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Characterization of Thrombin-Bound Dabigatran Effects on Protease-Activated Receptor-1 Expression and Signaling In Vitro

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ABSTRACT

Thrombin, the key effector protease of the coagulation cascade, drives fibrin deposition and activates human platelets through protease-activated receptor-1 (PAR1). These processes are critical to the progression of thrombotic diseases. Thrombin is the main target of anticoagulant therapy, and major efforts have led to the discovery of new oral direct inhibitors of thrombin. Dabigatran is the first oral anticoagulant licensed for the prevention of thromboembolisms associated with orthopedic surgery and stroke prevention in atrial fibrillation. Dabigatran is a direct thrombin inhibitor that effectively blocks thrombin's catalytic activity but does not preclude thrombin's exosites and binding to fibrinogen. Thus, we hypothesized that catalytically inactive thrombin retains the capacity to bind to PAR1 through exosite-I and may modulate its function independent of receptor cleavage and activation. Here, we report that dabigatran at clinically relevant concentrations is an effective and acute inhibitor of thrombin-induced PAR1 cleavage, activation, internalization, and β-arrestin recruitment in vitro. Interestingly, prolonged exposure to catalytic inactive thrombin incubated with dabigatran at 20-fold higher therapeutic concentration resulted in increased PAR1 cell-surface expression, which correlated with higher detectable levels of ubiquitinated receptor. These findings are consistent with ubiquitin function as a negative regulator of PAR1 constitutive internalization. Increased PAR1 expression also enhanced agonist-induced phosphoinositide hydrolysis and endothelial barrier permeability. Thus, catalytically inactive thrombin appears to modulate PAR1 function in vitro by stabilizing receptor cell-surface expression; but given the high clearance rate of thrombin, the high concentration of dabigatran required to achieve this effect the in vivo physiologic relevance is unknown.

Introduction

Thrombin is the main effector protease of the coagulation cascade, and it promotes hemostasis and thrombosis. Thrombin is generated at sites of vascular injury and inflammation when tissue factor is exposed to plasma coagulant proteases. Tissue factor binds to factor VII, supporting both its activation to VIIa and activation of factor X. Factor Xa acts with its cellular cofactor Va to generate thrombin, which drives conversion of fibrinogen to fibrin and platelet activation (Davie et al., 1991; Butenas and Mann, 2002). Activated platelets also provide a surface for assembly of the tenase and prothrombinase complexes that produce more thrombin, resulting in amplification of the coagulation pathway.

Thrombin contains a catalytic site and two exosites. Exosite-I acts as a binding site for fibrinogen, which facilitates thrombin cleavage and fibrin generation, whereas exosite-II binds heparin (Gandhi et al., 2011). Thrombin also interacts with and cleaves protease-activated receptors (PARs), a family of G protein–coupled receptors (GPCRs) expressed on the cell surface, to elicit cellular responses. Protease-activated receptor-1 (PAR1), the family prototype, is the predominant mediator of thrombin signaling in most cell types, including human platelets, endothelial cells, and fibroblasts (Coughlin, 2005; Soh et al., 2010). The PAR1 N-terminal LDPR41-S42 sequence is recognized by thrombin's catalytic site (Vu et al., 1991b). A second interaction occurs between thrombin's anion-binding exosite-I and an N-terminal acidic region of PAR1 and increases thrombin's affinity for and remarkable potency toward PAR1 (Vu et al., 1991b). PAR3 also contains an N-terminal acidic region and is a high-affinity thrombin receptor (Ishihara et al., 1997). Intriguingly, thrombin's exosite-I interaction with PAR3 is sufficient to maintain the protease bound to the receptor (Bah et al., 2007). This highly acidic region is similar to a sequence present in the leech anticoagulant peptide hirudin and is termed the *hirudin-like domain* (Rydel et al., 1991). Once bound, thrombin's catalytic site facilitates the cleavage of PAR1 at the N-terminal R41–S42 peptide bond, exposing the tethered ligand domain that binds intramolecularly to the receptor to promote transmembrane signaling (Chen et al., 1994). Synthetic peptides that mimic the

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ABBREVIATIONS: BRET, bioluminescence resonance energy transfer; BSA, bovine serum albumin; DMSO, dimethylsulfoxide; ELISA, enzyme-linked immunosorbent assay; GPCR, G protein–coupled receptor; PAR, protease-activated receptor; PEI, polyethylenimine; PI, phosphoinositide; Rluc, *Renilla* luciferase; WT, wild-type; YFP, yellow fluorescence protein.

tethered ligand sequence can activate PAR1 independent of thrombin and proteolytic cleavage to promote cellular signaling.

Thrombin is the main target of anticoagulant therapy indicated in thromboembolism, which is often associated with atrial fibrillation and other thrombotic diseases (Hirsh et al., 2008). The vitamin K antagonist warfarin and its derivatives have been the standard for anticoagulant therapy for the past 50 years. Vitamin K antagonists function by modulating newly synthesized coagulant factors, rendering them inactive, and has a slow onset and offset of action, large interindividual variability, and a narrow therapeutic window (Hirsh et al., 2008). Consequently, major efforts have been made to develop new oral active, direct thrombin inhibitors. Dabigatran is a specific and potent reversible direct thrombin inhibitor that binds to the catalytic site, and not to the exosites of thrombin, and has been approved for the prevention of strokes and blood clots associated with nonvalvular atrial fibrillation (van Ryn et al., 2013). Because dabigatran binds selectively to the active site of thrombin, it does not affect thrombin interaction with fibrinogen via exosite-I (Hogg and Bock, 1997; Sanford and Plosker, 2008; van Ryn et al., 2008). These findings raise the possibility that catalytically inactive thrombin-bound dabigatran may also interact with cell-surface localized PAR1 and modulate its function.

PAR1 is a member of the class A family of rhodopsin-like GPCRs. GPCRs are dynamic molecules assuming multiple conformational states, many of which are "active" as defined by their ability to modulate cellular activities. Similar to other GPCRs, PAR1 is allosterically modulated by different proteases, by receptor dimerization, and by its localization to specific plasma membrane microdomains (Canto et al., 2012). It is not known, however, whether thrombin inhibited with dabigatran can bind to PAR1 and affect its function. Here we report that dabigatran is an effective and acute inhibitor of thrombin-mediated PAR1 cleavage, internalization, β-arrestin recruitment, and downstream signaling assessed in vitro using HeLa cells and cultured human endothelial cells. Intriguingly, prolonged incubation with catalytically inactive thrombin caused an increase in PAR1 surface expression that enhanced agonist-dependent PAR1 signaling, suggesting that inactive thrombin binding to PAR1 can modulate its function.

Materials and Methods

Reagents and Antibodies. Human α-thrombin at 3117 NIH U/ml specific activity was purchased from Enzyme Research Laboratories (South Bend, IN). The PAR1 peptide agonists SFLLRN and TFLLRNPNDK were synthesized as the carboxyl amide and purified by reverse phase high-pressure liquid chromatography at the Tufts University Core Facility (Boston, MA). Dabigatran was obtained from Boehringer Ingelheim Pharma (Biberach, Germany). Polyethylenimine (PEI) transfection reagent was purchased from Polysciences (Warrington, PA). Rabbit polyclonal anti-FLAG antibody was purchased from Rockland Immunochemicals (Gilbertsville, PA). Mouse monoclonal anti–β-actin was purchased from Sigma-Aldrich (St. Louis, MO). The anti-PAR1 C5433 rabbit polyclonal antibody has been described previously (Paing et al., 2004). Mouse monoclonal anti-ubiquitin antibody (P4D1) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-PAR1 WEDE mouse antibody was from Beckman Coulter (Fullerton, CA). Anti-p44/42 extracellular signal regulated kinase-1/2 (ERK1/2), anti–phospho-p44/42 ERK1/2, phospho-protein kinase B (Akt) serine 473, and anti-Akt antibodies were from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase–conjugated secondary goat anti-mouse and anti-rabbit antibodies were obtained from Bio-Rad (Hercules, CA).

Cell Lines and cDNAs. HeLa cells expressing the FLAG-tagged PAR1 wild-type (WT) or lysine (K) "0K" mutant were previously described (Trejo et al., 2000; Wolfe et al., 2007). PAR1 lysine residues within the intracellular loops and C terminus were converted to arginine to generate the 0K mutant (Wolfe et al., 2007). HeLa cells stably expressing PAR1 were plated at 1×10^5 cells per well of a 24-well dish and 5×10^5 cells per well of six-well dishes coated with fibronectin and grown overnight at 37°C. Human umbilical vein endothelial-derived EA.hy926 cells were maintained as described (Russo et al., 2009). COS7 cells were grown in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin.

