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Research Article

Cardiovascular and biochemical studies on the effects of thrombin and dabigatran and the interaction with vasopressor molecules

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ABSTRACT

Background: The effect of serine protease thrombin and its directly acting inhibitor dabigatran were evaluated on the heart rate, blood pressure, and phospholipase C (PLC) enzyme activity and the intracellular calcium levels in the platelets.
Methods: Heart rate and blood pressure were estimated using electrophysiology equipment.
Results: While thrombin was unable to significantly affect the heart rate and blood pressure, the inhibitor dabigatran was able to reduce the heart rate appreciably but its effects on the blood pressure were minimal. The thrombin induced increase in PLC enzyme activity, and intracellular calcium levels were attenuated by dabigatran in the platelets. The posterior pituitary hormone, vasopressin, and the adrenergic agonist noradrenaline were used to stimulate the PLC and calcium levels in platelets.
Conclusion: The thrombin inhibitor, dabigatran reduces vascular oxidative stress and inflammation, improves endothelial function, and decreases atherosclerosis in rodents.

Keywords: Thrombin, Dabigatran, Phospholipase C, Blood pressure, Heart rate

INTRODUCTION

Thrombin is one of the key players in the coagulation cascade.[1] On one hand, thrombin has been suggested to increase endothelial-dependent vasorelaxation and nitric oxide bioavailability,[2] while on the other hand thrombin may increase vascular inflammation, alter endothelial cell phenotype and decrease endothelial function, possibly cause contraction and proliferation of vascular smooth muscle cells and thereby accelerate atherogenesis.[3] Thrombin-dependent platelet activation and aggregation have been shown to be heightened in the setting of angioplasty and stenting, which may cause clinical complications including acute myocardial infarction and death. The high-affinity thrombin receptor protease activated receptor 1 (PAR1) has been recognized as an obvious candidate for therapeutic intervention in patients with acute coronary syndromes.[4] One of the prominent direct inhibitors of thrombin is dabigatran. Dabigatran etexilate is a low-molecular-weight pro-drug that exhibits no pharmacological activity. After oral administration, dabigatran etexilate is converted to its active form, dabigatran, a potent, competitive, and reversible direct inhibitor of the active site of thrombin, the final effector in blood coagulation.[5] Thrombin has an active site and two secondary binding exosites. Exosite 1 acts as a dock for substrates like fibrin to promote orientation for active site binding. Exosite 2 is the heparin-binding domain. Dabigatran is a univalent direct thrombin inhibitor that binds to the active site, thereby inactivating both fibrin-bound and unbound (i.e., free) thrombin.[6] Indirect thrombin inhibitors such as unfractionated heparin and low-molecular-weight heparin cannot inhibit fibrin-bound thrombin. The ability to inhibit fibrin-bound thrombin is an important theoretical advantage of dabigatran over the heparins because bound thrombin can continue to trigger thrombus expansion. By inhibiting thrombin, dabigatran prevents the conversion of fibrinogen into fibrin, positive feedback amplification of coagulation activation, cross-linking of fibrin monomers, platelet

activation, and inhibition of fibrinolysis. Platelets activated by agonists like thrombin undergo a complex cascade of events that results in shape change, degranulation, and aggregation. After ligand-receptor binding, phosphatidyinositol-4,5-bisphosphate (PIP_2) is hydrolyzed by phospholipase C (PLC) to generate two important intracellular second messengers, inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG).[7] IP_3 mobilizes calcium (Ca^{++}) from intracellular stores to permit activation of a Ca^{++}-calmodulin dependent protein kinase that phosphorylates the 20-Kd light chains of myosin (p20).[8] Alternatively, DAG activates protein kinase C (PKC), resulting in the phosphorylation of a platelet protein of approximately 47 Kd (p47). Although Ca^{++} mobilization and PKC activation are independently associated with platelet aggregation and secretion, these two signaling pathways function synergistically to produce a maximal biologic effect. Thrombin may have effects on the cardiovascular homeostasis. And dabigatran has been shown to regulate cardiovascular activity through effect on blood pressure and myocardial contractility.[9] It has been shown to improve nonvalvular atrial fibrillation as per recent studies conducted in Germany.[10]

This study was henceforth undertaken to evaluate the effects of thrombin and its inhibitor dabigatran on cardiovascular parameters such as the heart rate and blood pressure, as well as effects on intracellular calcium and PLC activity.

