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Syed Waseem Bihaqi
Thomas Jefferson University

Haripriya Vittal Rao
Wake Forest Alzheimer's Disease Research Center

Abhik Sen
ICMR-Rajendra Memorial Research Institute of Medical Sciences

Paula Grammas
University of Rhode Island

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Dabigatran reduces thrombin-induced neuroinflammation and AD markers in vitro: Therapeutic relevance for Alzheimer's disease

Syed Waseem Bihaqi[a,d,*], Haripriya Vittal Rao[a,c], Abhik Sen[a,e], Paula Grammas[a,b]

[a] George & Anne Ryan Institute for Neuroscience, University of Rhode Island, Kingston, RI 02881, United States
[b] Department of Biomedical and Pharmaceutical Sciences, University of Rhode Island, Kingston, RI 02881, United States
[c] Wake Forest Alzheimer's Disease Research Center, Winston Salem, NC 27101, United States
[d] Department of Pathology, Anatomy and Cell biology, Thomas Jefferson University, Philadelphia, PA 19107, United States
[e] ICMR-Rajendra Memorial Research Institute of Medical Sciences, Patna, India

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ABSTRACT

Background: Vascular risk factors such as atherosclerosis, diabetes, and elevated homocysteine levels are strongly correlated with onset of Alzheimer's disease (AD). Emerging evidence indicates that blood coagulation protein thrombin is associated with vascular and non-vascular risk factors of AD. Here, we examined the effect of thrombin and its direct inhibitor dabigatran on key mediators of neuro-inflammation and AD pathology in the retinoic acid (RA)-differentiated human neuroblastoma cell line SH-SY5Y.

Methods: SH-SY5Y cells exposed to thrombin concentrations (10–100 nM) +/- 250 nM dabigatran for 24 h were analyzed for protein and gene expression. Electrophoretic mobility shift assay (EMSA) was used to determine DNA binding of NFkB. Western blotting, qRT-PCR and ELISA were used to measure the protein, mRNA, and activity levels of known AD hallmarks and signaling molecules.

Results: Dabigatran treatment attenuated thrombin-induced increase in DNA binding of NFκB by 175% at 50 nM and by 77% at 100 nM thrombin concentration. Thrombin also augmented accumulation of Aβ protein expression and phosphorylation of p38 MAPK, a downstream molecule in the signaling cascade, expression of pro-apoptotic mediator caspase 3, APP, tTau and pTau. Additionally, thrombin increased BACE1 activity, GSK3β expression, and APP, BACE1, Tau and GSK3β mRNA levels. Co-incubation with dabigatran attenuated thrombin-induced increases in the protein, mRNA, and activities of the aforesaid molecules to various extents (between −31% and −283%).

Conclusion: Our data demonstrates that thrombin promotes AD-related pathological changes in neuronal cultures and suggests that use of direct oral anticoagulants may provide a therapeutic benefit against thrombin-driven neuroinflammation and downstream pathology in AD.

1. Introduction

Alzheimer's disease (AD) is a progressive neurological disorder involving aberrant protein processing characterized by both intraneuronal protein aggregates of hyperphosphorylated tau (neurofibrillary tangles) and extracellular protein aggregates (senile plaques) composed of amyloid beta (Aβ) [1]. Additionally, vascular dysfunction and neuroinflammation have been consistently demonstrable in AD pathology [2]. Several population based studies have highlighted the association between vascular risk factors such as hypertension and diabetes with the onset of AD. Up to 84% of aged subjects show morphological substrates of cerebrovascular disease in addition to AD pathology [3]. Vascular comorbidity has been established as a risk factor for the onset of AD in 40% -80% of AD patients [4]. Various research groups have documented inflammation as an invariant feature and a likely driver of pathologic processes in the AD brain. Large-scale genome-wide association studies point out several genetic variants linked to neuroinflammation suggesting that neuroinflammation is a common nexus for the onset of many underlying pathologies of A [5].

Numerous inflammatory mediators are elevated in the AD brain microvasculature including tumor necrosis factor alpha (TNFα), transforming growth factor-β (TGFβ), interleukins (ILs) and the multifunctional inflammatory protein thrombin [6,7]. Thrombin is especially relevant for AD due to its ability to trigger both tau and Aβ accumulation as

* Corresponding author at: Department of Pathology, Anatomy and Cell biology, 1020 Locust Street, Jefferson Alumni Hall, Thomas Jefferson University, Philadelphia, PA 19107, United States.
E-mail address: swb140@jefferson.edu (S.W. Bihaqi).

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well as to stimulate proinflammatory signaling cascades. Thrombin has been implicated as a mediator of interest in AD since the 90swhen it was detected in senile plaques and in neurofibrillary tangles [8,9]. Elevated levels of thrombin as well as its specific receptor, PAR-1, have been identified in the AD brain [10,11]. Persistent thrombin signaling induces tau aggregation and related hippocampal degeneration [12,13]. There is cross talk between Aβ and thrombin where Aβpromotes thrombin generation through factor XII-mediated factor XI activation and thrombin induces secretion of amyloid precursor protein (APP) [14]. Thrombin can also regulate many of the proinflammatory signaling mechanisms relevant for AD including NFκB and mitogen activated protein kinases(MAPKs).The transcription factor NFκB is considered a primary regulator of inflammatory processes and several studies have shown that inhibiting NFκB pathway attenuates neuroinflammation [15,16]. NFκB signaling has been also implicated as a factor that is abundant in neurons as well as in microglia and plays a pivotal role as a transcriptional factor of various AD associated genes [17,18]. Postmortem studies examining brains from AD patients demonstrated upregulation of NFκB activity in cells involved in the neurodegenerative process [19]. In addition, increased immunoreactivity of NFκB- p65 in neurons and astrocytes in the proximity of Aβ plaques has been documented [20,21].

Furthermore, *in vivo* studies have reported that the β-site APP cleaving enzyme 1 (BACE1) promoter contains an NFκB binding element and that overexpressed Aβ stimulates BACE1 promoter activity via NFκB-dependent pathways [22]. Like the involvement of NFκB in many AD-related processes, MAPKs have been also implicated in AD signaling cascades. In this regard, Aβ toxicity, tau hyperphosphorylation, apoptosis and deregulated autophagy have been partly attributable to MAPK and MAPK signaling [23]. The p38 MAPK is of special interest due to its involvement in the regulation of Aβ toxicity, glutamate excitotoxicity, disruption of synaptic plasticity and NFκB activation [23].

