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Effects of aprotinin on platelet aggregation and cytosolic free calcium in swine platelets[1]

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ABSTRACT Aprotinin inhibited platelet aggregation induced by thrombin (0.25 U·ml^{-1}) with IC_{50}200 kIU·ml^{-1}, and inhibited the rise of cytosolic free calcium concentration in platelets stimulated by thrombin (0.1 U·ml^{-1}) in the absence and in the presence of Ca^{2+} 0.5 mmol·L^{-1} (IC_{50} 117 and 50 kIU·ml^{-1}, respectively), but had no effect on the amounts of actin and myosin heavy chain associated with cytoskeletons. These suggest that aprotinin is an anti-platelet agent and may exert its action through inhibiting the Ca^{2+} flux.

KEY WORDS aprotinin; thrombin; platelet aggregation inhibitors; calcium; cytoskeletal proteins; protease inhibitors

Some protease inhibitors inhibit phosphoinositide turnover, secretion, and aggregation induced by thrombin in platelets[1-3]. Aprotinin, M_r 6500, inhibits trypsin, chymotrypsin, kallikrein, and plasmin[4], but its effects on platelet function remain unkown. In the present study, we investigated the effect of aprotinin on thrombin-induced platelet aggregation.

MATERIALS AND METHODS

Bovine thrombin and egtazic acid (Sigma); Fura-2-acetoxy methylester (Fura-2/AM) (Dojindo, Japan); Triton X-100 (Merck); bovine serum albumin (BSA) (Fluka); aprotinin (The Biochemical Pharmaceutical Factory of Shanghai). All other chemicals were AR.

Swine blood were collected in plastic tubes and anticoagulated with 0.15 volume of ACD (trisodium citrate 86, glucose 111, citric acid 53 mmol·L^{-1}) or 0.1 volume of EDTA buffer (NaCl 120, Tris 50, edetic acid 50 mmol·L^{-1}, pH7.4).

Platelet aggregation Swine blood anticoagulated with ACD was centrifuged at 160×*g* for 15 min, and the supernatant was then centrifuged at 700×*g* for 10 min to yield a platelet pellet. The cells were resuspended gently at 2×10^8 platelets·ml^{-1} in Tyrode-HEPES buffer (NaCl 140, KCl 3, $MgSO_4$ 1, HEPES 10, glucose 10 mmol·L^{-1}, pH 7.4). Platelet aggregometry was carried out in the presence of $CaCl_2$ 0.5 mmol·L^{-1} by optical method using a SPA-4 aggregometer (Shanghai). The platelet suspensions (0.2 ml) were incubated with aprotinin for 2 min, and then stimulated with thrombin for 5 min.

Cytosolic free calcium Platelets obtained from ACD-anticoagulated blood were suspended at 2×10^8 platelets·ml^{-1} in a Tyrode-Hepes buffer (pH 6.7) containing 0.3% bovine serum albumin (BSA) and egtazic acid 0.2 mmol·L^{-1}, and incubated with Fura-2/AM 5 μmol·L^{-1} at 37℃ for 40 min. The cell suspension was then centrifuged at 700×*g* for 10 min. The pellet was washed once with Tyrode-Hepes buffer (pH 6.7) containing 0.3% BSA, and then resuspended at (3—4)$\times10^7$ platelets·ml^{-1} in a Tyrode-Hepes buffer (pH 7.4) containing 0.3% BSA. Fluorescence (λ_{ex} 340 nm; λ_{em} 500 nm) was measured at 23℃ using a Hitachi MPF-4 spectrofluorometer. Cytosolic free calcium concentration ($[Ca^{2+}]_i$) was calculated:

$$[Ca^{2+}]_i = K_d(F-F_{min})/(F_{max}-F),$$

K_d = 224 nmol·L^{-1}

Maximum fluorescence (F_{max}) was determined by lysing the cells in the presence of $CaCl_2$ 1 mmol·L^{-1} with 0.1% Triton X-100; minimum fluorescence (F_{min}) was derived from cell autofluorescence by quenching the signal with $MnCl_2$ 2 mmol·L^{-1}.

Cytoskeletal proteins Platelet-rich plasma (PRP) was prepared by centrifuging the EDTA-anticoagulated blood at 160×*g* for 15 min. PRP was divided into 0.4-ml aliquots and incubated at 37℃ for 60 min. The aliquots were treated with aprotinin for 2 min, and then stimulated with thrombin 0.5 U·ml^{-1}

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for 1 min. Platelet cytoskeletons were prepared as described by Markey *et al*[5]. The cytoskeletal proteins were solubilized and applied to SDS-polyacrylamide gel electrophoresis. Proteins were stained with Coomassie brilliant blue R250, and the relative amounts of actin and myosin heavy chain present were determined as Wallace *et al*[6].

Data were expressed as $\bar{x} \pm s$, and statistical significance was analyzed by t test.

