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Nitric oxide derived from endothelial cells inhibits Na^+/H^+ exchange in rabbit platelets activated by thrombin[1]

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KEY WORDS nitric oxide; blood platelets; sodium-hydrogen antiporter; hydrogen-ion concentration; calcium; thoracic aorta; vascular endothelium; thrombin; aspirin; nitroarginine

ABSTRACT

AIM: To study the effect of nitric oxide (NO) derived from endothelial cells on Na^+/H^+ exchange in rabbit platelets activated by thrombin. **METHODS**: Intracellular Ca^{2+} ($[Ca^{2+}]_i$) and intracellular pH (pH_i) were measured by the dual-wavelength fluorophotometer with the fluorescent probes Fura-2 and 2′,7′-biscarboxyethyl-5,6-carboxyfluorescein (BCECF). Effects of NO on rabbit platelets were tested by cultured bovine endothelial cells (BAEC). **RESULTS**: BAEC ($0.1 - 1 \times 10^9 \cdot L^{-1}$) inhibited thrombin ($100\ U \cdot L^{-1}$)-induced platelet aggregation in a concentration-dependent manner. This inhibiting effect was abolished by preincubating BAEC with N^G-nitro-*L*-arginine 1 $mmol \cdot L^{-1}$. When the $[Ca^{2+}]_i$ store was depleted with ionomycin in the presence of egtazic acid (EGTA), the increase in pH_i induced by thrombin was inhibited. Refilling intracellular Ca^{2+} store partially reversed this effect. BAEC $2 \times 10^8 \cdot L^{-1}$ inhibited thrombin ($100\ U \cdot L^{-1}$)-induced elevation of pH_i and mobilization of intracellular Ca^{2+} store ($P < 0.01$). No direct effect of endothelial cells on unstimulated rabbit platelets was observed. **CONCLUSION**: NO derived from endothelial cells inhibited thrombin-induced rabbit platelet activation by inhibiting thrombin-induced $[Ca^{2+}]_i$ mobilization and then inhibiting the consequent Na^+/H^+ exchange in rabbit platelets.

INTRODUCTION

Nitric oxide inhibits platelet aggregation induced by several platelet activators[1,2]. This effect was mediated by NO to inhibit the activation of PIP_2-specific phospholipase C, thereby suppress the consequent activation of PKC and the intracellular Ca^{2+} release in thrombin-induced platelet aggregation[3].

Na^+/H^+ exchange plays an important role in the processes such as shape change, aggregation and granule secretion that initiate specific platelet function[4]. The regulation of platelet Na^+/H^+ exchange is closely related with many steps of signal transduction in platelets[5]. The crucial question is whether the concentration of free Ca^{2+} ions affects Na^+/H^+ exchange or *vice versa*[4,6].

The present study was to study the effect of NO on Na^+/H^+ exchange and whether the effect of NO on intracellular Ca^{2+} store mobilization was essential for this process in rabbit platelet activated by thrombin.

MATERIALS AND METHODS

Rabbit Newzealand rabbits (number of ♀ equals number of ♂, weighing 2 kg ± 0.5 kg, from Department of Experimental Animals, Hunan Medical University, Grade Ⅱ, Certificate № 20-009).

Drugs and reagents 2′,7′-Biscarboxyethyl-5,6-carboxyfluorescein acetoxymethylester (BCECF-AM), Fura-2 acetoxymethylester (Fura 2-AM) purchased from Molecular Probe Co were diluted by Me_2SO and stored at −20 ℃. Medium 199, thrombin, aspirin (Asp), HEPES, Triton-X-100,

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bovine serum albumin (BSA), ionomycin, egtazic acid (*EGTA*), *edetic acid* (*EDTA*), and N^G-nitro-*L*-arginine were purchased from Sigma Chemical Co.

Preparation of bovine endothelial cells (BAEC) BAEC were isolated from the thoracic aorta of fetal bovine by trypsin digesting method[7]. Cells in 3rd to 10th passage were used. Asp 100 μmol·L^{-1} was added 1 h before collection to prevent the synthesis of prostanoids. BAEC were collected with 0.1 % trypsin containing 0.02 % EDTA and resuspended in HEPES buffer (NaCl 145, KCl 5, HEPES 10, Na_2HPO_4 0.5, glucose 6, $MgSO_4$ 1 mmol·L^{-1}) containing Asp 100 μmol·L^{-1}.