METHODS

Chemicals used

The following chemicals and reagents were used for this study. Heparin (Gland Pharma, India), lysis buffer (Sucrose, Tris HCl, $MgCl_2$, Triton X100), Hank's balanced salt solution (HBSS) (Sigma Limited). The following chemicals used were of analytical grade i.e., trichloroacetic acid, sodium hydroxide, ammonium formate and formic acid, pyridyl-azo-resorcinol, triethanolamine, dowex resin, lithium chloride (Fisher Scientific), norepinephrine, arginine vasopressin (AVP), Fura-2 (Henan Tianfu chemicals Co., China), thrombin (Sigma Limited), dabigatran, reagents for Tyrode's solution, water for injection.

Blood sampling from animals

Blood was collected from goats after sacrifice in anticoagulant-containing bottles.

Preparation of platelets

1.0 ml of the lysis buffer was added to the 8.0 ml of blood and mixed. This was centrifuged for 15 mins at 2000 rpm at 22°C. Platelet rich plasma was removed, and the blood was re-centrifuged for 20 mins at 8000 rpm at 22°C. Afterward, the platelet pellet formed was washed twice in saline solution and finally suspended in 2.0 ml of HBSS.

Blood pressure evaluation

Wistar albino rats were anesthetized with either thiopentone (40 mg/kg) or ketamine (75-100 mg/kg) given intraperitoneally. The carotid artery was cannulated, and the animal was heparinized with 0.4 ml of 1000 IU/ml of heparin. The cannula was then connected to a three-way stopcock, which was finally connected to the pressure transducer and a syringe filled with heparinized saline. Heparinized saline was used to apply a positive pressure and maintain it at the baseline value. The three-way stopcock worked as a passage connecting the pressure transducer and carotid cannula. The jugular vein was cannulated to infuse drugs intravenously. The help of data acquisition system was used to record the mean arterial pressure recordings which were noted.[11,12]

Estimation of heart rate

The animals were anesthetized using ketamine (10 mg/kg i.m) and diazepam (4 mg/kg i.p). Electrocardiography (ECG) was conducted using the limb lead II on a physiograph (INCO, India) using the speed of 10 mm/sec for the control heart rate readings. Heart rate was estimated from the ECG tracings by counting the number of "R" waves/min as per earlier described technique.[12]

Estimation of PLC activity

The PLC activity was determined by anion exchange chromatography as described in previous studies.[13] Briefly, the 1.0 ml of blood platelets sample was taken in tubes containing 2.0 ml of (HBSS) and 0.5 ml of 10 mM lithium chloride was added to it. This incubation mixture was incubated for 20 mins at 37°C. Pretreatment with dabigatran was done and incubated for 20 mins at 37°C. Either vasopressin (5 µg) or thrombin (10 U) or norepinephrine (20 µM) was later added and incubated at 37°C for 15 mins. After incubation period had got over, 0.5 ml 10% trichloroacetic acid was added to the solution. The volume was made to 5 ml with distilled water finally, and 0.1 ml of NaOH added. The contents were poured in the column containing Dowex resin and later eluted with 5 ml of 0.8M ammonium formate+0.1M formic acid. Finally, the elutant was collected in a beaker, and 0.5 ml PA-resorcinol was added along with 0.5 ml triethanolamine. The resultant eluates were used to measure the optical density at 410 nm using a spectrophotometer.

Calcium mobilization measurement

For calcium mobilization studies, the sensitized platelets were incubated at room temperature for 45 mins with 2 µM Fura-2-AM (Henan Tianfu, China) in Tyrode's buffer (10 mM Hepes, pH 7.4, 110 mM NaCl, 2.7 mM KCl, 0.4 mM NaH_2PO_4, 1.6 mM $CaCl_2$, 1.1 mM $MgCl_2$, 2 mM glucose, and 1% BSA). In our study, fura-2 fluorescence was monitored continuously using monochromator settings of 340/380 nm (excitation) and 510 nm (emission). The ($Ca2^+$), levels were

calculated using the general formula: $Ca2^{+}$=Kd(F-Fmin/(Fmax-F). Where Kd is the dissociation constant for $Ca2^{+}$ binding to the indicator and F is arbitrary fluorescent units. For fura-2, Kd=224 nM.[14] Thrombin mediated calcium mobilization was measured using a spectrophotometer (T60 LAB INDIA UV-VIS) set for dual excitation at 340 and 380 nm and emission at 510 nm.