RESULTS

Effects of aprotinin on thrombin-induced platelet aggregation Stimulation of platelets with thrombin 0.25 U·ml^{-1} resulted in the aggregation of 70.9±5.6% ($n=4$) of platelets. Aprotinin inhibited the platelet aggregation induced by thrombin concentration-dependently, with IC_{50}200 kIU·ml^{-1}(Fig 1).

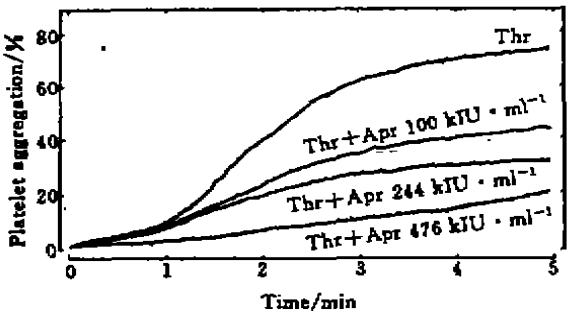

Fig 1. Effects of aprotinin on thrombin-induced platelet aggregation.

Effects of aprotinin on thrombin-induced rise in cytosolic free Ca^{2+} Thrombin (0.1 U·ml^{-1}) stimulated the rise in $[Ca^{2+}]_i$ in the absence and in the presence of Ca^{2+} 0.5 mmol·L^{-1} (data not shown), and the effects were inhibited by aprotinin in a concentration-dependent manner, with IC_{50}117 and 50 kIU·ml^{-1}, respectively. The inhibition in the presence of Ca^{2+} was much stronger than that in the absence of Ca^{2+}.

Effects of aprotinin on actin and myosin heavy chain associated with cytoskeletons Exposure of platelets to thrombin resulted in an increase in the amounts of actin and myosin heavy chain associated with cytoskeletons by 45% ($P<0.05$) and 85% ($P<0.01$), respectively, and aprotinin (200 kIU·ml^{-1}) had no effects on the changes in the cytoskeletal proteins induced by thrombin ($P>0.05$).

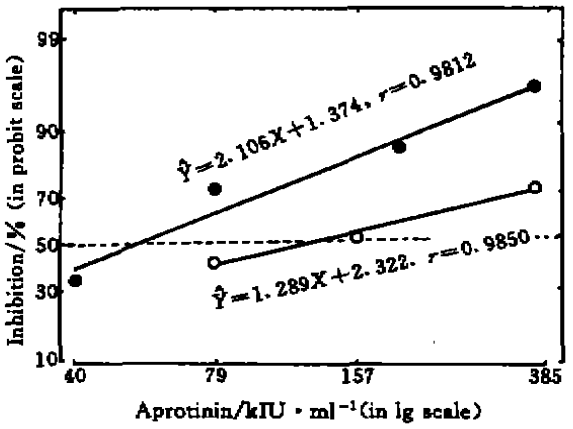

Fig 2. Effects of aprotinin on thrombin-induced rise in cytosolic free calcium in the absence (○) and presence (●) of Ca^{2+} 0.5 mmol·L^{-1}. $n=3$.

DISCUSSION

The results demonstrated that aprotinin inhibits thrombin-induced platelet aggregation in a concentration-dependent manner. These and other evidence[1-3] indicate that protease inhibitors may be one type of anti-platelet agents.

Cytoskeletal proteins involve in platelet activation[7], and the increase in the amounts of cytoskeleton-associated actin is independent of the rise in cytosolic free calcium concentration[8]. Data presented showed that aprotinin had no effect on the amounts of actin and myosin heavy chain associated with cytoskeletons. The results suggest that the inhibition of aprotinin on platelet aggregation makes no difference with the cytoskeletons.

Elevation of cytosolic free calcium concentration is thought to be important to many aspects of blood platelet function, including shape change, secretion and aggregation[9-11].

The results showed that aprotinin inhibited the rise in cytosolic free calcium concentration induced by thrombin either in the absence or in the presence of Ca^{2+}. The inhibition on the rise in cytosolic free calcium in the presence of Ca^{2+} was much stronger than that in the absence of Ca^{2+}. The effect of aprotinin on cytosolic free calcium could be related to its inhibition on platelet aggregation. These suggest that aprotinin may exert its action on thrombin-induced platelet aggregation through inhibiting the Ca^{2+} flux.

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抑肽酶对猪血小板聚集和胞浆游离钙的影响

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A **摘要** 抑肽酶抑制凝血酶(0.25 U·ml^{-1})诱导的血小板聚集(IC_{50}=200 kIU·ml^{-1})。在EGTA 1 mmol·L^{-1}或Ca^{2+} 0.5 mmol·L^{-1}，抑肽酶对凝血酶(0.1 U·ml^{-1})诱导的胞浆游离钙浓度升高都有抑制作用(IC_{50}分别为117和50 kIU·ml^{-1})。抑肽酶不影响细胞骨架蛋白。表明抑肽酶具有抑制血小板聚集的作用，可能与抑制钙有关。

关键词 抑肽酶；凝血酶类；血小板聚集抑制剂；钙；细胞骨架蛋白；蛋白酶抑制剂

Instructions to authors

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