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The effect of some ε-aminocaproic acid derivatives on platelet responses★

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ε-Aminocaproic acid (EACA) is a synthetic low molecular drug with antifibrinolytic activity. However, treatment with this drug can be incidentally associated with an increased thrombotic tendency. The aim of the present work was to test synthetic EACA derivatives for their antiplatelet activities. We investigated the effect of three EACA derivatives with antifibrinolytic activity: I. ε-aminocaproyl-L-leucine hydrochloride (HCl•H-EACA-L-Leu-OH), II. ε-aminocaproyl-L-(*S*-benzyl)-cysteine hydrochloride (HCl•H-EACA-L-Cys(S-Bzl)-OH) and III. ε-aminocaproyl-L-norleucine (H-EACA-L-Nle-OH) on platelet responses (aggregation and adhesion) and on their integrity. It was found that: 1. as judged by LDH release test, none of the tested compounds, up to 20 mM, was toxic to platelets, 2. in comparison with EACA, all the synthetic derivatives inhibited much stronger the ADP- and collagen-induced aggregation of platelets suspended in plasma (platelet rich plasma) and aggregation of these cells in whole blood, 3. EACA and its derivatives exerted a similar inhibitory effect on the thrombin-induced adhesion of platelets to fibrinogen-coated surfaces. Since platelet activation and blood coagulation are tightly associated processes, the antiplatelet properties of EACA derivatives are expected to indicate reduced thrombotic properties of these derivatives compared to EACA.

ε-Aminocaproic acid (6-aminohexanoic acid), EACA, is the first synthetic representative of a new class of drugs, antifibrinolytic agents (Markwardt, 1978; Verstraete, 1985; Hardy, 1992). Its antifibrinolytic effect is related to the reversible complex formation

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Abbreviations: BSA, bovine serum albumin; EACA, ε-aminocaproic acid; LDH, lactate dehydrogenase; PBS, phosphate-buffered saline; PRP, platelet-rich plasma.

with plasminogen and with the active protease plasmin. Saturation of the lysine binding site of plasminogen with these drugs displaces plasminogen from the surface of fibrin. Even if plasminogen is transformed into the active plasmin, it cannot bind to fibrin and its fibrinolytic action is inhibited (Verstraete, 1985). Thus EACA is thought to act by preventing the premature dissolution of normal fibrin clot. It was also reported that high concentrations (higher than therapeutical) of EACA are able to inhibit platelet aggregation induced by collagen and ADP (Green *et al.*, 1985). EACA was used to control or to prevent bleeding in patients undergoing open-heart surgery (Verstraete, 1985; Hardy, 1992). However, there are reports that treatment with this drug is incidentally associated with an increased thrombotic tendency (Verstraete, 1985; Mannucci, 1998). According to the cell-based model of coagulation, the rate of thrombin generation in the blood, and thus the rate of blood coagulation strongly depend on platelets (Monroe *et al.*, 2002; Heemskerk *et al.*, 2002). We have shown that ε-aminocaproyl derivatives of α-amino acids with bulky, hydrophobic side chain significantly inhibit the fibrinolytic activity of plasmin and euglobulin fraction of plasma (Midura-Nowaczek *et al.*, 1996; 1998). The high antifibrinolytic activity of these compounds can be a result of formation of an antifibrinolytically active conformation (Maciejewska *et al.*, 2002). The present studies were performed to determine how the above-mentioned EACA derivatives affect platelet aggregation and adhesion.

It was found that ε-aminocaproyl amino acids with high antifibrinolytic activity: I. ε-aminocaproyl-L-leucine hydrochloride (HCl•H-EACA-L-Leu-OH), II. ε-aminocaproyl-L-(*S*-benzyl)-cysteine hydrochloride (HCl•H-EACA-L-Cys(S-Bzl)-OH) and III. ε-aminocaproyl-L-norleucine (H-EACA-L-Nle-OH) demonstrate antiplatelet properties much stronger than EACA.

MATERIALS AND METHODS

Materials. Hepes, EGTA, apyrase, thrombin, ADP disodium salt, bovine fibrinogen, bovine serum albumin (BSA), *p*-nitrophenyl-phosphate, Triton X-100 were purchased from Sigma Chemical Co (St. Louis, MO, U.S.A.). Collagen (fibrillar, from equine tendon) was from Hormon Chemie (Munich, Germany). EACA (ε-aminocaproic acid, 6-aminohexanoic acid) used as a reference compound was from Ziołolek, Poznań (Poland). The examined compounds: I. ε-aminocaproyl-L-leucine hydrochloride (HCl•H-EACA-L-Leu-OH), II. ε-aminocaproyl- L-(*S*-benzyl)-cysteine hydrochloride (HCl•H- EACA-L-Cys(S-Bzl)-OH) and: III. ε-aminocaproyl-L-norleucine (H-EACA-L-Nle-OH) were obtained as described earlier (Midura-Nowaczek *et al.*, 1998).

