart diseases and is often associated with inflammatory process. In the heart, excessive accumulation of extracellular matrix (ECM) components, especially the type I, III, and V collagens, is responsible for myocardial fibrosis. Changes in tissue structure and cardiac dysfunction following myocardial fibrosis contribute to heart failure, arrhythmia, sudden cardiac death and other serious cardiovascular events. The molecular mechanisms of myocardial fibrosis are complex, on which there are still controversies. Previous studies suggested that myocardial fibrosis is closely related to the increase of endogenous mineralocorticoid. However, the roles of some molecular signaling pathways in mineralocorticoid induced myocardial fibrosis remain unclear. Various cytokines and growth factors have been demonstrated to play crucial roles in the development of myocardial fibrosis. These factors include transforming growth factor ß (TGFß)[1], interleukin-1 (IL-1)[2], tumor necrosis factor α (TNFα)[3], and platelet-derived growth factor (PDGF)[4]. PDGF is a member of growth factor family and has been found to promote the division and proliferation of fibroblasts and smooth muscle cells. There is evidence showing that high protein expressions of PDGFs may lead to myocardial fibrosis, which suggests that PDGF-PDGFR signaling pathways may serve as important molecular mechanisms of myocardial fibrosis[5].

Imatinib is a tyrosine kinase receptor inhibitor that is mainly used in the treatment of tumor in clinic currently and has been confirmed to exert inhibitory effect on the PDGF receptor (PDGFRα and PDGFRβ) activity. PDGF-PDGFR signaling pathways are regulated by PDGFR[5] , and thus imatinib can modulate the cell biological response induced by the PDGF-PDGFR signaling pathways. In this study, the effects of imatinib on the myocardial inflammation and fibrogenic response, monocyte/macrophage infiltration,

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and the protein expressions of PDGF-A, PDGF-C, PDGFRα, p-PDGFRα, procollagen I (PI), procollagen III (PIII), tenascin-C and fibronectin were investigated in deoxycorticosterone-acetate (DOCA)/salt-induced hypertensive rats, aiming to examine whether imatinib can effectively attenuate myocardial fibrosis and explore the relationship between PDGFRα signaling pathway and myocardial fibrosis.

Materials and methods

Animal Model

Sixty male Sprague Dawley (SD) rats weighing 200 ~ 250 g were purchased from the Animal Experimental Center affiliated to Anhui Medical University and housed in specific pathogen free environment. Before experiment, animals were anesthetized by intraperitoneal injection of 10% chloral hydrate (400 mg·kg^{-1}) and right nephrectomy was performed. One week after surgery, rats were fed with water containing 1% NaCL and 0.2% KCL, and randomly divided in three groups (n=20 per group): 1) control group (CON group): distilled water was intragastrically administered daily, and subcutaneous injection of sesame oil was given once every 4 days; 2) deoxycorticosterone group (DOCA group): deoxycorticosterone (60 mg.kg^{-1}.4d^{-1}) was subcutaneously given, and distilled water intragastrically administered daily; 3) deoxycorticosterone plus imatinib group (DOCA + IMA group): deoxycorticosterone (60 mg.kg^{-1}.4d^{-1}) was subcutaneously given once every 4 days, and imatinib (60 mg.kg^{-1}.d^{-1}) intragastrically administered daily simultaneously. The volume of distilled water used in the control group was identical to the volume of distilled water containing drugs in the remaining 2 groups. In addition, the volume of sesame oil used in the control group was equivalent to the volume of sesame oil containing drugs in the other 2 groups. Treatment with drugs lasted for 4 weeks.

Main Reagents

Deoxycorticosterone, sesame oil (Sigma, U.S.), imatinib (Novartis, Switzerland), Sirius red dye (Beijing Hede Biocompany), hematoxylin, SP immunostaining kits (Beijing Goldenbridge Biotechnology CO., Ltd), ED-1 antibody (Beijing Biosynthesis Biotechnology CO., Ltd), PDGF-A, PDGF-C, PDGFRα, p-PDGFRα, PI, PIII, Fibronectin, Tenascin-C and β-actin primary antibodies and secondary antibodies (Santa Cruz Inc) and ECL kits (Pierce Inc) were used in the present study.

Systolic Blood Pressure Measurement and Sample Collection

The systolic blood pressure (SBP) was measured on 1d, 14d and 28d by using the tail-cuff method. On the 14th day and 28th day after drug intervention, animals (n=10) were intraperitoneally anesthetized with 10% chloral hydrate (400 mg·kg^{-1}) and then killed. The hearts were obtained, the bilateral atria, right ventricle, great vessels and connective tissues were removed and the apical portion of left ventricle was collected for further examinations. The samples collected on the 14th day were fixed in 4% paraformaldehyde, embedded in paraffin followed by sectioning and immunostaining. The samples obtained on the 28th day were divided into 2: one was fixed in 4% paraformaldehyde for HE staining and Sirius red collagen staining, and the other was stored at - 80 ºC for western blot assay.

