Effects of ebelactone B on cathepsin A activity in intact platelets and on platelet activation

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Abstract

Purpose: Previous in vitro studies have demonstrated that a potent antihypertensive agent ebelactone B inhibits cathepsin A/deamidase activity. The aim of our studies was to assess the effects of this inhibitor on cathepsin A activity in intact platelets and on platelet activation events.

Material and methods: PRP or washed human platelets from healthy volunteers were pre-incubated with different concentrations of ebelactone B (1-10 μM) for 10-60 min. Cathepsin A activity in platelets was assayed colorimetrically using Cbz-Phe-Ala at pH 5.5. Expression of platelet activation markers GpIIb/IIIa and P-selectin on non-activated or agonist-activated platelets (ADP, TRAP) was measured by flow cytometry.

Results: Pre-treatment of platelets for up to 60 minutes with 10 μmol/l ebelactone B, that effectively inhibits cathepsin A activity in platelet lysate, did not affect this activity in intact platelets. Exposure of PRP to 10 μmol/l ebelactone B alone, or before platelet activation with ADP or TRAP caused only a small but non-significant increase in P-selectin and GpIIb/IIIa expression on the platelet surface, as demonstrated by flow cytometry analysis.

Conclusions: The lack of cathepsin A inhibition by ebelactone B in intact platelets indicates that this inhibitor does not enter cells. Therefore, a potential antihypertensive significance of this compound may be through the inhibition of cathepsin A/deamidase released from activated or damaged cells. In vitro ebelactone B seems to exert no effect on platelet activation. Further studies are underway to determine whether ebelactone B administration affects platelet activation events in experimental model of hypertension in rats.

Key words: ebelactone B, cathepsin A, human platelets, platelet activation markers.

Introduction

Cathepsin A (EC 3.4.16.1) is a lysosomal serine peptidase present in all examined mammalian cells and tissues [1], including human blood platelets [2,3]. In vitro it hydrolyzes various synthetic and natural biologically active peptides both at acidic pH (carboxypeptidase activity) and at neutral pH (deamidase and esterase activities) [4]. For example, human platelet cathepsin A deamidates substance P, endothelin 1 and enkephalinamides, converts angiotensin I to angiotensin II, and also cleaves bradykinin [2]. Therefore, it is postulated that after secretion from activated platelets or other cells such as macrophages, cytotoxic lymphocytes NK and LAK cathepsin A/deamidase may play a role in the local metabolism of the biologically active peptides [2,5,6]. Moreover, earlier in vitro studies have demonstrated that deamidase activity of cathepsin A is inhibited by an effective antihypertensive agent ebelactone B [5], which is a potent inhibitor of urinary carboxypeptidase Y-like kininase [7,8]. However, the role of cathepsin A/deamidase and the significance its inhibition in cell physiology is still unknown.

In the present study we investigated whether ebelactone B can inhibit cathepsin A activity in intact blood platelets. We also investigated the effect of ebelactone B on platelet activation, because some of the antihypertensive drugs are known to reduce platelet hyperactivity in hypertension [9-13]. Cathepsin A activity was assayed at pH 5.5 using a highly specific synthetic substrate Cbz-Phe-Ala, which is frequently employed for determination of this activity in crude extracts. The level of platelet activation induced by ADP or TRAP was assessed by determining P-selectin and GpIIb/IIIa expression on platelet surface using the flow cytometric method.

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Material and methods

Cbz-Phe-Ala, ebelactone B, and ninhydrin reagent solution were purchased from Sigma Chemical Co, St. Louis, MO USA. ADIAflow Gp (Platelet) kit containing mouse IgG-Fluorescein isothiocyanate (FITC) and antibodies against GpIIb/IIIa complex (CD41a) and P-selectin (CD62P), TRAP (thrombin receptor agonist peptide), calibration beads solution, fixative reagent was purchased from American Diagnostica; ADP was from Sigma (St. Louis, MO, USA).

PRP or washed human platelets (5×10^8 cells/ml) in HEPES--Tyrode's buffer, pH 7.4 (136 mmol/l NaCl, 2.68 mmol/l KCl, 1 mmol/l $MgCl_2$, 0.36 mmol/l NaH_2PO_4, 11.9 mmol/l $NaHCO_3$, 3.4 mmol/l HEPES, 0.1% glucose, 0.35% BSA) were incubated without or with 1.5 or 10 μmol/l ebelactone B at 37°C for 15-60 min. After centrifugation, platelet pellets were washed 3-times to remove the inhibitor, and finally resuspended in „assay buffer" (50 mmol/l sodium acetate buffer containing 100 mmol/l NaCl, 500 mmol/l sucrose and 1 mmol/l EDTA at pH 5.5) containing 0.5% Triton X-100 for determination of cathepsin A activity.

