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Original Article

Correlation between platelet-derived growth factor signaling pathway and inflammation in desoxycorticosterone-induced salt-sensitive hypertensive rats with myocardial fibrosis

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Abstract: Objective: To investigate whether inflammation could excessively activate platelet-derived growth factor (PDGF) signaling pathway in desoxycorticosterone (DOCA) induced salt-sensitive hypertensive rats with myocardial fibrosis (MF). Methods: A total of 30 male SD rats underwent right nephrectomy and then bred with 1% sodium chloride and 0.1% potassium chloride for 2 weeks. These animals were randomly divided into 3 groups: CON group, DOCA group and DOCA+FAS group. Systolic blood pressure (SBP) was measured once every 2 weeks; HE staining was done to observe myocardial inflammation; immunohistochemistry was done to detect expressions of monocyte-macrophage antigen (ectodermal dysplasia 1, ED-1), PDGFRα and PDGFRβ in the myocardium; real time fluorescence quantitative PCR was employed to detect the mRNA expressions of DGF-A, PDGF-B, PDGF-C, PDGF-D, PDGFRα and PDGFRβ. Results: The SBP in DOCA group and DOCA+FAS group increased markedly when compared with CON group (*P<0.01*), but there was no marked difference between DOCA group and DOCA+FAS group (*P>0.05*). At 14 days, in DOCA group, the myocardial inflammation was obvious, ED-1 expression increased markedly, the mRNA expressions of PDGF-A, PDGF-B, PDGF-C, PDGFRα and PDGFRβ increased to different extents, protein expressions of PDGFRα and PDGFRβ also elevated markedly (P<0.01), but the PDGF-D mRNA expression remained unchanged, when compared with CON group. After treatment with fasudil (a drug with anti-inflammatory activity), myocardial inflammation was significantly attenuated, mRNA expressions of PDGF-A, PDGF-B, PDGF-C and PDGFRα as well as PDGFRα protein expression reduced dramatically (*P<0.01*), but the mRNA and protein expressions of PDGFRβ remained unchanged (P>0.05) when compared with DOCA group. Conclusion: In DOCA/salt induced hypertensive rats with MF, excessive activation of PDGF/PDGFR signaling pathway is involved in myocardial inflammation.

Keywords: Desoxycorticosterone, platelet-derived growth factor, platelet-derived growth factor receptor, fasudil, inflammatory response

Introduction

Myocardial fibrosis (MF) is a common pathological feature of some cardiovascular diseases at the end stage. Studies have shown that the increase of mineralocorticoid may induce the occurrence of MF [1, 2], but the mechanisms are complex and still poorly understood. To inhibit infiltration of monocytes/macrophages is helpful to attenuate MF [1], which suggests that inflammation plays an important role in MF. Platelet-derived growth factor (PDGF), a growth factor that can promote cell division and proliferation, has chemotactic activity. The binding of PDGF to PDGF receptor (PDGFR) may stimulate growth, differentiation and migration of cells [3, 4]. Previous studies have shown that, in rats with DOCA/salt induced MF, the PDGF/PDGFR signaling pathway is involved in the occurrence of MF, and the inflammatory response deteriorates in the myocardium [2]. However, no study has been conducted to investigate the relationship between inflammatory response and PDGF/PDGFR signaling pathway. This study aimed to investigate infiltration of monocytes/macrophages and detect the expressions of PDGF and PDGFR in the myocardium of rats with DOCA/salt induced MF. At the same time, anti-inflammatory therapy was done in these rats with fasudil [1], aiming to explore

Table 1. Primers used in RT-PCR

Gene		Primer sequences
PDGF-A	Sense	CCAACCTGAACCCAGACCAT
	Anti-sense	AGGAGAACAAAGACCGCACG
PDGF-B	Sense	TGGAGTCGAGTCGGAAAGCT
	Anti-sense	GAAGTTGGCATTGGTGCGAT
PDGF-C	Sense	TTAGGACGCTGGTGTGGTTC
	Anti-sense	TGAAGGGGGCAACACTGAAG
PDGF-D	Sense	CGGATACAGCTGGCCTTTGA
	Anti-sense	TCTTCCTCTGACAACGGTGC
PDGFRα	Sense	GAGACCCTCCTTCTACCACCT
	Anti-sense	GTTGTCAGAGTCCACACGCAT
PDGFRβ	Sense	GCACCGAAACAAACACACCTT
	Anti-sense	ATGTAACCACCGTCGCTCTC
GADPH	Sense	TGGGAAGCTGGTCATCAAC
	Anti-sense	GCATCACCCCATTTGATGTT