Inflammatory Pathological Changes and Immunohistochemistry

Two pathologists who were blinded to the study evaluated the sections. Upon the 14th and 28th days, the apical portion of the left ventricles was subjected to H&E staining for the examination of cardiac inflammation. The infiltration of myocardial macrophages was observed by immunohistochemistry at 14 days after drug intervention. ED-1 is a kind of specific antigen on the surface of monocyte/macrophage. Immunohistochemistry was performed to detect the ED-1 expression in the heart according to manufacturer's instructions. Macrophages with brown membrane were regarded to be positive for ED-1. Ten fields in the positive region were randomly selected at a magnification of ×400. Image-Proplus 6.0 software was employed to count the number of positive cells followed by calculation of the number of macrophage per mm^2 visual field. Then, average was obtained for each section.

Detection of Myocardial Fibrosis and Vascular Remodeling

Myocardial collagen volume faction (CVF) and myocardial peripheral vessel collagen area (PVCA) were measured by picric acid-Sirius red staining on the 28th day. The calculation method was stated below: Nikon photographic system was used, Image-Proplus 6.0 software was employed for analysis. CVF was expressed as the ratio of myocardial collagen area to whole area of visual field. Eight fields (×400) were selected randomly and the corresponding CVFs were measured in each section, and the average was considered as the final CVF for each section. PVCA was expressed as the ratio of peripheral collagen area around the small artery-to-artery lumen area. Four small arteries (×400) were chosen randomly for detection in each section, and the average was considered as the final PVCA for the corresponding section.

Western Blot Assay

The protein expressions of PDGF-A, PDGF-C, PDGFRα, p-PDGFRα, PI, PIII, fibronectin and tenascin-C in the heart were detected by western blot assay on the 28th day after drug intervention. The total protein was extracted using cell lysis buffer(Main components: 20mM Tris (pH7.5), 150mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 1mM PMSF, 2mg/mL leupeptin, 1 mM EDTA, sodium pyrophosphate, β-glycerophosphate and Na_3VO_4), the supernatant containing proteins was collected by centrifugation, and the protein concentration was detected with BCA method. All the samples (One sample stand for one) were diluted into the same concentration. Three samples were randomly selected from each group and compared with those from the remaining two groups. Then, 50 μg of proteins were subjected to polyacrylamide gel electrophoresis,

transferred onto PVDF membrane, and blocked in skim milk overnight. Then, the membrane was incubated with primary antibody for 8~12 h and with secondary antibody for 2 h. Visualization was done with ECL kit. The bands were scanned with Bio-Rad imaging system, and Quantity-one software was employed for the detection of optical density. β-actin served as an internal reference. The optical density of target protein was normalized by that of β-actin representing the relative amount of target protein.

Statistical Analysis

SPSS version 13.0 software program was employed for statistical analysis. Data were presented as mean ± standard error (SEM). The means among multiple groups were compared with one-way analysis of variance (ANOVA). The ratios were compared with chi square test. A value of $P<0.05$ was considered statistically significant.

Results

Changes in SBP

There was no significant difference in SBP among groups prior to experiment. However, SBP in the DOCA and DOCA+IMA groups were significantly higher than that in the CON group on the 14th and 28th days. No statistical significance in the SBP was noted between DOCA group and DOCA+IMA group regarding levels (Table 1).

Myocardial Inflammation

H&E staining revealed evident infiltration of inflammatory cells and mild fibrosis in the heart on the 14th day in the DOCA group. A large amount of inflammatory cells infiltrated the heart in the DOCA+IMA group while fibrosis was absent. Twenty-eight days after drug intervention, myocardial cells were regularly arranged, and infiltration of inflammatory cells and severe fibrosis were noted in the DOCA group. In the DOCA+IMA group, myocardial cells were regularly arranged. Although there were inflammatory cells infiltrating the heart, fibrotic tissues were not found. The myocardial cells in the control group were normal throughout the experiment, and neither infiltrating inflammatory cells nor fibrotic tissues were found (Figure 1).

Infiltration of Monocytes/macrophages

The number of ED-1 positive cells reflects the extent of monocyte/macrophage infiltration. Immunohistochemistry for ED-1 showed that the number of ED-1 positive cells in

Table 1 – SBP in three groups

		Groups		
		CON	DOCA	DOCA+IMA
SBP (mm Hg)	Day 0	130 ± 2	130 ± 3	131 ± 2
	Day 14	129 ± 2	156 ± 6*	157 ± 5*
	Day 28	131 ± 2	198 ± 5**	195 ± 7**

*Data were expressed as means ± SEM. Chi square test was used. *p <0.05, **p <0.01 vs CON group. n=20 on days 0 and 14, n=10 on day 28.*