The pleiotropic effects of thrombin in several cell types on proinflammatory signaling pathways including NFκB and MAPK, suggests that thrombin could contribute to pathological events in the AD brain. This idea is supported by studies showing that inhibiting thrombin reduces AD pathology. A recent study showed that treatment of TgCRND8 transgenic AD mice with the direct thrombin inhibitor dabigatran improved spatial memory deficits, reduced neuroinflammation and amyloid plaque formation [24]. We have previously documented that dabigatran reduces oxidative and inflammatory stress in the brain microvasculature of the triple transgenic AD mouse model [25]. Although these data demonstrate beneficial effects of inhibiting thrombin in the whole brain and cerebral vasculature, respectively, the possible direct effects on AD-related pathology in neurons has not been determined. The objective of the current study is to determine the effects of thrombin in the presence or absence of dabigatran on key mediators of neuroinflammation and AD pathology in the retinoic acid (RA) differentiated SH-SY5Y neuronal cell line.

2. Material and methods

2.1. Cell culture

Human neuroblastoma cells (SH-SY5Y) procured from American type cell culture (ATCC, VA) were seeded at a density of 27×10^3 in cell culture flask for routine culture. Cells were cultured in growth medium consisting of Dulbecco's Modified Eagle Medium (DMEM)/F12 medium (Invitrogen, MD) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine in a humidified atmosphere of 5% CO_2 in air at 37 °C.

2.2. SH5Y differentiation and treatments

Cells at the density of 10^6 cells/ml were sub-cultured in 100mm culture dishes (Cyto-one, USA Scientific, FL) containing 5 ml each of growth media. Twenty-four hours (h) after seeding, the medium was changed to differentiation medium consisting of DMEM/F-12 medium supplemented with, 1% FBS, 1 mM l-glutamine, 100 U penicillin/mL, 100 μg streptomycin/mL and 10 μM of all-trans-retinoic acid (Sigma Aldrich, MO). The differentiation medium was changed every 3rd day for the duration of 7 days. Treatment medium consisted of DMEM (Invitrogen, CA) containing 1% Bovine Serum Albumin (BSA, Sigma Aldrich, MO), sodium pyruvate, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine. Differentiated cells were treated with 0, 10, 50, and 100 nM human alpha-thrombin (EMD Millipore, MA), with or without dabigatran (250nM) for 24 h at 37°C in a 5% CO_2 incubator. Concentration of the dabigatran stock solution was 1 mM (dissolved in DMSO), whereas the concentration of the thrombin stock solution was 10μM (dissolved in sterile water). The concentration of DMSO in the cell culture media was maintained at 0.1% for control and across all treatment groups.

2.3. Preparation of total cell extract

Following treatment, the cells were rinsed with cold phosphate buffer saline (PBS, pH 7.4) and harvested and lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate) containing 1% protease and phosphatase inhibitors (Sigma Aldrich, MO), followed by incubation on ice for 30 min. The cell debris was removed by centrifugation at 10,000 g for 10 min at 4°C and the supernatant was collected, protein concentration was quantified by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL)

2.4. Nuclear protein extraction and Electrophoretic Mobility Shift Assay (EMSA)

Using the NE-PER Nuclear Cytoplasmic Extraction Reagent kit (Pierce, Rockford, IL), nuclear extracts were prepared according to the manufacturer's instruction. Treated cells were rinsed with cold PBS and centrifuged at 500 g for 5 min. The resulting cell pellet was suspended in 200 μL of cytoplasmic extraction reagent I by vortexing. This was followed by incubation of the suspension on ice for 10 min and 11 μL of a second cytoplasmic extraction reagent II was added, vortexed for 5 s, incubated on ice for 1 min and centrifuged for 5 min at 16,000 g. The cytoplasmic extract (supernatant fraction) was transferred to a pre-chilled tube. The insoluble pellet fraction, containing crude nuclei, was resuspended in 100 μL of nuclear extraction reagent by vortexing for 15 s and incubated on ice for 10 min followed by centrifugation for 10 min at 16, 000 g. The resulting supernatant, constituting the nuclear extract, was used for subsequent experiments. EMSA was performed in accordance with the manufacturer's protocol for Light Shift Chemiluminescent EMSA kit (Thermo-Fisher, MA). Single stranded 3'-biotin labeled consensus oligonucleotides for NF⊇β 5´-AGTTGAGGGGACTTTCCCA GGC-3 was purchased from Integrated DNA Technologies (Coralville, IA). DNA binding reactions were performed in 20 μL reaction mix containing biotin-labeled oligonucleotides and nuclear extracts. Additional unlabeled oligonucleotides were added for competition. Reaction products were then separated by electrophoresis. Thereafter, the protein-DNA complexes were transferred onto a positively charged nylon membrane (Millipore, MA) and detected by chemiluminescence.

2.5. Western blot

Protein extracts from different treatment groups were mixed with equal volumes of sodium dodecyl sulphate (SDS) buffer [0.125 M Tris–HCl, pH 6.8, 2% SDS, 0.5% (v/v) 2-mercaptoethanol, 1% bromophenol blue, and 19% glycerol] and boiled for 5 min. For determining the protein expressions, 30μg of the total protein was resolved by SDS-PAGE on 4-20% Novex gradient gels (Invitrogen, CA) at 90V constant under reducing conditions and transferred using dry blotting (iBlot, Invitrogen,

Table 1
List of primary antibodies used for the western blot analysis.

Antibody/ Catalog number	Source	Supplier	Isotype	Dilution
APP (E8B3O) XP/ 29765 S	Rabbit	Cell Signaling Technology	IgG	1:1000
Tau (Tau-46)/ 4019S	Mouse	Cell Signaling Technology	IgG1	1:1000
Phospho-Tau (Thr181) (D9F4G)/ 12885S	Rabbit	Cell Signaling Technology	IgG	1:1000
Phospho-Tau (Ser404) (D2Z4G)/20194S	Rabbit	Cell Signaling Technology	IgG	1:1000
p38 MAPK (D13E1) XP/8690S	Rabbit	Cell Signaling Technology	IgG	1:1000
Phospho-p38 MAPK (Thr180/ Tyr182) (D3F9) XP/ 4511S	Rabbit	Cell Signaling Technology	IgG	1:1000
Caspase-3 (D3R6Y)/14220S	Rabbit	Cell Signaling Technology	IgG	1:1000
β-Actin (8H10D10)/ 3700S	Mouse	Cell Signaling Technology	IgG2b	1:3000

Table 2
Primers used in qPCR for the measuring gene expresssion.

