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Inhibition of platelet aggregation by bovine endocardial apyrase

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KEYWORDS apyrase; endocardium; adenosine diphosphate; aspirin; methylene blue

ABSTRACT

AIM: To study the anti-aggregatory effect of bovine endocardial endothelial cell (EEC)-associated apyrase. **METHODS**: Cultured bovine EEC was used. Adenosine diphosphate (ADP) was analyzed by reversed phase HPLC, and rabbit platelet aggregation was measured turbimetrically. **RESULTS**: Incubation of EEC with ADP 500 $\mu mol \cdot L^{-1}$ resulted in a progressive decrease in ADP concentration, which was paralleled by the decrease in platelet aggregating potential of the unmetabolized ADP. In the presence of aspirin (Asp 1 $mmol \cdot L^{-1}$)-treated EEC 1×10^9 cells $\cdot L^{-1}$, the aggregation of Asp (1 $mmol \cdot L^{-1}$) and methylene blue (10 $\mu mol \cdot L^{-1}$)-treated platelets in response to thrombin 500 $U \cdot L^{-1}$ and platelet activating factor (PAF 1 $nmol \cdot L^{-1}$) was markedly inhibited and was reversible, which was very similar to that in apyrase-treated platelets. The supernatants of EEC had no effect on platelet aggregation. EEC inhibited ADP (5 $\mu mol \cdot L^{-1}$)-induced platelet aggregation, but failed to inhibit adenosine 5'-*O*-(2-thiodiphosphate) (ADP-β-S, an unmetabolizable structural analog of ADP, 15 $\mu mol \cdot L^{-1}$)-induced platelet aggregation. **CONCLUSION**: ADP hydrolysis by EEC-associated apyrase is a major anti-thrombotic mechanism of bovine EEC.

INTRODUCTION

Platelet activation and aggregation are important factors in the mediation of vascular and cardiac inflammation including endocarditis and valvulitis[1-4]. Among the various agonists of platelet activation, adenosine diphosphate (ADP) is the most important for recruitment and further amplification of platelet aggregation[2,5].

It is now clear that vascular endothelial cells contain at least three separate "thromboregulatory" systems: epoprostenol (PGI_2), NO, and endothelial cell ecto-enzymes termed ATP diphosphohydrolases (apyrase, EC 3.6.1.5), which is the most important and can hydrolyze extracellular inflammatory mediators ATP and ADP to AMP, thus inhibiting the formation of platelet thrombi[2,5,6]. Moreover, Our recent studies showed that apyrase could cause aggregated platelet deaggregation by decreasing the elevated $[Ca^{2+}]_i$[7].

Like vascular endothelial cells, endocardial endothelial cells (EEC) possess the ability to synthesize epoprostenol and NO[8,9]. Recently, we found that EEC also had an NaN_3-sensitive apyrase activity[10]. The present work was to study the anti-aggregatory effect of bovine EEC-associated apyrase.

MATERIALS AND METHODS

Materials ADP, M199 medium, bovine serum albumin (BSA), adenosine 5'-*O*-(2-thiodiphosphate) (ADP-β-S), thrombin, platelet activating factor (PAF), aspirin (Asp), methylene blue, edetic acid, and HEPES were obtained from Sigma Chemical Co. Fetal bovine serum (FBS) was purchased from Sijiqing Biologic Engineering Co, Hangzhou. Apyrase was prepared from potatoes as described previously[7]. Newborn bovine hearts were purchased from a local slaughterhouse.

Cell culture Bovine EEC were harvested and cultured in M199 medium with 20 % heat-inactivated FBS under air containing 5% CO_2[9]. These cells showed typical morphology, and were identified by electron microscopy. Cells (from passage 3 to 9) were used.

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Measurement of the hydrolysis of ADP with HPLC EEC were plated for experiments in 24-well culture dishes at a density of 1×10^5 cells·cm^2. The ability to hydrolyze ADP 500 μmol·L^{-1} represented apyrase activities. The ADP was analyzed by reversed phase HPLC, as reported previously[7].

Preparation of washed platelets (WP) New Zealand white rabbits ($n = 32$, 2.3 kg ± s 0.4 kg, ♀ and ♂) were provided by the Animal Center of Hunan Medical University (Certificate № 001). Rabbit platelets were prepared[7]. The final suspending medium was Tyrode-HEPES solution containing NaCl 140, KCl 2.5, $MgCl_2$ 1.0, $CaCl_2$ 1, $NaHCO_3$ 10.0, $NaHPO_4$ 0.5, HEPES 10.0 mmol·L^{-1}, and 0.1 % glucose, 0.2 % BSA, pH 7.4.