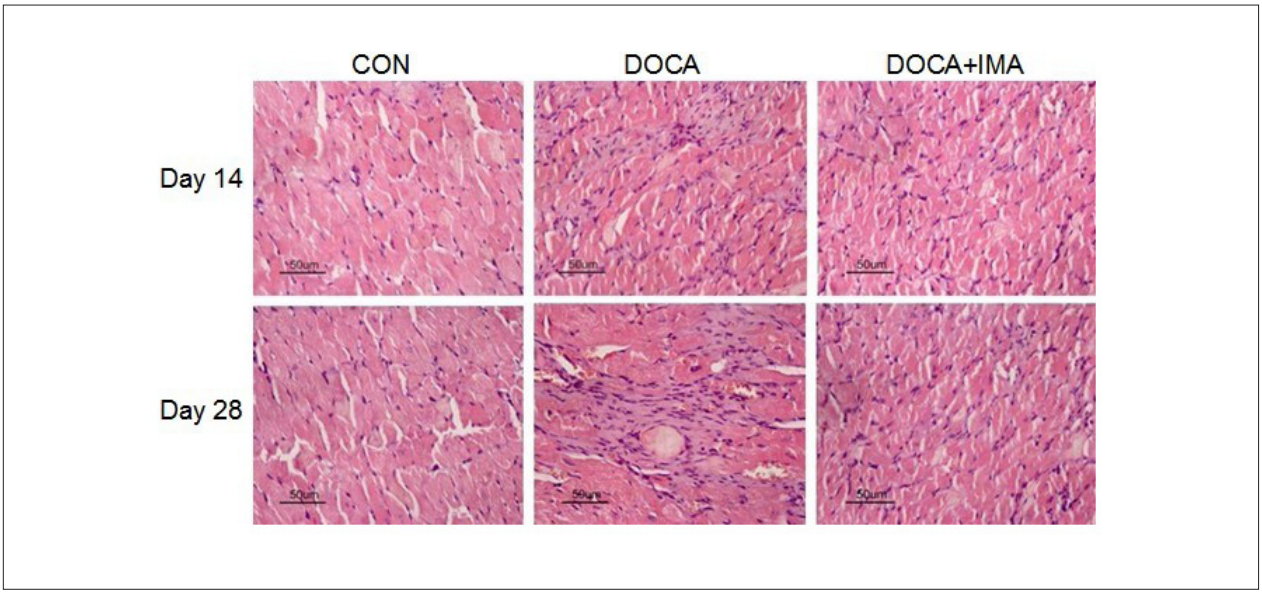

Figure 1 – *Myocardial inflammation and fibrotic scar in the two treatment groups on day 14 and day 28 compared with the CON group (HE staining, ×400)*

the DOCA group and DOCA+IMA group was significantly higher than that in the CON group (DOCA group: 6.3±0.4/mm^2; DOCA+IMA group: 6.0±0.3/mm^2; CON group: 1.1±0.2/mm^2 [Figure 2]).

Myocardial Fibrosis

At 28 days after intervention, Sirius red staining showed that the myocardial collagen became light red, and the remaining cardiac tissues presented yellow. Sirius red staining revealed that the myocardial fibrosis in the DOCA group was the most severe among all groups, characterized by the highest level of interstitial collagen. Additionally, the CVF and PVCA in the DOCA group were also significantly increased when compared with those in the CON group. The CVF and PVCA in the DOCA_IMA group were also higher than those in the CON group, whereas markedly lower than those in the DOCA group (Table 2, Figures 3 and 4).

