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Original article

Possible role of rivaroxaban in attenuating pressure-overload-induced atrial fibrosis and fibrillation

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ABSTRACT

Background: Coagulation factor Xa (FXa) promotes thrombus formation and exacerbates inflammation via activation of protease-activated receptor (PAR)-2. We tested the hypothesis of whether administration of direct oral anticoagulant, rivaroxaban, would attenuate transverse aortic constriction (TAC)-induced atrial inflammatory fibrosis and vulnerability to atrial fibrillation (AF) in mice.

Methods: Ten-week-old male CL57/B6 mice were divided into a sham-operation (CNT) group and TAC-surgery group. These two groups were then subdivided into vehicle (VEH) and rivaroxaban (RVX) treatment (30 μg/g/day) groups. We assessed PAR-2 expression in response to TAC-related stimulation using rat cultured cells.

Results: TAC-induced left atrial thrombus formation was not observed in the TAC-RVX group. Cardiac PAR-2 upregulation was observed in both TAC groups. In the quantitative analysis of mRNA levels, cardiac PAR-2 upregulation was attenuated in the TAC-RVX group compared to TAC-VEH group. In histological evaluation, the TAC-VEH group showed cardiac inhomogeneous interstitial fibrosis and abundant infiltration of macrophages, which were attenuated by RVX administration. Electrophysiological examination revealed that AF duration in the TAC group was shortened by RVX administration. TAC-induced protein overexpression of monocyte chemoattractant protein-1, and mRNA overexpression of tumor necrosis factor-α, interleukin (IL)-1β and IL-6 in the left atrium was suppressed by RVX treatment. In cardiac fibroblasts, persistent intermittent stretch upregulated PAR-2, which was suppressed by RVX pre-incubation.

Conclusions: These observations demonstrate that coagulation FXa inhibitor probably has a cardioprotective effect against pressure-overload-induced atrial remodeling.

Introduction

Atrial fibrillation (AF) is the most common arrhythmia observed in the clinical setting [1,2]. Atrial inflammatory profibrotic signals induce interstitial atrial fibrosis, which plays a critical role in AF pathogenesis [3–5]. For instance, hypertension, characterized by pressure overload, stimulates inflammatory profibrotic signals, leading to enhanced vulnerability to AF [6–12]; indeed, hypertension is a major risk factor for AF [13].

In addition, AF can be important because of the high risk of stroke. Vitamin K antagonist (VKA), warfarin, has been used for the prevention of stroke in patients with non-valvular AF (NVAF). However, long-term administration of warfarin induces endothelial dysfunction and calcification through inhibition of Vitamin K2 [14–17]. Recently, the use of direct oral anticoagulants (DOACs) has been significantly increased instead of warfarin. DOACs, which are represented by direct thrombin inhibitor and factor Xa (FXa) inhibitor, have been reported to show anti-inflammatory effects by inhibiting the activation of the protease-activated receptor (PAR)-1 or PAR-2 signaling pathway [18–20]. It has been reported that

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dabigatran, which is one of the oral direct thrombin inhibitors, attenuates bleomycin-induced pulmonary fibrosis in mice [18]. In addition, it has been reported that direct FXa inhibitor, rivaroxaban (RVX), attenuates atherosclerotic plaque progression and destabilization in mice [20]. However, there is a limited amount of evidence to substantiate the pleiotropic effects of direct FXa inhibitors.

The PAR family comprises four members (PAR1–PAR4) with a proteolytic cleavage-based activation mechanism [21]. It has been reported that FXa-dependent PAR-1 and PAR-2 cleavage might play a role in tissue fibrosis and remodeling [22,23]. Indeed, PAR-2 overexpression induces cardiac hypertrophy in mice. In contrast, cardiac remodeling is inhibited after the induction of myocardial infarction in PAR-2 knockout (KO) mice [23]. However, the association of the PAR-2 and pressure-overload-induced atrial remodeling has not been investigated.

Hence, we investigated whether pressure overload activates FXa in plasma, and upregulates PAR-2 expression in the heart. In addition, we also investigated whether RVX attenuates atrial inflammatory fibrosis and vulnerability to AF in a transverse aortic constriction (TAC) model.

Materials and methods

All experimental procedures were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Physiological Society of Oita University, Japan, which follow the guidelines established by the US National Institutes of Health. This study was approved by Institutional Animal Use and Care Committee of Oita University.