Gene	Sense Strand	Anti-Sense Strand
APP	5- GCCAAAGAGACATGCAGTGA-3	5′- CCAGACATCCGAGTCATCCT′-3′
BACE1	5-AGGGAGCATGATCATTGGAG-3	5′-AACACTTTCTTGGGCAAACG-3′
GSK3β	5-ATTACGGGACCCAAATGTCA-3	5'-TGCAGAAGCAGCATTATT GG-3'
MAPT	5-AAGATCGGCTCCACTGAGAA -3	5′- GGACGTGGGTGATATTGTCC -3′
β-actin	5-GGGAAATCGTGCGTGACAT-3	5'-CAGGAGGAGCAATGATCTTG-3

CA). Non-specific binding was blocked by incubating the PVDF membrane with 5% BSA in Tris-buffered saline with 0.05% Tween 20 (TBS-T) with gentle shaking for 1 h at room temperature (RT). Immunoblotting was performed after overnight exposure to the antibodies (Cell Signaling Technology, MA) listed in Table 1, diluted in TBST with gentle agitation on a shaker at 4°C. On the following day the membranes were exposed for 1 h to IRDye® 680 LT or IRDye® 800LT infrared dye (LI-COR Biotechnology, NE) goat anti-mouse/goat anti-rabbit diluted at 1: 10,000 after washing with TBS-T. Finally, Odyssey® Infrared Imaging System (Li-COR Biotechnology, NE) was used to detect and quantify infrared signal of protein bands. Loading of the samples was assessed by staining the gels with Bio-safe Coomassie blue stain (Bio-Rad, CA) after the transfer to PVDF membranes.

2.6. Enzyme-linked immunosorbent assays for Aβ (1–42) and BACE1 levels

The levels of Aβ were measured in cell lysate and in conditioned media using human *Aβ (1–42)* assay kit. The conditioned media was concentrated using spin columns (Thermo-scientific, MA) prior to use. Protein concentration of the column media concentrate, and cell lysate was determined by BCA protein assay (Pierce, Rockford, IL). This ELISA kit is based on solid-phase sandwich enzyme-linked immunosorbent assays with two kinds of highly specific antibodies, which are 100% reactive with human Aβ (1–42). The assay was conducted according to the manufacturer's instructions (Immuno-Biological Laboratories, Gunma, Japan) with minor modifications. Assay standards and 100 μgof protein sample in 100 μL of EIA buffer were added to 96-well plates precoated with anti-human Aβ IgG monoclonal antibody. The plates were incubated overnight at 4°C and were washed seven times using the 40X diluted wash buffer supplied with the kit (0.05% Tween 20 in phosphate buffer). Next, 100 μL labeled antibody was added to the samples and standards, followed by incubation at 4°C for 1 h. After incubation, the plates were washed nine times followed by the addition of 100 μL tetramethyl benzidine (TMB) as a coloring agent. The plates were incubated in the dark for 30 minutes at room temperature. Lastly, 100 μL of stop solution was pipetted in each well to stop the reaction, and absorbance was measured at 450 nm using a Spectra Max UV/Vis Spectrometer (GMI, Inc.). The levels of Aβ in the test samples were calculated relative to the standard curve generated for each plate.

BACE1 levels were measured with a Human Beta-secretase-1 ELISA Kit (Invitrogen, CA) according to the manufacturer's protocol. The enzymatic reaction for measuring secretase levels was carried out in an anti-BACE1-coated 96-well plate. Assay standards and 100 μg of protein samples in 100 μL of 1X Assay Diluent were added to 96-well plates. The wells were covered and incubated for 2.5 h at RT with gentle shaking. The solution was aspirated from the wells and washed four times with a wash buffer. This was followed by the addition of 100μL of 1X prepared biotinylated antibody to each well and incubated for 1 h at RT with gentle shaking. The solution was pipetted out and wells were washed four times with a wash buffer. Afterwards 100μL of Streptavidin-HRP solution was pipetted in each well and incubated for 45 min at RT with gentle shaking. After discarding the solution and washing the wells 4 times with a wash buffer 100μL of TMB substrate was added to each well followed by incubation for 30 min at RT in the dark with gentle shaking. Lastly, 50 μl of Stop Solution to each well and the absorbance was measured at 450nm and 550 nm using a plate reader. The levels of BACE1 were presented in nanograms per milliliter (ng/ml).

2.7. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) analysis

The SH-SY5Y cells were treated as mentioned above. Cells were harvested and the total RNA was extracted using TRIzol reagent (Invitrogen, CA) according to the manufacturer's protocol. RNA concentrations were estimated using the Nanodrop (Thermo Scientific, DE). First-strand complementary DNA (cDNA) was synthesized from 1.5 μg of total RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) as described earlier [26]. The PCR reaction (40 cycles) were carried out using a SYBR green PCR master mix (Applied Biosystem, CA) and was performed using the ViiA7 Real-Time PCR system (Applied Biosystem). Table 2 shows the list of Sense and anti-Sense primers designed using Primer-3 plus. Expression data were reported using $2^{-\otimes\otimes}$Ct method and β-actin mRNA as endogenous control.

2.8. Statistical analysis

Western blot bands were quantified by using the LI-COR/ Odyssey infrared image system. All measurements were made in triplicate and all values are presented as mean ± standard error of the mean (S.E.M). The significance of the difference among means of the experimental groups was obtained with one-way analysis of variance, the Tukey-Kramer multiple-comparison post-test, and the student Newman-Keuls comparison post-test, using GraphPad Prism version 8.0 computer software (La Jolla, CA, USA). The level of significance was set at $p < 0.05$.