RESULTS

The results of our study are shown in the Tables 1 and 2 and Figures 1 and 2. It is clear that the directly acting thrombin inhibitor dabigatran was able to attenuate the effects of thrombin on the blood pressure and heart rate, but to a lesser extent the effects mediated by norepinephrine. On the other hand, in the platelets loaded with Fura-2, dabigatran showed a similar trend with virtually no change in the calcium levels increased by norepinephrine but almost 50% reduction in the effect mediated by the serine protease thrombin. In the case of the PLC activity estimated in the platelets, all the stimulants, i.e., thrombin, norepinephrine, and AVP caused an increase in the PLC activity. The thrombin inhibitor dabigatran was able to appreciably reduce the effects of thrombin on inositol triphosphate levels, i.e., from about 3.11 fmol/100 µg to about 2.44 µg/100 µg platelet protein levels. Dabigatran also reduced the levels of IP_3 generated by vasopressin. However, it did not appreciably reduce the NE-induced effects on the PLC activity.

Table 1: Basal values of mean blood pressure: 91.52 mm of Hg and heart rate: 316.68 beats/min

Pre-treatment	Treatment	Percentage change in blood pressure	Percentage change in heart rate
Saline	Norepinephrine	7.38↑	12.91↑
Saline	Thrombin	5.39↑	3.27↑
Dabigatran	Norepinephrine	5.51↑	10.48↑
Dabigatran	Thrombin	4.58↓	6.53↓

Influence of drugs on mean blood pressure and heart rate in Wistar albino rats. The blood pressure recordings were done using data acquisition equipment. The values represent the percentage change from basal values of blood pressure, i.e., mm of Hg and heart rate, i.e., beats/min. The data are representative of 5 different experiments (n=5). ↑ values indicate increase and ↓ indicate decrease in blood pressure and heart rate

Table 2: Basal values of mean calcium levels: 373.41 nM.

Pre-treatment	Treatment	Percentage change in calcium levels
-	Thrombin	24.33↑
-	Norepinephrine	6.11↑
Thrombin	Norepinephrine	27.47↑
Dabigatran	Thrombin	12.39↑
Dabigatran	Norepinephrine	5.37↑

Effect of drugs on calcium levels in the platelet cells loaded with Fura-2 dye for 45 min. The data are representative of 5 different experiments (n=5). ↑ Values indicate increase and ↓ indicate decrease in calcium levels.

DISCUSSION

The prominent blood cells, i.e., the platelets, are small anuclear cells that play a critical role in hemostasis. They adhere to the injured vessel wall and recruit other platelets to form a hemostatic plug that is critical in limiting blood loss and initiating vascular repair. On the other hand, platelet-platelet interactions can lead to inordinate thrombus growth, which is a major patho-mechanism in the development of acute ischemic disorders, including stroke and myocardial infarction.[15,16] The directly acting inhibitor, dabigatran has a predictable pharmacokinetic profile, allowing a

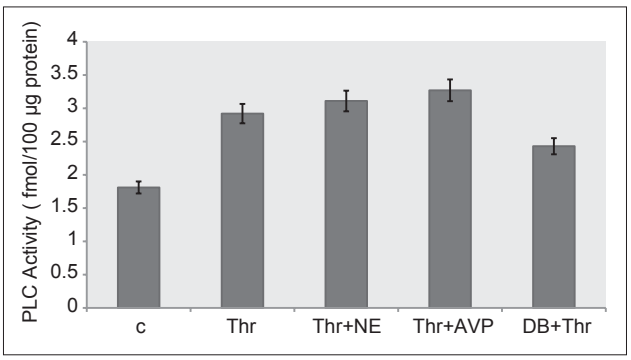

Figure 1: Effect of drugs on inositol triphosphate levels in blood platelets. The platelets were treated with sterile water as a control group: C, Thr: Thrombin, NE: Norepinephrine, AVP: Arginine vasopressin, DB: Dabigatran, alone or in combination. The values are mean±Standard error of six experiments represented as fmol/100 µg of platelet protein.