Human PAR1 WT and R41A mutant containing an N-terminal FLAG epitope were cloned into the pcDNA vector as described previously (Lin et al., 2013). The PAR1 arginine (R) at position 41 was converted to alanine (A) to disrupt the thrombin cleavage site (Vu et al., 1991a). Full-length PAR1 WT fused to yellow fluorescence protein (YFP) at the C terminus cloned into the pRK6 vector was obtained from Dr. Jean-Philippe Pin (Montpellier University, Montpellier, France). The PAR1 R41A mutant fused to YFP was generated by QuickChange site-directed mutagenesis (Agilent Technologies, Santa Clara, CA) and confirmed by dideoxy sequencing (Retrogen Inc., San Diego, CA). β-arrestin1/2 fused to *Renilla* luciferase (Rluc) at the C terminus was provided by Dr. Kathryn DeFea (University of California, Riverside, Riverside, CA).

Cell Transfections. Cells were transiently transfected using varying amounts of cDNA plasmids diluted in PEI (1 mg/ml) and combined with OptiMEM (ThermoFisher Scientific, Grand Island, NY) at a 1:6 ratio before the addition to cells. Cells were transfected for 48 hours.

PAR1 Cleavage. Cleavage of FLAG-tagged PAR1 was determined as previously described (Ishii et al., 1993). Briefly, HeLa cells expressing PAR1 with an N-terminal FLAG epitope were grown in 24-well plates, washed, incubated with or without thrombin-bound dabigatran, and fixed with 4% paraformaldehyde; the amount of uncleaved PAR1 on the cell surface was detected with a polyclonal anti-FLAG antibody and enzyme-linked immunosorbent assay (ELISA).

PAR1 Internalization. PAR1 internalization was examined as previously described (Paing et al., 2002). HeLa cells stably expressing FLAG-tagged PAR1 WT or R41A were grown in 24-well plates and serum starved for 1 hour at 37°C. Cells were then treated with or without agonist preincubated with dabigatran for varying times at 37°C. Cells were fixed with 4% paraformaldehyde, and the amount of PAR1 remaining on the cell surface was detected with a polyclonal anti-PAR1 antibody and ELISA.

Phosphoinositide Hydrolysis. HeLa cells expressing FLAG-tagged PAR1 grown in 24-well plates were labeled with 1 μCi/ml myo-[^{3}H]inositol (American Radiolabeled Chemicals, St. Louis, MO) overnight at 37°C. Cells were then treated with or without thrombin pretreated with or without dabigatran in the presence of 20 mM lithium chloride for 60 minutes at 37°C and accumulated [^{3}H]inositol phosphates were measured as previously described (Paing et al., 2002). To assay phosphoinositide (PI) hydrolysis after prolonged agonist exposure, cells were treated with thrombin-bound dabigatran for 36 hours at 37°C in media only and then incubated with media containing agonist and 1 μCi/ml myo-[^{3}H]inositol overnight. Cells were washed and treated with or without 10 nM thrombin for 60 minutes, and [^{3}H]inositol phosphate s were measured as described herein.

ERK1/2 and Akt Activation. HeLa cells stably expressing FLAG-tagged PAR1 WT or R41A mutant or endothelial cells were grown in 24-well plates and serum-starved overnight. Cells were then treated with thrombin for 5 minutes at 37°C or activated protein C (APC) for 60 minutes at 37°C with or without dabigatran-thrombin. Cells were lysed in 2× Laemmli buffer, resolved by SDS-PAGE, transferred to membranes, and immunoblotted with anti–phospho-ERK1/2 or Akt and anti-ERK1/2 or Akt antibodies. Membranes were developed using chemiluminescence and quantified with ImageJ (National Institutes of Health).

Bioluminescence Resonance Energy Transfer Assay. COS-7 cells were transfected with PAR1 WT or R41A fused to YFP at the

C terminus together with β-arrestin fused to Rluc at acceptor:donor ratio of 10:1 using PEI transfection reagent for 48 hours, detached with Cellstripper (Mediatech, Manassas, VA), washed with phosphate-buffered saline (PBS), and resuspended in PBS containing 0.5 mM $MgCl_2$ and 0.1% glucose at a density of 0.5–1 × 10^6 cells/ml. An aliquot (80 μl) of cells was added to a 96-well microplate in triplicate, and 10 μl of coelenterazine *h* substrate was then added at a final concentration of 5 μM. After an 8-minute delay, cells were stimulated with or without thrombin-bound dabigatran, and the bioluminescence resonance energy transfer (BRET) signal was determined with a TriStar LB 941 plate reader (Berthold Biotechnologies, Schwarzwald, Germany) using two filter settings: 480 nm for Rluc and 530 nm for YFP. The BRET ratio was calculated as emission at 530 nm/emission at 480 nm, and net BRET was determined by subtracting the background BRET ratio (BRET ratio from cells expressing the Rluc construct only) using MicroWIN 2000 software (Berthold Technologies). The YFP signal was determined by excitation at 485 nm, and emission was detected at 535 nm. Total luminescence was measured by integrating the signal for 1 second/well without filter selection.

Cell-Surface ELISA. HeLa cells stably expressing FLAG-tagged PAR1 WT or 0K mutant cells were grown in 24-well plates as described already, and human cultured EA.hy926 endothelial cells expressing endogenous PAR1 were plated at 3.5 × 10^5 cells/well in 12-well plates. Cells were incubated with thrombin-bound dabigatran or dimethylsulfoxide (DMSO) for 24–72 hours at 37°C. After treatment, cells were placed on ice for 10 minutes and washed once with ice-cold PBS. Cells were fixed in 4% paraformaldehyde for 5 minutes on ice, washed twice with PBS, and then incubated with anti-FLAG antibody or anti-PAR1 antibody for 60 minutes at room temperature. Cells were washed twice with medium and incubated with horseradish peroxidase–conjugated secondary antibody for 60 minutes at room temperature. After incubation, cells were washed three times with PBS before incubating with 1-step 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)-diammonium salt solution (ThermoFisher Scientific, Rockford, IL). The absorbance of an aliquot was determined at 405 nm using a Molecular Devices SpectraMax Plus microplate reader (Sunnyvale, CA).

PAR1 Immunoprecipitation. HeLa cells stably expressing FLAG-tagged PAR1 WT, 0K, or R41A mutants were grown in six-well dishes and treated with or without thrombin-bound dabigatran for 24–48 hours at 37°C. Cells were placed on ice, washed with PBS, and lysed with Triton X-100 lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, 50 mM NaF, 10 mM sodium pyrophosphate, and 1% Triton X-100) supplemented with 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml soybean trypsin inhibitor, 1 μg/ml pepstatin, and 10 μg/ml benzamidine. Cell lysates were cleared by centrifugation, protein concentrations were determined using the bicinchoninic acid assay (ThermoFisher Scientific), and equal amounts of lysates were immunoprecipitated with anti-PAR1 antibody. Immunoprecipitates were washed three times with lysis buffer, and proteins were eluted in 2× Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 5% SDS, 0.01% bromophenol blue) containing 0.2 M dithiothreitol. Cell lysates and immunoprecipitates were resolved by SDS-PAGE, transferred to polyvinylidene fluoride membranes, and immunoblotted with the appropriate antibodies. Membranes were developed by chemiluminescence and quantified with ImageJ software (National Institutes of Health).

PAR1 Ubiquitination. Human cultured EA.hy926 endothelial cells were plated at 2 × 10^6 cells/dish in 60-mm dishes and grown overnight. Cells were treated with or without thrombin-bound dabigatran for 72 hours at 37°C and lysed in buffer containing 5 mM EDTA, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 0.1% (v/v) Nonidet P-40, 20 mM *N*-ethylmaleimide, 10 μg/ml of aprotinin, 10 μg/ml of leupeptin, and 10 μg/ml of pepstatin A. Equivalent amounts of cell lysates were immunoprecipitated with anti-PAR1 WEDE antibody. Immunoprecipitates were resolved by SDS-PAGE and transferred to polyvinylidene fluoride membranes. Endogenous ubiquitination of PAR1 was detected using an anti-ubiquitin P4D1 antibody, and PAR1 was detected using an anti-PAR1 antibody.