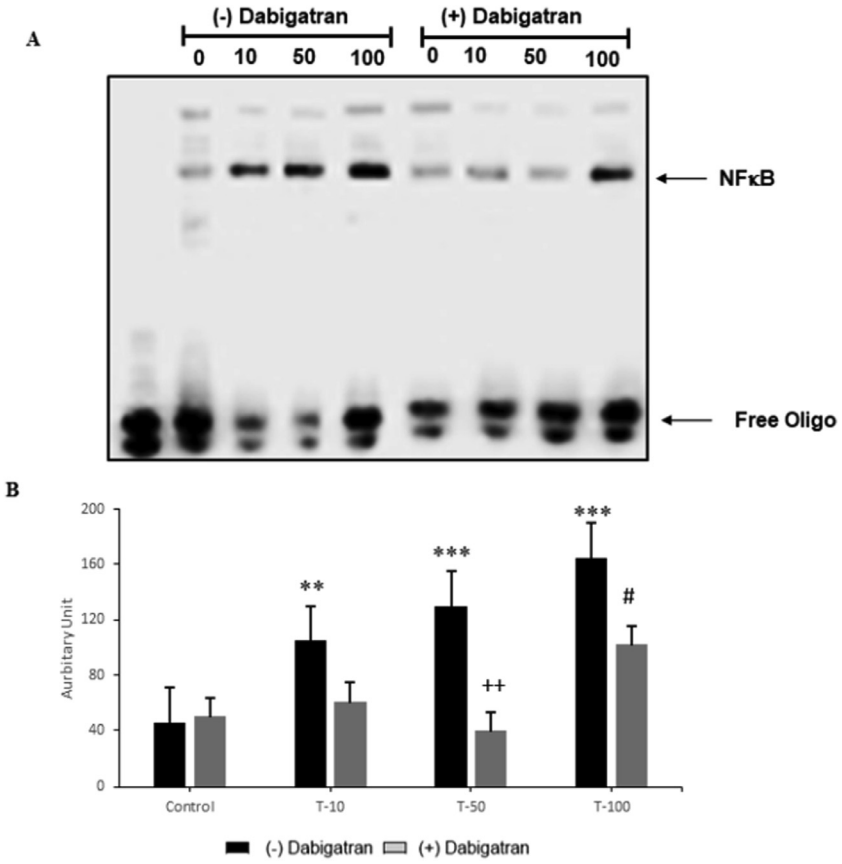

Fig 1. Dabigatran attenuates thrombin concentration-dependent NF-/B p65 DNA-binding activity.
Nuclear extract of RA differentiated SH5Y cells treated with a series of thrombin concentrations (0-100nM) with or without dabigatran (250 nM) for 24 h, were incubated with the biotin-labeled oligonucleotides probe corresponding to binding of NF/B and subjected to Electrophoresis Mobility Shift Assay. Untreated cells were used as Control. (A) Representative DNA: protein shifts for dabigatran (-) and dabigatran (+). (B) Quantification of bound to free DNA determined that thrombin induced concentration dependent higher association of nuclear proteins with the NFκB. $^{**}p < 0.05$, $^{***}p < 0.001$. Data are represented as mean± SEM, $n = 3$ (represent three independent experiments).

3. Results

3.1. Dabigatran attenuates thrombin-induced NF/B activation

The impact of dabigatran on thrombin-induced activation of NF/B was studied in the nuclear extract of RA differentiated SH-SY5Y cells by EMSA. Our results show a significant up-regulation of NFκB DNA-binding after 24 h exposure to a series of thrombin concentrations (10–100 nM), compared to untreated control cells (Fig 1A). Differentiated SH-SY5Y cells exposed to thrombin and co-treated with dabigatran (250nM) displayed a significant decrease in NF/B activation (reduced NFκB DNA-binding) at 50nM ($p < 0.01$; −175.11% ± 26.24% and 100 nM ($p < 0.05$; −77.05% ±14.60%) compared to cells treated with only thrombin. No effect on NF/B activation was observed when cells were exposed to dabigatran (250nM) alone (Fig. 1B). These results were verified by western blot that showed a similar trend of reduced expression of NFκB in cells co-treated with thrombin and dabigatran (data not shown).

3.2. Expression of neuroinflammation-related signaling proteins and proapoptotic protein increased by thrombin is blocked by dabigatran

Western blot analysis using antibodies directed against p38 and the phosphorylated form of p38 (Pp38) were used to study the expression of these signaling proteins in the neuroblastoma cell line. RA differentiated SH-SY5Y cells were exposed to a series of thrombin concentrations (10-100 nM) with or without dabigatran (250 nM) (Fig 2A). Thrombin increased expression of the target proteins, but the effective dose was variable. For p38, insignificant increase was observed at 50 nM but was statistically significant at 100 nM thrombin ($p < 0.001$, 61.36% ± 10.77%). On the other hand, thrombin significantly increased the expression of Pp38 at 50nM ($p < 0.01$, 55.42 ± 10.25%) and 100 nM ($p < 0.001$, 105.3% ± 20.36%) concentrations. Treatment of dabigatran in thrombin-exposed cells significantly lowered signaling protein expression of p38 at 50nM ($p < 0.01$, 46.51% ± 1.60%) and 100nM ($p < 0.001$, −84.87 ± 4.75%), Reduction in the protein expression of Pp38 was observed at 50nM ($p < 0.01$, −52.83% ± 5.26%) and at 100nM ($p < 0.001$, −115.46% ± 17.46%) by dabigatran co-treatment (Fig. 2B and C).

Expression of the apoptosis-related protein, caspase-3 increased with 50 nM and 100 nM thrombin exposure ($p < 0.05$, 29.56% ± 3.86% and $p < 0.01$, 49.33% ± 7.67%) compared to control, which was significantly ($p < 0.01$, −64.33 ± 1.01% and $p < 0.001$, −98.26 % ± 4.53%) reversed by inclusion of 250 nM dabigatran in the treatments (Fig. 2D).

3.3. Dabigatran attenuated APP protein expression as well as BACE1 and Aβ levels in neuronal cells exposed to thrombin

APP protein expression was increased by all concentrations of thrombin and significantly ($p < 0.01$, 53.51% ± 3.70%) at the 100 nM, that was significantly reverted to control levels by co-treatment with 250 nM dabigatran ($p < 0.05$, − 68.28% ± 18.61%) (Fig. 3B). A similar pattern was demonstrable for expression levels of Aβ 1-42 in cell lysate (Fig. 3C) as well as in conditioned media (Fig. 3D). Thrombin increased expression of Aβ 1-42 in both lysate ($p < 0.01$, 102.39% ± 21.20%) and conditioned media ($p < 0.05$, 87.03% ± 19.21%) only at 100 nM concentration. Cotreatment of dabigatran significantly attenuated thrombin response in lysate ($p < 0.05$, −91.04% ± 7.0%) as well in conditioned media ($p < 0.05$, −55.18%), significant reduction was only observed in cell exposed to 100 nM thrombin.

Enzyme-linked immunosorbent assay was used to evaluate the changes in the levels of BACE1 in cell lysate. Levels of BACE1 were significantly elevated in differentiated SH-SY5Y cells exposed to thrombin concentrations of 50nM ($p < 0.05$, 43.19% ± 6.52%) and 100 nM ($p < 0.05$, 35.39% ± 5.57%) for 24 h. As was observed with APP and Aβ 1-42, dabigatran significantly ($p < 0.05$, −51.57%±5.94%) decreased