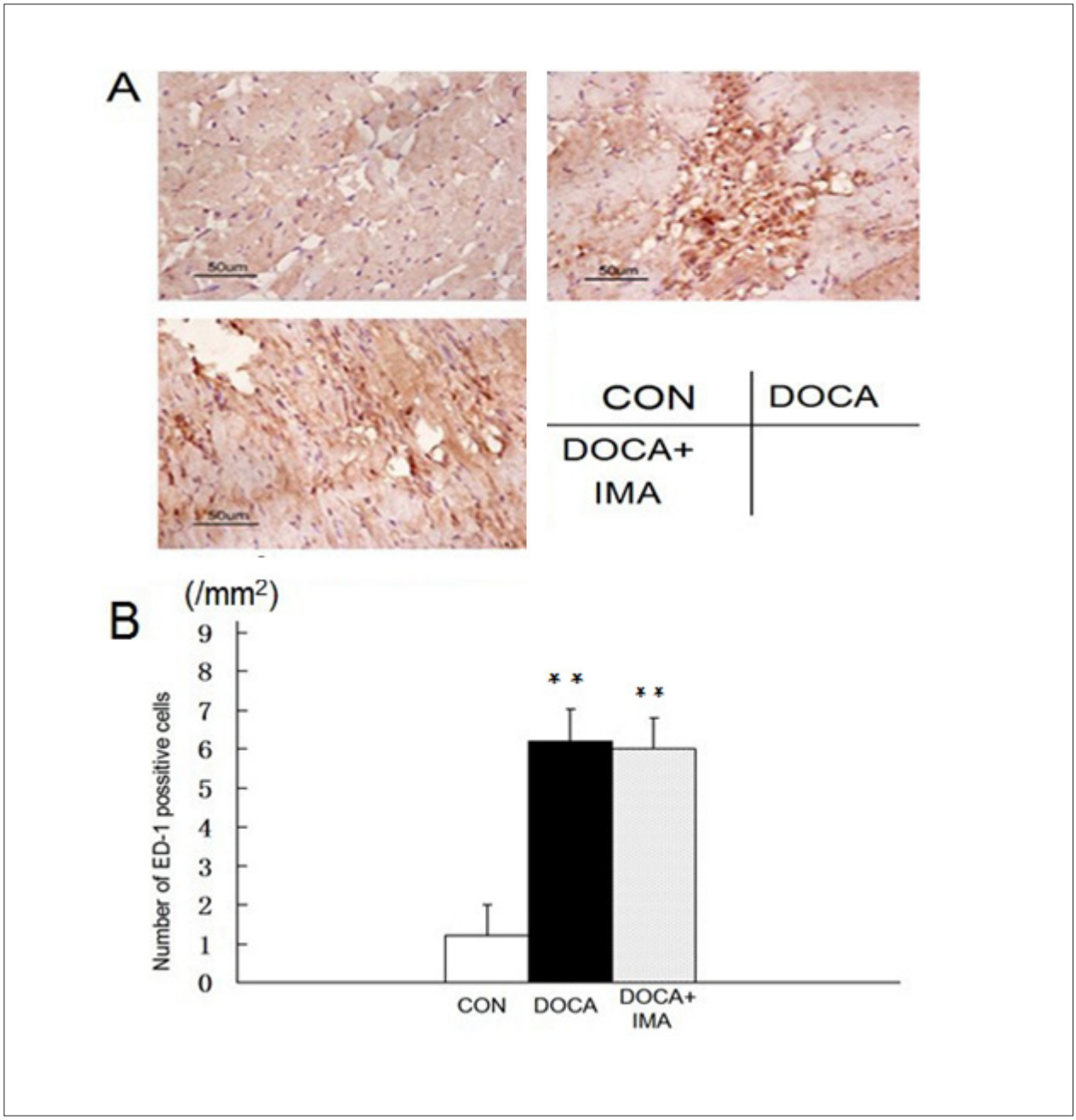

Figure 2 – *Number of ED-1 positive cells in different groups on day 14 (immunohistochemistry, ×400). Data were expressed as means ± SEM (n=10). Chi square test was used. **p<0.01 vs. CON group*

Table 2 – CVF and PVCA in different groups

	CVF (%)	PVCA (%)
CON	5.7 ± 1.2	19.5 ± 2.1
DOCA	28.5 ± 2.3**	39.6 ± 3.8**
DOCA+IMA	11.6 ± 1.5*#	25.4 ± 3.0*#

*Data were expressed as means ± SEM (n=10). Chi square test was used. *p<0.05, **p<0.01 vs CON group. #p<0.05 vs DOCA group.*

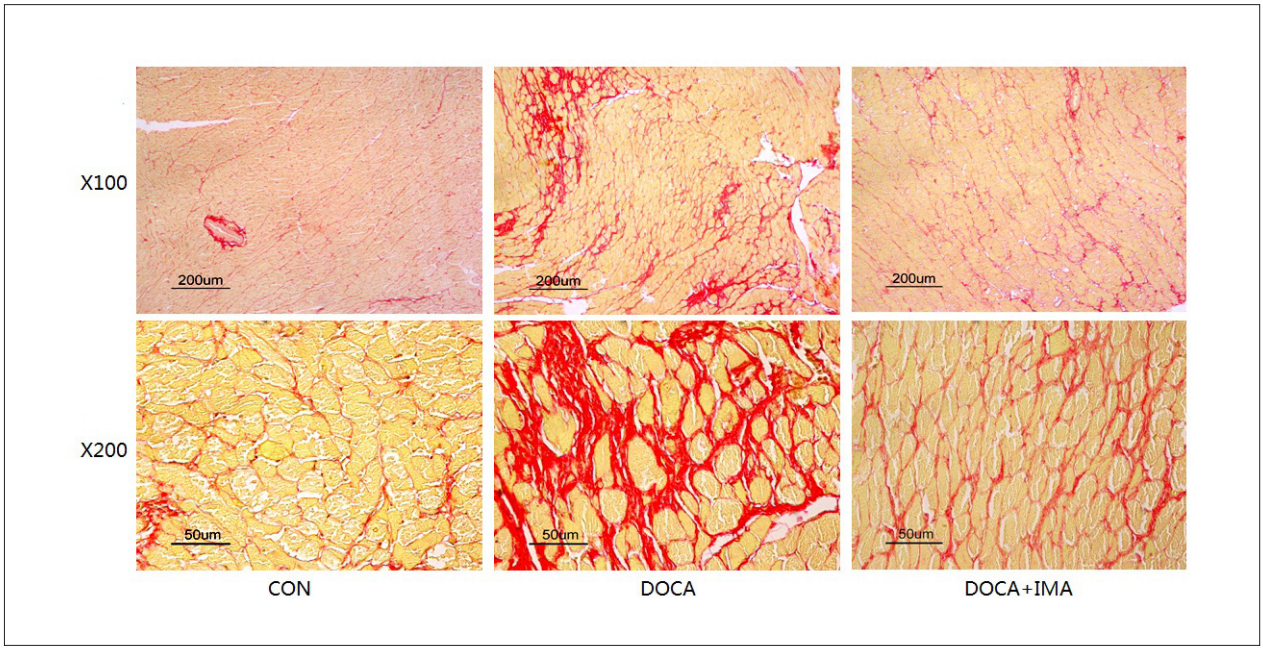

Figure 3 – *Myocardial fibrosis in the two treatment groups on day 28 compared with the CON group (Sirius-Red staining, ×100 and ×400)*