Animals, surgery, and treatment

Ten-week-old male CL57/B6 mice were purchased from SLC Japan (Hamamatsu, Japan). Mice were allocated into four groups: pressure overload induced by TAC operation with and without RVX treatment (30 μg/g/day for 2 weeks) (TAC-RVX and TAC-VEH), and sham-operated counterparts (Sham-RVX and Sham-VEH). RVX was supplied by Bayer Pharma AG (Elberfeld, Germany) and administered by oral gavage once a day. RVX-treatment is started on postoperative day 1. TAC operation was performed as described previously [24]. Mice were anesthetized by intraperitoneal injection of a mixture of ketamine (60 mg/kg) and xylazine (10 mg/kg). The depth of anesthesia was confirmed from a stable heart rate and a lack of flexor responses to a paw-pinch. At the age of 12 weeks the hearts were excised.

Evaluation of the effect of RVX

We measured the prothrombin time (PT) and tail vein bleeding time (TVBT) to confirm the effect and bioavailability of RVX by using a test device (CoagChek XS; Roche Diagnostics, Mannheim, Germany).

Electrophysiological studies

Electrophysiological studies in isolated perfused hearts were conducted using a Langendorff apparatus as previously described [3,25]. AF was defined as a rapid irregular atrial rhythm with irregular R–R intervals lasting for at least 1 s. The duration of AF was measured from the end of burst pacing to the first P wave detected after the rapid irregular atrial rhythm.

Histology

Isolated left atria and ventricles were fixed in 4% paraformaldehyde, embedded in paraffin, and then cut into 5-μm sections. Masson's trichrome staining was used to evaluate interstitial fibrosis and thrombi in the left atrium (LA). Images were acquired and digitized on a BZ-9000 Biolevo epifluorescence microscope (Keyence, Osaka, Japan) with an attached digital camera. Fibrotic and normal myocardial tissue areas were analyzed at 400× magnification using the associated software (Keyence). The percentage of fibrosis was determined by calculating the ratio of areas of fibrotic to normal myocardial tissue. Four images per atrium were analyzed from eight animals per group to obtain the mean values. To avoid selection bias of the views, the blind randomized selection and evaluation of images obtained from each group were performed by two investigators.

Immunohistochemistry

Isolated left atria were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5-μm sections that were labeled with primary antibodies against PAR-2, CD68 and CD206 (Abcam, Cambridge, UK) and the appropriate biotin-conjugated secondary antibody (ABC reagent; Vector Laboratories, Burlingame, CA, USA). Immunoreactivity of PAR-2 was visualized by treating sections with diaminobenzidinetetrahydrochloride (NacalaiTesque, Kyoto, Japan). In immunofluorescence staining, the sections labeled with primary antibody were followed by the appropriate Alexa 488- or 594-conjugated secondary antibodies (Invitrogen, Carlsbad, CA, USA). Sections were counterstained with 4′6-diamidino-2-phenylindole (DAPI) to label nuclei, then mounted and examined under an epifluorescence microscope. The number of total macrophages ($CD68^+$ cells) was calculated as a percentage of the total number of DAPI-labeled cells. Images were acquired and digitized on a BZ-9000 Biolevo epifluorescence microscope with an attached digital camera.

Quantitative reverse transcription-polymerase chain reaction of left atrial tissue

The transcript expression level was determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) as previously described [4]. Total RNA was extracted using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany) from mouse left atrial tissue and single-stranded cDNA was transcribed using the QuantiTect Reverse Transcription Kit (Roche Diagnostics). The amplification was performed with the Universal Probe Library on a LightCycler 480 probe master instrument (Roche Diagnostics) using the TaqMan method [4]. The transcript level was measured relative to that of actin, which was obtained from a standard curve.

Western blot analysis of whole mouse left atria, cardiac myocytes, and fibroblasts

Whole-heart excision was performed on Day 14 and cells were collected after 12 h, followed by western blotting to determine protein expression levels of collagen-1, collagen-3, monocyte chemoattractant protein (MCP)-1 and PAR-2, as previously described [25]. Equal amounts of total protein were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and were then electrophoretically transferred to polyvinylidene fluoride membranes (PVDF) (Bio-Rad, Hercules, CA, USA). After blocking to minimize nonspecific binding, PVDF membranes were incubated with antibodies against collagen-1, collagen-3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), MCP-1, PAR-2 (Abcam) and glyceraldehyde 3-phosphate dehydrogenase (Sigma–Aldrich, St. Louis, MO, USA). After washing, the PVDF membranes were incubated with horseradish-peroxidase-conjugated secondary antibodies (GE Healthcare, Amersham, UK), and the protein bands

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