Endothelial Barrier Permeability. Endothelial barrier permeability was quantified by measuring the flux of Evans blue–bound bovine serum albumin (BSA) as previously described (Russo et al., 2009). Briefly, endothelial EA.hy926 cells were plated on 12-mm-diameter transwell dishes coated with 2% gelatin (3-μm pore size polycarbonate filter) at 1.2 × 10^5 cells/well. The upper and lower chambers contained 500 and 1500 μl growth medium, respectively. Cells were first treated with or without thrombin-bound dabigatran for 3 days at 37°C. The day before the experiment, cells were starved with medium supplemented with 0.2% fetal bovine serum in the absence or presence of dabigatran-bound thrombin. On the day of the experiment, cells were treated with 100 μM of TFLLRNPNDK for 10 minutes at 37°C. After stimulation, the medium in the upper chamber was replaced with 0.67 mg/ml Evans blue-BSA diluted in medium containing 4% BSA; after 20–30 minutes, the optical density at 650 nm was measured in a 1:2 diluted 75-μl sample from the lower chamber using a Molecular Devices SpectraMax plate reader.

Data Analysis. Data were analyzed using GraphPad Prism 4.0 (GraphPad Software, La Jolla, CA). Statistical analysis was determined by performing Student's *t* test, one-way analysis of variance, Dunnett's multiple comparison test, or two-way analysis of variance and Bonferroni's post-test.

Results

To assess the capacity of dabigatran to inhibit thrombin function at PAR1 in vitro, we first examined receptor cleavage using an antibody that detects the N-terminal FLAG epitope of PAR1 and ELISA (Fig. 1A). HeLa cells expressing FLAG-tagged PAR1 were incubated with 1 or 10 nM thrombin pretreated with varying concentrations of dabigatran or DMSO for 30 minutes. Dabigatran-inhibited thrombin was added to cells for 5 minutes at 37°C. Cells were fixed, and the amount of PAR1 N-terminal FLAG epitope remaining was determined by ELISA. This assay provides an accurate measure of PAR1 cleavage by thrombin (Fig. 1A) (Ishii et al., 1993; Soto and Trejo, 2010). A 5-minute incubation with 1 nM thrombin alone resulted in ~50% cleavage of PAR1, whereas a 5-minute incubation with 10 nM thrombin preincubated with DMSO resulted in nearly complete cleavage of the receptor: ~95% (Fig. 1B); however, thrombin preincubated with increasing concentrations of dabigatran resulted in progressive inhibition of PAR1 cleavage compared with thrombin treated with DMSO vehicle (Fig. 1B). The effective concentration of dabigatran to inhibit PAR1 cleavage by 1 or 10 nM thrombin was 2.68 ± 0.47 nM and 17.7 ± 3.17 nM, respectively (Fig. 1B, insert), significantly lower than the 100 to 500 nM concentrations of dabigatran used therapeutically (van Ryn et al., 2010). The time course of inhibition of PAR1 cleavage by dabigatran using a cell-surface ELISA was also examined. A brief 5-minute incubation with 10 nM thrombin at 37°C resulted in complete PAR1 cleavage, whereas thrombin preincubated with 100 or 300 nM dabigatran failed to cleave PAR1 at 5 minutes (Fig. 1C); however, cleavage of PAR1 was more evident after 30–60 minutes of thrombin preincubated with 100 nM but not with 300 nM dabigatran concentration (Fig. 1C), suggesting that residual thrombin activity exists at the lower concentration, at least when examined in vitro (Fig. 1C). These data indicate that dabigatran acts as a potent inhibitor of thrombin cleavage of PAR1, consistent with direct inhibition of the active site of the coagulant protease.

Although catalytically inactive thrombin-bound dabigatran is not effective at cleaving PAR1, it may interact with the

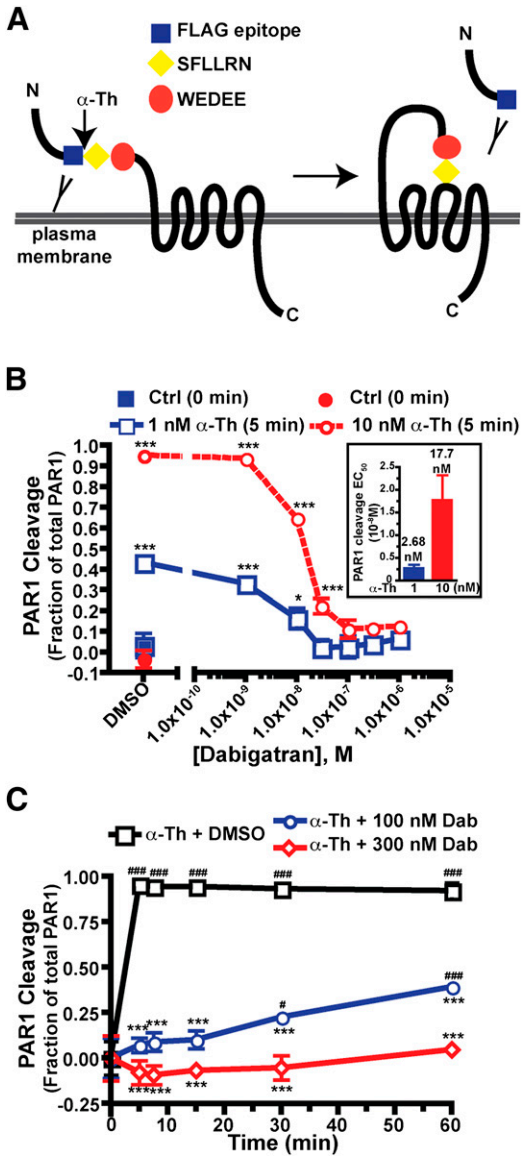

Fig. 1. Dabigatran inhibition of thrombin-induced PAR1 cleavage. (A) The anti-FLAG antibody detects a FLAG epitope in the N terminus of PAR1 that is cleaved off by thrombin and is used to measure receptor cleavage. PAR1-expressing HeLa cells were treated with or without thrombin preincubated with varying concentrations of dabigatran (Dab) or DMSO for 5 minutes (B) or various times (C) at 37°C. Cells were fixed, and the amount of PAR1 N-terminal FLAG epitope present on the cell surface was determined by ELISA. These data are representative of three independent experiments. The inset shows the EC_{50} of dabigatran inhibition of thrombin-induced PAR1 cleavage and is representative of three independent experiments. The differences in PAR1 cleavage induced by 1 nM thrombin preincubated with 0–10 nM dabigatran or 10 nM thrombin preincubated with 0–30 nM dabigatran compared with no thrombin treated control (Ctrl) at 5 minutes were statistically significant ($^{*}P < 0.05$; $^{***}P < 0.001$) as determined by two-way analysis of variance (ANOVA) and Bonferroni's post-tests. The differences in PAR1 cleavage stimulated with 10 nM thrombin preincubated with dabigatran compared with DMSO at various times were statistically significant ($^{***}P < 0.001$) as determined by two-way ANOVA and Bonferroni post-tests. The differences in PAR1 cleavage stimulated with 10 nM thrombin preincubated with DMSO for 5–60 minutes and 10 nM thrombin preincubated with 100 nM dabigatran for 30–60 minutes compared with 0 minute were statistically significant ($^{\#}P < 0.05$; $^{\#\#\#}P < 0.001$) as determined by two-way ANOVA and Bonferroni post-tests.

receptor via exosite-I and modulate PAR1 activity. To test this possibility, we first determined whether catalytically inactive thrombin affected PAR1 internalization using a cell-surface ELISA and an anti-PAR1 antibody that can detect the cleaved and uncleaved receptor (Fig. 2A). HeLa cells expressing FLAG-tagged PAR1 were incubated with 10 nM thrombin pretreated with or without dabigatran for varying times at 37°C. Cells were fixed, and the amount of PAR1 remaining on the cell surface was determined by ELISA. A 5-minute incubation with 10 nM thrombin resulted in a rapid and substantial increase in PAR1 internalization (Fig. 2B), consistent with that observed in previous studies (Paing et al., 2006; Wolfe et al., 2007). PAR1 internalization was significantly inhibited in cells treated with dabigratran-thrombin at 5 minutes, but at later times, an increase of ~20% in receptor internalization became evident in cells treated with the 100 nM dabigatran concentration (Fig. 2B). This effect is likely due to residual thrombin activity resulting from dabigatran dissociation at the lower concentration observed in vitro. To test whether receptor cleavage is necessary for internalization, a PAR1 R41A mutant that cannot be cleaved by thrombin was examined (Vu et al., 1991a). PAR1 R41A failed to internalize after treatment with thrombin or dabigatran-thrombin (Fig. 2C). To ensure that activated PAR1 retained the capacity to internalize in the presence of dabigatran, we used the synthetic agonist peptide SFLLRN, which bypasses thrombin and receptor cleavage and directly activates PAR1 (Vu et al., 1991a). Activation of PAR1 with SFLLRN resulted in robust receptor internalization regardless of dabigatran pretreatment (Fig. 2D), whereas internalization of PAR1 was significantly inhibited in cells incubated with dabigatran-thrombin. These results suggest that PAR1 cleavage by thrombin is necessary for internalization and catalytically inactive thrombin is not sufficient to promote receptor internalization independent of receptor cleavage.

