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Full Length Article

Dabigatran but not rivaroxaban or apixaban treatment decreases fibrinolytic resistance in patients with atrial fibrillation

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ABSTRACT

Introduction: Most anticoagulants stimulate fibrinolysis in vitro through mechanisms dependent on and independent of thrombin activatable fibrinolysis inhibitor (TAFI). We evaluated the effect of dabigatran, rivaroxaban and apixaban treatment on plasma fibrinolysis in patients with non-valvular atrial fibrillation.
Methods and results: Patients treated with dabigatran etexilate (n = 22), rivaroxaban (n = 24) or apixaban (n = 22) were studied. Plasma was obtained before (trough) and 2 h after drug intake (peak). Fibrinolytic resistance of clots exposed to exogenous tissue plasminogen activator was significantly lower in peak than in trough samples and correlated with drug concentration only in dabigatran group. Moreover, fibrinolytic resistance at peak was lower in dabigatran than in rivaroxaban and apixaban groups. This difference disappeared if the TAFI pathway was inhibited. Thrombin generation and TAFI activation were markedly lower in peak than in trough samples in all three groups. However, TAFIa levels in trough and peak samples were significantly lower in dabigatran group than in rivaroxaban and apixaban groups. Circulating levels of prothrombin fragment F1 + 2 (reflecting in vivo thrombin generation) and plasmin-antiplasmin complex (reflecting plasmin generation) were not or barely influenced by drug levels in all groups.
Conclusions: Our data suggest that dabigatran, contrary to rivaroxaban and apixaban, reduces fibrinolytic resistance by virtue of its greater impact on TAFI activation. The profibrinolytic effect of dabigatran may play a role locally, at sites of fibrin formation, by making the nascent thrombus more susceptible to plasminogen-dependent degradation.

1. Introduction

Dabigatran, rivaroxaban and apixaban are target-specific direct oral anticoagulants (DOACs). Dabigatran is a reversible inhibitor of thrombin, which is formed in vivo after intake of the prodrug, dabigatran etexilate [1]. Rivaroxaban and apixaban are highly specific reversible inhibitors of factor Xa [2,3]. Several large clinical trials have documented the efficacy and safety of DOACs in diverse thromboembolic diseases [4], and the drugs are now licensed for the prevention of venous thromboembolism after knee and hip replacement, the prevention of stroke and systemic embolism in patients with non-valvular atrial fibrillation and for the treatment of acute venous thromboembolism.

In vitro, most anticoagulants, including dabigatran [5] and rivaroxaban [6], have been shown to decrease fibrinolytic resistance of plasma and/or blood clots through the blockade of the antifibrinolytic effects brought about by thrombin, suggesting that the hastening of fibrin removal might contribute to the antithrombotic activity of the drugs [7]. Thrombin, besides its role in platelet activation and fibrin formation, is a key player in the protection of the hemostatic plug against premature lysis. In fact, thrombin is able to delay or even halt the fibrinolytic process through multiple mechanisms, which include: 1) a direct effect on fibrin structure, whereby the higher the thrombin concentration the more tightly packed and lysis-resistant the clot [8]; 2) the activation of factor XIII, which is essential for fibrin cross-linking and for the covalent binding of α2-antiplasmin to fibrin, which is one of the most powerful anti-fibrinolytic mechanisms [9,10]; 3) the activation of thrombin activatable fibrinolysis inhibitor (TAFI), a plasma procarboxypeptidase (also known as plasma procarboxypeptidase U or B) that, once activated (TAFIa), removes the C-terminal lysines from partially degraded fibrin, thereby reducing the binding of tissue-type plasminogen activator (t-PA) and plasminogen to the clot and, therefore, plasmin formation [11]. Whether DOACs decrease fibrinolytic resistance in clinical setting is unknown and hard to anticipate because patients under anticoagulation are generally old and frequently affected by several diseases, and thus may present with a compromised

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fibrinolytic system [12,13]. Moreover, the expression of thrombomodulin (TM) by vascular endothelial cells might interfere with the activity of direct thrombin inhibitors, as suggested by in vitro studies. It has indeed been reported that dabigatran and other thrombin inhibitors make the clots more resistant to fibrinolysis if TM is present in the clot lysis model [5,14,15]. Moreover, direct thrombin inhibitors have been shown to enhance thrombin generation in plasma containing TM [16,17] and in some animal models [17,18], raising concerns about a possible prothrombotic risk associated with dabigatran treatment. We performed a study in patients with non-valvular atrial fibrillation treated with dabigatran, rivaroxaban or apixaban to investigate 1) the effect of DOACs on fibrinolytic resistance; 2) the possible influence of TM; 3) the in vivo changes of fibrinolysis markers.

2. Materials and methods

2.1. Patients and sample collection

Sixty-eight consecutive patients with non-valvular atrial fibrillation (NVAF), older than 18 years, were enrolled at the Hemostasis and Thrombosis Center of Azienda Ospedaliera "Istituti Ospitalieri", Cremona, Italy. Patients received dabigatran etexilate (Pradaxa®, n = 22), rivaroxaban (Xarelto®, n = 24), or apixaban (Eliquis®, n = 22) according to AIFA (Agenzia Italiana del Farmaco) prescription criteria for the prevention of stroke and systemic embolism in NVAF. Exclusion criteria were alcohol abuse, infections and cancer. Venous blood was collected into 0.109 M citrate using Becton Dickinson coagulation tubes (BD, USA). Two samples were collected in the same morning, one just before drug intake (trough sample) and one 2 h after drug intake (peak sample). Blood was immediately centrifuged at 1200 g for 15 min at room temperature, and the resulting plasma was collected, snap frozen and stored at −80 °C until analysis.

