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Thrombin induces protease-activated receptor 1 signaling and activation of human atrial fibroblasts and dabigatran prevents these effects☆,☆☆

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ABSTRACT

Background: Data with animal cells and models suggest that thrombin activates cardiac fibroblasts (Fib) to myofibroblasts (myoFib) via protease-activated receptor 1 (PAR1) cleavage, and in this way promotes adverse atrial remodeling and, thereby, atrial fibrillation (AF).
Objective: Here, we explored the effects of thrombin on human atrial Fib and whether they are antagonized by the clinically available direct thrombin inhibitor, dabigatran.
Methods: Fib isolated from atrial appendages of patients without AF undergoing elective cardiac surgery were evaluated for PAR expression and treated with thrombin with or without dabigatran. PAR1 cleavage, downstream signaling and myoFib markers were investigated by immunofluorescence and Western blot. Collagen synthesis, activity of matrix metalloprotease (MMP)-2 and proliferation were assessed by Picro-Sirius red staining, gelatinolytic zymography and BrdU incorporation, respectively. Fib function was studied as capability to contract a collagen gel and stimulate the chemotaxis of peripheral blood monocytes from healthy volunteers.
Results: Primary human atrial Fib expressed PAR1, while levels of the other PARs were very low. Thrombin triggered PAR1 cleavage and phosphorylation of ERK1/2, p38 and Akt, elicited a switch to myoFib enriched for αSMA, fibronectin and type I collagen, and induced paracrine/autocrine transforming growth factor beta-1, cyclooxygenase-2, endothelin-1 and chemokine (C-C motif) ligand 2 (CCL2); conversely, MMP-2 activity decreased. Thrombin-primed cells displayed enhanced proliferation, formed discrete collagen-containing cellular nodules, and stimulated the contraction of a collagen gel. Furthermore, their conditioned medium caused monocytes to migrate. All these effects were prevented by dabigatran.
Conclusion: These results with human cells complete the knowledge about thrombin actions on cardiac Fib and strengthen the translational potential of the emerging paradigm that pharmacological blockade of thrombin may counteract molecular and cellular events underlying AF.

1. Introduction

Even very short episodes of atrial fibrillation (AF) activate the coagulation cascade with possible formation of intra-atrial thrombi [1–3]. As a result, AF carries an intrinsic risk of cardioembolism and, especially when a patient presents additional risk factors for stroke and systemic embolism, it is an indication for anticoagulant therapy [4]. The drugs of choice for prevention of AF-related cardioembolism are oral direct inhibitors of factor Xa or thrombin. The former group includes apixaban, rivaroxaban and edoxaban, while dabigatran is the only direct thrombin inhibitor clinically available at present [5].

☆ All the authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

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Thrombin plays a central role in the coagulation system, as it converts fibrinogen into fibrin, which constitutes the thrombus core. Furthermore, it activates platelets by cleaving the transmembrane G protein-coupled receptor, protease-activated receptor 1 (PAR1), at the N-terminus and unmasking a tethered ligand that initiates downstream intracellular signaling [6].

PAR1 is expressed by cardiac cells, on which, therefore, thrombin may act after being generated during the coagulation response [7–9]. Cardiac fibroblasts (Fib) isolated from mouse and rat hearts express PAR1, and in the last years thrombin has been shown to stimulate the transition of these cells to collagen-secreting myofibroblasts (myoFib) [10,11].

Very recently, it has been proposed that thrombin and AF are linked to each other in a vicious circle, where AF triggers thrombin production and thrombin sustains AF, by recruiting myoFib via PAR1 and causing atrial remodeling, inflammation and fibrosis, which in turn predispose to arrhythmia [12,13].

Here, we investigated the effects of thrombin on primary human atrial Fib and whether they are antagonized by dabigatran, in order to integrate these studies with information of potential translational value.

2. Methods

For full Methods see Supplementary material.

Labware and reagents were purchased from Sigma-Aldrich (Munich, Germany) unless otherwise specified.