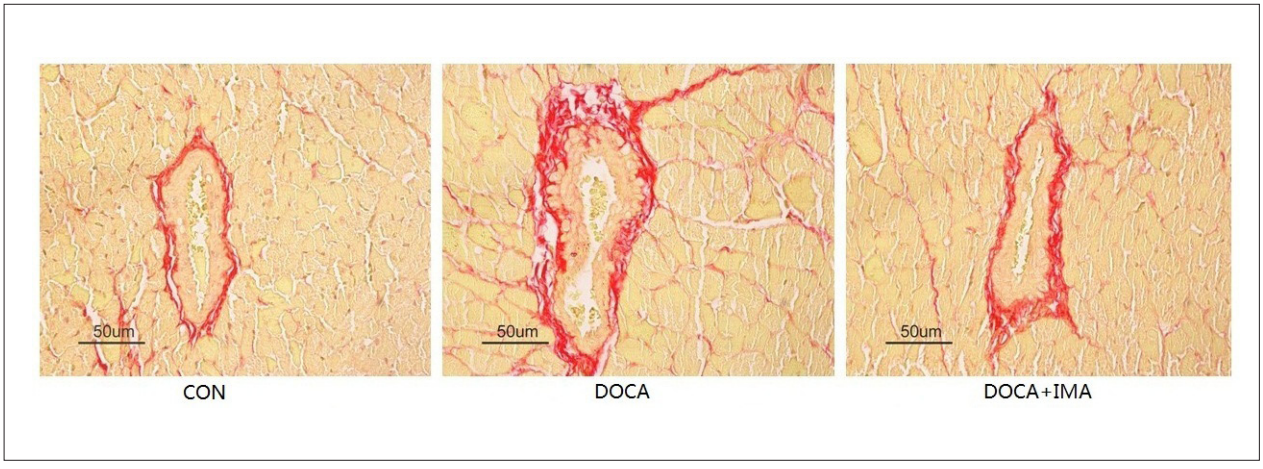

Figure 4 – *Perivascular fibrosis in the two treatment groups on day 28 compared with the CON group (Sirius-Red staining, ×400)*

Protein expressions of PDGF-A, PDGF-C, PDGFRα and p-PDGFRα

When compared with the CON group, the protein expressions of PDGF-A, PDGF-C and PDGFRα in the DOCA group and DOCA+IMA group were remarkably increased. The p-PDGFRα protein expression in the DOCA group was significantly elevated, but that in the DOCA+IMA group was markedly decreased as compared to the Con group. Western blot assay (Figure 5) showed that deoxycorticosterone-acetate treatment (DOCA group and DOCA +IMA group) significantly up-regulated the protein expressions of PDGF-A (0.61±0.12-fold and 0.59±0.09-fold, respectively, $p<0.01$) and PDGF-C (0.58±0.11-fold and 0.55±0.06-fold, respectively, $p<0.01$) when compared with the CON group (0.17±0.04-fold and 0.15±0.02-fold, respectively). The PDGFRα protein expression (Figure 5) was significantly increased in the DOCA group (0.41±0.08-fold, $p<0.01$) and DOCA+IMA group (0.43±0.10-fold, $p<0.01$) when compared with the CON group (0.12±0.03-fold). PDGFRα is a kind of tyrosine kinase receptor, which shows activity when intracellular tyrosine residue is phosphorylated, p-PDGFRα is the phosphorylated form of PDGFRα, and increased p-PDGFRα level suggested an increase in the activation of PDGF signaling pathway. Results showed that the DOCA group has the highest p-PDGFRα protein expression (0.38±0.06-fold, $p<0.01$) when compared with the CON group (0.08±0.02-fold) and the DOCA+IMA group (0.11±0.02-fold), and the p-PDGFRα protein expression in the DOCA+IMA group was higher than that in the CON group, but without significant difference (Figure 5).

Protein expressions of PI, PIII, fibronectin and tenascin-C

PI and PIII, the accurate and direct indexes of myocardial fibrosis, are the main components of collagen protein. The increased protein expressions of fibronectin and fenascin-C have also been found in severe fibrotic myocardium. In the present study, the protein expressions of PI, PIII, fibronectin and tenascin-C in the DOCA group were higher than those in the CON group and DOCA+IMA group. Western blot assay (Figure 6) showed that deoxycorticosterone-acetate treatment (DOCA group) significantly up-regulated the protein expressions of PI (2.33±0.21-fold, $p<0.01$) and PIII (1.81±0.16-fold, $p<0.05$) when compared with the CON group (0.55±0.11-fold and 0.42±0.08-fold, respectively) and the DOCA+IMA group (1.13±0.09-fold and 0.89±0.07-fold, respectively). In addition, the DOCA group had higher protein expressions of fibronectin (0.80±0.15-fold) and tenascin-C (0.31±0.05-fold) than the CON group (0.07±0.02-fold and 0.05±0.01-fold, respectively, $p<0.01$) and the DOCA+IMA group (0.10±0.03-fold [$p<0.01$] and 0.18±0.04-fold, [$p<0.05$], respectively) (Figure 6).

