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The direct factor Xa inhibitor Rivaroxaban reduces platelet activation in congestive heart failure

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ABSTRACT

Background: Platelet activation in congestive heart failure (CHF) contributes to an increased risk for thromboembolic complications. Rivaroxaban, the first oral direct FXa inhibitor is approved in Europe for prevention and treatment of venous thrombosis, pulmonary embolism, and prevention of thromboembolic events in atrial fibrillation. As heart failure is an important risk factor for thromboembolism and increased platelet activation is common in heart failure, we investigated the potential effect of Rivaroxaban treatment on platelets in an experimental CHF model.
Methods and results: Chronic myocardial infarction was induced in male Wistar rats by coronary ligation. Rats were randomized to placebo or Rivaroxaban (3 and 10 mg/kg once daily). After 10 weeks platelet activation was assessed. Platelet-bound fibrinogen, detected by flow-cytometry, was significantly increased in CHF-Placebo ($p < 0.05$) and reduced following treatment with Rivaroxaban ($p < 0.05$ vs. CHF-Placebo). ADP-induced aggregation was significantly enhanced in CHF-Placebo vs. sham-operated animals ($p < 0.05$) and normalized following chronic FXa inhibition ($p < 0.05$ vs. CHF-Placebo). In separate in vitro experiments, attenuated platelet aggregation was present after incubating whole blood directly with Rivaroxaban but absent when the experiment was performed in platelet-rich plasma only. Thus, a direct effect on platelets could be excluded.
Conclusion: Chronic direct factor Xa inhibition using Rivaroxaban reduces platelet activation in CHF rats by attenuating the secondary phase of ADP-induced platelet aggregation. Thus, Rivaroxaban may constitute a useful approach to prevent thromboembolic complications and reduce platelet activation in CHF at the same time.

1. Introduction

Systolic congestive heart failure (CHF) is characterized by impaired left ventricular function, which increases the risk for embolic stroke. Prevention of embolism is routinely intended by therapeutic anticoagulation. However, prophylactic anticoagulation is only recommended in CHF patients who are hospitalized and immobilized, or in CHF patients with a history of thrombembolic events, atrial fibrillation or a prosthetic heart valve [1,2]. Patients suffering from CHF are at risk of sustaining thrombembolic events partly due to increased activity of procoagulant factors [3], stasis in the dilated, hypokinetic heart and stasis in peripheral blood vessels [2].

Similar to several other cardiovascular diseases such as peripheral artery disease [4], coronary artery disease [5], cerebrovascular disease [6], hypercholesterolemia [7], hypertension [8] and diabetes [9], which are associated with increased platelet activation, activated platelets are also part of the cardiovascular phenotype of CHF [10,11] and contribute to cerebral and/or peripheral thrombembolic events [12].

Once activated, platelets themselves activate factor X (FX) to FXa on their surface leading to further thrombin generation and platelet activation [13]. Also, tissue factor (TF)-induced platelet aggregation decreases concentration-dependently following in vitro incubation of platelet-rich plasma (PRP) with direct factor Xa inhibitors [14]. Considering that platelets play an important role within coagulation and take part in coagulation factor activation, we assumed a potential influence of FXa inhibition on platelet activation in CHF.

Rivaroxaban, the first oral direct FXa inhibitor, was approved in Europe for the prevention of venous thromboembolism after elective hip and knee replacement surgery in adults in 2008 [15,16]. In

Abbreviations: BSA, bovine serum albumin; CHF, congestive heart failure; FITC, fluorescein isothiocyanate; FX, factor X; GPCR, G-protein coupled receptor; LVEDP, left ventricular end-diastolic pressure; MFI, mean fluorescence intensity; PBS, phosphate-buffered saline; PRP, platelet-rich plasma; TF, tissue factor; WB, whole blood; PMA, platelet–monocyte aggregates; PLA, platelet–leukocyte aggregates; DMSO, dimethyl sulfoxide.

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the meantime, the drug was also authorized for the treatment of symptomatic deep vein thrombosis [17], the prevention of ischemic events due to non-valvular atrial fibrillation [18] and treatment of pulmonary embolism [19]. Recently, Rivaroxaban was evaluated in addition to antiplatelet treatment in patients with coronary artery disease [20] showing a reduction in risk for myocardial infarction and stroke. However, the drug increased the risk of major bleeding and intracranial hemorrhage, which was not surprising as Rivaroxaban was compared to placebo in this study.

Based on the cellular model of the coagulation cascade, where the coagulation process is divided into three phases, initiation, amplification and propagation, cells and cell surfaces are considered to be important elements within coagulation. Platelets in particular play a pivotal role in this model. They become activated through small amounts of thrombin which is generated in the initiation and amplification phase by FXa. FX activation, however, only takes place on cell surfaces. Because thrombin, the final mediator of the coagulation cascade, induces both plasmatic coagulation as well as platelet activation, reducing thrombin generation by inhibiting FXa seems to be a promising approach to achieve simultaneous inhibition of both plasmatic coagulation as well as platelet activation.