Preparation of Asp and methylene blue-treated platelet (AM-platelet) and Asp-treated EEC (Asp-EEC) The influence of prostenoids and NO was determined by evaluating EEC inhibitory responses in the presence of cyclooxygenase inhibitors (Asp and indometacin) and by methylene blue that prevents effects of NO by inhibiting soluble guanylate cyclase[11]. To avoid cross-metabolize endoperoxides, both platelet and EEC were treated with Asp. Specifically, platelet suspensions were treated with Asp 1 mmol·L^{-1} and methylene blue 10 μmol·L^{-1} for 30 min followed by twice centrifugations at 1450 × g at 4 ℃ for 15 min. After resuspension, indometacin 10 μmol·L^{-1} was added before study. The platelet count was adjusted to 5×10^{11} platelets·L^{-1}. EEC were treated with Asp 1 mmol·L^{-1} at 37 ℃ for 30 min. Cells were washed in Ca^{2+} and Mg^{2+}-free Tyrode-HEPES solution and detached with 0.05% trypsin/edetic acid. The cells were centrifuged at 500 × g for 10 min, and finally resuspended in Asp-free Tyrode-HEPES solution. Indometacin 10 μmol·L^{-1} was then added. Cell count was adjusted to 4×10^9 cells·L^{-1}.

Platelet aggregation assay Platelet aggregation was quantified by light transmission at 37 ℃ in an aggregometer (Danyang Institute of Electrical Research, Suzhou, China). Maximal change in light transmission was assumed to represent maximal platelet aggregation. As for aggregation studies with combined suspensions of AM-platelets (3×10^{11} platelets·L^{-1}) and Asp-EEC (1×10^9 cells·L^{-1}), combined suspensions were preincubated in plastic cuvettes containing stirring bar. Control cuvettes contained equal numbers of EEC and the platelet-EEC combinations, to correct for light absorption by the nonaggregating EEC[11].

Statistical analysis Data were presented as $\bar{x} \pm s$. Paired t test was employed for statistical analysis.

RESULTS

Concurrent metabolism of ADP by EEC and loss of its potential as a platelet agonist ADP 500 μmol·L^{-1} was added as a final concentration to 24-well culture dishes where EEC 2×10^5 cells/well were cultured. At regular intervals 3 μL of the solution was taken and added as platelet agonist to 300 μL of platelet suspensions. Incubation of EEC with ADP resulted in a progressive decrease in ADP concentration ($n = 6$). After incubation with EEC for 10, 30, 60, 90, and 120 min, the ADP concentration decreased to (451 ± 19), (350 ± 33), (215 ± 34), (150 ± 46), and (110 ± 30) μmol·L^{-1}, respectively (Fig 1, A), which was

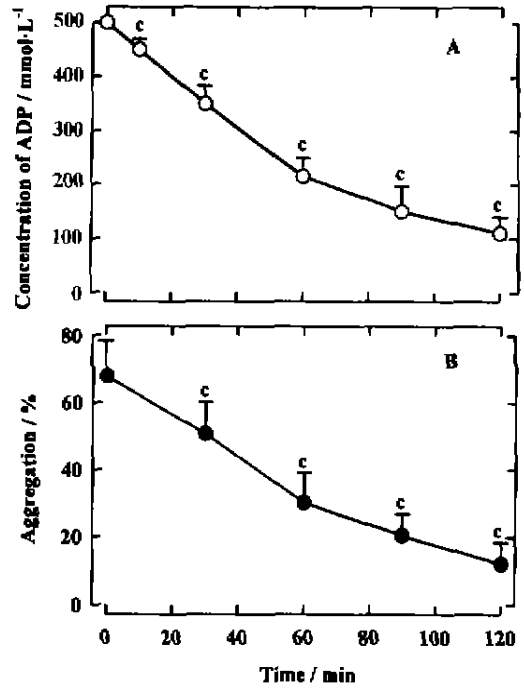

Fig 1. Concurrent metabolism of ADP by bovine EEC 1×10^9 cells·L^{-1} and loss of its potential as a platelet agonist. (A) Time course of metabolism of ADP by EEC monolayers. (B) Platelet aggregation induced by ADP that was incubated with EEC for indicated time. $\bar{x} \pm s$. $n = 6$ experiments (each was from 4 bovine hearts and assayed in triplicate). $^cP < 0.01$ vs 0 time.

paralleled by the decrease in platelet aggregating potential of the unmetabolized ADP ($n = 6$). At 0 time of the incubation, platelet aggregation was 68 % ± 10 %. While at 30, 60, 90, and 120 min of the incubation, platelet aggregation decreased to 51 % ± 9 %, 30 % ± 9 %, 20 % ± 6 %, and 12 % ± 6 %, respectively (Fig 1, B).

Asp-EEC inhibited AM-platelet aggregation When control AM-platelets were stimulated with standard agonists, aggregation was a consistent event. However, in the presence of ASP-EEC, the aggregation of platelets in response to thrombin 500 U · L^{-1} and PAF 1 nmol · L^{-1} was markedly inhibited and was reversible, which was very similar to that of apyrase (100 mg · L^{-1})-treated platelets (Fig 2). The EEC-associated factor that inhibited platelet aggregation was found to be cell associated, since the supernatants of EEC had no effect on platelet aggregation.