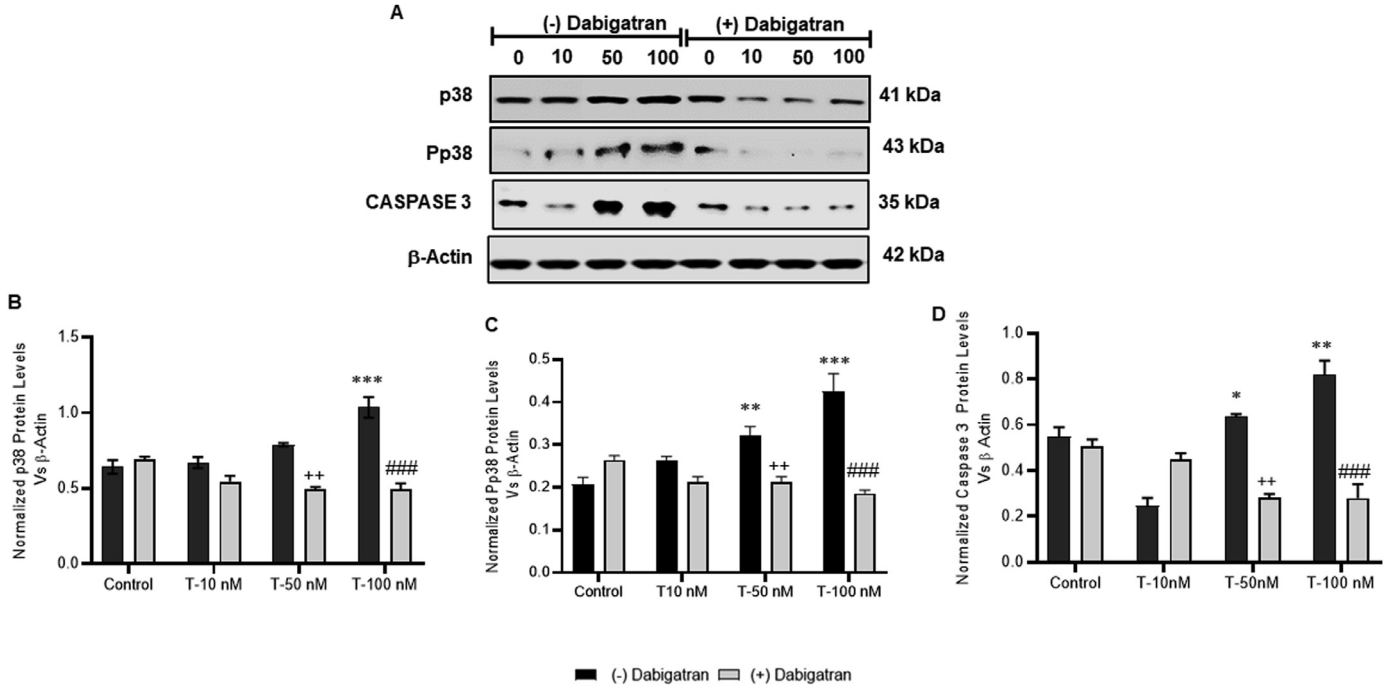

Fig 2. Dabigatran alleviates thrombin induced dose-dependent change in p38 MAPK, P38 MAPK and Caspase 3.
RA differentiated SH5Y were pre-incubated with thrombin (10nM, 50nM and 100nm) with (+) or without (-) dabigatran (250 nM) for 24h h in treatment medium. Protein expression was measured by western blotting as stated in section 2.4. (A) Representation of the protein expressions of p38, Pp38, Caspase3 and housekeeping protein ®-actin. (B-D) Individual data points are represented on the graph, as well as the means for each time point ($n = 3$) and significance is represented as $*p < 0.01$, $**p < 0.05$, $***p < 0.001$ compared to untreated control, $+p <+ 0.01$ compared to 50nM thrombin, $\#\#\#p < 0.001$ compared 100nM thrombin.

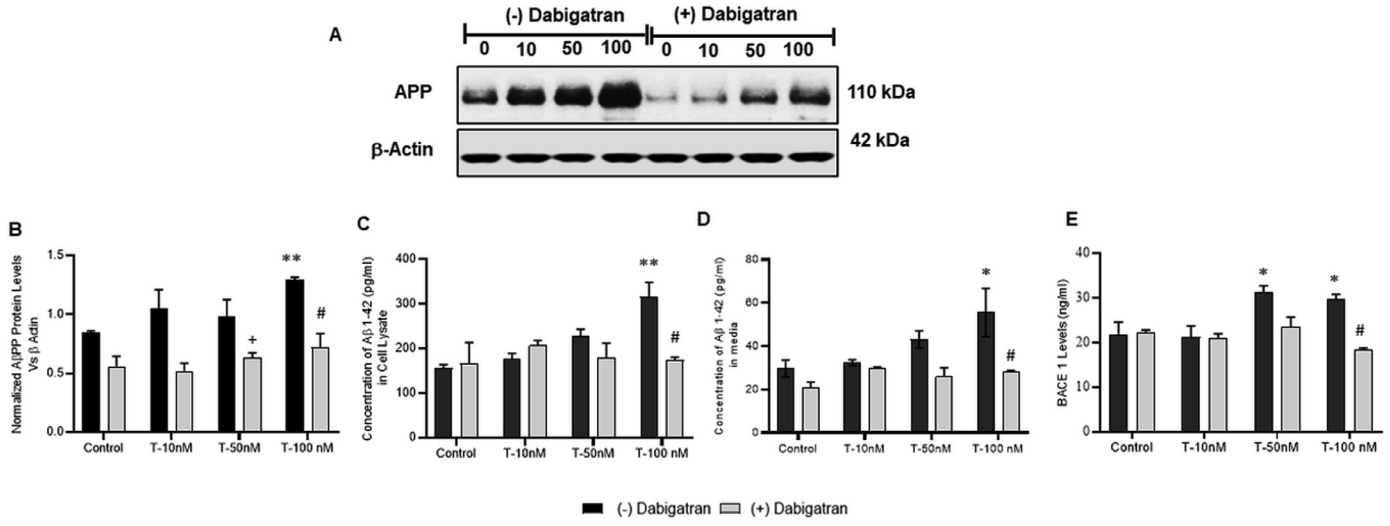

Fig 3. Dabigatran rectifies thrombin-induced change in the expression or levels of proteins associated with amyloid pathway.
RA differentiated SH5Y were pre-incubated with thrombin (10nM, 50nM and 100nm) with (+) or without (-) dabigatran (250 nM) for 24h h in treatment medium. Western blotting and ELISA were carried out for the measurement of protein levels. (A) Immunoblot representation of APP protein expressions, (B) Quantification of APP western blot bands normalized to β-actin, (C-E) Aβ-42 and BACE1 levels were determined by ELISA. Histograms represent quantification of protein expression and chemilucent signal. Data shown are the mean ± SEM ($n = 3$) and significance is represented as $*p < 0.05$, $**p < 0.01$ compared to untreated control, $+p <+ 0.05$ compared to 50nM thrombin, $\#p < 0.05$ compared 100nM thrombin.

BACE1 levels, which was statistically significant at 100 nM thrombin concentration (Fig. 3E).