Table 2. SBP in different groups ($\bar{\chi} \pm s$, n=10)

		CON	DOCA	DOCA+FAS
SBP (mmHg)	Day 0	128±12	138±11	137±11
	Day 14	137±5	158±5**	160±10**

**P<0.01 vs CON group.

the relationship between PDGF/PDGFR signaling pathway and inflammation in this animal model.

Materials and methods

Animal and grouping

Specific pathogen free male SD rats weighing 200-250 g (n=30) were purchased from Experimental Animal Center of Affiliated Provincial Hospital, Anhui Medical University. Animals were anesthetized intraperitoneally with 10% chloral hydrate at 400 mg/kg and the received right nephrectomy. 1 week after surgery, animals were bred with 1% sodium chloride and 0.2% potassium chloride. Then, rats were randomly assigned into 3 groups (n=10 per group): 1) CON group: Animals were subcutaneously treated with soybean oil once every 4 days and intragastrically with distilled water twice daily; 2) DOCA group: rats were subcutaneously treated with DOCA at 60 mg/kg/4d and intragastrically with distilled water twice daily; (3) DOCA+FAS group: rats were subcutaneously treated with DOCA at 60 mg/kg/4d and intragastrically with FAS at 10 mg/kg/d twice daily. In CON group, the volume of intragastric distilled water was equal to that of intragastric drugs in other two groups; in CON group, the volume of subcutaneous soybean oil was identical to that of subcutaneous drugs dissolved in soybean oil. Treatment was done for 14 days. Systolic blood pressure (SBP) was measured before and at 14 days after treatment. At 14 days, animals were anesthetized intraperitoneally with 10% chloral hydrate at 400 mg/kg and then sacrificed. The heart was harvested and the atriums, major vessels and connective tissues were removed. The ventricular tissues were divided into 2 parts: one (apex) was stored at -80 °C for PCR; one was fixed in 4% paraformaldehyde followed by embedding in paraffin for HE staining and immunohistochemistry.

Main reagents and instrument

The following reagents were used in the present study: DOCA (Sigma, USA), fasudil (Selleck; USA), soybean oil for injection (Zhejiang Tianyushan Soybean Oil Development Co., Ltd), SP kit for immunohistochemistry, DAB (Beijing Zhongshan Golden Bridge Biotech Co., Ltd), ED-1 antibody (Millipore, USA), antibodies against PDGFRα or PDGFRβ (Abcam, USA), citric acid solution for antigen retrieval (Shanghai Beyotime Institute of Biotechnology), polylysine coated slides, and phosphate buffer solution (PBS) (Wuhan Boster Biotech Co., Ltd). The primers for PDGF-A, PDGF-B, PDGF-C, PDGF-D, PDGFR-α, PDGFR-β and GADPH (**Table 1**) were designed with Primer-Blast software of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/tools/primer-blast) and synthesized in Shanghai Sangon Biotech. RNeasy Mini Kit for mRNA extraction (Qiagen Germany), PrimeScript™ RT reagent Kit with gDNA Eraser for reverse transcription, and SYBR® *Premix Ex Taq*™ II for real time quantitative PCR (Takara, Japan) were used for PCR. Sirius red dye, hematoxylin - eosin, formaldehyde, microtome (Leica), and microscope camera system (Nikon eclipse 80i, Japan) were provided by the comprehensive laboratory of Basic Medicine of Anhui Medical University. Thermal cycler (Applied Biosystems Step One Plus System) was provided by the Department of Parasitology of Basic Medicine, Anhui Medical University.