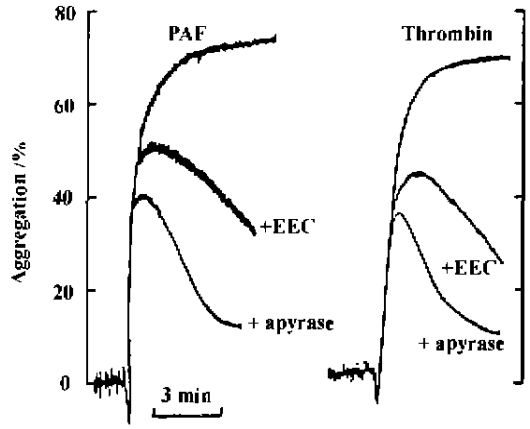

Fig 2. Inhibition of platelet aggregation by EEC suspensions (middle curves) or by apyrase (lower curves). The upper curves (controls) represented the response of washed platelets to thrombin 500 U · L^{-1} and PAF 1 nmol · L^{-1}, respectively. The middle and the lower curves depicted inhibited platelet responsiveness when EEC 1 × 10^9 cells · L^{-1} or apyrase 100 mg · L^{-1} were present. Representatives of 6 independent experiments.

Inhibitory effect of EEC on ADP-induced platelet aggregation ADP-β-S was used to induce aggregation of AM-platelet, both alone and in combination with Asp-EEC. The profound inhibitory effect of these EEC on platelet aggregation was noted when ADP 5 μmol · L^{-1} was used as the platelet agonist. However, this pattern of inhibition of platelet aggregation by EEC was not observed when ADP-β-S 15 μmol · L^{-1} was added (Fig 3). These data suggested that the hydrolysis of ADP by apyrase was responsible, at least in part, for the inhibitory potential of EEC.

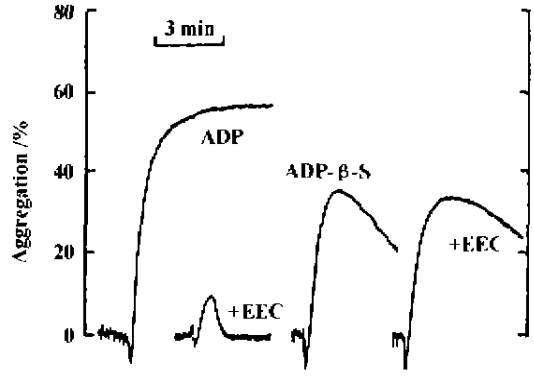

Fig 3. Effect of EEC 1 × 10^9 cells · L^{-1} on platelet aggregation induced by ADP 5 μmol · L^{-1} and ADP-β-S 15 μmol · L^{-1}. Representatives of 6 independent experiments.

DISCUSSION

The present studies showed that the incubation with EEC resulted in a progressive decrease in ADP concentration, which was paralleled by the decrease in platelet aggregating potential of the unmetabolized ADP.

In order to evaluate the anti-thrombotic effect of apyrase independently, the influence of prostenoids and NO was prevented by Asp and indometacin to eliminate the production of any cyclooxygenase-derived eicosanoids, and by methylene blue to eliminate the effects of NO by inhibiting soluble guanylate cyclase[11]. The present experiments showed that when platelets were preincubated with EEC, thrombin or PAF-induced platelet aggregation was inhibited and reversible, which might be due to the hydrolysis of released ADP since the reversal aggregation shape was very similar to that of apyrase-treated platelets. The EEC-induced inhibition was cell-associated since the inhibitory activity was not present in supernatants from EEC.

ADP-β-S, a structural analog of ADP, can

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activate platelet receptors but is not hydrolyzed by the apyrase[11]. ADP-induced platelet aggregation was markedly inhibited in the presence of EEC, while ADP-β-S-induced aggregation was not affected by EEC. These results provided further evidence for ADP hydrolysis by EEC as a major mechanism underlying their inhibitory effect on stimulated platelets.

In conclusion, our results demonstrated that cell-associated and Asp-insensitive apyrase was a major antithrombotic mechanism of bovine EEC.

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牛心内膜腺苷三磷酸双磷酸酶对血小板聚集的抑制作用

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关键词 腺苷三磷酸双磷酸酶；心内膜；腺苷二磷酸；阿司匹林；亚甲蓝

目的：研究牛心内膜内皮细胞（EEC）腺苷三磷酸双磷酸酶的抗血小板聚集效应。**方法**：培养牛 EEC，以高效液相色谱法测定 ADP，用比浊法测血小板聚集。**结果**：ADP 500 μmol·L^{-1}与 EEC 温孵后，引起 ADP 浓度进行性地降低。与此相适应的是，未代谢的 ADP 诱导血小板聚集的能力也降低。在阿司匹林处理过的 EEC 1×10^9 cells·L^{-1}存在下，凝血酶 500 U·L^{-1}及 PAF 1 nmol·L^{-1}诱导的经阿司匹林 1 mmol·L^{-1}及亚甲基蓝 10 μmol·L^{-1}处理过的血小板聚集明显受到抑制，且聚集是可逆的；EEC 的此种作用类似于腺苷三磷酸双磷酸酶的作用。EEC 明显抑制 ADP 5 μmol·L^{-1}诱导的血小板聚集，但不能抑制 ADP-β-S 15 μmol·L^{-1}诱导的血小板聚集。**结论**：腺苷三磷酸双磷酸酶水解 ADP 是牛 EEC 重要的抗血栓机制。

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