Discussion

In this study, our findings showed: 1) The DOCA-salt induced hypertensive rats presented apparent inflammation in the myocardial cells. More macrophages infiltrated in the myocardium, accompanied by over-expressions of PDGF-A, PDGF-C and PDGFRα. These findings suggest that the occurrence of myocardial fibrosis is closely associated with increased activation of PDGF signaling pathway. 2) When there was no difference in SBP before experiment, and the CVF and PVCA in the DOCA+IMA group were dramatically decreased when compared with those in the DOCA group, indicating that imatinib is able to relieve myocardial fibrosis independent of lowering SBP. 3) The p-PDGFRα protein expression was significantly declined in the DOCA+IMA group when compared with the DOCA group. As an inhibitor of tyrosine kinase receptor, imatinib can suppress the activity of PDGFRα. The inhibitory effect on myocardial fibrosis is possibly attributed to the compromised proliferation of fibroblasts following inhibition of PDGF signaling pathway. (4) Fibronectin and tenascin-C are regarded as the indexes of the degree of fibrosis. Our results showed the protein expressions of fibronectin and tenascin-C were proportionally related to the degree of cardiac muscular fibrosis.

Inflammation and Myocardial Fibrosis

Previous studies suggested that mineralocorticoid-induced myocardial fibrosis was accompanied by early-stage acute inflammation in the myocardium, suggesting that inflammation is an initial event in the myocardial fibrosis. In addition, chronic inflammation at the mid- and late-stage aggravates the fibrosis process[6-8], which had been confirmed in the present study. On the 14th and 28th days after intervention, HE staining revealed the infiltration of inflammatory cells in the heart of the DOCA group. The increased infiltration of mononuclear macrophages during inflammation is a major characteristic and key step in tissue fibrosis[9,10]. Moreover, multiple cytohormones and growth factors secreted by mononuclear cells and macrophages are critical for the myocardial fibrosis. Removal of mineralocorticoid receptors (MR) on the mononuclear cells and macrophages by knock-out technique or inflammatory effusion and mononuclear macrophage infiltration in drug-dependent manner can significantly alleviate the DOCA-induced myocardial fibrosis[8,11,12]. Besides, mineralocorticoid is able to regulate the differentiation and maturation of mononuclear cells and macrophages by activating MR on the mononuclear cells and macrophages. Thus, MR inhibitors, such as antisterone, are able to exert anti-inflammation and anti-myocardial fibrosis effects[13,14]. In this study, the DOCA-treated rats presented obvious myocardial inflammation and increased amount of infiltrated macrophages when compared with the control group. Therefore, it can be reasonably assumed an explicit relationship between mineralocorticoid-induced myocardial fibrosis and subsequent inflammation in the myocardium. The aggravated infiltration of interstitial macrophages is of significance towards the occurrence and development of myocardial fibrosis.

PDGF Signaling Pathways and Myocardial Fibrosis

PDGFs act as vital growth factors, are able to promote the division and proliferation of fibroblasts and vascular smooth muscle cells and play a chemotactic role. Recent