Detection of myocardial inflammation

14 days after treatment, the left ventricle was fixed in 4% paraformaldehyde for 24 h, dehy-

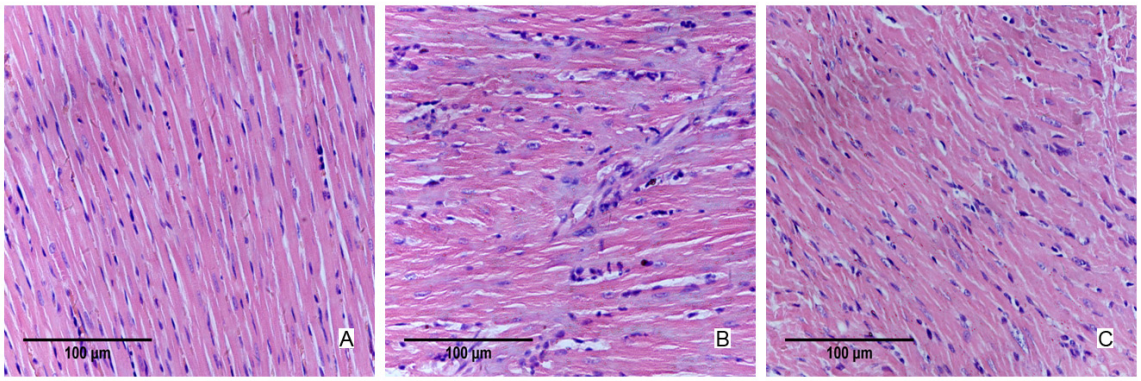

Figure 1. Myocardial inflammation in different groups (HE staining, ×200). A: CON; B: DOCA; C: DOCA+FAS.

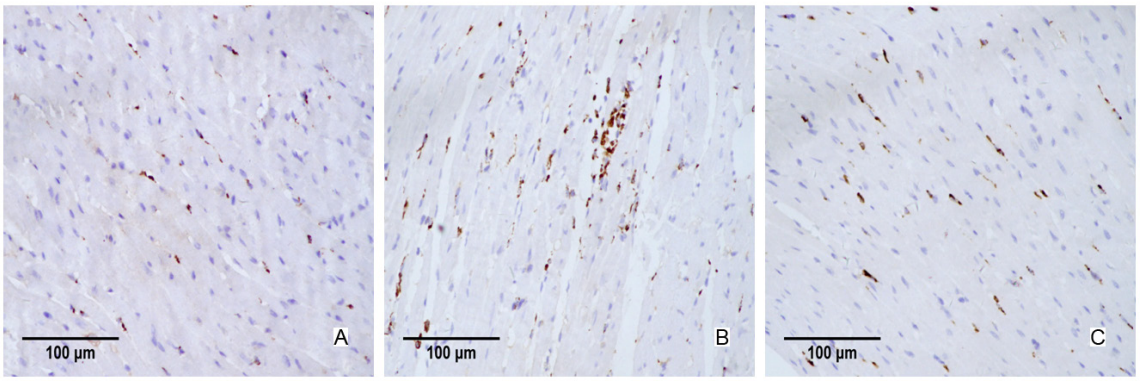

Figure 2. ED-1 positive cells in different groups at 14 days after intervention (immunohistochemistry, ×200). A: CON; B: DOCA; C: DOCA+FAS.

drated and embedded in paraffin followed by sectioning (4 μm) onto polylysine coated slides and subsequent heating. Then, HE staining was done according to routine protocol and the myocardial inflammation was observed.

Detection of macrophage infiltration and expressions of PDGFRα and PDGFRβ

The left ventricle was embedded in paraffin and deparaffinized. After antigen retrieval in citric acid solution for 10 min at 95 °C, sections were treated with 3% H_2O_2 at 37 °C for 10 min and then with 10% normal goat serum at 37 °C for 30 min. Subsequently, these sections were incubated with primary antibodies independently (PDGFRα: 1:400; PDGFRβ: 1:200; ED-1: 1:200) at 4 °C overnight and then with biotinylated secondary antibody and streptavidin at 37 °C for 25 min followed by visualization with DAB. Washing was performed between procedures (5 min in each) and a final counterstaining was done with hematoxylin for 6 min. After dehydration and transparentization, mounting was done. In the negative control group, the primary antibody was replaced with PBS. Under light microscope, cells with brown cytoplasm were regarded as positive. Ten fields were randomly selected from positive area at a high magnification (×200), and photographs were captured for the detection of protein expressions of ED-1, PDGFRα and PDGFRβ. The ED-1 positive cells were counted, and the Image Pro Plus 6.0 software was employed to detect the integrated optical density (IOD) of PDGFR. Average was obtained.