We next assessed the effect of dabigatran-inhibited thrombin on PAR1 signaling by examining PI hydrolysis. In these experiments, PAR1 expressing HeLa cells were labeled with myo-[^{3}H]inositol, which is incorporated into PIs enriched at the plasma membrane and serves as a substrate for phospholipase C-β. Activated PAR1 stimulates G_q-mediated phospholipase C to promote PI hydrolysis (Soh et al., 2010). Cells were incubated with or without thrombin pretreated with varying concentrations of dabigatran. A 60-minute incubation with 10 nM thrombin resulted in a significant increase in [^{3}H]inositol phosphate accumulation compared with untreated control cells (Fig. 3A). In contrast, the capacity of thrombin preincubated with increasing concentrations of dabigatran to promote PI hydrolysis was progressively inhibited and virtually ablated in cells exposed to thrombin treated with 300 nM dabigatran (Fig. 3A); however, thrombin failed to promote a signaling response in PAR1 R41A expressing cells (Fig. 3B), whereas the peptide agonist SFLLRN elicited an increase in PI hydrolysis comparable to that observed with WT receptor (Fig. 3B). These data suggest that PAR1 cleavage is critical for G_q-mediated PI signaling and dabigatran is a potent inhibitor of thrombin activity.

To confirm these findings, we examined thrombin activated PAR1-stimulated ERK1/2 signaling in HeLa cells expressing comparable levels of PAR1 WT or the R41A mutant. We previously showed that activated PAR1 promotes ERK1/2 signaling via G_q and G_i signaling pathways (Trejo et al., 1996). Activation of PAR1 WT with 10 nM thrombin caused a marked increase in phosphorylation of ERK1/2 after 5 minute of agonist incubation compared with untreated control cells

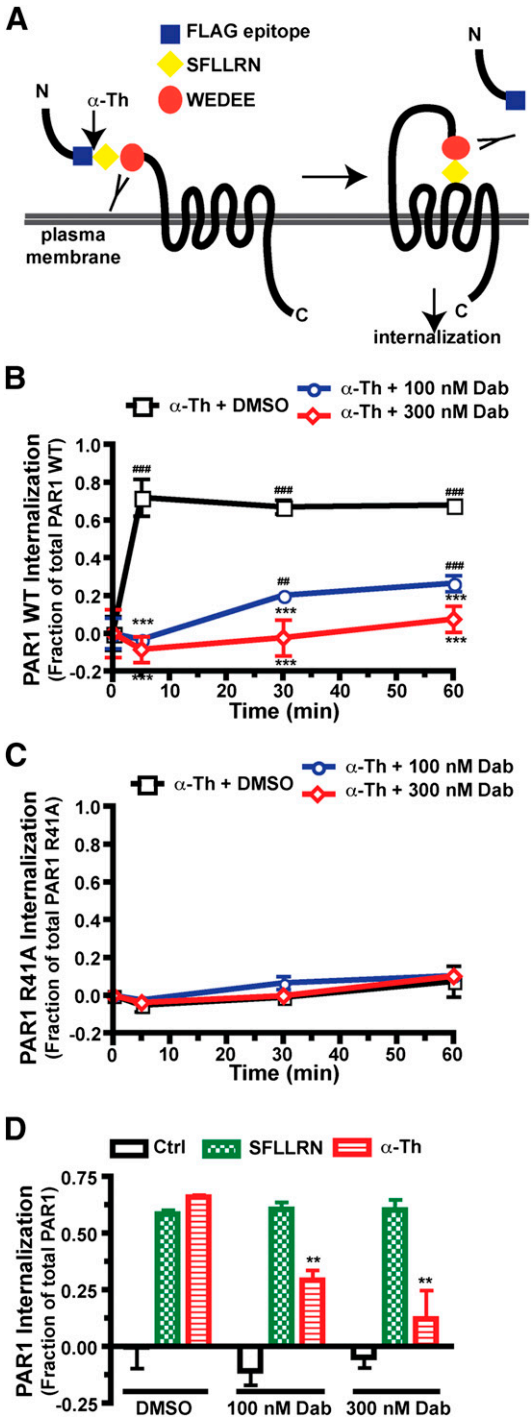

Fig. 2. Inhibition of thrombin-induced PAR1 internalization by dabigatran. (A) The anti-PAR1 antibody detects cleaved and uncleaved PAR1 and was used to assess internalization of PAR1. (B) PAR1 WT or (C), R41A-expressing HeLa cells were incubated with 10 nM thrombin preincubated with dabigatran (Dab) at 100 or 300 nM or DMSO for varying times at 37°C. Cells were fixed, and the amount of PAR1 WT or R41A remaining at the cell surface was determined by ELISA. These data are representative of three independent experiments. The differences in PAR1 WT internalization stimulated with thrombin preincubated with dabigatran or DMSO were statistically significant (***$P < 0.001$). The differences in PAR1 WT internalization stimulated with thrombin preincubated with DMSO for 5–60 minutes and thrombin preincubated with 100 nM dabigatran for 30–60 minutes were statistically significant ($^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$) as determined by two-way analysis of variance (ANOVA) and Bonferroni post-tests. (D) PAR1-expressing cells were stimulated with 100 μM SFLLRN or 10 nM thrombin after pretreatment with dabigatran at 100 or 300 nM or DMSO for 30 minutes at 37°C. Cells were fixed, and the amount of PAR1 internalization was determined. Similar findings were observed in multiple independent experiments. The differences in PAR1 internalization stimulated with thrombin preincubated with dabigatran or DMSO were statistically significant (**$P < 0.01$) as determined by one-way ANOVA and Dunnett's multiple comparison tests. Ctrl, control.

(Fig. 4A, lanes 1 and 2). Thrombin-induced ERK1/2 phosphorylation was ablated in cells expressing PAR1 R41A mutant as expected (Fig. 4A, lanes 3 and 4), indicating that PAR1 cleavage is essential for activation of the ERK1/2 signaling pathway. Thrombin pretreated with 300 nM dabigatran attenuated ERK1/2 activation compared with cells treated with thrombin only (Fig. 4B, lanes 2 and 6). A similar effect on ERK1/2 activation was observed with thrombin pretreated with 100 nM dabigatran (Fig. 4B). These results suggest that thrombin cleavage of PAR1 is necessary for ERK1/2 activation and is effectively blocked by dabigatran pretreatment.

In addition to G protein coupling, activated PAR1 can affect cellular function via interaction with β-arrestins, which are multifunctional adaptor proteins that serve diverse functions (Lefkowitz and Shenoy, 2005). To determine the effect of catalytically inactive thrombin on other PAR1-mediated responses, we examined the kinetics of β-arrestin recruitment by BRET. BRET is a very sensitive and widely used assay to monitor GPCR protein-protein interaction in living cells and relies on the oxidation of coelenterazine by Rluc, which releases photons that can excite a fluorophore such as YFP that is in close proximity (Lin et al., 2013). In these assays, full-length PAR1 fused in frame to YFP was coexpressed with β-arrestin-2 fused to Rluc and the change in BRET was monitored over 60 minutes. Activation of PAR1 WT but not the R41A mutant with 10 nM thrombin resulted in a robust increase in a BRET response between PAR1 and β-arrestin-2 (Fig. 5A), indicating that PAR1 cleavage by thrombin is essential for β-arrestin-2 recruitment. Thrombin preincubated with either 100 or 300 nM dabigatran resulted in significant inhibition of WT PAR1–β-arrestin-2 interaction at 5 minutes, but the response appeared to recover over time (Fig. 5A); however, β-arrestin recruitment was not observed with the PAR1 R41A mutant stimulated with 100 or 300 nM dabigatran-pretreated thrombin (Fig. 5A), indicating that PAR1 cleavage is required for the residual β-arrestin recruitment and is likely due to thrombin activity. A high 10 μM concentration of dabigatran, which is 20-fold greater than therapeutic doses (van Ryn et al., 2010), was required to completely inhibit thrombin-induced β-arrestin-2 recruitment to PAR1 assessed by BRET in vitro assessed over 60 minutes (Fig. 5B). Similar results were observed with β-arrestin-1 (data not shown). These findings suggest that dabigatran inhibits thrombin's catalytic activity and effectively blocks PAR1 cleavage, internalization, signaling, and β-arrestin recruitment.