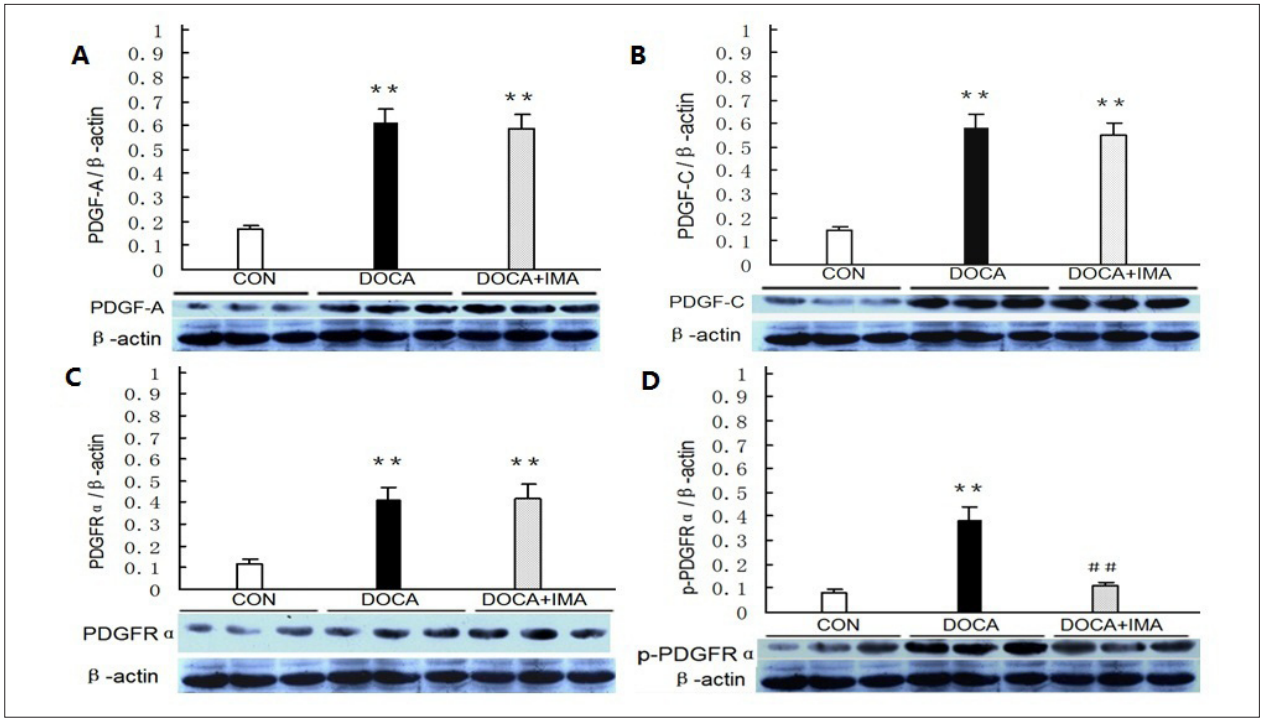

Figure 5 – *Expressions of PDGF-A (A), PDGF-C (B), PDGFRα (C), and p-PDGFRα (D) in different groups on day 28 (Western blot assay). Imatinib treatment significantly reduced the p-PDGFRα expression in the DOCA + IMA group. Data were expressed as means ± SEM (n=10). Chi square test was used. **p<0.01 vs CON group, ##p<0.01 vs DOCA group*

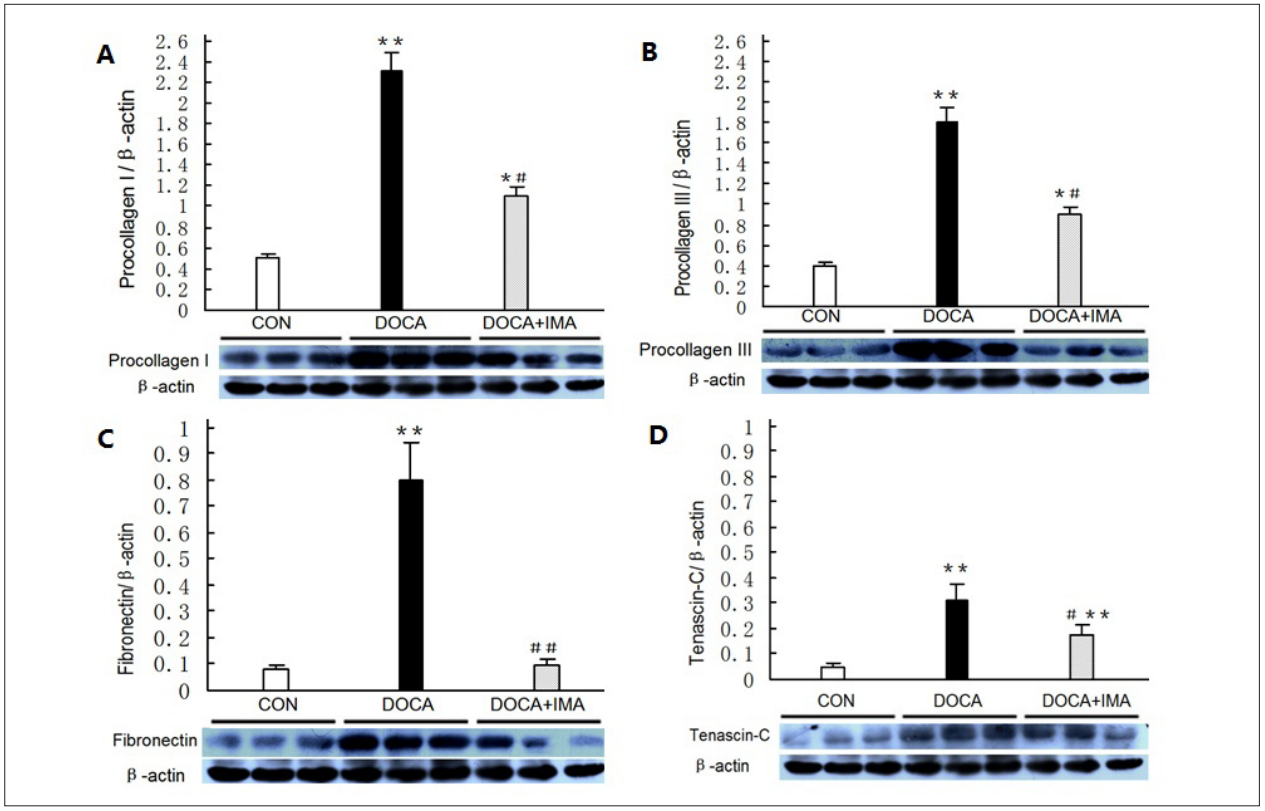

Figure 6 – *Expressions of procollagen I (A), procollagen III (B), fibronectin (C), and tenascin-C (D) in different groups on day 28 (Western blot assay). Imatinib treatment significantly reduced the expressions of procollagen I, procollagen III, fibronectin and tenascin-C in the DOCA+IMA group. Data were expressed as means ± SEM (n=10). Chi square test was used. *p <0.05 and **p<0.01 vs CON group, #p<0.05 and ##p<0.01 vs DOCA group*