Preparation of platelets Washed rabbit platelets were prepared from Newzealand rabbit and resuspended in HEPES buffer containing Asp 100 μmol·L^{-1}. The platelet count was adjusted to 2.5×10^{11}·L^{-1}.

Platelet aggregation The rate of aggregation was monitored using a two-channel aggregometer (DAM-1, The Electronic Research Institute of Danyang, Jiangsu) by a turbidimetric method. The rate was measured in light transmission (%)[8].

$[Ca^{2+}]_i$ and pH_i measurement $[Ca^{2+}]_i$ and pH_i were measured by the dual-wavelength fluorophotometer (RF-5000, Shimadzu) with Fura-2 and BCECF[9].

Experimental protocol In platelet aggregation test, 0.2 mL platelet (2.5×10^{11}·L^{-1}) containing extracellular Ca^{2+} 1.25 mmol·L^{-1} was placed in aggregometer. BAEC suspension of different concentration ($0.5-5 \times 10^9$·L^{-1}) or control solution (HEPES buffer containing Asp 100 μmol·L^{-1}) 50 μL was added before the thrombin 100 U·L^{-1}[10].

N^G-nitro-*L*-arginine 1 mmol·L^{-1} was added to endothelial cells for 5 min to demonstrate the role of NO in the antiaggregatory action of endothelial cells.

The effect of endothelial cell supernatant on platelet aggregation was tested to exclude the influence of any soluble factors besides NO.

In $[Ca^{2+}]_i$ and pH_i measurement, EGTA 2 mmol·L^{-1} was added to chelate extracellular Ca^{2+}, so the elevation of $[Ca^{2+}]_i$ was ascribed to intracellular Ca^{2+} mobilization. Depleting intracellular Ca^{2+} store by preincubating platelet with ionomycin 1 μmol·L^{-1} in the presence of EGTA 1 mmol·L^{-1} and then refilling it with extracellular Ca^{2+} 1 mmol·L^{-1}, the variation of pH_i and $[Ca^{2+}]_i$ activated by thrombin 100 U·L^{-1} were observed to evaluate the relationship between pH_i and $[Ca^{2+}]_i$ mobilization[11].

The role of NO on pH_i and $[Ca^{2+}]_i$ activated by thrombin was investigated by adding endothelial cells (2×10^8·L^{-1}) into platelet before thrombin 100 U·L^{-1}.

Statistical analysis Data were expressed as $\bar{x} \pm s$ and compared by paired t test.

RESULTS

Effect of BAEC on thrombin-induced rabbit platelet aggregation 70 % platelet aggregation induced by thrombin 100 U·L^{-1} were used to obtain the optimal antiaggregatory effects of endothelial cells. BAEC added to platelets immediately before thrombin inhibited thrombin-induced aggregation. The antiaggregant effect was dependent on the number of cells used (Fig 1).

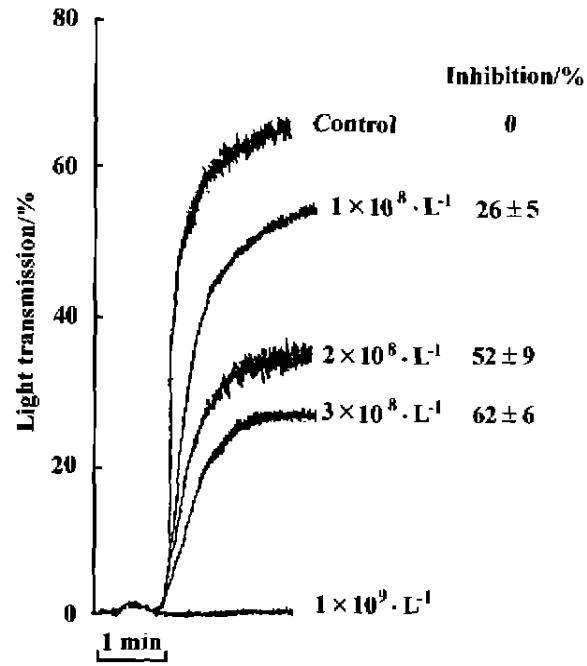

Fig 1. Effects of cultured bovine endothelial cells on thrombin (100 U·L^{-1})-induced platelet aggregation. $n=9$. $\bar{x} \pm s$.

This antiaggregating effect was substantially prevented by preincubation of BAEC with N^G-nitro-*L*-arginine. The supernatant of the endothelial cells did

not show any antiaggregating property (Tab 1).