To evaluate whether catalytically inactive thrombin could affect PAR1 function after prolonged exposure in vitro, we examined whether PAR1 protein expression was affected after 24-hour exposure to thrombin inhibited with dabigatran. HeLa cells were treated for 24 hours with or without thrombin preincubated with dabigatran or DMSO, and PAR1 was immunoprecipitated and analyzed by immunoblotting. In control conditions, PAR1 migrated as a broad ~75-kDa band as a result of extensive glycosylation (Fig. 6A, lanes 1 and 3)

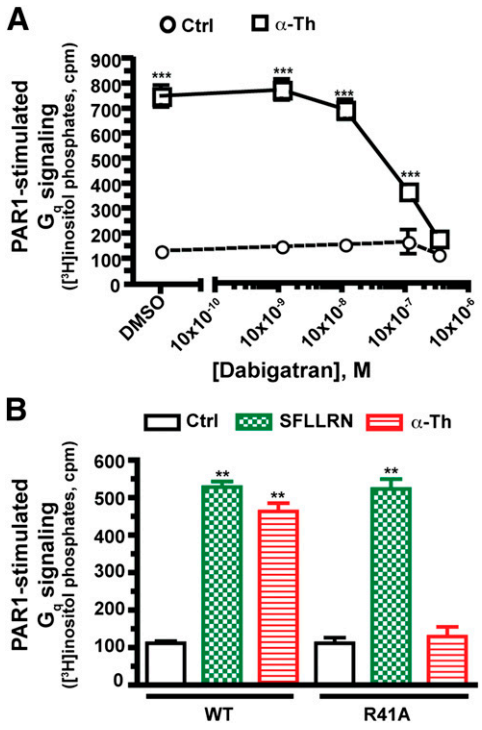

Fig. 3. Dabigatran inhibition of thrombin-activated PAR1-stimulated PI signaling. (A) PAR1-xpressing HeLa cells labeled with myo-[^{3}H]inositol were incubated in the absence (Ctrl) or presence of 10 nM thrombin preincubated with various concentrations of dabigatran or DMSO for 60 minutes at 37°C. The differences in thrombin-stimulated signaling compared with control were statistically significant (***$P < 0.001$) as determined by two-way analysis of variance (ANOVA) and Bonferroni's post-tests. (B) HeLa cells transiently expressing comparable surface PAR1 WT (mean ± S.D. = 0.584 ± 0.017) or R41A mutant (mean ± S.D. = 0.486 ± 0.022) were labeled with myo-[^{3}H]inositol and incubated in the absence (Ctrl) or presence of 10 nM thrombin or 100 μM SFLLRN for 60 minutes at 37°C. Cells were processed, and the amounts of [^{3}H]inositol phosphates accumulation were then measured. These data are representative of three independent experiments. The differences in thrombin- and SFLLRN-stimulated signaling compared with control were statistically significant (**$P < 0.01$) as determined by one-way ANOVA and Dunnett's post-tests.

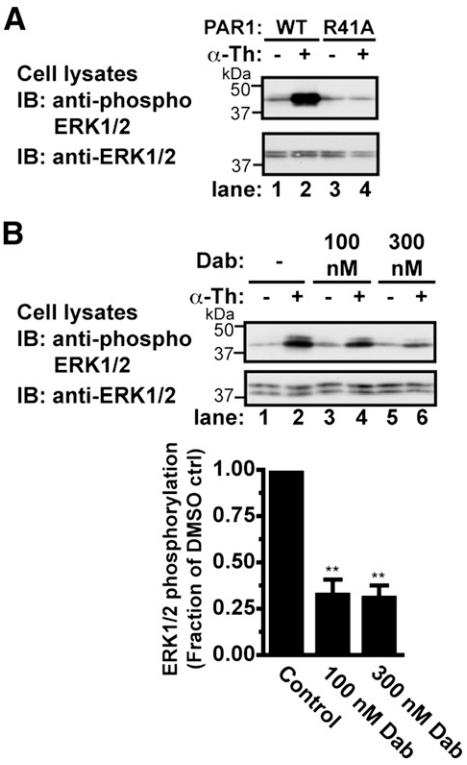

Fig. 4. Dabigatran inhibition of thrombin-induced ERK1/2 activation. (A and B) PAR1 WT or R41A mutant-expressing HeLa cells were serum-starved overnight and stimulated for 5 minutes at 37°C with 10 nM thrombin preincubated with or without 100 or 300 nM dabigatran (Dab) or DMSO. Cells were lysed, and equivalent amounts of cell lysates were subjected to SDS-PAGE, transferred to polyvinylidene fluoride membranes, and immunoblotted (IB) with anti-ERK1/2 antibody. Membranes were stripped and reprobed with anti-phospho ERK1/2 antibody. ERK1/2 phosphorylation from three independent experiments was quantified by densitometry using ImageJ. The differences in phosphorylation of ERK1/2 stimulated with thrombin preincubated with dabigatran compared with DMSO were statistically significant (**$P < 0.01$) as determined by one-way analysis of variance and Dunnett's post-tests.

(Soto and Trejo, 2010). In cells incubated with thrombin pretreated with 10 μM dabigatran, however, a significant ~20% increase in PAR1 WT protein expression was detected compared with cells treated with dabigatran or DMSO alone (Fig. 6A, lanes 1, 3, and 4). In contrast, cells treated with thrombin only resulted in a substantial loss of detectable PAR1 (Fig. 6A, lane 2). We also examined the effect of catalytically inactive thrombin on a PAR1 "0K" lysine mutant, which cannot be ubiquitinated and displays enhanced constitutive internalization (Wolfe et al., 2007). Similar to PAR1 WT, incubation with dabigatran-thrombin for 24 hours resulted in a marked increase in PAR1 0K protein expression compared with control treated cells (Fig. 6A, lanes 5, 7, and 8). Incubation with thrombin alone nearly abolished detection of the ~75-kDa PAR1 0K-positive band (Fig. 6A, lane 6), consistent with efficient cleavage of the receptor.

To determine whether the effect of dabigatran-thrombin on PAR1 expression was recapitulated with a cleavage-defective receptor mutant, we examined PAR1 R41A mutant expression. PAR1 R41A migrates as a broad band similar to WT receptor (Fig. 6B, lanes 9 and 10). Although prolonged exposure to thrombin failed to cleave PAR1 R41A, a significant increase of PAR1 protein levels was detected (Fig. 6B, lanes 9, 10, and 11). A similar effect was observed with thrombin pretreated with low 10 nM or high 10 μM dabigatran concentrations (Fig. 6A, lanes 9, 12, and data not shown). These results suggest that thrombin binding to PAR1 without cleavage may be sufficient to enhance receptor expression.

We next examined whether catalytically inactive thrombin affected PAR1 expression at the cell surface using ELISA. HeLa cells stably expressing comparable amounts PAR1 WT or 0K mutant were treated with or without dabigatran-thrombin or vehicle control. A prolonged incubation with 10 μM dabigatran-treated thrombin caused a significant increase in PAR1 WT and 0K mutant detected at the cell surface compared with cells treated with dabigatran or DMSO only at steady state (Fig. 6C). As expected, the addition of thrombin caused a substantial loss of detectable PAR1 WT and 0K mutant expression at the cell surface (Fig. 6C). PAR1 R41A mutant expression at the cell surface was also markedly increased after prolonged treatment with thrombin preincubated with vehicle control or 10 nM dabigatran (Fig. 6D). These findings indicate that prolonged exposure to catalytically inactive thrombin in vitro or thrombin incubation with a cleavage-defective PAR1 mutant increases receptor protein levels and surface expression in HeLa cells.

