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Platelet activating factor-induced P-selectin expression in platelets and its related signal transduction[1]

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KEY WORDS platelet activating factor; P-selectin; amiloride; genistein; egtazic acid; flow cytometry; blood platelets

ABSTRACT

AIM: To study the intracellular signal transduction mechanisms of platelet activating factor (PAF)-induced platelet P-selectin expression. **METHODS**: Human blood platelets were used to test the effect of PAF-induced P-selectin expression using flow cytometry. **RESULTS**: PAF 20 nmol·L^{-1} elicited a moderate up-regulation of P-selectin expression [(47.5 ± 1.3) % *vs* control (3.8 ± 0.9) %, $P < 0.01$]. Pretreatment with egtazic acid (EGTA) 2 mmol·L^{-1} and 5, 5'-dimethyl-bis-(o-aminophenoxy)-ethane-*N*, *N*, *N'*, *N'*-tetracetic acid (BAPTA) 200 μmol·L^{-1} to block Ca^{2+} influx or chelate the intracellular calcium, respectively, reduced P-selectin expression in response to PAF [(13.3 ± 0.9) % and (16.8 ± 1.9) % *vs* (47.5 ± 1.3) % of PAF group, $P < 0.01$]. Inhibition of Na^+/H^+ exchange with amiloride (Ami) 400 μmol·L^{-1} resulted in an inhibition of P-selectin expression [(37.5 ± 2.1) % *vs* (47.5 ± 1.3) % of PAF group, $P < 0.01$]. Genistein (Gen) 300 μmol·L^{-1} to inhibit protein tyrosine phosphorylation showed similar effect [(29 ± 4) % *vs* (47.5 ± 1.3) % of PAF group, $P < 0.01$]. **CONCLUSION**: Multiple signal transduction pathways, including protein tyrosine phosphorylation, Na^+/H^+ exchange, and Ca^{2+} mobilization, mediated PAF-induced P-selectin expression.

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INTRODUCTION

Adhesion molecules, such as P-selectin, are potential markers for evaluating platelet activation and studying the role of cell-cell interactions in numerous biological processes related to hemostasis and inflammation[1,2]. Surface-expressed P-selectin mediates the binding of activated platelets to neutrophils and monocytes involved in early inflammatory reactions[3,4]. There is also growing interest in role of α-granule release in modulation of vascular injury and repair mechanisms[5]. While the regulatory mechanism for P-selectin expression in α-granule of activated platelets is poorly understood, it apparently involves a complex sequence of activation processes including structural transformation and translocation of α-granules and secretion[6]. This study aims to investigate the intracellular signal transduction pathways, including Na^+/H^+ exchange, protein tyrosine phosphorylation, and Ca^{2+} mobilization, on the secretion of α-granules and P-selectin expression in response to platelet activating factor (PAF).

MATERIALS AND METHODS

Drugs and reagents PAF, amiloride (Ami), genistein (Gen), [5, 5'-dimethyl-bis-(o-aminophenoxy)-ehtane-*N*, *N*, *N'*, *N'*-tetracetic acid] (BAPTA), egtazic acid (EGTA), indomethacin, bovine serum albumin (BSA), edetic acid (EDTA) were purchased from Sigma Chemical Co. Mouse monoclonal antibodies against human P-selectin (CD62, GMP-140, or PADGEM) was from Suzhou Medical College. The secondary antibody (goat anti-mouse IgG-phycoerythrin conjugate) was from Huamei Biotechnology Inc.

Platelet preparation and stimulation 20 healthy volunteers (12 male, 8 female), aged 28 a ± *s* 4 a, and weighing 52 kg ± *s* 7 kg, have not received

any aspirin for at least 1 a. Platelets were prepared from fresh human blood of these volunteers[7]. The final platelet suspensions contained Ca^{2+} 1 mmol·L^{-1}, indomethacin 10 μmol·L^{-1} and apyrase 1 mg·L^{-1}. Platelets were incubated with PAF 20 nmol·L^{-1} at 37 ℃ for 3 min without stirring. In other experiments, platelets were treated with Ami 400 μmol·L^{-1}, Gen 300 μmol·L^{-1}, BAPTA 200 μmol·L^{-1} or EGTA 2 mmol·L^{-1}, respectively, for 10 min before PAF stimulation. After incubation, platelets were fixed by 1 % paraformaldehyde in phosphate buffer solution (PBS) at pH 7.4 at 4 ℃ for 60 min and washed twice with PBS containing 0.02 % BSA, then incubated with the primary anti-P-selectin at 4 ℃ for 30 min. After washed with PBS, platelets was then incubated with the secondary antibody at a 1 : 100 dilution for 20 min. The stained platelets were washed twice and immediately analyzed by flow cytometry (Coulter Epics).