Tab 1. Effects of cultured bovine endothelial cells (BAEC) preincubated with N^G-Nitro-*L*-arginine on platelet aggregation. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* thrombin 100 U·L^{-1}.

Treatments	*n*	Platelet aggregation/ %
Thrombin 100 U·L^{-1}	9	67 ± 8
BAEC 2×10^8·L^{-1} + Thrombin 100 U·L^{-1}	9	32 ± 5^c
Preincubating BAEC 2×10^8·L^{-1} + N^G-nitro-*L*-arginine 1 mmol·L^{-1} + thrombin 100 U·L^{-1}	6	62 ± 6^a
Supernatant of BAEC + thrombin 100 U·L^{-1}	6	67 ± 4^a

Effect of emptying and refilling intracellular Ca^{2+} store on pH_i When extracellular calcium was chelated with EGTA 2 mmol·L^{-1}, the basal level of platelet $[Ca^{2+}]_i$ was (39 ± 4) nmol·L^{-1} and pH_i 7.43 ± 0.11. Thrombin 100 U·L^{-1} induced an elevation in $[Ca^{2+}]_i$ and pH_i. Platelets were preincubated with ionomycin in the presence of EGTA and then thrombin-induced intracellular Ca^{2+} mobilization was abolished. This effect was partially reversed by incubating the same preparation to extracellular calcium 1 mmol·L^{-1} and chelating remnant extracellular calcium with EGTA 2 mmol·L^{-1} just before the measurement which demonstrated that depleting of the intracellular Ca^{2+} store was achieved. By this treatment to deplete intracellular Ca^{2+} store, thrombin-induced cytosolic alkalization was markedly inhibited. This effect was also partially reversed by refilling the intracellular Ca^{2+} store with extracellular calcium 1 mmol·L^{-1} (Tab 2, Fig 2).

Tab 2. Thrombin-evoked shifts in $[Ca^{2+}]_i$ and pH_i with different treatment. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control. $^fP < 0.01$ *vs* depletion of $[Ca^{2+}]_i$ with ionomycin.

Treatments	*n*	$\Delta[Ca^{2+}]_i$/ nmol·L^{-1}	ΔpH_i
Control	6	150 ± 17	0.20 ± 0.09
Depletion of $[Ca^{2+}]_i$ with ionomycin	4	0	0.05 ± 0.05
Refilling with $[Ca^{2+}]_o$ 1 mmol·L^{-1}	4	86 ± 11^f	0.130 ± 0.017^f
Endothelial cells 2×10^8·L^{-1}	6	67 ± 8^c	0.07 ± 0.05^c

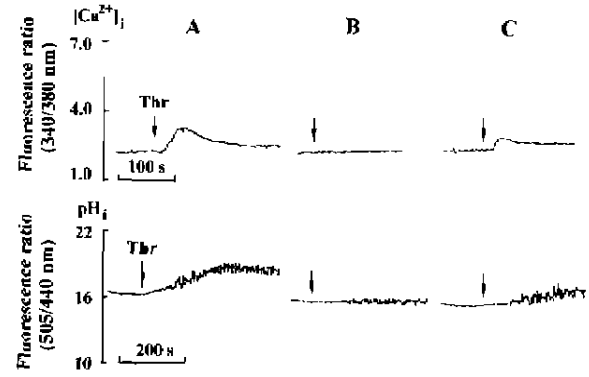

Fig 2. Effects of depleting or refilling intracellular Ca^{2+} stores on $[Ca^{2+}]_i$ and pH_i induced by thrombin. A) Control, B) depleting intracellular Ca^{2+} stores by ionomycin and EGTA treatment, C) refilling the intracellular Ca^{2+} stores with extracellular Ca^{2+} 1 mmol·L^{-1}.

Effect of BAEC on thrombin-induced elevation of pH_i and mobilization of intracellular calcium The elevation of pH_i and $[Ca^{2+}]_i$ induced by thrombin 100 U·L^{-1} was inhibited by BAEC (2×10^8·L^{-1}) which had 50 % inhibiting effect on platelet aggregation (Tab 2, Fig 3). No direct effect of BAEC on sedate platelet was observed.