To assess whether increased expression of PAR1 at the cell surface affects downstream signaling, we examined

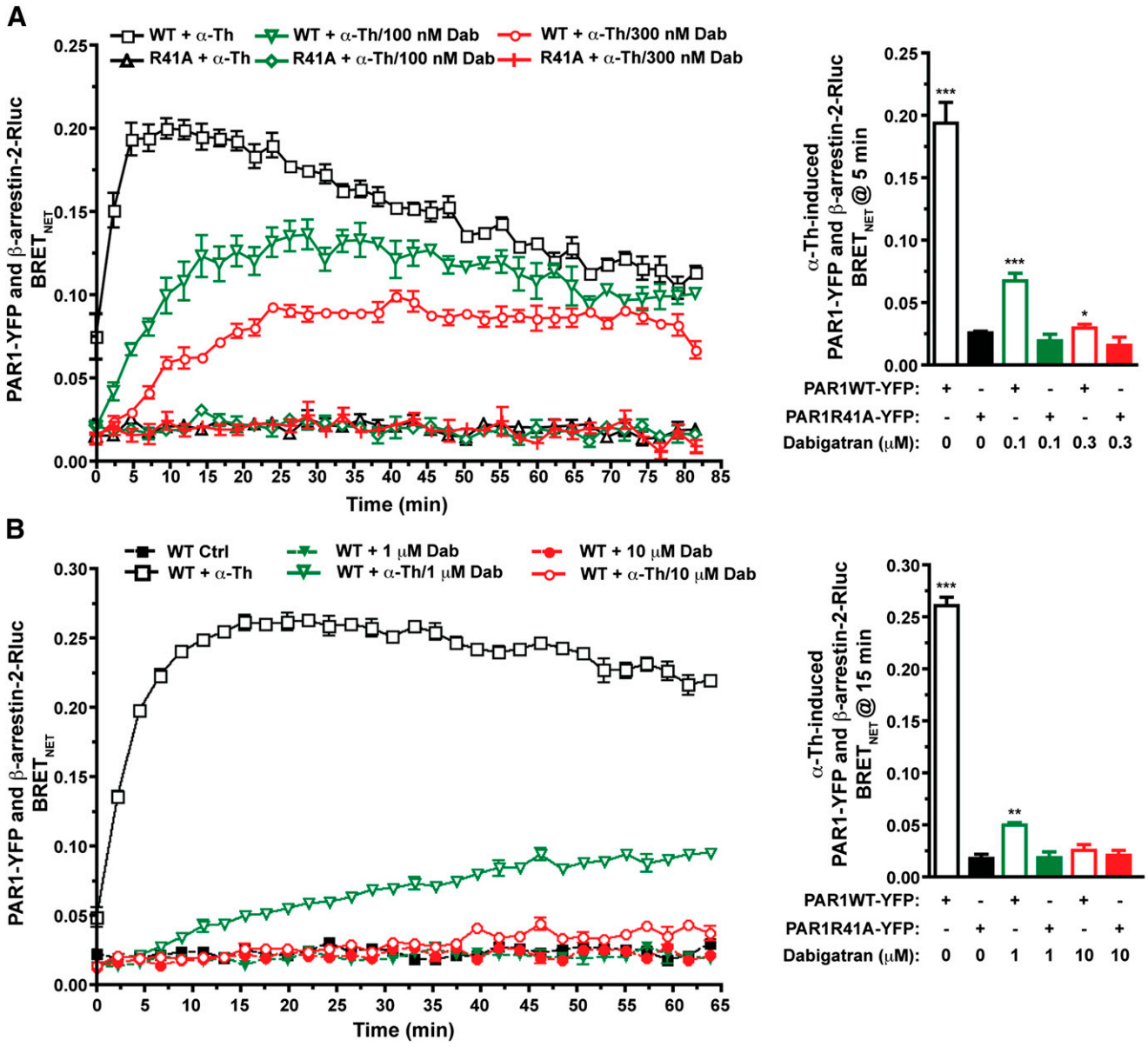

Fig. 5. Inhibition of thrombin-induced β-arrestin-2 recruitment to PAR1 by dabigatran. (A and B) COS-7 cells expressing comparable amounts of PAR1 WT-YFP (mean ± S.D. = 7522 ± 445 fluorescence A.U.) or PAR1 R41A-YFP (mean ± S.D. = 9147 ± 1342 fluorescence A.U.) together with β-arrestin-2-Rluc were stimulated with 10 nM thrombin pretreated with 100 nM to 10 μM dabigatran (Dab) or DMSO, and the BRET signal was determined. Similar results were observed in separate experiments. BRET signals at peak β-arrestin recruitment to PAR1 WT are shown in bar graphs. Thrombin pretreated with up to 1 μM dabigatran stimulated statistically significant (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$) β-arrestin recruitment as determined by student's t test. Ctrl, control.

PAR1-stimulated PI hydrolysis in cells after prolonged treatment with dabigatran-thrombin. HeLa cells expressing PAR1 WT were incubated with or without thrombin pretreated with 10 μM dabigatran or vehicle control for 24 hours and then labeled with myo-[^{3}H]inositol at 37°C overnight. Cells were washed to remove thrombin-bound dabigatran and stimulated with thrombin only for 1 hour at 37°C, and the [^{3}H]inositol phosphate accumulation was measured. In cells pretreated with dabigatran or DMSO, thrombin induced a comparable ~2-fold increase in PI hydrolysis (Fig. 6E). In contrast, thrombin promoted a significantly greater ~3-fold increase in PI hydrolysis in cells pretreated with dabigatran-thrombin compared with untreated control cells (Fig. 6E). Taken together, these data suggest that increased PAR1 cell-surface expression in HeLa cells after prolonged treatment with high concentrations of dabigatran in complex with catalytically inactive thrombin enhances receptor signaling.

Next, we investigated whether dabigatran-inhibited thrombin blocked PAR1 activation by other proteases, specifically APC using human cultured endothelial cells. APC binds to and cleaves the N terminus of PAR1, albeit at a site distinct from thrombin and elicits cytoprotective signaling, including Akt activation (Mosnier et al., 2012). APC induced a marked increase in Akt phosphorylation (Fig. 6F, lanes 1 and 2), whereas neither thrombin alone nor thrombin-dabigatran affected Akt phosphorylation (Fig. 6F, lanes 3, 5, 7, and 9). Interestingly, thrombin inhibited by dabigatran using varying concentrations also failed to affect APC-induced Akt activation (Fig. 6F, lanes 4, 6, and 8). These data suggest that thrombin-bound dabigatran does not block activation of PAR1 by APC.