Cathepsin A activity was assayed using Cbz-Phe-Ala, as described earlier [3]. Briefly, the reaction mixture (500 μl) contained the substrate (5 mmol/l), cell lysate (50-100 μg of total protein) and "assay buffer". After 60 minutes of incubation at 37°C, the reaction was stopped by the addition of 500 μl of 10% TCA. The aliquots were taken to determine the amount of the released C-terminal alanine by the colorimetric ninhydrin method [14]. Enzyme activity was expressed as μmoles of the alanine released from the substrate per min per mg of protein.

For flow cytometry analysis, we used platelet rich plasma (PRP) obtained by centrifugation (150 μg, 15 min, 23°C) of fresh human blood from healthy volunteers as described in ref. 15. Samples were preincubated with ebelactone B (1-10 μmol/l), or vehlicle (DMSO 0.01%) for 10 or 60 minutes. Then the surface expression of GpIIb/IIIa or P-selectin on non-activated platelets or on platelets activated with ADP (4 μg/ml) for 2 minutes or with TRAP was determined according to the manufacture procedures using flow cytometer (Becton-Dickinson).

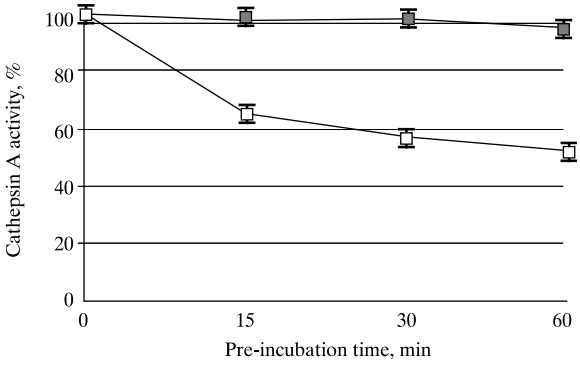
Protein concentration in platelet lysates was determined by the method of Bradford [16] using Bio-Rad reagent with bovine albumin as a standard.

Statistical analysis of the data was performed by using Student's t-test. All results are expressed as mean ± standard deviations. $P < 0.05$ was considered statistically significant.

Results

The effect of ebelactone B on cathepsin A activity in platelet lysate or in intact platelets pretreated with this inhibitor for 15-60 minutes is presented in *Fig. 1*. Cathepsin A activity was effectively inhibited by 10 μmol/l ebelactone B in platelet lysate. The percentage of this inhibition was about 37% and 50% after 15 and 60 minutes of preincubation with this inhibitor, respectively. The lower concentrations of the ebelactone B (1 or 5 μmol/l) were less effective (not shown). In contrast, exposure of intact platelets to 10 μmol/l ebelactone B for up to 60 minutes, followed by washing of the platelet pellets to remove the inhibitor, before lysis, did not result in a decrease in cathepsin A activity. The same results were obtained during preincubation of PRP with ebelactone B, followed by washing of the platelet pellets to remove extracellular inhibitor before lysis (not shown).

Figure 1. Comparison of cathepsin A activity in platelet lysate and in platelets pretreated with ebelactone B. Means ± SD.



The intact platelets (-■-) or platelet lysate (-□-) were incubated with 10 μmol/l ebelactone B (remove) at 37°C for 15, 30 or 60 minutes. After centrifugation of intact platelets, the platelet pellets were washed 3-times to remove the inhibitors, and finally resuspended in "assay buffer" containing 0.5% Triton X-100. Cathepsin A activity was assayed at pH 5.5 using 5 mmol/l Cbz-Phe-Ala after 60 min incubation. The maximal cathepsin A activity in the absence of the inhibitor (4.8 U/mg) was designated as 100%.