3.4. Dabigatran lowers expression of total tau and phosphorylated tau species induced by thrombin

Levels of total tau (tTau) protein as well as the site-specific phosphorylated (pTau)species Tau-Ser-404 and Thr-181 were evaluated in SH-SY5Y cells exposed to various concentrations of thrombin (Fig. 4A). Thrombin treatment resulted in a significant increase in tTau at 50nM ($p < 0.01$, 64.64% ± 19.11%) and 100 nM ($p < 0.01$, 85.95% ± 20.36%) compared to untreated control. Co-treatment of cells with dabigatran significantly ($p < 0.05$, −45.08% ± 19.66%) alleviated the increased expression of tTau protein evoked by 100 nM thrombin (Fig. 4B). Antibodies directed against select serine and threonine residues were used to evaluate the effect thrombin on site-specific phosphorylated species

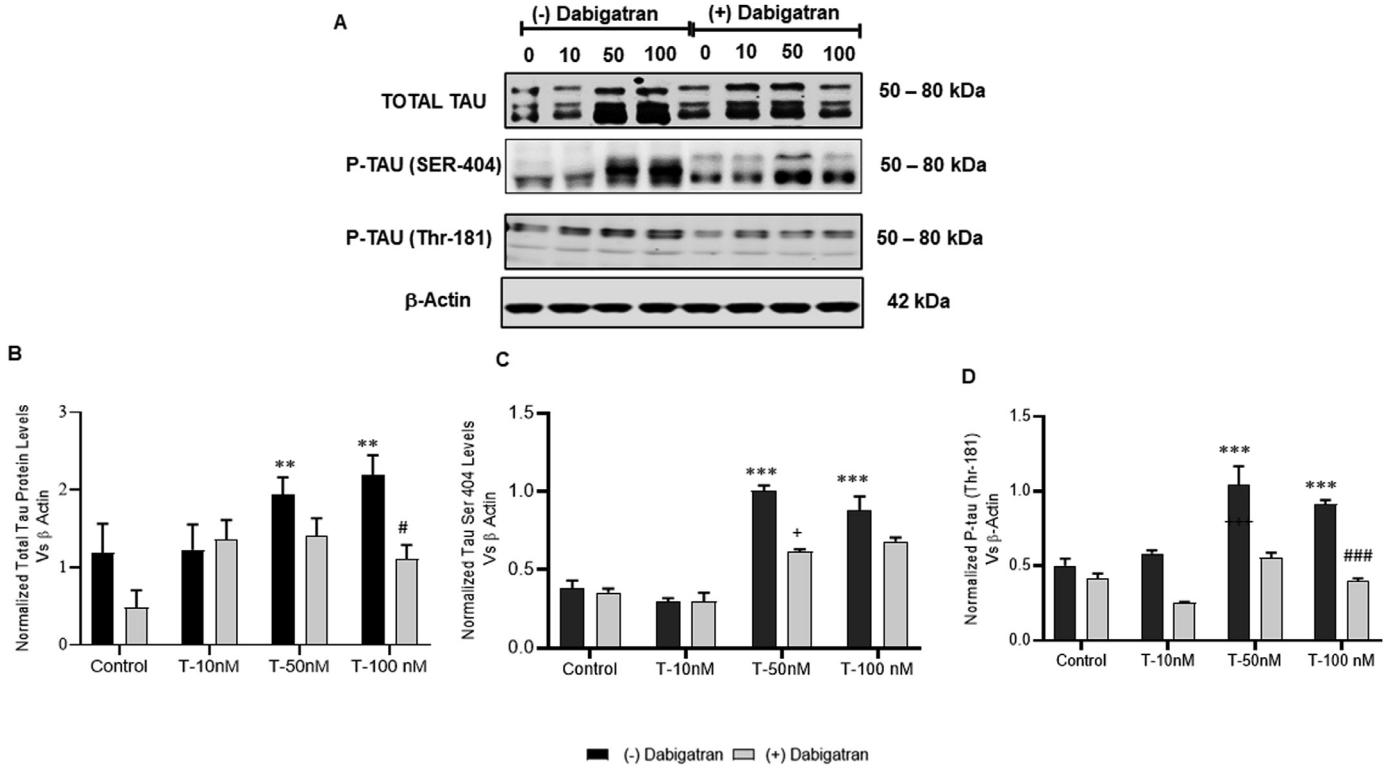

Fig 4. Dabigatran treatment reduces thrombin induced Total Tau and site-specific Tau Phosphorylation.
RA differentiated SH5Y were pre-incubated with thrombin (10nM, 50nM and 100nm) with (+) or without (-) dabigatran (250 nM) for 24h h in treatment medium. Protein expression was measured by western blotting as stated in section 2.4. (A) Representative Tau and site-specific tau (Ser-404 and Thr-181) proteins levels. The RIPA cell lysate was used, and proteins were measured by western blot analysis as described in the methods section. (B-D) Histograms represent quantification after normalization to β-actin levels. Data shown are the mean±SEM ($n = 3$) and significance are represented as $**p < 0.01$, $***p < 0.001$ compared to untreated control, $+p$ <+ 0.05, $+p$ <+ 0.01 compared to 50nM thrombin, $\#\#\#p < 0.001$ compared 100nM thrombin.

pTau Ser-404 and pTau Thr-181. The effect of thrombin on these phosphorylated tau species was like that observed for tTau. Thrombin-treated cells displayed significant increase in the protein expression of pTau Ser-404 at 50nM ($p < 0.001$, 160.62% ± 8.91%) and 100 nM ($p < 0.001$, 130.28 ± 20.09%), significant increase in protein expression of pTau Thr-181 was also observed in cells exposed to thrombin at 50nM ($p < 0.001$, 108.52% ± 25.38%) and 100nM ($p < 0.001$, 82.67% ± 5.81%) compared to non-treated control. Co-treatment with dabigatran was able to mitigate the thrombin-induced elevation in pTau Ser-404 at 50 nM ($p < 0.05$, −101.79% ± 4.81%), whereas pTau Thr-181 was reduced significantly ($p < 0.001$, −102.74% ± 2.68%) at 100 nM (Fig. 4C and D).

3.5. Dabigatran modulates thrombin-induced kinases protein expression

We explored the effects of thrombin on GSK-3β, a kinase implicated in AD pathogenesis by its multiple effects on tau phosphorylation, amyloid-β production, memory, neurogenesis, and synaptic function. Treatment of the neuroblastoma cell line SH-SY5 with thrombin evoked an increase in GSK-3β expression that was significant at 50nM ($p < 0.01$, 180.30% ± 11.35%) and at 100 nM thrombin ($p < 0.001$, 250.98% ± 17.88%) (Fig. 5A). Exposure of cells to dabigatran reduced GSK-3β levels that were statistically significant at 50nM ($p < 0.001$, −196.44% ± 11.24%) and 100nM ($p < 0.001$, −283.78% ±11.24%) as compared to thrombin treated counterparts (Fig. 5B).

3.6. Effect of dabigatran on AD-related gene expression in thrombin exposed differentiated SH-SY5Y cells

We further explored whether the effect of thrombin on protein expression was also demonstrable at the transcriptional level. mRNA levels of *APP, BACE1, tau* and *GSK3β* normalized to β-actin, were evaluated by PCR in control and thrombin exposed cells treated with or without dabigatran. Our results indicated that thrombin at 50 nM ($p < 0.01$, 160.86% ± 7.59%) and 100 nM ($p < 0.01$, 150.97% ± 29.21%) caused a significant upregulation in mRNA levels for APP (Fig. 6A), BACE 1 at 50nM ($p < 0.01$, 84.30% ± 31.48%) and 100nM ($p < 0.001$, 149.69% ± 25.82%) (Fig. 6B) and tau at 50nM ($p < 0.05$, 106.85% ± 16.73%) and 100nM ($p < 0.05$, 132.22 % ± 37.77%) (Fig. 6C), whereas thrombin was able to elicit a significant increase of GSKβ mRNA levels even at 10 nM ($p < 0.05$, 130.57% ± 9.97%), increase was also observed at 50nM ($p < 0.001$, 250. 20% ± 27.85%) and 100nM ($p < 0.001$, 270.94% ± 33.06%) (Fig. 6D). For all four AD-related mediators, dabigatran was able to significantly mitigate the increase in message evoked by thrombin.

