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Apixaban attenuates ischemia-induced myocardial fibrosis by inhibition of Gq/PKC signaling

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ABSTRACT

It was previously found that patients with symptom of myocardial dysfunction had increased levels of thrombin. Apixaban is one of the novel oral anticoagulant drugs widely used in clinic. As the inhibitor of FXa (prothrombin), it inhibits prothrombin conversion into thrombin leading to thrombin deficiency *in vivo*. However, the effects of apixaban on myocardial fibrosis were still unclear, and the concomitant molecular mechanisms remain to be investigated. Here, we showed that myocardial fibrosis-bearing mice induced by continuous myocardial ischemia (MI) had higher levels of thrombin. Orally administration of apixaban significantly abrogated fibrosis condition and thrombin levels. *In vitro*, thrombin induced collagen deposition in primary cardiac fibroblasts in a dose-dependent manner. Mechanistic experiments showed that thrombin induced collagen deposition by activation of the Par-1-coupled Gq/PKC signaling. Genetic ablation of Gq or pharmacological inhibition of PKC effectively blunted thrombin-induced collagen deposition in cardiac fibroblasts. Moreover, administration of PKC inhibitor or Gq antagonist obviously blocked MI-induced myocardial fibrosis in mice. To conclude, apixaban attenuates MI-induced myocardial fibrosis by inhibition of thrombin-dependent Par-1/Gq/PKC signaling axis.

1. Introduction

Myocardial fibrosis often occurs as a result of hypertension, ischemic injury, myocardial infarction, and other cardiovascular diseases, and it also serves as a crucial aspect of cardiac remodeling following these disorders [1]. As the disease progresses, myocardial fibrosis often develops into cardiac dysfunction, coronary artery anomalies, and arrhythmia, with left ventricular diastolic dysfunction and increase in myocardial stiffness [2]. Generally, myocardial fibrosis is featured with activation of cardiac fibroblasts (CFs), which synthesize and excrete excessive collagen I (Col1a1) and III (Col3a1), resulting in deposition of collagen proteins [3]. Hence, the activity of CFs and the deposition of collagen are closely associated with the progression of myocardial fibrosis, and they also reflect the pathological state of myocardial fibrosis.

Thrombin, a multifunctional serine protease, acts through the receptor protease-activated receptor 1 (Par-1) to modulate many cellular functions such as proliferation and differentiation in several cell types [4]. The increased synthesis of thrombin is closely related to cardiac hypertrophy and heart failure [5]. Therefore, the regulation on thrombin synthesis and activity has been a promising strategy for treatment of myocardial dysfunction. Par-1 is a pleiotropic G protein coupled receptor capable of activating members of the Gq, Gi, and G12/13 families of G proteins [6]. And the sustained activation of Gq signaling has been confirmed to be implicated in cardiac hypertrophy and heart failure [7]. Moreover, the activation of Gq signaling is likely to result in activation of the protein kinase C (PKC) [8], which contributes to promoting myocardial fibrosis through inducing CFs proliferation and collagen deposition [9]. By now, it can be speculated reasonably that the thrombin may exert an impact on pathological development of myocardial fibrosis via Par-1 and the downstream effector Gq/PKC signaling. The above findings reveal the significance of thrombin in the development of

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myocardial fibrosis, while the underlying mechanisms still remain unclear.

Recently, several inhibitors of thrombin synthesis such as the Dabigatran, Ximelagatran, and Argatroban have been developed for treatment of myocardial fibrosis in clinic [10]. Dong et al. demonstrated that Dabigatran attenuated pressure overload-induced cardiac fibrosis and dysfunction through suppressing the activity of thrombin and down-regulating Par-1 [11]. Apixaban is a novel oral anticoagulant drug with activities of inhibiting blood coagulation factor Xa (FXa) leading to lower thrombin generation [12,13]. Apixaban has been applied in clinical for treatment of the acute and chronic disease with thrombus. However, the effects of Apixaban on myocardial fibrosis and the molecular mechanisms still to be clarified. In current study, the mouse model of myocardial ischemia-induced myocardial fibrosis was established. We investigated the effects of apixaban on myocardial fibrosis and explored the molecular mechanisms in cardiac tissue-derived primary cardiac fibroblasts.