Blood collection. Blood was drawn *via* venipuncture from healthy volunteers, who had not taken any medication for at least ten days, into tubes containing a 1:9 volume of 3.8% (w/v) trisodium citrate.

Preparation of washed platelets. Platelet-rich plasma (PRP) was prepared by centrifugation of whole blood at 200 ***g*** for 20 min. After acidification to pH 6.5 with 1 M citric acid, the suspension was centrifuged at 1500 ***g*** for 20 min to obtain a pellet which was resuspended in a Ca^{2+}-free Tyrode/Hepes buffer containing: 138 mM NaCl, 2.8 mM KCl, 8.9 mM $NaHCO_3$, 0.8 mM KH_2PO_4, 0.8 mM $MgCl_2$, 5.6 mM glucose, 10 μM EGTA, bovine serum albumin (3.5 mg/ml), apyrase (2 U/ml) and 10 mM Hepes (pH 7.4). The platelets were washed once with the above buffer and suspended in the same buffer without apyrase and EGTA. The platelet concentration was standardized to 3×10^8/ml by dilution with autologous platelet poor plasma or the Tyrode/Hepes buffer. Platelet number was determined under a phase contrast microscope according to the standard laboratory technique.

Assay of platelet aggregation in platelet rich plasma. Platelet aggregation was followed turbidimetrically by recording light transmission through a stirred platelet suspension in the plastic cuvette of an aggregometer (Elvi, Logos, Milan, Italy) at 37°C (Born, 1963). Samples of PRP (300 μl) were incubated with stirring for 2 min at 37°C. Then threshold concentrations of the stimulus were added to induce aggregation. For each platelet preparation, the threshold aggregating concentrations, defined as the minimum amount of the stimulus that induced at least a 70% increase in light transmission within 3 min were determined. The substances tested were added to the platelet suspension two minutes prior to the addition of the stimulator. The extent of aggregation was expressed as the percentage of the maximum change of light transmission between platelet suspension and suspending medium. Quantification of aggregation tracings was performed by measuring the maximal increase in light transmission from the peak of shape change to the highest transmission level (the extent of platelet aggregation). All experiments were performed at least in quadruplicate using 4–5 different platelet preparations.

Measurement of platelet aggregation in whole blood. Platelet aggregation in whole blood was measured with a Chrono-Log lumiaggregometer (Chrono-Log, 1987) as described by Cardinal & Fower (1980). The instrument passes a low electric current between two electrodes immersed in a sample of blood. On contact with the blood, the electrodes become coated with a monolayer of platelets. When an aggregating agent is added, platelets aggregate to the monolayer coating the electrodes and increase the impedance. The change of impedance is recorded as a function of time. All procedures were conducted in accordance with Chrono-Log manual (1987).

Measurement of platelet adhesion. A colorimetric procedure measuring the activity of acid phosphatase was used for the determination of the adhesion of human platelets to fibrinogen-coated surfaces (Bellavite *et al.*, 1994). Plastic 96-well microtiter plates were coated overnight with 0.2 mg/ml human fibrinogen in phosphate-buffered saline (PBS) followed by a 30-min incubation with 3 mg/ml BSA in PBS (previously heat-inactivated for 3 min at 80°C) and washed twice with physiological saline. Immediately after coating and washing, the wells were supplemented with 25 μl of the compound under investigation (3 × final desired concentration, in PBS containing 2 mM $CaCl_2$ and 2 mM $MgSO_4$). Plates were then brought to 37°C, and 25 μl of platelets (2.5×10^6 cells) suspended in PBS and pre-warmed at 37°C was added to each well. Platelets were incubated for 10 min at 37°C, then 25 μl of thrombin (3 × final desired concentration) dissolved in PBS supplemented with 1 mM $CaCl_2$ and 1 mM $MgSO_4$) was added to each well. The incubation was carried out for a further 60 min, then plates were transferred to an automatic washer (Tecan, Austria) and subjected to two washing cycles with PBS at room temperature. After washing, the wells containing adherent platelets were rapidly supplemented with 150 μl of 0.1 M citrate buffer, pH 5.4, containing 5 mM *p*-nitrophenyl phosphate and 0.1% Triton X-100. After incubation at room temperature for 60 min, the reaction was stopped and the color was developed by the addition of 100 μl of 2 M NaOH. The *p*-nitrophenol produced by the reaction was measured with a microtiter plate reader (Tecan, Austria) at 405 nm against a platelet-free blank. The percentage of adherent cells was calculated on the basis of a standard curve obtained with a defined number of platelets from the same donor.