Detection of mRNA expressions of PDGF and PDGFR by real time fluorescence quantitative PCR

In brief, the heart tissues were homogenized, followed by extraction of mRNA with RNeasy Mini Kit according to according to manufacturer's instructions. Then, mRNA was mixed with genomic DNA-free gDNA Eraser followed by reverse transcription into cDNA with Prime-

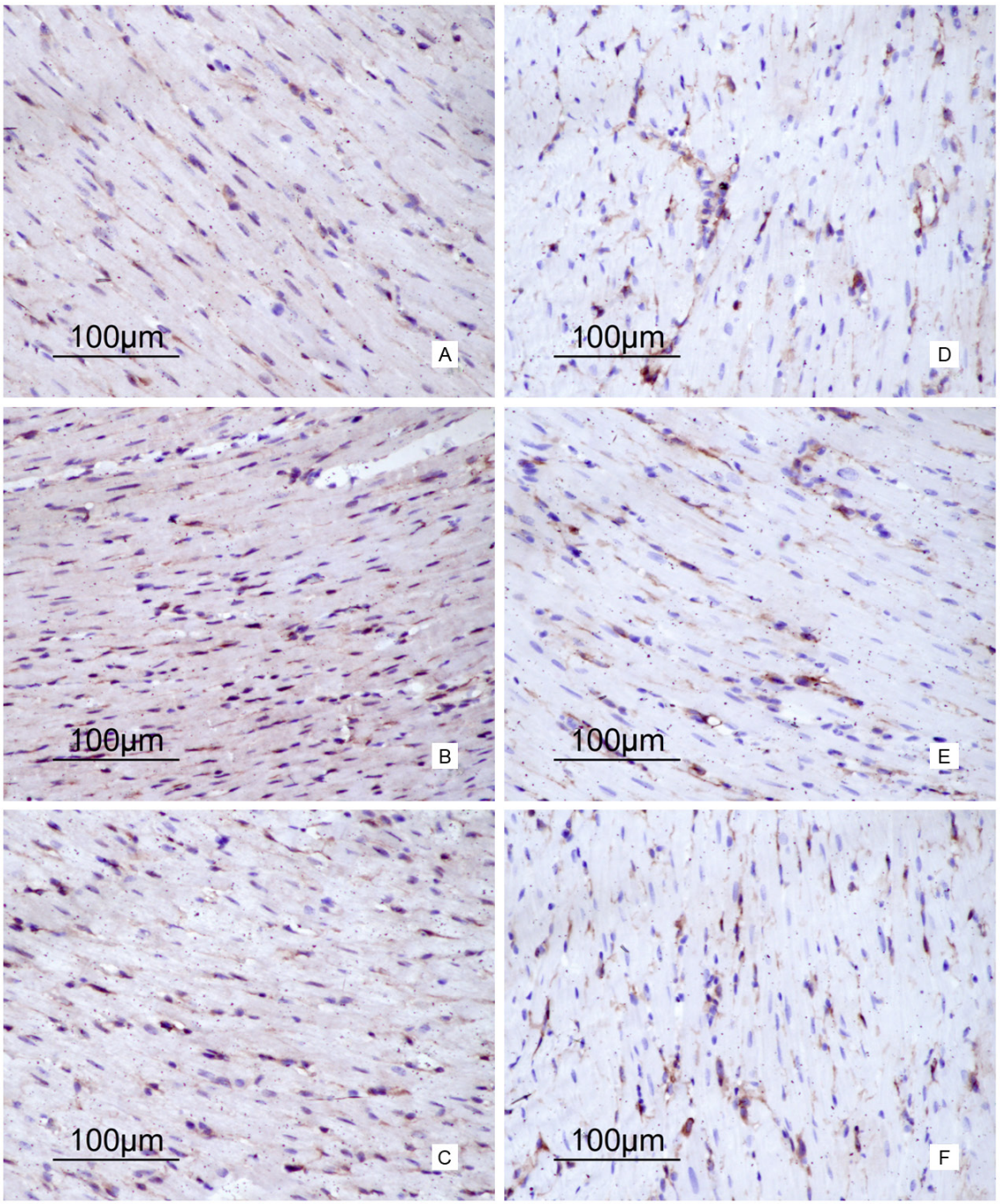

Figure 3. Protein expressions if PDGFRα and PDGFRβ in different groups (immunohistochemistry, ×200). A-C: PDGFRα, A: CON, B: DOCA, C: DOCA+FAS; D-F: PDGFRβ, D: CON; E: DOCA, F: DOCA+FAS.

Script™ RT reagent Kit with gDNA Eraser kit. Amplification of cDNA was done on thermal cycler (Applied Biosystems Step One Plus System). The reaction mixture was 20 μl in volume, and amplification was performed according to manufacturer's instructions (SYBR® Premix Ex Taq™ II PCR kit and Applied Biosystems