findings indicate that PDGF family members participate in the fibrosis of multiple organs and tissues. Obvious interstitial inflammation and increased PDGFs secretion by inflammatory cells (mononuclear cells and macrophages) have been observed in the fibrosis of the lung, heart, liver and kidney[15-17], accompanied by elevated PDGFR expression on the fibroblasts[18-21]. Some investigations suggested that myocardial fibrosis was associated with excessive activation of PDGF signaling pathways and elevated proliferation of fibroblasts[22-24]. PDGF family members includes PDGF-A, PDGF-B, PDGF-C and PDGF-D. PDGF-A and PDGF-C are considered to be closely related to myocardial fibrosis[25-28]. PDGF-AA is the dimeric isoform of PDGF-A which has been demonstrated as a mitogen for myocardial fibroblasts and is implicated in the pathogenesis of myocardial fibrosis[29,30]. PDGF-C, a newly discovered ligand for PDGFRα[31, 32], also plays an important role in the development of myocardial fibrosis. PDGFs function by binding to the receptors of transmembrane protein-tyrosine kinases (PDGFRα and PDGFRβ). PDGF signaling pathways have been divided into PDGFRα and PDGFRβ signaling pathways based on the type of PDGFR[33]. It has been proved that myocardial fibrosis is mainly related to the PDGFRα signaling pathway[5,34]. Besides, PDGF signaling pathway is mainly regulated by PDGFR. The inhibition of PDGFR can completely block the cellular and biological responses mediated by this pathway[5,35]. Results in the present study confirmed the findings mentioned above. Western blot assay revealed that the protein expressions of PDGF-A, PDGF-C and PDGFR-α in the DOCA group and DOCA + IMA group were dramatically increased when compared with those in the CON group, and p-PDGFRα protein expression was positively associated with the myocardial fibrosis.

Mechanism of Cardioprotective Effect of Imatinib

Because PDGFs and their receptors are major mediators for the proliferation, survival and migration of fibroblasts, PDGFR inhibitors may become promising drugs for the treatment of myocardial fibrosis. Imatinib is an inhibitor of tyrosine kinase receptor. Previous studies suggested that inhibiting the transformation from PDGFRs into activated PDGFRα was able to block the PDGF signaling pathway, eventually preventing the occurrence of myocardial fibrosis[36-38]. In this study, the fibrosis of myocardium and tissues surrounding blood vessels in the DOCA + IMA group were markedly alleviated, and the protein expressions of PI and PIII significantly decreased when compared with the DOCA group. Fibronectin and tenascin-C are also major components of fibrotic tissues[39,40]. Tenascin-C has been found to be involved in the adhesion between myocardial cells and fibrotic tissues, and to regulate the fibroblast recruitment after myocardial injury. Thus, tenascin-C has been regarded as a novel index of myocardial fibrosis and ventricle remodeling. Additionally, tenascin-C can also be used to evaluate the function of left ventricle. The protein expressions of fibronectin and tenascin-C in the DOCA + IMA group were significantly decreased when compared with the DOCA group. These findings confirmed that imatinib is able to inhibit myocardial fibrosis in mineralocorticoid induced hypertensive rats.

Role of PDGF Signaling Pathways In Mineralocorticoid induced Myocardial Fibrosis

Our findings demonstrated that PDGF signaling pathway participates in the mineralocorticoid induced myocardial fibrosis. Additionally, PDGFs play a role as succeeding factors of macrophages. Thus, we speculate that mineralocorticoid induces the myocardial inflammation, activates MR on the macrophages, and recruits the macrophages into the myocardium leading to the production of a large amount of PDGFs. Then, PDGFs bind to and activate PDGFRα on the myocardial fibroblasts, which promotes the infiltration and proliferation of fibroblasts into inflammatory sites and facilitates the synthesis and secretion of excessive collagens, eventually resulting in myocardial fibrosis[22,36]. Imatinib exert anti-myocardial fibrosis effect through blocking the PDGF signaling pathway. Thus, animals in the DOCA + IMA group presented obvious myocardial inflammation, and elevated expressions of PDGF-A, PDGF-C, and PDGF-α. However, severe myocardial fibrosis was absent.

Conclusion

This study confirms that PDGF signaling pathway participates in the mineralocorticoid induced myocardial fibrosis. Imatinib is able to effectively suppress the myocardial fibrosis by inhibiting the PDGFRα activity and eventually blocking the PDGF signaling pathway.

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Potential Conflict of Interest

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

Sources of Funding

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Study Association

This study is not associated with any post-graduation program.

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