Assay of platelet integrity. The extent of platelet lysis following incubation with the studied compounds was estimated in suspension of washed platelets by measuring the activity of lactate dehydrogenase (LDH) lost from the cells into the suspending fluid. The

activity of LDH was measured according to Bergmeyer *et al.*, (1965).

Data analysis. Data reported in this paper are the mean (±S.D.) of the indicated (n) number of determinations. Statistical analysis was performed by the Student's *t*-test, elaborating experimental data by means of Slide Write plus (Advanced Graphics Software, Inc, Carlsbad, CA, U.S.A.)

RESULTS AND DISCUSSION

Figure 1 illustrates the results of experiments performed to determine whether synthetic derivatives of EACA are able to affect platelet integrity. The potential disrupting effect to platelet plasma membrane was measured by the LDH release test. A ten minute

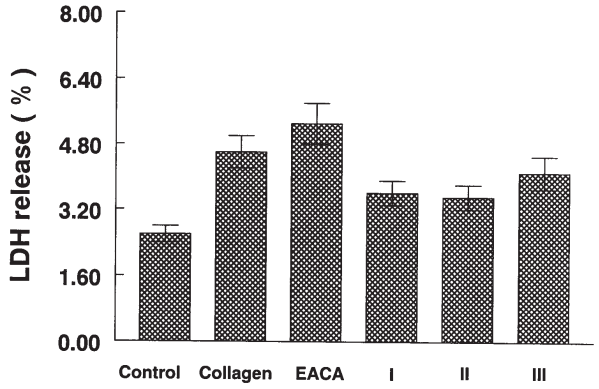

Figure 1. The effect of EACA and its derivatives on the platelet plasma membrane integrity.

Samples of washed platelets (0.5 ml) were incubated for 10 min at 37°C without (control) and with compounds tested added to a final concentration of 20 mM. Incubation was stopped by centrifugation at 11 000 ***g*** for 3 min. LDH activity was determined in the supernatants. To estimate total LDH activity platelets were lysed with Triton X-100 added to a final concentration of 10% (v/w).

incubation of platelets with high (20 mM) concentrations of synthetic derivatives (I, II and III) resulted in only very limited (below 6%) LDH release, which was even less than that produced by EACA and similar to that evoked by collagen, a physiological platelet stimulator. This indicates that EACA and all the derivatives tested even at very high concentrations do not disrupt the platelet membrane.

Table 1 shows the effects of increasing concentrations of EACA and its derivatives (I, II and III) on collagen-induced platelet aggregation in PRP. A two minute preincubation of platelets with 0.2 to 20 mM of the tested substances inhibited, in a dose-dependent manner, the aggregation induced by threshold concentrations of ADP and collagen. The estimated IC_{50} values for EACA were higher than 20 mM and those for compounds I, II, and III 9.8, 9.0 and 9.8 mM, respectively. Near total inhibition of the collagen-evoked platelet aggregation was observed after the treatment of platelets with 20 mM compounds I, II, and III. By contrast, incubation of platelets with 20 mM EACA reduced the aggregation to about 56%.

Table 2 shows the results of similar experiments with ADP as the platelet stimulator. A two-minute exposition of platelets to EACA or its derivatives (0.2–20 mM) resulted in a dose-dependent inhibition of aggregation induced by threshold concentrations of ADP. The estimated IC_{50} values were as follows: >20 mM (EACA), 9.8 mM (comp. I), 7.1 mM (II) and 9.6 mM (III). Incubation of platelets with 20 mM EACA or its derivatives reduced the ADP-evoked platelet aggregation to: 51% (EACA), 2.3% (comp. I), 1.7% (II) and 1.5% (III).