Step One Plus System). The reaction conditions were as follows: pre-denaturation at 95 °C for 30 s and 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The melt curve was employed to determine the specificity of products. The supporting software was used to analyze the Ct value of products. According to the

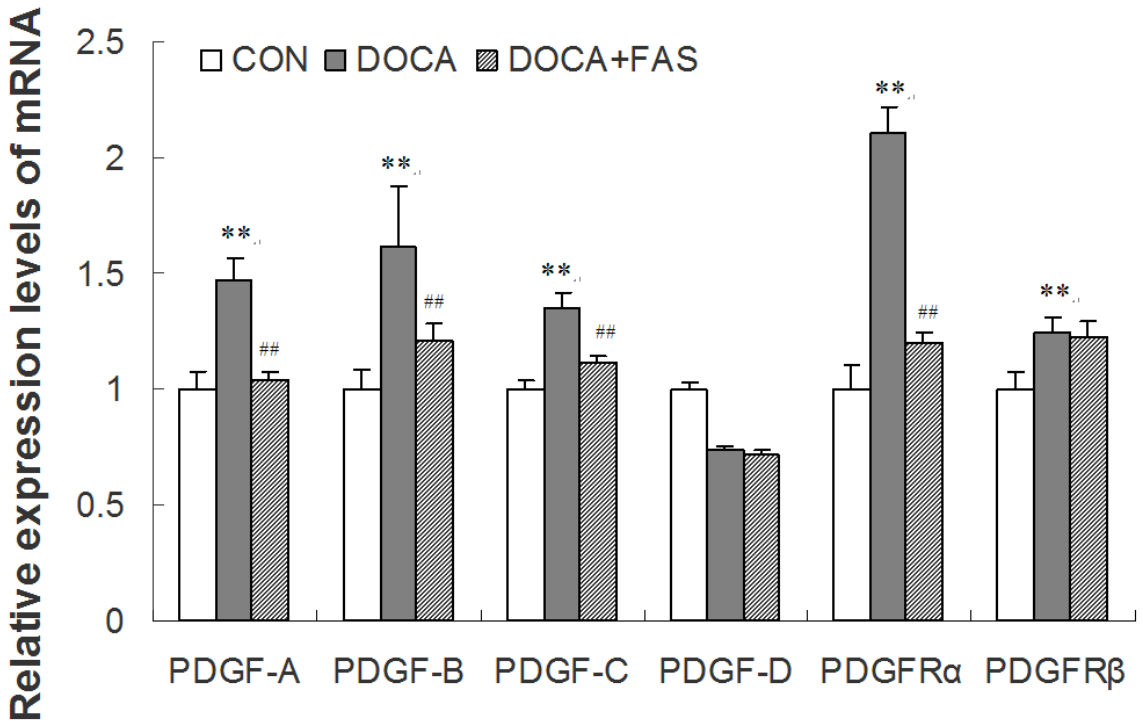

Figure 4. mRNA expressions of PDGF-A, PDGF-B, PDGF-C, PDGF-D, PDGFRα and PDGFRβ in different groups. $\bar{\chi} \pm s$, n=10. **P<0.01 vs CON. ##P<0.01 vs DOCA.

following formula: $\Delta Ct = Ct_{target\ gene} - Ct_{internal\ reference}$, the ΔCt was calculated in two groups. The Ct value of target gene is negatively proportional to the copies of this gene, and thus, the larger the ΔCt, the lower the gene expression is. Then, $2^{-\Delta\Delta Ct}$ method was employed to calculate the relative mRNA expression of target genes.