2. Materials and methods

2.1. Establishment of myocardial fibrosis model and drug treatment

All the animal experiments were approved by the ethics committee of the First Affiliated Hospital of Soochow University, and performed in accordance with the Guide for the Care and Use of Laboratory Animals proposed by the Chinese National Institutes of Health. The C57BL/6 mice (6–8 weeks old) were purchased from the Shanghai Lab. Animal Research Center, Shanghai, China, and were provided with a standard diet and free access to water. The continuous myocardial ischemia (MI) was induced by ligating the left anterior descending coronary artery according to the previous study [14]. Briefly, the mouse was anesthetized by 4% isoflurane and 1.5% isoflurane. Keep on a heating pad to prevent hypothermia, the mouse was fixed with tape in supine position. With the exposure of the left side of the heart, the left atrium, the left ventricle, and the left anterior descending coronary artery are visible under the stereomicroscope. Perform a ligation of the left anterior descending coronary artery with a single 6-0 prolene ligature about 1 mm under the tip of the left atrium. Successful ligation of the left anterior descending coronary artery induces immediate discoloration, resulting in a pale appearing myocardium in the affected territory. The ligation was not conducted in the sham group, while the other procedures were performed similarly with mice in myocardial ischemia (MI) group. The mice were divided into several groups with 5 mice in each group. At the next day of MI surgery, mice were orally administrated with lower dosage of 30 μg/g/day (MI + Api-L) or higher dosage of 60 μg/g/day (MI + Api-H) for 4 weeks. Meanwhile, the mice in sham or MI group were orally administrated with the same volume of normal saline.

2.2. Hematoxylin&eosin (H&E) staining

Myocardial fibrosis was evaluated by hematoxylin-eosin (HE) staining. Mice were killed with 2 ml of pentobarbital (40 mg/kg) by intraperitoneal injection, and the heart was excised. The left ventricular myocardium was fixed in 10% formalin for overnight and dehydrated with ethanol. Then the cardiac muscle tissue was embedded in paraffin and cut into 4 μm thick sections. They were mounted on glass slides and stained with hematoxylin and eosin, respectively, and eight separate views were selected by light microscopy to analysis the myocardial fibrosis.

2.3. Cardiac functional measurements

The right carotid artery was isolated to allow insertion of a catheter containing 0.05% heparin saline to the left ventricle, and the other end of the catheter was connected to the PowerLab polygraph recorder to monitor the changes of the left ventricular mean systolic pressure (LVSP), left ventricular end diastolic pressure (LVEDP). After the mice euthanized, pre-cooled saline was infused into the left ventricle of the mice until the heart and kidney paled. The heart was rapidly excised and rinsed in cold saline. Subsequently, the left and right ventricles were isolated and weighted, and the left and right ventricular weight indices (LVWI and RVWI) were calculated with the left and right ventricular free full mass (mg) divided by body mass (g), respectively.

2.4. Real-time quantitative RT-PCR

To determine the mRNA expression of Col1a1and Col3a1, total RNA was extracted from cardiac tissues by using the Trizol reagent (Invitrogen) according to the manufacture's manual. And cDNA was synthesized with 2 μg of total RNA by using a SuperScript Reverse Transcription Kit (Invitrogen). Then the quantitative RT-PCR was conducted on an ABI PRISM7900 Sequence Detection System (Applied Biosystems) with SYBR Green Master Mix (Applied Biosystems). The specific oligonucleotide primers were designed and synthesized by Sangon Biotech (Shanghai, China). GAPDH served as the internal control and the relative expression was analyzed with the $2^{-\Delta\Delta Ct}$ method. The primer sequences used in the present study were as follows:

COL1A1: Forward: 5′-CGCCATCAAGGTCTACTGC-3′
Reverse: 5′-ACGGGAATCCATCGGTCA -3′
COL3A1: Forward: 5′-GCCCACAGCCTTCTACACCT -3′
Reverse: 5′-GCCAGGGTCACCATTTCTC -3′
PAR-1: Forward: 5′-CCTATGAGACAGCCAGAATC-3′
Reverse: 5′-GCTTCTTGACCTTCATCC-3′
GAPDH: Forward: 5′-TATGTCGTG GAG TCT ACT GGC GTC-3′
Reverse: 5′-GAATGGGAGTTGCTGTTGAAGTCA-3′

2.5. Western blot

The expression of collagen proteins (Col1a1and Col3a1) was analyzed by western blot. Cardiac tissues were treated with RIPA lysis buffer (Beyotime, China) for protein extraction, and the concentration of protein was measured with a BCA protein assay kit (Beyotime, China). Total protein was separated with 12% SDS-PAGE and transferred onto the polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The membrane was then blocked with Tris-buffered saline Tween-20 (TBST) containing 5% nonfat milk for 1 h at RT, and then incubated with the primary antibodies: rabbit anti-mouse Col1a1(Santa cruz biotechnology), rabbit anti-mouse Col3a1(Santa cruz biotechnology, MA, US), and rabbit anti-mouse β-actin (Cell signaling technology, MA, US) for overnight at 4 °C. The membrane was then washed by 1×TBST and incubated with HRP-bounded secondary antibodies for 1 h at RT and the proteins were visualized with an ECL Plus Western Blot Substrate (Thermo Fisher).

2.6. Thrombin activity detection

Thrombin activity was measured with the Enzyme-linked immuno sorbent assay (ELISA) by using an ELISA system kits (R&D Systems). Following sacrifice, the peripheral blood of each mouse were collected and placed into the 96-well black microplate

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