4. Discussion

The serine protease thrombin, associated with coagulation and inflammation, has the potential to orchestrate adverse series of cellular events mediated through activation of its widely expressed protease-activated receptors (PARs) [27]. Accumulating evidence has shown an association between elevated thrombin levels and increased PAR activity in AD brains suggesting a contributory role in disease onset and/or progression [10,11]. Previously we have shown that administration of thrombin directly in rat brains induced neuronal cell death, glial scarring, and cognitive deficits in these rat brains [28]. Thrombin inhibition is a new tactic that has been proposed to curb the noxious cycle of feed-forward cycle responsible for the onset of neuroinflammation in AD [29]. Dabigatran, a member of the direct oral anticoagulants (DOACs) family of thrombin inhibitors, is widely utilized for sev-

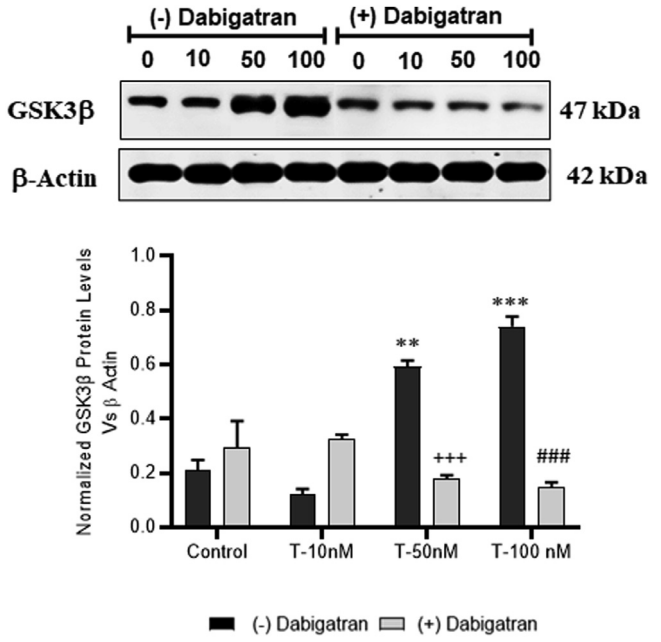

Fig 5. GSK3® protein expression after Dabigatran treatment in thrombin exposed human neuroblastoma cells.
RA differentiated SH5Y were pre-incubated with thrombin (10nM, 50nM and 100nm) with (+) or without (-) dabigatran (250 nM) for 24h h in treatment medium. Protein expression was measured by western blotting as stated in section 2.4. (A) Representative GSK3β levels in RA differentiated SH5Y cells preincubated in thrombin (10nM, 50nM and 100nm) with (+) or without (-) dabigatran (250 nM) for 24h h. The RIPA cell lysate was used, and proteins were measured by western blot analysis as described in the methods section. (B) Histograms represent quantification after normalization to β-actin levels. Data shown are the mean±SEM (n = 3) and significance are represented as $***p < 0.001$compared to untreated control, $++p$ <+ 0.001 compared to 50nM thrombin, $###p < 0.001$ compared 100nM thrombin.

eral clinical applications including prevention of stroke in patients with non-valvular atrial fibrillation and the treatment of venous thromboembolism [30,31].

In the present study, we used an *in vitro* neuronal model the RA differentiated SH-SY5Y human neuroblastoma cells to demonstrate that the direct thrombin inhibitor dabigatran at a concentration of 250 nM, was efficacious in preventing thrombin-induced increase in DNA-binding of the inflammation-mediating transcription factor NFκB. We also demonstrated that dabigatran attenuates thrombin-induced expression levels of signaling protein p38, its phosphorylated form Pp38, APP, AD biomarkers Aβ and Tau, as well as proteins involved in their processing or modification, namely BACE1 and GSK3β, and the apoptotic protein caspase-3.

Several studies have documented that inhibiting the NF/B pathway is central to mitigating neuroinflammation [15,16]. Our EMSA studies revealed a thrombin concentration-dependent increase in NF/B-DNA binding, with 100nM thrombin displaying the maximum NF/B activation, which was prevented by dabigatran. Recently we reported that human endothelial cells exposed to a series of thrombin concentrations like this study had elevated protein expression of cytokines IL-6, IL-1® and TNFα [32], that are known to be transcriptionally regulated by NFκB [33], and we showed that this increase was attenuated by dabigatran [32]. Consistent with our earlier findings on inflammatory proteins, dabigatran curbed the increased activation of NF/B induced by thrombin which was evident by decrease in the DNA binding.

Several reports have shown that interaction of thrombin with APP contributes to cerebral A® formation in AD patients and in other related disorders. Smith and coworkers observed localized immunoreactivity of thrombin at the sites of A® sites in AD brain [34]. Seminal studies by Igarashi and colleagues demonstrated the potential of thrombin in cleaving purified full-length APP at a site just upstream from the amino terminus of the Aβ domain between residues Arg556 and Ile567 [35]. Consistent with earlier research, our data revealed elevated APP protein expression and upregulated *APP* and *BACE1* gene levels following exposure to a series of thrombin concentrations for 24 h. A®-42 and BACE1 levels measured by ELISA revealed a similar increase in their levels. Treatment of cells with dabigatran (250nM) attenuated the increased levels of APP protein and downregulated APP mRNA, these changes were also reflected by a significant decrease in the levels of BACE1 and A®-42 in cell lysate and conditioned media. Accumulating evidence from basic and clinical research indicate inflammation induced by A®has been implicated in neuronal degeneration [36,37]. It has been reported that the levels of proinflammatory cytokines are significantly elevated in brains of AD patients, which suggests that inflammation might contribute to the pathogenesis of AD [38]. A vicious cycle of inflammation between Aβ accumulation, activated microglia, and microglial inflammatory mediators enhances Aβ deposition and neuroinflammation [39]. Our findings show that thrombin is involved independently in both arms of the pathological cascade, namely, inflammation and Aβ formation, which can create a feed forward pathological cycle.