The study was approved by the local Ethics Committee and the patients and healthy donors gave written informed consent to the use of their primary cells for the experiments presented here.

2.1. Cells and treatments

Primary Fib were isolated from human right atrial appendages collected during elective cardiac surgery, when the venous cannula was placed for extracorporeal circulation, and grown in Endothelial Growth Medium-2 (EGM-2; Lonza, Walkersville, MD, USA) to limit the tendency to undergo spontaneous activation, as previously reported [14]. Only atrial fragments from subjects without a history of AF were used (for clinical details see Full Methods in Supplementary Material).

Cells from passage 2 to passage 5 were washed twice with Endothelial Basal Medium-2 (EBM-2; Lonza) and then were treated with 1 nM thrombin (human α-thrombin at 3117 NiH U/mL specific activity; Enzyme Research Laboratories, South Bend, IN, USA), 500 nM dabigatran active compound (Boehringer Ingelheim International GmbH, Biberach, Germany) for 30 min and then 1 nM thrombin, or 500 nM dabigatran alone. The concentration of thrombin was chosen as it was the one maximally inducing the synthesis of collagen, as assessed by Picro-Sirius red staining, among those tested in a preliminary analysis (Supplementary Fig. 1A). Preliminary experiments also showed that Fib from passages 2 to 5 responded similarly to 1 nM thrombin (Supplementary Fig. 1B). In fact, biological replicates throughout the study were done with Fib at random passages between 2 and 5, and no substantial variations in results were noted in spite of being cells at different stages of culture. Dabigatran was used at 500 nM because this value falls within the blood levels normally attained in patients receiving approved doses of the drug [15]. Treatments were carried out in EBM-2 and cells left in EBM-2 for the duration of the experiments were used as control.

To determine the role of autocrine/paracrine transforming growth factor beta 1 (TGF-β1), atrial Fib were pre-incubated with 10 μM GW788388 (Selleckchem, Munich, Germany), a TGF-β receptor phosphorylation inhibitor, for 40 min before adding thrombin.

Human monocytes were isolated from the buffy coats of healthy volunteers.

2.2. PAR1 cleavage and signaling

Total PAR1 was evaluated after 15 min of no treatment or exposure to thrombin, thrombin preceded by dabigatran, or dabigatran alone by immunofluorescence with a mouse monoclonal primary antibody against Arg27-Thr102, which spans the tethered ligand, and Ser375-Thr425 of human PAR1 (clone 731,115, R&D Systems, Minneapolis, MN, USA), and quantified as signal area normalized for the number of nuclei. Cleavage of PAR1 was analyzed after 5 min of incubation with thrombin and/or dabigatran, by means of Western blotting with a rabbit polyclonal antibody against a fragment of human PAR1 containing Ser42, where thrombin cleavage occurs (Sigma-Aldrich) [16].

Western blotting was also used to study the phosphorylation of the kinases transducing intracellular PAR1 signals after 5 and 15 min-exposure to thrombin and/or dabigatran, and the levels of TGF-β1 and cyclooxygenase-2 (COX-2) after 24 h-treatment with thrombin and/or dabigatran, with the following primary antibodies: rabbit monoclonal anti-phosphorylated p38 (clone D3F9, Cell Signaling Technology) and rabbit polyclonal anti-p38 (Santa Cruz Biotechnology, Dallas, TX, USA); rabbit polyclonal anti-phosphorylated Erk1/2 (Cell Signaling Technology) and mouse monoclonal anti-ERK1/2 (clone C-9, Santa Cruz Biotechnology); rabbit monoclonal anti-phosphorylated Akt Ser^{473} (clone D9E, Cell Signaling Technology) and mouse monoclonal anti-Akt1 (clone B-1, Santa Cruz Biotechnology); mouse monoclonal anti-actin (clone C4, Santa Cruz Biotechnology); mouse monoclonal anti-TGF-β1 (clone TB21, Santa Cruz Biotechnology); and rabbit polyclonal anti-COX-2 (Elabsciences Biotechnology Inc., Houston, TX, USA). Levels of phosphorylated proteins or other proteins of interest were expressed as optical density of the corresponding bands normalized for the one of total protein or actin, respectively, as internal control.