Statistical analysis

Statistical analysis was done with SPSS version 19.0. Data were expressed as mean ± standard deviation ($x \pm s$). Comparisons of means between two groups were done with independent t test. A value of P<0.05 was considered statistically significant.

Results

Change in SBP in different groups

Before interventions, the SBP was comparable among groups (P>0.05). 14 days after intervention, the SBP in DOCA group and DOCA+FAS group were significantly higher than those in CON group (P<0.01), but there was no significant difference between DOCA group and DOCA+FAS group (P>0.05) (**Table 2**).

Myocardial inflammation and monocyte-macrophage infiltration

In CON group, the myocytes arranged regularly, and only a few inflammatory cells were observed in the myocardial interstitium. In DOCA group, the myocytes arranged irregularly, and a large number of inflammatory cells were observed in the myocardial interstitium, accompanied by the formation of fibrotic scars. After FAS treatment (DOCA+FAS group), the myocytes arranged regularly, and infiltration of a few inflammatory cells was noted in the myocardial interstitium (**Figure 1**). The number of ED-1 positive cells reflects the infiltration of monocyte-macrophages. Results showed the number of ED-1 positive cells was 39.45±3.27 in DOCA group, which was markedly higher than that in CON group (11.95±1.50) (*P<0.01*). After FAS treatment, the number of ED-1 positive cells was 20.45±2.46, which was significantly reduced when compared with DOCA group (P<0.01) (**Figure 2**).

Protein expressions of PDGFRα and PDGFRβ

Immunohistochemistry revealed that PDGFRα and PDGFRβ were mainly expressed in the

myocardial interstitium. Image analysis revealed that the IODs of PDGFRα and PDGFRβ were 4747.0361±2060.6887 and 3048.1416±714.8241, respectively, in DOCA group, which were significantly higher than those in CON group (1774.8462±559.1295 and 2307.6851±960.7840, respectively; *P<0.01*). After anti-inflammatory therapy with FAS (DOCA+FAS group), the IODs of PDGFRα and PDGFRβ were 1997.9807±1212.9807 and 2981.5969±1390.9339, respectively. Significant difference in PDGFRα protein expression was observed between DOCA group and DOCA+FAS group (P<0.01), but the PDGFRβ protein expression was comparable between them (P>0.05) (**Figure 3**).

mRNA expression of PDGF and PDGFR

After real time PCR, the Ct value and number of cycles were employed for delineation and the amplification curve of mRNA was obtained. Results showed there was good repeatability and the amplification efficiency was consistent. $2^{-\Delta\Delta Ct}$ method was used to determine the relative expressions of target genes. Results revealed that the mRNA expressions of PDGF-A, PDGF-B, PDGF-C, PDGFRα and PDGFRβ were 1.4703±0.0981, 1.6175±0.2592, 1.3491±0.0661, 2.1012±0.1131 and 1.2426±0.0698, respectively in DOCA group, which were markedly higher than those in CON group (1.0024±0.0726, 1.0025±0.0787, 1.0004±0.0327, 1.0045±0.1000 and 1.0024±0.0726, respectively; P<0.01). In DOCA group, the PDGF-D mRNA expression was 0.736±0.0191 and comparable to that in CON group (1.0005±0.0321; P>0.05). After FAS treatment, the mRNA expressions of PDGF-A, PDGF-B, PDGF-C and PDGFRα were 1.0343±0.0402, 1.2076±0.0799, 1.1173±0.0276 and 1.2014±0.047, respectively, which were dramatically lower than those in DOCA group (P<0.01). However, the PDGFRβ mRNA expression was similar between DOCA group and DOCA+FAS group (1.226±0.0645; P>0.05) (**Figure 4**).