As it is seen from Table 3, a two-minutes incubation of platelets with EACA or its derivatives at the concentration range of 0.2 to 20 mM results in inhibition of collagen-induced platelet aggregation measured in whole blood. The inhibitory effect was dose dependent. The estimated IC_{50} values were as follows: >20 mM (EACA), 9.5 mM (comp. I), 9.7 mM (II) and 10.0 mM (III). Incubation of platelets with 20 mM EACA or its derivatives reduced the collagen-evoked platelet aggregation to: 62% (EACA), 1.1% (comp. I), 2.1% (II) and 1.5% (III).

Table 1. The effect of EACA and its derivatives on collagen-induced aggregation of platelets measured in PRP

Inhibitor	Inhibitor concentration (mM)			IC_{50}
	0.2	2	20	(mM)
EACA	96.0±2.9	73.3±3.8	55.6±2.30	
HCl·H-EACA-L-Leu-OH (I)	95.0±2.6	49.3±2.5	1.60±0.08	9.8
HCl·H-EACA-L-Cys(S-Bzl)-OH (II)	88.5±3.2	58.0±3.7	2.40±0.12	9.0
H-EACA-L-Nle-OH (III)	96.5±2.5	72.1±3.7	2.20±0.10	9.8

PRP (0.6 ml) was incubated with stirring (800 r.p.m.) for 2 min at 37°C in the cuvette of aggregometer without (control) or with EACA or its derivatives (I, II, III) added to the final concentration as indicated. Aggregation was initiated by the addition of threshold concentrations of collagen (10–15 μg/ml). The extent of platelet aggregation was measured 3 min after the addition of the agonist and the maximum extent of aggregation was taken as 100%. All experiments were performed at least in quadruplicate using 5–7 different platelet preparations.

The results presented in Table 4 show that both EACA and its derivatives at the concentration range of 0.2 to 20 mM inhibit thrombin-induced platelet adhesion in a dose dependent manner. The estimated IC_{50} values were as follows: 1.2 mM (EACA), 1.2 mM (comp. I), 1.1 mM (II), and 1.4 mM (III). Incubation of platelets with 20 mM EACA or its derivatives reduced thrombin-evoked platelet adhesion to: 1% (EACA), 2% (comp. I), 0% (II) and 4% (III).

The results presented here indicate that, in comparison with EACA, synthetic ε-aminocaproyl amino acids demonstrate a significantly stronger antiplatelet activity. Their antiaggregatory effect was observed both in the PRP and in whole blood. As judged by the LDH release test, they were non toxic to the cells up to 20 mM, and in addition to being antiaggregatory, they were able to inhibit platelet adhesion with a potency similar to that of EACA. Since previous studies have shown that synthetic ε-aminocaproyl amino acids demonstrate antifibrinolytic properties stronger than EACA (Midura-Nowaczek *et al.*, 1996), it means that they have the potency to

Table 2. The effect of EACA and its derivatives on ADP-induced aggregation of platelets measured in PRP

Inhibitor	Inhibitor concentration (mM)			IC_{50}
	0.2	2	20	(mM)
EACA	95.0±2.6	71.4±3.4	51.0±2.60	
HCl·H-EACA-L-Leu-OH (I)	96.0±2.4	88.6±3.2	2.30±0.12	9.8
HCl·H-EACA-L-Cys(S-Bzl)-OH (II)	76.1±2.2	57.1±3.0	1.70±0.09	7.1
H-EACA-L-Nle-OH (III)	94.0±2.5	52.9±2.8	1.50±0.08	9.6

PRP (0.6 ml) was incubated with stirring (800 r.p.m.) for 2 min at 37°C in the cuvette of aggregometer without (control) or with EACA or its derivatives (I, II, III) added to the final concentration as indicated. Aggregation was initiated by the addition of threshold concentrations of ADP (8–13 μM). The extent of platelet aggregation was measured 3 min after the addition of the agonist and the maximum extent of aggregation was taken as 100%. All experiments were performed at least in quadruplicate using 5–7 different platelet preparations.