2.3. Fibroblast activation and collagen secretion

Expression of the myoFib markers, α-smooth muscle actin (αSMA) and fibronectin, as well as of type I collagen was demonstrated by immunofluorescence with mouse monoclonal anti-human αSMA (clone 1A4, Dako, Denmark), mouse monoclonal anti-human fibronectin (clone IST-4, Sigma-Aldrich) and rabbit polyclonal anti-type I collagen (Proteintech, Manchester, UK) antibodies. Signal intensity was assessed as gray level normalized for total cell number (arbitrary units/cell). Collagen synthesis was also analyzed by Picro-Sirius red staining of Fib cultured in 8-well chamber slides; red signal intensity was quantified in images taken with a conventional light microscope.

2.4. Fibroblast proliferation

Proliferation of Fib left untreated or exposed to thrombin and/or dabigatran for 24 h was measured as BrdU incorporation by means of the Cell Proliferation ELISA BrdU kit (Roche Diagnostics).

2.5. Gelatinolytic zymography

Activity of matrix metalloprotease (MMP)-2, the main MMP in human cardiac Fib [17], was investigated by zymography of the conditioned media collected after 24 h-treatment of Fib [18]. MMP-9, which is readily detectable in the plasma of patients with heart failure [18], but it is not expressed by human adult cardiac Fib [19], served as internal control.

2.6. Collagen gel contraction

Control or treated Fib were mixed in a collagen gel, which was then allowed to polymerize [20]. After 24 h, the gel was mechanically separated from the wall of the well in which it was placed, in order to allow contraction, if any. After 96 h, the area was measured.

2.7. RT-PCR

RT-PCR was performed by using Taqman Gene Expression Assay MTO from Life Technologies (assays ID in Supplementary Table 1). The expression of the genes of interest was normalized against that of *RPLP0* and analyzed with the comparative DDCt method.

Endothelin-1 and chemokine (C-C motif) ligand 2 (CCL2) mRNA levels were investigated after 4 h-exposure to thrombin and/or dabigatran.

2.8. Expression of chemokine (C-C motif) ligand 2/C-C chemokine receptor type 2 by human atrial fibroblasts and monocytes

Protein expression of CCL2 in atrial Fib was confirmed by Western blot with a rabbit polyclonal anti-CCL2 antibody (Novus Biological, Littleton, CO, USA) after 24 h-treatment with thrombin and/or dabigatran, while the presence of the receptor for CCL2, C-C chemokine receptor type 2 (CCR2), in human monocytes was documented by immunofluorescence with a rabbit polyclonal anti-CCR2 antibody (Novus Biological).

2.9. Migration of monocytes

Monocyte migration was investigated by means of a microchemotaxis chamber (Neuro Probe Inc., Gaithersburg, MD, USA), with monocytes in the upper wells and 10 nM chemokine (C-C motif) ligand 3 (CCL3; R&D Systems), as positive control, or concentrated conditioned media of Fib, untreated or exposed to thrombin and/or dabigatran for 24 h, in the lower wells [21]. The effects of direct incubation with thrombin and/or dabigatran for 24 h were also studied. The chemotaxis index was calculated as the ratio between the number of cells migrated towards the Fib medium or CCL3 and the number of cells migrated in the absence of any stimulus.

2.10. Statistical analysis

Each experiment was repeated at least three times. Data are presented as mean ± SEM of absolute values or fold change vs. control and were compared by one-way ANOVA followed by post-hoc Tukey's multiple comparisons test or Kruskal-Wallis test followed by post-hoc Dunn's multiple comparisons test, respectively, by using GraphPad Prism Version 6.0a (GraphPad Software). Statistical significance was set at $P < 0.05$.

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