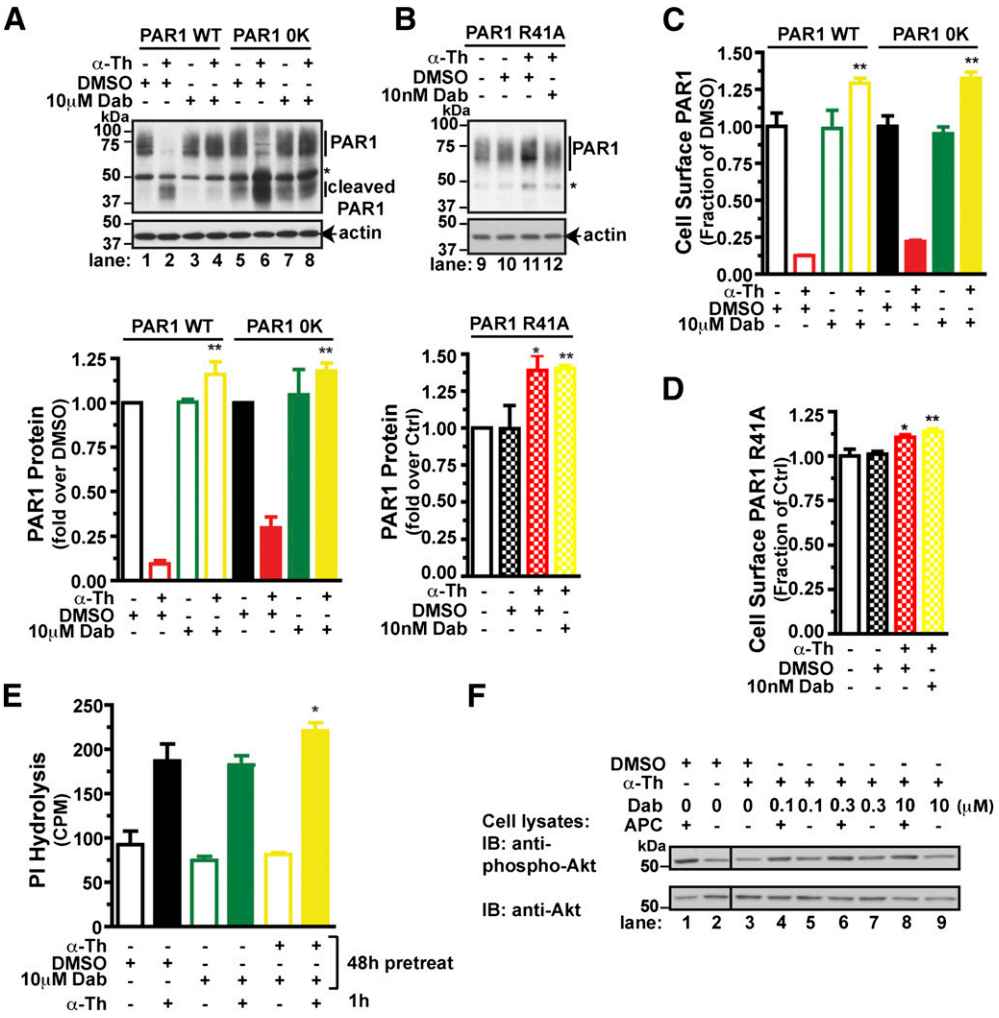

Fig. 6. PAR1 expression and signaling are enhanced after a prolonged treatment of thrombin-bound dabigatran in HeLa cells. (A) HeLa cells stably expressing PAR1 WT or 0K mutant were incubated in the absence or presence of 10 nM thrombin pretreated with or without 10 μM dabigatran (Dab) for 24 or 48 hours at 37°C. (B) HeLa cells transiently expressing PAR1 R41A mutant were incubated the absence or presence of 10 nM thrombin pretreated with or without 10 nM dabigatran for 48 hours at 37°C. Cells were lysed after 24 or 48 hours of thrombin-bound dabigatran exposure, and equivalent amounts of lysates were immunoprecipitated with anti-PAR1 antibody, immunoblotted (IB), and quantified with ImageJ densitometry. The differences in PAR1 protein detected after prolonged thrombin-bound dabigatran or thrombin exposure compared with control in three independent experiments was statistically significant ($^*P < 0.05$; $^{**}P < 0.01$) as determined by one-way analysis of variance (ANOVA) and Dunnett's multiple comparison tests. Cell lysates were immunoblotted with anti-actin antibody as a control. *Indicates a nonspecific band. (C and D) Cell-surface expression of PAR1 WT and mutants was determined by ELISA after 24 or 48 hours of thrombin-bound dabigatran incubation. The differences in PAR1 expression detected at the cell surface calculated from three experiments were statistically significant ($^*P < 0.05$; $^{**}P < 0.01$) as determined by one-way ANOVA and Dunnett's multiple comparison tests. (E) Cells were labeled with myo-[^{3}H]inositol overnight, washed, stimulated with 10 nM thrombin for 1 hour at 37°C, processed, and [^{3}H]inositol phosphates accumulation was measured. These data are representative of at least three independent experiments. The difference in thrombin-induced signaling in cells pretreated with dabigatran versus DMSO was statistically significant ($^*P < 0.05$) as determined by one-way ANOVA and Dunnett's multiple comparison tests. (F) Cells were treated with 20 nM APC for 1 hour at 37°C alone or with thrombin preincubated with varying concentrations of dabigatran. Cells were lysed and immunoblotted with antiphospho-Akt antibodies, stripped, and reprobed with anti-Akt antibody. Similar results were observed in three independent experiments.

To determine whether catalytically inactive thrombin modulated endogenous PAR1 function, we used human EA.hy926 endothelial cells. Endothelial cells were treated with or without thrombin preincubated with a high concentration of 10 μM dabigatran or DMSO vehicle control for 72 hours at 37°C and cell-surface expression was detected by ELISA. Endothelial cells subjected to prolonged thrombin exposure displayed a significant loss in PAR1 expression from the cell surface compared with cells treated with dabigatran or DMSO (Fig. 7A). In contrast, a modest but significant increase in endogenous PAR1 surface expression was detected after prolonged incubation with thrombin-bound dabigatran compared with control cells (Fig. 7A), consistent with results observed in HeLa cells (Fig. 6). To determine the mechanism by which catalytically inactive thrombin modulates PAR1 surface expression, we examined basal ubiquitination of PAR1. We previously reported that basal ubiquitination of PAR1 negatively regulates constitutive internalization and enhances receptor expression at the cell surface (Wolfe et al., 2007). Endothelial cells were treated with or without thrombin pretreated with 10 μM dabigatran or DMSO for 72 hours at 37°C. Cells were lysed, PAR1 was immunoprecipitated, and ubiquitination was assessed by immunoblotting as described (Chen et al., 2011). In endothelial cells exposed to dabigatran or DMSO, immunoprecipitates of PAR1 revealed a modest amount of ubiquitination compared with control IgG immunoprecipitates (Fig. 7B, lanes 1–3).

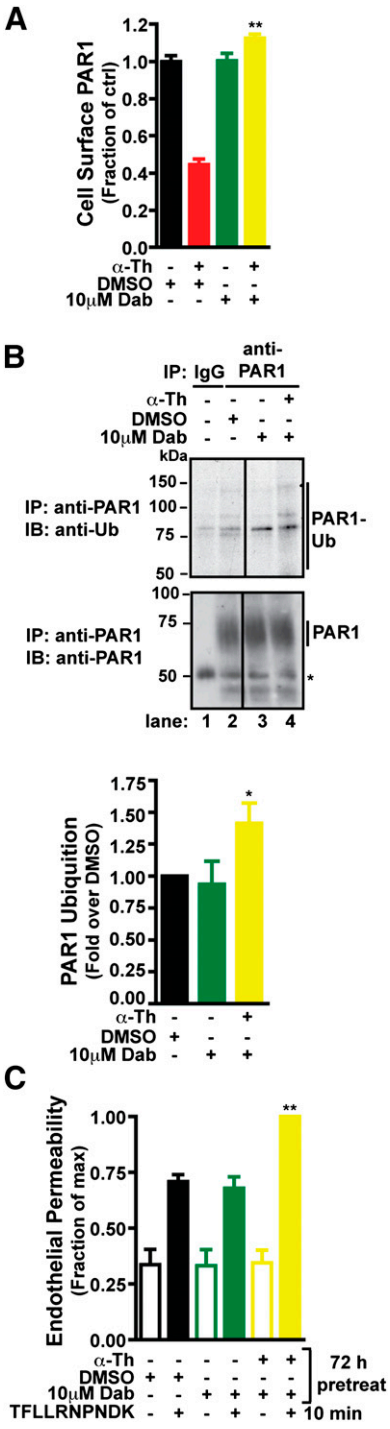

Fig. 7. PAR1 expression, ubiquitination, and endothelial permeability after a prolonged treatment of thrombin-bound dabigatran in endothelial cells. Endothelial cells expressing endogenous PAR1 were incubated in the absence or presence of 10 nM thrombin pretreated with or without 10 μM dabigatran (Dab) for 72 hours at 37°C. (A) After treatment, cell-surface PAR1 was determined by ELISA. The difference in cell-surface expression of PAR1 observed in three different experiments was statistically significant (**$P < 0.01$) as determined by one-way analysis of variance (ANOVA) and Dunnett's multiple comparison tests. (B) Cells were lysed, and equivalent amounts of lysates were immunoprecipitated (IP) with anti-PAR1 antibody. PAR1 was then immunblotted (IB) with an anti-PAR1 antibody and receptor ubiquitination (Ub) determined using an anti-ubiquitin antibody. *Indicates a nonspecific band. Similar results were observed in three independent experiments. Ubiquitination of PAR1 was quantified, and the difference in PAR1 ubiquitination observed in α-Th– and dabigratan-treated cells versus DMSO control was statistically significant (*$P < 0.05$) as determined by Student's t test. (C) After treatment, cells were incubated with 100 μM TFLLRN for 10 minutes at 37°C, and endothelial barrier permeability was assessed. The difference in TFLLRN-stimulated endothelial permeability observed in three independent experiments was statistically significant (**$P < 0.01$) as determined by one-way ANOVA and Dunnett's multiple comparison tests.

However, cells exposed to prolonged thrombin-bound dabigatran exhibited a marked increase in PAR1 ubiquitination (Fig. 7B, lane 4). These findings suggest that ubiquitination of PAR1 may contribute to enhanced expression at the cell surface.

To examine the functional consequences of increased PAR1 expression induced by prolonged incubation with catalytically inactive thrombin, we examined PAR1-stimulated endothelial barrier permeability. Endothelial cells form a semipermeable barrier through cell-cell contacts mediated by adherens junctions. Thrombin activation of PAR1 triggers intracellular signaling that induces disassembly of adherens junctions and reorganization of the actin cytoskeleton and barrier disruption (Komarova et al., 2007). Endothelial cells were grown in transwell dishes and treated with or without thrombin preincubated with 10 μM dabigatran or DMSO for 72 hours at 37°C. Cells were then stimulated with the PAR1-specific peptide agonist TFLLRNPNDK for 10 minutes at 37°C. Endothelial barrier permeability was monitored by examining the flux of Evans blue conjugated to BSA for varying times as previously described (Russo et al., 2009; Soh and Trejo, 2011). Direct stimulation of PAR1 with agonist peptide caused a marked increase in endothelial barrier permeability (Fig. 7C), consistent with PAR1-mediated disruption of the endothelial barrier as previously reported (Russo et al., 2009; Soh and Trejo, 2011). Intriguingly, however, activation of PAR1 by agonist peptide resulted in a significantly greater increase in endothelial barrier permeability in cells treated with thrombin-bound dabigatran versus cells incubated with only dabigatran or DMSO (Fig. 7C). Taken together, these findings suggest that prolonged exposure to thrombin inhibited with supratherapeutic concentrations, 20-fold higher than peak therapeutic concentrations of dabigatran, leads to upregulation of PAR1 surface expression and enhances the capacity for agonist-induced receptor signaling.