So far, there are few data published about the impact of direct factor Xa inhibitors on platelet function. It could be shown that Rivaroxaban dose-dependently inhibited thrombin generation on platelet surfaces [21,22]. In terms of aggregation, Rivaroxaban had no effect on collagen-induced platelet aggregation [23,24].

In the present study we investigated the effect of chronic Rivaroxaban treatment on platelet activation and aggregation in an experimental CHF model. Moreover, we addressed the impact of acute Rivaroxaban treatment as well as in vitro incubation with the direct factor Xa inhibitor on platelet activation.

2. Methods

Experimental procedures on animals met the requirements of the German legislation on protection of animals and were approved by the Government of Bavaria, Germany, and are in accordance with EU Directive 2010/63/EU.

2.1. Human blood samples

For some in vitro experiments, blood samples were collected from healthy donors who gave informed consent and who had not taken any medication within the last 10 days.

2.2. Animals and induction of CHF

Sham-operation or left coronary artery ligation was performed in adult male Wistar rats (250–300 g, obtained from Harlan-Winkelmann, Borchen, Germany) as previously described [25]. Hemodynamic studies were performed after ten weeks to ensure that the animals were in the chronic stable phase of heart failure [26]. CHF was defined by elevated left ventricular end-diastolic pressure (LVEDP, >15 mmHg) and impaired left ventricular function.

Rats were randomized to placebo or Rivaroxaban (3 and 10 mg/kg once daily given by gavage). After 10 weeks platelet activation was assessed for experiments under chronic treatment, for experiments under acute conditions platelet activation was measured after 2 h.

2.3. Platelet sampling

General anesthesia was induced using isoflurane. The abdominal cavity was opened under deep anesthesia, determined by total absence of reaction to pain under spontaneous respiration, and blood was taken by direct puncture of the inferior caval vein into a tube containing 3.8% sodium citrate. Pulmonary edema was assessed by net fluid weights. The lungs were placed in a drying oven for 1 week at 40 °C, and the difference between the wet and dry weights yielded the pulmonary fluid accumulation values.

2.4. Flow cytometry

Whole blood was diluted with PBS (free of Ca^{2+} and Mg^{2+}, enriched with D-glucose (5.5 mmol/L) and 0.5% BSA). Platelet bound fibrinogen, which requires activated glycoprotein IIb/IIIa on the platelet surface, was determined by incubation with a FITC-labeled anti-fibrinogen antibody (WAK-Chemie, Steinbach, Germany).

Following incubation with the antibody, platelets were fixed with methanol-free formaldehyde (1.5%) for 10 min, and subsequently analyzed in a Becton Dickinson FACSCalibur at a low flow rate. The platelet population was identified on its forward and side scatter distribution, and 20,000 events were analyzed for mean fluorescence using CELLQuest software, version 3.1f. Unspecific binding was arbitrarily adjusted to a mean fluorescence of 10.

To evaluate platelet–leukocyte aggregates and leukocyte activation, respectively, citrated whole blood was pre-incubated with Rivaroxaban (1 μM) or vehicle (DSMO) for 15 min, stimulated with 20 μM ADP and stained with the appropriate antibodies. CD61-FITC (Beckman Coulter) was used to define the platelet population, CD45-PE (BD Biosciences) as pan leukocyte marker and CD14-APC (BD Biosciences) as monocyte marker. To analyze leukocyte activation the following antibodies were used: CD11b-APC (MAC-1; BD Biosciences), CD61-PerCP and CD45-FITC (both antibodies BD Biosciences). After staining, blood samples were lysed with BD FACS lysing solution, washed twice and fixed with BD cell fix.

Flow cytometry of lysed blood samples was performed on a FACSCanto (BD Biosciences), 2500 $CD14^+$ events were acquired for each sample to evaluate platelet–leukocyte/–monocyte aggregate formation. Overall platelet–leukocyte aggregates were defined as $CD61^+/CD45^+$ events, platelet–monocyte aggregates within the afore-mentioned population were further specified as $CD14^+$ events. 30,000 $CD45^+$ cells were analyzed to evaluate mean fluorescence intensity (MFI) of CD11b.

Analysis was performed using FACSDiva software.

2.5. Platelet aggregation

Platelet aggregation was analyzed using a platelet aggregation profiler (PAP-8, MöLab, Hilden, Germany). Citrated whole blood was centrifuged at 180 × *g* for 10 min to obtain platelet-rich plasma (PRP), which was diluted with PBS to obtain a final platelet concentration of 250,000/μl. For in vitro studies, samples of PRP or whole blood respectively were pre-incubated with Rivaroxaban (1 μM, 30 min) and afterwards stimulated with ADP.

2.6. Substances

Unless stated otherwise, all chemicals were obtained from Sigma (Deisenhofen, Germany) in the highest purity available. Rivaroxaban was kindly provided by Bayer Vital GmbH (Leverkusen, Germany).

2.7. Statistics

Data are presented as means ± SEM and analyzed using Student's *t*-test or one-way ANOVA with a Tukey post hoc test where appropriate. A repeated measures ANOVA was applied for dose response curves. A $p < 0.05$ was considered statistically significant.