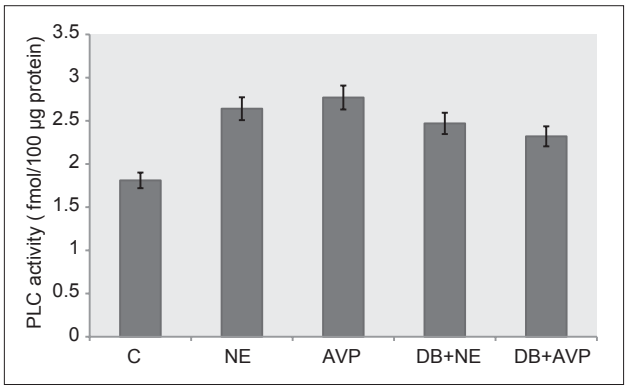

Figure 2: Effect of drugs on inositol triphosphate levels in blood platelets. The platelets were treated with sterile water as a control group: C, NE: Norepinephrine, AVP: Arginine vasopressin, DB: Dabigatran, alone or in combination. The values are mean±Standard error of six experiments represented as fmol/100µg of platelet protein.

fixed-dose regimen without the need for routine coagulation monitoring.[17,18] Studies in patients, e.g., stroke prevention in atrial fibrillation, showed a close correlation between plasma dabigatran concentration and the degree of anticoagulant effect.[19]

In this study, thrombin mediated an increase in PLC activity in platelets and an increase in intracellular calcium levels in platelets as shown in Figure 1 and Table 2. Thrombin is known to rapidly increase PIP_2 metabolism in humans.[20] Two major PLC isoforms are expressed in the blood, and they become activated by different signaling pathways.[21,22] PLCβ becomes activated downstream of G-protein (Gq)-coupled receptors, which are mainly triggered by soluble agonists such as adenosine diphosphate and TxA_2 or locally produced thrombin with only short interaction times in flowing blood.[23] In contrast, PLCγ also present in the platelets is activated by signaling pathways involving tyrosine phosphorylation cascades downstream of receptors that predominantly interact with immobilized ligands and may trigger sustained signaling events. The best characterized PLCγ-activating receptors in platelets are the immunoreceptor tyrosine-based activation motif-coupled collagen receptor GPVI, FcγRIIa, ligand-occupied integrins, and possibly also GPIb. In addition, the recently identified C-type lectin-like receptor-2 also strongly activates PLCγ in platelets, and mediates powerful cellular activation. Like PARs expressed on platelets, endothelial PARs serve as sensors of extracellular proteases and transmit signals after cleavage by proteases such as thrombin and factors VIIa and Xa.[24] Activation of endothelial thrombin receptors leads to calcium mobilization and secretion of Weibel-Palade bodies, which harbor von Willebrand factor (vWF) multimers and the P-selectin adhesion molecule.[25] Exposure of endothelial cell-anchored vWF to circulating platelets provides an initial means of tethering platelets to the blood vessel wall.[26] PAR1 in the vascular endothelium therefore complements the functions of platelet PAR1 during normal hemostasis by localizing the thrombus to the site of vascular injury.[27] Thrombin receptor activation is known to cause a rapid, transient increase in cytosolic IP_3 levels in this megakaryocytic cell line.[28] Endothelial PAR1 is also involved in acute inflammatory responses and vessel repair. Akin to the $G_{12/13}$-dependent shape change in platelets, thrombin activation of PAR1 causes Rho-dependent cytoskeletal rearrangements in endothelial cells and induces cell contraction and rounding. Based on the results of our study, it can be stated that thrombin-mediated effects in the platelets are largely mediated by increased calcium levels probably also subsequent to PLC activity and dabigatran is able to attenuate these biochemical responses. These data are supported by previous studies conducted by Michel et al.[29] and Imai et al.[30] However, thrombin alone does not seem to appreciably modulate effects on the heart rate and blood pressure although these were also reduced by dabigatran. However, dabigatran did potentiate the vasopressin-induced bradycardia (data not shown) thus suggesting some direct interaction at the cardiovascular level or through vagal nerve modulation. Dabigatran, however, was unable to significantly alter the responses on heart rate and blood pressure mediated by norepinephrine.

CONCLUSION

This experimental research study suggests that thrombin activates the PLC activity and intracellular calcium levels in platelets which were attenuated by dabigatran. Thus, suggesting a downstream effect of dabigatran on signal transduction. It also implies potentiating effect of thrombin on the phospholipase activation by vasopressin in the platelets.

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Ethical approval: The study was approved by the Institutional Animal Ethics Committee

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