Discussion

In the present study, we found that dabigatran functions as a direct inhibitor of thrombin effects on PAR1. Thrombin preincubated with dabigatran in a concentration-dependent manner was no longer capable of cleaving the N-terminal domain of PAR1. Dabigatran also blocked thrombin-induced PAR1 internalization in a concentration-dependent manner, whereas direct activation of PAR1 with peptide agonist bypassed receptor cleavage and induced receptor internalization. Thrombin-elicited phosphoinositide hydrolysis, ERK1/2 signaling, and recruitment of β-arrestins were also significantly inhibited acutely by dabigatran treatment; however, PAR1 signaling or β-arrestin recruitment observed at 30 or 60 minutes with thrombin treated with lower concentrations of dabigatran in vitro was apparently due to residual thrombin activity since the effect was lost in cells expressing the PAR1 R41A mutant not cleavable by thrombin. However, prolonged treatment with catalytically inactive thrombin pretreated

with 10 μM dabigatran, a supratherapeutic concentration, resulted in increased PAR1 cell-surface expression and enhanced signaling and correlated with increased receptor ubiquitination. We hypothesize that dabigatran-thrombin interacts with the "hirudin-like" domain in the N terminus of PAR1 via thrombin's exosite-I and after long-term treatment stabilizes a receptor conformation that enhances retention on the cell surface. Consequently, increased expression of PAR1 at the cell surface results in the capacity of agonist to enhance signaling.

In a series of elegant studies in search for a receptor that conferred thrombin signaling, Coughlin and colleagues discovered PAR1, originally dubbed the "thrombin receptor" (Vu et al., 1991b). The N-terminal region of PAR1 contains an LDPR/S sequence and is similar to a sequence found in the thrombin substrate zymogen Protein C. The PAR1 LDPR/S sequence is cleaved by thrombin (Vu et al., 1991b). A second highly acidic region carboxyl to the cleavage site of PAR1 resembles a region in the leech anticoagulant hirudin and was shown to bind to exosite-I of thrombin (Rydel et al., 1991; Vu et al., 1991b). A crystal structure of thrombin bound to the N-terminal region of PAR1 revealed extensive interactions between PAR1 and both the active site and exosite-I of thrombin (Gandhi et al., 2011), indicating that thrombin recognizes the N terminus of PAR1 over an extended surface. Thrombin cleavage of PAR1 was confirmed using a mutant receptor in which the critical R41 was converted to proline, which rendered the receptor insensitive to thrombin cleavage and incapable of signaling. In the same study, a synthetic peptide SFLLRN, which represented the first six amino acids of the newly cleaved N terminus of the receptor activated PAR1 independent of thrombin and proteolytic cleavage (Vu et al., 1991b). Thus, it was hypothesized that the new N-terminal domain of PAR1 generated by thrombin acts as a tethered ligand and binds intramolecularly to the receptor and promotes signaling (Vu et al., 1991b). Consistent with these studies, we found that dabigatran bound to the catalytic site of thrombin is incapable of facilitating PAR1 cleavage, internalization, β-arrestin recruitment, and effector signaling. Moreover, the residual effects observed with thrombin-bound dabigatran were virtually ablated in cells expressing the PAR1 R41A noncleavable mutant. Thus, dabigatran functions as a direct inhibitor of thrombin function at PAR1.

Dabigatran binds directly to the active site of thrombin and inhibits its proteolytic activity, but it leaves the exosites of thrombin unencumbered (van Ryn et al., 2013). These findings suggest that thrombin-bound dabigatran retains the capacity to bind to substrates such as fibrinogen and PARs. Previous studies investigated the capacity of dabigatran to inhibit fluid and fibrin bound thrombin using platelet-rich plasma (van Ryn et al., 2008). Increasing concentrations of dabigatran was found to effectively inhibit fluid and clot bound thrombin similarly. These findings suggest that dabigatran effectively inhibits thrombin activity but not its capacity to bind to fibrin or other substrates through exosite-I. The idea that thrombin exosite-I interaction with substrate is sufficient to retain interaction is also supported by X-ray crystallography studies using the N-terminal fragment of PAR3 and thrombin. This work showed that the cleaved PAR3 N-terminal fragment remains bound to exosite-I of thrombin, leaving thrombin's active site free and accessible to other substrates (Bah et al., 2007). Taken together, these studies indicate that dabigatran-thrombin is fully capable of interacting with the PAR1 N-terminal hirudin-like domain via exosite-I and may modulate its function.

GPCRs are allosterically modulated, and PAR1 is no exception. Allosteric modulation of GPCRs occurs via the binding of different ligands to allosteric sites on the receptor, interaction with other transmembrane proteins, the plasma membrane microenvironment, and dimerization. Several studies have described allosteric modulation of PAR1 by many of these different factors. A previous study using human dermal microvascular endothelial cells showed that the tethered and the soluble peptide agonist differ in their capacity to promote G_q versus $G_{12/13}$ signaling (McLaughlin et al., 2005). PAR1 is also differentially activated by distinct proteases. The best example of differential activation of PAR1 is by thrombin versus activated protein C, which results in opposite effects on endothelial barrier permeability (Russo et al., 2009; Soh and Trejo, 2011). Interestingly, we found that dabigatran-thrombin did not affect the capacity of APC-activated PAR1 to signal, suggesting that the proteases may require distinct determinants for receptor recognition. Moreover, compartmentalization of PAR1 in caveolae, lipid-raft microdomains enriched in cholesterol, is important for differential activation by activated protein C (Bae et al., 2008; Russo et al., 2009). These studies suggest that the localization of PAR1 in plasma membrane microdomains may restrict activation to a subset of signaling effectors to elicit protease-specific signaling responses. PAR1 dimerization with PAR3 examined in HEK293 cells also appears to modulate the efficiency of G_{13} signaling (McLaughlin et al., 2007). Taken together, these studies strongly suggest that PAR1 is primed for allosteric modulation.

Our findings support the idea that dabigatran-thrombin binds to and modulates PAR1 by inducing a distinct conformation that stabilizes the receptor at the cell surface. The thrombin-bound dabigatran-induced stable conformation of PAR1 also displays enhanced ubiquitination. These findings are consistent with our previous work showing that basal ubiquitination of unactivated PAR1 is important for retaining receptor expression at the cell surface (Wolfe et al., 2007), whereas deubiquitinated PAR1 exhibits enhanced constitutive internalization. Ubiquitination of PAR1 is thought to preclude binding of the clathrin adaptor protein complex-2 to a canonical tyrosine-based motif and thereby blocks internalization through clathrin-coated pits (Paing et al., 2006). It is possible that dabigatran-thrombin is preferably recognized by E3 ubiquitin ligases or fails to be recognized by the deubiquitination machinery and consequently increases receptor ubiquitination; however, the E3 ubiquitin ligases and deubiquitinating enzymes that target PAR1 for ubiquitination are not known. In addition, the increase in PAR1 surface expression induced by dabigatran-thrombin is sufficient to promote effects on cellular signaling assessed in HeLa cells and endothelial cells.

In summary, we found that dabigatran is an effective inhibitor of acute thrombin-mediated PAR1 cleavage, internalization, signaling, and β-arrestin recruitment. However, prolonged exposure to thrombin pretreated with supratherapeutic concentrations of dabigatran caused a significant increase in PAR1 surface expression and enhanced agonist-induced signaling in vitro. However, the in vivo physiologic relevance of this effect is not known since the studies were not performed in plasma containing antithrombin. These findings suggest that catalytically inactive thrombin can bind to and modulate PAR1

function in vitro, but whether these effects are translated in vivo is not known, and given the rapid clearance of thrombin and metabolism of dabigatran (Stangier, 2008; van Ryn et al., 2013), it seems unlikely in patients with normal excretion and metabolic processes (Connolly et al., 2013).

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Authorship Contributions

Participated in research design: Chen, Soto, Goss, van Ryn, Trejo.

Conducted experiments: Chen, Soto, Coronel.

Contributed new reagents or analytic tools: Goss, van Ryn.

Performed data analysis: Chen, Soto, Coronel, Trejo.

Wrote or contributed to the writing of the manuscript: Chen, Goss, van Ryn, Trejo.

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