Discussion

Studies have shown that PDGF is a potent growth factor that can promote the division and proliferation of cells and has the chemotactic activity, and it plays important roles in the proliferation, survival and migration of interstitial cells [4, 5]. Increasing evidence shows that PDGF/PDGFR signaling pathway is involved in the pathological fibrosis of multiple organs [6-8]. Our previous study [2] confirmed that the PDGF/PDGFR signaling pathway was up-regulated in DOCA/salt induced hypertensive rats, which was consistent with MF. This implies that PDGF/PDGFR signaling pathway is related to the progression of active fibrosis. The present study further confirmed that the up-regulation of this pathway may be used as an important indicator reflecting the progression of active MF.

MF is characterized by the reticular deposition of extracellular matrix in the myocardial interstitium, which finally leads to heart failure [9]. A variety of studies have shown that the progression of MF is accompanied by inflammatory response [10]. In the heart of DOCA/salt induced hypertensive rats, the inflammatory response is obvious in the myocardial interstitium, and it has been regarded that inflammation is involved in the mineralocorticoid induced MF [1]. In addition, infiltration of inflammatory cells (mainly monocytes/macrophages) precedes the MF [11]. Increasing evidence demonstrates that monocytes/macrophages can secret a lot of pro-fibrotic factors to influence the differentiation of fibroblasts into myofibroblasts, which may exert regulatory effect on MF [12, 13]. Moreover, both monocytes and macrophages play pivotal roles in the initiation and development of fibrosis [14]. In addition, monocytes/macrophages can produce and secret a lot of pro-inflammatory factors, such as interleukin-1 (OL-1), IL-6, tumor necrosis factor-α (TNF-α) and monocyte chemoattractant protein (MCP-1), which may induce the myocardial interstitial inflammation [14, 15]. After anti-inflammatory therapy, the infiltration of monocytes/macrophages is attenuated, the deposition of ECM in the myocardial interstitium also reduces and the severity of MF also reduced [16, 17]. Our results showed, in the heart of DOCA/salt induced hypertensive rats, the myocardial interstitial inflammation and the infiltration of monocytes/macrophages were more severe than those in CON group. On the basis of these findings and previous results, we postulated that the myocardial inflammation has causal relationship with the mineralocorticoid induced MF.

Fasudil is a specific inhibitor of Rho kinase (ROCK). ROCK is a downstream effector protein

of the small GTPase [18]. Nuclear factor –κB (NF-κB), NADPH, IL-6, MCP-1, macrophage migration inhibitory factor (MMF) and interferon-γ have been found to up-regulate ROCK expression [19], which is involved in the pathogenesis of inflammation. Fasudil can significantly inhibit the activation of NF-κB, IL-6 and MCP-1 to attenuate the infiltration of monocytes/macrophages [1, 18]. In addition, no studies have shown that fasudil can influence the PDGF/PDGFR signaling pathway. In the present study, results showed the infiltration of monocytes/macrophages and inflammation in the heart of DOCA/salt induced hypertensive rats increased, accompanied by increased expressions of PDGF-A, PDGF-B, PDGF-C, PDGFRα and PDGFRβ. After fasudil treatment, the infiltration of monocytes/macrophages and inflammation in the myocardial interstitium were attenuated, accompanied by reduction in the expressions of PDGF-A, PDGF-B, PDGF-C and PDGFRα. However, the expressions of PDGF-D and PDGFRβ were not influenced by fasudil. These suggest that PDGFRα may be a downstream component of inflammation. We speculate that the excessive activation of mineralocorticoid receptor on the monocytes/macrophages may induce the infiltration of monocytes/macrophages and inflammatory response in the myocardial interstitium of DOCA/salt induced hypertensive rats [15]. The proliferation of inflammatory cells including monocytes/macrophages may cause the secretion of PDGF by these cells. The activated PDGF binds to PDGFRα, leading to the activation of PDGF/PDGFR signaling pathway, which is involved in the development of MF.

Taken together, inflammation and PDGF/PDGFR signaling pathway are involved in the mineralocorticoid induced MF, and to attenuate myocardial inflammation may inhibit the expressions of components of PDGF/PDGFR signaling pathway. These suggest that the excessive activation of PDGF/PDGFR signaling pathway is closely related to the myocardial inflammation.

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Disclosure of conflict of interest

None.

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