Dabigatran concentration in patients' plasma was measured by diluted thrombin time (STA-thrombin, Diagnostica Stago, Asnieres, France) on a magneto-mechanical coagulation analyzer (STA-R, Diagnostica Stago) as described [19]. Rivaroxaban and apixaban concentrations were measured by a chromogenic anti-Xa assay (STA®-Liquid Anti-Xa, Diagnostica Stago). The study conformed to the Declaration of Helsinki and informed written consent was obtained from each patient. The study protocol was approved by the institutional Ethic Committee (42-2014-OSS_FARM-CR27).

2.2. Proteins and reagents

Single-chain recombinant t-PA was from Boehringer Ingelheim (Florence, Italy); human thromboplastin (Recombiplastin) was from Instrumentation Laboratory (Milan, Italy); bovine fibrinogen and potato tuber carboxypeptidase inhibitor (PTCI) were from Sigma (Milan, Italy). Rabbit thrombomodulin (TM) and reptilase ST were from American Diagnostica (Pfungstadt, Germany).

2.3. Plasma clot lysis assay

The lysis of tissue factor-induced plasma clots exposed to exogenous t-PA was studied with a turbidimetric assay as described [5], with minor modifications. One hundred μL plasma, 10 μL thromboplastin (1:1000, final dilution, corresponding to approximately 6 pM tissue factor), 10 μL t-PA (30 ng/mL, final concentration, f.c.), and 20 μL Tris-NaCl buffer, were added to microplate wells, after which the clotting reaction was started with 100 μL $CaCl_2$ (8.3 mM, f.c.). The plate was incubated at 37 °C, and the changes in optical density at 405 nm were measured every minute in a microplate reader (Multiskan FC; Thermo Fisher Scientific, Waltham, MA, USA). Clot lysis time was defined as the interval between the midpoint of the clear to maximum turbidity transition and the midpoint of the maximum turbidity to clear transition. Where indicated, experiments were performed in the presence of the specific TAFIa inhibitor PTCI (25 μg/mL, f.c.), or upon addition of the TAFI activation cofactor TM (4 nM, f.c.). All samples were also tested with the thrombin-like enzyme reptilase (1:50, final dilution) [5] as substitute for thromboplastin and calcium to avoid thrombin generation and TAFI activation.

2.4. TAFIa generation

Thrombin-mediated TAFIa generation was assessed in plasma by a two-stage functional assay as previously described [20], with minor modifications. Plasma was defibrinated by reptilase (1:50) for 1 h at 37 °C. Then, a mixture similar to that used for clot lysis assay, except for the absence of t-PA, was prepared in a test tube and incubated at 37 °C. After 10 min, unless otherwise specified, an aliquot was withdrawn, mixed with hirudin (200 U/mL, f.c.; Abbott GmbH, Ludwigshafen, Germany) to stop TAFI activation, and kept on melting ice until tested. TAFIa activity was evaluated as the ability to prolong the lysis time of purified fibrin clots. Thirty-five μL of sample were added to a microplate well along with 25 μL bovine fibrinogen (830 μg/mL, f.c.), 10 μL t-PA (30 ng/mL, f.c.) and 40 μL Tris-buffer, after which clot formation was induced by 10 μL reptilase (1:50, final dilution). The plate was read every minute at 405 nm at room temperature (to reduce the temperature-dependent TAFIa decay) and lysis times were calculated as described above. PTCI served as a reference for the absence of TAFI activity and results were expressed as prolongation of lysis time over the PTCI-containing sample. Experiments on pooled normal plasma containing increasing amounts of TAFIa showed a linear correlation between PTCI-sensitive lysis time prolongation and TAFIa concentration (r = 0.994).

2.5. Thrombin generation

Thrombin generation was evaluated by the calibrated automated thrombinography (CAT) method developed by Hemker et al. [21]. Where indicated, experiments were performed in the presence of 4 nM TM. Because dabigatran inhibited thrombin bound to α2-macroglobulin [22], neither the sample-specific calibrator (test plasma spiked with α2-macroglobulin-thrombin) nor the Thrombinoscope software could be used in dabigatran-containing plasma samples. Therefore, we calculated the thrombin generation curve manually, without an internal calibrator. Briefly, we first plotted the raw fluorescence values against time and then we calculated the first derivative of the curve (velocity of fluorescence increase), which resulted in a thrombin generation curve similar to those obtained by CAT. Then, thrombin generation parameters, namely lag time (time to thrombin increase), peak thrombin and area under the curve (endogenous thrombin potential, ETP) were calculated by the GraphPad Prism Software (La Jolla, CA, USA). Due to the lack of calibrators, the latter two parameters were expressed in arbitrary units (AU). Manual calculations could not be used for rivaroxaban and apixaban samples because of the bias caused by the cleavage of the fluorogenic substrate by thrombin-α2-macroglobulin complex. Therefore, CAT parameters in these samples were calculated by the Thrombinoscope software.

2.6. ELISA assays

The following biomarkers were measured in patients' plasma by commercially available ELISAs according to the manufacturers' instructions: activated TAFI (Asserachrom TAFIa/ai, Diagnostica Stago); prothrombin F1 + 2 (Enzygnost F1 + 2, micro, Siemens Healthcare Diagnostics Products, Marburg, Germany); and plasmin/α2-antiplasmin complex (Technozyme PAP, Technoclone GmbH, Vienna, Austria).