Flow cytometry The determination of the percentage of platelet expressing P-selectin was carried out[8]. Mean P-selectin fluorescence for the entire platelet population was expressed in arbitrary fluorescence units.

Statistics Results were expressed as $\bar{x} \pm s$ and compared by ANOVA and t-test.

RESULTS

Platelet P-selectin expression Preliminary experiments were performed to identify the time course of peak P-selectin expression in response to PAF, the percentage of P-selectin-positive platelets was found to plateau at 3 min after addition of PAF. The baseline percentage of platelet expressing P-selectin in the PRP before PAF or diluent was (3.8 ± 0.9) %. The P-selectin expression was increased to (47.5 ± 1.3) % after PAF stimulation (Tab 1).

Effects of amiloride, genistein, BAPTA and EGTA on P-selectin expression Inhibition of Na^+/H^+ exchange with Ami 400 μmol·L^{-1} suppressed platelet P-selectin expression in response to PAF. Platelet aggregation was also completely inhibited at the same concentration. Gen 300 μmol·L^{-1} showed similar inhibitory effect on P-selectin expression. To determine whether inhibition of intracellular Ca^{2+} elevation and Ca^{2+} entry affected α-granule release, platelets were treated with BAPTA 200 μmol·L^{-1} and EGTA 2 mmol·L^{-1}, the results showed that the percentage of P-selectin-positive platelets was reduced (Tab 1).

Tab 1. Effects of various treatments on PAF-stimulated P-selectin expression. $n = 4$ independent experiments, $\bar{x} \pm s$. $^cP < 0.01$ *vs* control, $^fP < 0.01$ *vs* PAF.

Treatments	P-Selectin-positive platelet/ %
Basal control	3.8 ± 0.9
PAF 20 nmol·L^{-1}	47.5 ± 1.3^c
Ami 400 μmol·L^{-1} + PAF	37.5 ± 2.1^f
Gen 300 μmol·L^{-1} + PAF	29 ± 4^f
BAPTA 200 μmol·L^{-1} + PAF	16.8 ± 1.9^f
EGTA 2 mmol·L^{-1} + PAF	13.3 ± 0.9^f

DISCUSSION

Based on our previous study, PAF appears to activate platelets by multiple signal transduction pathways[9,10]. Intracellular Ca^{2+} mobilization and Na^+/H^+ exchange are both early event in PAF-induced activation[9-11]. The blockade of Ca^{2+} influx and the inhibition of Ca^{2+} elevation effectively suppressed aggregation and dense-granule release. We found that P-selectin expression was also inhibited with the same treatment. Ami, as a specific inhibitor of Na^+/H^+ exchange, similarly inhibited P-selectin expression. This may result from its effect on the activation of phospholipase A_2, or the regulation of phosphoinositide cycle in platelets[12,13]. The involvement of protein tyrosine kinase (PTK) in P-selectin expression was further suggested in our experiments by the observation with Gen, a PTK inhibitor, also suppressed P-selectin expression.

In conclusion, multiple signal transduction pathways, including protein tyrosine phosphorylation, Na^+/H^+ exchange, and Ca^{2+} mobilization, mediated PAF-induced P-selectin expression.

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血小板活化因子诱导血小板 P-选择素表达及相关信号传导机制[1]

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关键词 血小板激活因子；P-选择素；阿米洛利；4,5,7-三羟异黄酮；依他酸；流式细胞术；血小板

目的：研究 PAF 诱导 P-选择素表达的细胞内信号机制．**方法**：置备人血小板悬液，用流式细胞术测定 PAF 诱导的 P-选择素表达．**结果**：用依他酸 2 mmol·L^{-1}和 BAPTA 200 μmol·L^{-1}分别阻断外钙内流和络合内钙，显著抑制 PAF 诱导的 P-选择素表达［(13.3 ± 0.9) %；(16.8 ± 1.9) % *vs* PAF 对照组(47.5 ± 1.3) %，$P < 0.01$］．用阿米洛利 400 μmol·L^{-1}抑制 Na^+/H^+ 交换，Genistein 300 μmol·L^{-1}抑制蛋白酪氨酸磷酸化也均可抑制 PAF 引起的 P-选择素上调［(37.5 ± 2.1) %；(29 ± 4) % *vs* PAF 对照组(47.5 ± 1.3) %，$P < 0.01$］．**结论**：蛋白酪氨酸磷酸化，细胞内钙动员及 Na^+/H^+ 交换激活中介 PAF 诱导的血小板 P-选择素表达．

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