Table 3. The effect of EACA and its derivatives on collagen-evoked aggregation of platelets measured in whole blood

Inhibitor	Inhibitor concentration (mM)			IC_{50}
	0.2	2	20	(mM)
EACA	96.5±2.9	85.9±2.3	62.5±3.1	
HCl·H-EACA-L-Leu-OH (I)	94.3±2.7	54.5±2.7	1.10±0.1	9.5
HCl·H-EACA-L-Cys(S-Bzl)-OH (II)	93.8±2.6	64.6±3.2	2.10±0.2	9.7
H-EACA-L-Nle-OH (III)	97.2±2.0	68.6±2.4	1.50±0.1	10.0

Whole blood samples were diluted with equal amounts of 0.9% NaCl solution. One-mililiter sample of diluted blood was incubated with stirring (800 r.p.m.) for 2 min at 37°C in the cuvette of an aggregometer without (control) or with EACA or its derivatives (I, II, III) added to the final concentration as indicated. Aggregation was initiated by the addition of threshold concentrations of collagen (10–15 μg/ml). The extent of platelet aggregation (maximum change in impedance) was measured 6 min after the addition of the agonist. The gain of the aggregometer was calibrated using standard 20 impedance, which corresponds to 100% aggregation.

inhibit simultaneously fibrinolysis and platelet activation better than EACA. Compounds with such properties may be very useful during the course of large surgical procedures (e.g. heart surgery) where there is a need for reduction of blood loss and prevention of the decrease of platelet number. This is because all large surgical procedures are associated with activation of platelets and their number in the blood decreases. A decrease of the platelet concentration below 50 000/μl prolongs bleeding time and augments blood loss. Another consequence of platelet activation due to surgical procedures is appearance on their surface of catalytic sites required for the assemble of prothrombinase complexes. The complexes formed are able to produce large (up to 800 nM) quantities of thrombin,

Table 4. The effect of ε-aminocaproic acid and its derivatives on the adhesion of platelets to fibrinogen coated surfaces

Inhibitor	Inhibitor concentration (mM)			IC_{50}
	0.2	2	20	(mM)
EACA	95±8	10±1	1±0.5	1.2
HCl·H-EACA-L-Leu-OH (I)	94±7	10±1	2±1.0	1.2
HCl·H-EACA-L-Cys(S-Bzl)-OH (II)	96±7	8±1	1±0.5	1.1
H-EACA-L-Nle-OH (III)	95±6	14±2	4±2.0	1.4

Aliquots of washed platelets were incubated at 37°C in fibrinogen coated wells of a microtiter plate without (control) or with EACA or its derivatives (I, II, III) added to the final concentration as indicated. Adhesion was initiated by the addition of thrombin (0.3 U/ml). The extent of platelet adhesion was measured 60 min after the addition of the agonist and the maximum extent of adhesion evoked by thrombin was taken as 100%. All experiments were performed at least in quadruplicate using 5–7 different platelet preparations.

adhesion to the uncovered collagen fibrills. Thus activated platelets form aggregates at the sites of injury and in consequence their which is responsible for the undesired and potentially lethal thrombotic events. Therefore, it is clear why drugs with antiplatelet proper-

ties may be useful to decrease the thrombotic potential of blood and to reduce the decrease of platelet number. This is confirmed by the observation that prophylactic administration of EACA to patients undergoing various selective operations with cardiopulmonary bypass reduced the postoperative blood loss and preserved platelet function (Markwardt, 1978; DelRossi *et al.*, 1989). The use of pharmacological therapies to reduce blood loss and blood transfusion in surgery has been restricted to a few drugs (Barrons & Jahr, 1996; Mannucci, 1998; Munoz *et al.*, 1999; Peters & Noble, 1999; Dunn & Goa, 1999; Porte & Leebeek, 2002). Among the antifibrinolytic agents aprotinin, EACA and tranexamic acid have the best evidence supporting their use especially in cardiac surgery, liver transplantation and some orthopedic surgical procedures. Meta-analyses of randomised, controlled trials in cardiac patients have suggested that all the above mentioned drugs exhibit a comparable therapeutic value (Munoz *et al.*, 1999; Porte & Leebeek, 2002). However, there is clinical evidence that supports the use of aprotinin in preference to the other drugs in patients at a high risk of haemorrhage, in those for whom transfusion is unavailable or who refuse allogenic transfusions (Peters & Noble, 1999). Aprotinin is a serine protease inhibitor isolated from bovine lung. The cost of isolation and purification of this protein remains high (Barrons & Jahr, 1996). These cost factors are actually likely to deter many health-care providers from using the drug. Taking this into account, improvement of aminocaproic acid or tranexamic acid seems to be more reasonable. In this context the new EACA derivatives exhibiting antiplatelet properties stronger than EACA may have potential clinical benefit. Further studies are needed to estimate whether the ε-aminocaproyl amino acids with the antifibrinolytic and antiplatelet activity stronger than EACA will reduce blood loss and preserve platelet function *in vivo* more efficiently than EACA.

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