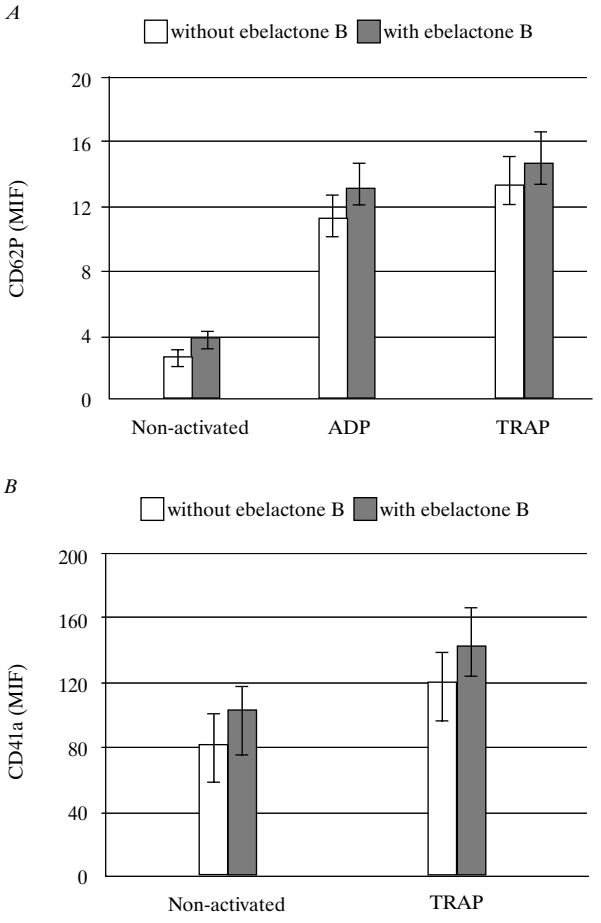
Next, we investigated whether ebelactone B affects platelet activation events using CD62P (P-selectin) and CD41a (GpIIb/IIIa) antibodies (*Fig. 2*). The expression of these markers on platelets pretreated with ebelactone B was measured before and after platelet activation with ADP or TRAP, and the mean fluorescence intensity values (MIF) were determined using flow cytometry. As seen in *Fig. 2*, in the control PRP, not treated with ebelactone B, activation of the platelets resulted in an increase in the platelet surface binding of monoclonal antibodies directed against P-selectin (CD62P) (*Fig. 2 A*) and GpIIb/IIIa (CD41a) (*Fig. 2 B*). After exposure of platelet rich plasma to 10 μmol/l ebelactone B, the mean fluorescence intensity values (MIF) of both P-selectin and GpIIb/IIIa were not statistically different between non-activated platelets and activated platelets.

Discussion

Ebelactone B is an effective antihypertensive agent that prevents the development of deoxycorticosterone acetate-salt hypertension in rats through the inhibition of urinary carboxypeptidase Y-like kininase [7,8]. In vitro it also inhibits cathepsin A/deamidase which shares many enzymatic properties with carboxypeptidase Y-like enzyme, including the ability to hydrolyze some bioactive peptides involved in the development of hypertension [2,4].

In the present study, we demonstrate that ebelactone B, at concentration of 10 μmol/l, which effectively inhibits cathepsin

Figure 2. Effects of ebelactone B on P-selectin (A) and GpIIb/ /IIIa (B) expression during platelet activation. Means ± SD.



PRP was preincubated for 60 minutes without or with 10 μmol/l ebelactone B, and then the platelets were activated by ADP (4 μg/ml) for 2 minutes or TRAP according to the manufacture procedure. Expression of CD41a and CD62P were measured by flow cytometry.

A activity in platelet lysate, does not inhibit this activity in intact platelets. It indicates that ebelactone B does not enter cells. Therefore, a potential antihypertensive significance of this compound may be through the inhibition of cathepsin A/deamidase released from activated or damaged cells, as was shown by others for urinary carboxypeptidase Y-like kininase, derived from the kidney [7,17]. The lack of cathepsin A inhibition by ebelactone B in intact platelets also indicates that this inhibitor cannot be used to study the role of cathepsin A in platelet physiology and pathology. The only known cell-permeable inhibitor, which almost completely inhibits cathepsin A activity in intact platelets and other cells, is a microbial product lactacystin/β-lactone [18,19]. However, it also inhibits a cytosolic multienzyme proteinase complex (proteasome), which is responsible for nonlysosomal proteolysis in all examined cells [20].

A number of reports have demonstrated that the antihypertensive drugs such as angiotensin II (AT) [9] receptor antagonist losartan or rilmenidine [21] are potent antiplatelet agents in hypertension [11-13]. In contrast, captopril and enalapril, the inhibitors of angiotensin-converting enzyme (ACE), have no effects on platelet activation events [22,23]. In aggreement with these studies, in our model system, cathepsin A/deamidase inhibitor ebelactone B seems to exert no effect on platelet activation. It causes only a small but non-significant increase in expression of P-selectin, a component of α-granule membrane that mediates the adhesion of activated platelets to monocytes and neutrophils. Ebelactone B has also no effect on expression of GpIIb/IIIa receptor that is critical for platelet aggregation. These markers are frequently used to test platelet activation by flow cytometric analysis under various pathological conditions, including hypertension [9,10,15]. Therefore, further studies are necessary to find out whether ebelactone B affects platelet activation events in experimental model of hypertension.

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