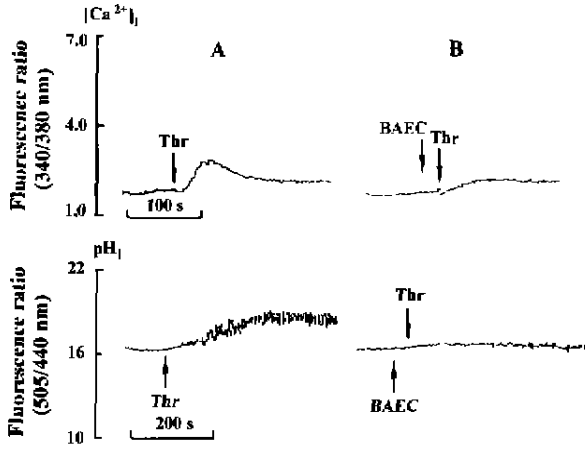

Fig 3. Effects of cultured bovine endothelial cells on $[Ca^{2+}]_i$ and pH_i induced by thrombin. A) Control, B) BAEC 2×10^8·L^{-1} added immediately before thrombin.

DISCUSSION

In the present study a model in which BAEC was

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directly in contact with rabbit platelets was used to test the effects of NO on platelets. Preincubating BAEC with asprin prevented prostacyclin release of BAEC. BAEC inhibited platelet aggregation in a concentration-dependent manner. This antiaggregant effect was abolished by preincubating BAEC with N^G-nitro-*L*-arginine, the most potent inhibitor of NO-synthase. Otherwise, the supernatant of BAEC showed no effect on platelet aggregation, which excluded the presence in our preparation of any prostanoid or other soluble compounds influencing platelet aggregation. So the antiaggregant effect of BAEC can be ascribed to NO [10].

The regulation of pH_i is not very clear now. Phosphorylation of Na^+/H^+ exchanger isoform 1 (NHE1, the most important Na^+/H^+ exchanger isoform in platelets) had been postulated as a very likely mechanism for the elevation of pH_i. The common phosphorilation pattern led us to propose the existence of a NHE1 kinase activated by a pathway integrating all extracellular stimuli. Now it is clearly demonstrated that phosphorylation of NHE1 can not fully account for its activation. Many findings suggests the existence of an activating mechanism that dose not require direct phosphorylation of NHE1. The simplest hypothesis is to postulate the existence of one or multiple regulatory protein(s). In 1995, Shigeo Wakabayashi's Group demonstrated that NHE1 was a Ca^{2+}/calmodulin binding protein whose activity may be regulated by rise in $[Ca^{2+}]_i$ [12].

To determine the relationship between activation of pH_i and intracellular calcium mobilization, platelets were preincubated with ionomycin, a Ca^{2+} inophone, in the presence of EGTA. Thrombin-induced Ca^{2+} mobilization was then abolished. This effect was partially reversed when the same preparation was exposed to extracellular Ca^{2+}. The similar effect was observed in pH_i, suggesting that thrombin-induced cytosolic alkalization of platelet was dependent on $[Ca^{2+}]_i$ mobilization.

This study demonstrated for the first time that NO significantly inhibited thrombin-induced cytosolic alkalinination in rabbit platelets. Our results also proved that NO inhibited thrombin-induced $[Ca^{2+}]_i$ mobilization at the same conditions. And we have known that thrombin-induced cytosolic alkalization of platelet was dependent on $[Ca^{2+}]_i$ mobilization. So we can considered that NO inhibiting cytosolic alkalization was mediated by the inhibiting effect of NO on $[Ca^{2+}]_i$ mobilization.

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内皮细胞衍生的一氧化氮抑制凝血酶激活的兔血小板 Na^+/H^+ 交换[1]

R973.1

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关键词 一氧化氮；血小板；钠-氢反向输运；氢离子浓度；钙；胸主动脉；血管内皮；凝血酶；阿司匹林；硝基精氨酸

目的：探讨内皮细胞衍生的 NO 对凝血酶激活的血小板内 Na^+/H^+ 交换的影响。**方法**：荧光双波长比值法。**结果**：内皮细胞（$0.1-1\times10^9\cdot L^{-1}$）数量依赖地抑制凝血酶诱导的血小板聚集。硝基精氨酸 1 mmol·L^{-1} 可取消这种作用。用依他酸及 ionomycin 耗竭细胞内钙池，凝血酶诱导的血小板胞浆碱化被取消，重新充填细胞内钙池部分恢复。内皮细胞（$2\times10^8\cdot L^{-1}$）显著抑制凝血酶诱导的兔血小板 pH_i 升高及内钙释放。**结论**：血管内皮细胞衍生的 NO 抑制凝血酶诱导的血小板活化是通过抑制凝血酶诱导的血小板内钙动员，继而抑制 Na^+/H^+ 交换的激活来介导的。

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