Activation of protease cascades within neurons has been associated with cell death by apoptosis. Thrombin has been identified to play a pivotal role in tissue injury by initiating an apoptotic mechanism coupled with activation of its receptor, PAR-1 [40]. Smirnova *et.al* demonstrated initiation of apoptosis in neuronal cell line exposed to nanomolar thrombin concentrations that was associated with increased levels of caspase-3 [40]. Seminal studies by Choi and coworkers demonstrated increased loss of dopaminergic neurons in the substantia nigra of rodents that were intracranially exposed to thrombin. Same results were also shown in mesencephalic cultures, following treatment with thrombin [41]. Similarly, Yuan and colleagues reported Aβ aggregation and activation of caspase-3 in the mouse neuroblastoma cell line expressing double-mutated human APP and PS1 [42]. Consistent with other research groups, our results also indicated a concentration dependent increase in protein and mRNA levels of caspase 3, which was attenuated by co-treatment with dabigatran.

Aβ-induced elevated Tau-phosphorylation and modified Tau conformation has been often linked with GSK3β activation [40]. In agreement with earlier reports, our data showed thrombin significantly elevated the protein and mRNA levels of GSK3β at 100nM concentration. Concurrent with increased GSK3β expression, t Tau as well as site-specific phosphorylation at p Tau Ser-404 (Fig 5C) and p Tau Thr-181 (Fig 5D) were increased by thrombin. These changes were also associated with an increase in the tau mRNA levels. Our results are in agreement with studies carried out by Suo *et.al*, who showed thrombin treated immortalized hippocampal neuronal cells (HT22 cells) displayed the development of thioflavin-S positive tau aggregates within 24 h, followed by an escalation in cell death at 72 h [13]. Our data revealed RA differentiated cells with prior exposure to thrombin and subsequently treatment with dabigatran reduced GSK3β mRNA and protein levels. These changes were also manifested by decrease in the tTau levels as well as site specific phosphorylation.

Neuroinflammation has been postulated as the common portal for the onset of many underlying pathologies of AD. Many of the key events namely, Aβ toxicity, tau hyperphosphorylation, apoptosis and deregulated autophagy have been partly explained by the activation MAPKs particularly p38 MAPK, justifying p38 MAPK as a potential target to break the vicious Aβ toxicity cycle [23]. In this study, thrombin induced significant increase in the expression of p38 and its phosphorylated isoform in RA-differentiated human neuroblastoma cells (Fig 2A and B). Several research groups have demonstrated that arresting p38 activity attenuates the transcriptional activity of the proinflammatory transcription factor NFκB [43]. Our data support these findings as we also observed decreased p38 MAPK expression in thrombin exposed SH5Y cells treated with dabigatran, these changes correlated with reduction

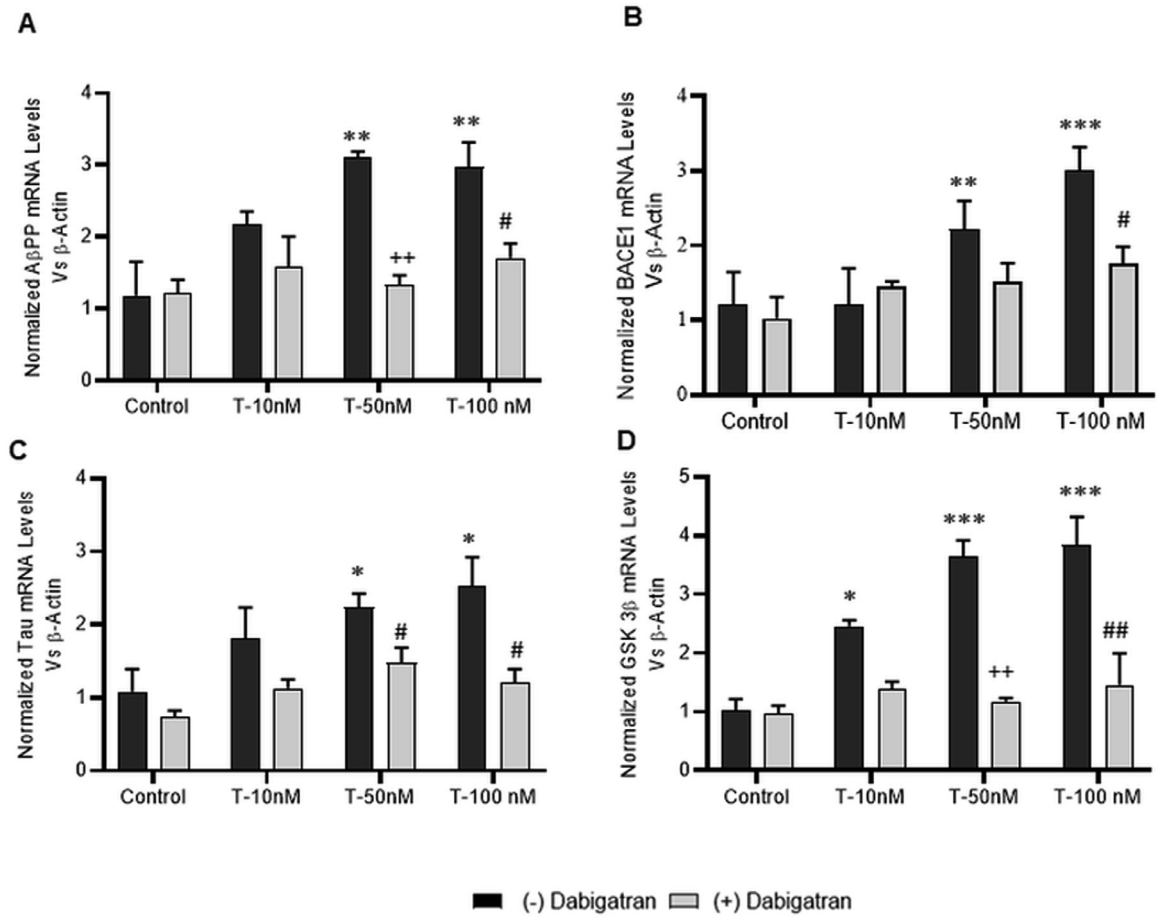

Fig 6. Dabigatran alleviates thrombin induced dose-dependent change in mRNA levels BACE 1, APP, Tau and GSK 3β.
RA differentiated SH5Y were pre-incubated with thrombin (10nM, 50nM and 100nm) with (+) or without (-) dabigatran (250 nM) for 24h h in treatment medium. mRNA levels of BACE 1, APP, Tau and GSK3β normalized against β-actin in qPCR. Histograms represent quantification after normalization. Data shown are the mean ± SEM ($n = 3$) and significance is represented as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$compared to untreated control, $+p <+ 0.01$ compared to 50nM thrombin, $\#p < 0.01$, $\#\#p < 0.01$ compared 100nM thrombin.

in NFκB binding as well as down regulation of protein and mRNA levels of AD associated genes.

This study, although *in-vitro*, is congruent with a growing literature documenting the extensive crosstalk between coagulation and inflammation. We suggest that repurposing the drug dabigatran, known to decrease inflammation and infarct volume in experimental stroke, could also provide therapeutic benefit against thrombin-driven neuroinflammation and downstream pathology in AD.

Deceleration of Competing Interest

None.

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