ISSN 0253-9756 Acta Pharmacologica Sinica 中国药理学报 1999 Feb; 20 (2): 157 - 161
http://www.simm.ac.cn; www.chinainfo.gov.cn/periodical

Regulatory role of protein tyrosine phosphorylation in platelet activating factor-induced signal transduction in platelets[1]

ZENG Shan, YI Fu-Xian, GUO Zhao-Gui[2]
(*Laboratory of Molecular Pharmacology, Hu-nan Medical University, Changsha 410078, China*)

KEY WORDS platelet activating factor; genistein; tyrosine; phosphorylation; platelet aggregation; serotonin; calcium; sodium-hydrogen antiporter; blood platelets; Western blotting

ABSTRACT

AIM: To study the role of protein tyrosine phosphorylation (PTP) in platelet activating factor (PAF)-induced platelet signal transduction cascade. **METHODS**: Washed rabbit platelets were used to test the inhibitory effect of genistein (Gen) on platelet aggregation and serotonin secretion. Intracellular Ca^{2+} ($[Ca^{2+}]_i$) and pH (pH_i) were measured by a dual wavelength fluorophotometer with Fura 2-AM and BCECF-AM. PTP was determined with a specific anti-phosphotyrosine monoclonal antibody by Western blotting. **RESULTS**: Pretreatment with Gen (100 and 200 $\mu mol \cdot L^{-1}$) inhibited PAF (20 $nmol \cdot L^{-1}$)-stimulated platelet serotonin release by 23.7 % ± 2.0 % and 41 % ± 8 %, respectively. Similar inhibitory effects of Gen were observed on PAF-evoked increase of $[Ca^{2+}]_i$ and intracellular alkalization. PAF also elicited a pronounced increase in PTP of several bands with M_r 70 000, 60 000, 50 000, 42 000/40 000, and 34 000, which were suppressed markedly by Gen 200 and 400 $\mu mol \cdot L^{-1}$. Pretreatment with staurosporine (Sta) 20 $nmol \cdot L^{-1}$, BAPTA 200 $\mu mol \cdot L^{-1}$, and egtazic acid 2 $mmol \cdot L^{-1}$ to inhibit PKC activation, $[Ca^{2+}]_i$ elevation, and Ca^{2+} influx respectively, also showed an inhibitory effects on the formation of PTP. **CONCLUSION**: PTP is involved in multiple signal transduction pathways induced by PAF, on which PKC activation and calcium mobilization play a regulatory role.

INTRODUCTION

There is an increasing body of evidence for the important role of protein tyrosine phosphorylation (PTP) facilitated by tyrosine kinases in the regulation of cell functions. Platelets possess a high level of nonreceptor protein-tyrosine kinases (PTK) activity, and the activation of platelets are accompanied by rapid tyrosine-specific phosphorylation of many platelet proteins[1]. The increased tyrosine phosphorylation is involved in signal transduction pathways, such as the elevation of Ca^{2+}, PKC activation, and regulation of arachidonic acid release[2,3]. But the precise role of PTP and the regulatory mechanism involved have not yet clearly elucidated, especially about protein phosphorylation-associated intracellular signaling processes. Platelet activating factor (PAF), as a pro-inflammatory mediator, activates platelets via G-protein coupled receptors. Recent evidence suggested that an association existed among the G-protein coupled receptor with PTK, phosphatidylinositol 3-kinase (PI-3 kinase). PTK is also involved in PAF receptor-coupled phospholipase C activation[4,5].

[1] Project supported by the National Natural Science Foundation of China, No 39570816.
[2] Correspondence to Prof GUO Zhao-Gui.
Phn 86-731-447-4411, Ext 2797. Fax 86-731-447-1339.
E-mail guozg@public.cs.hn.cn
Received 1998-04-10 Accepted 1998-07-24

ISSN 0253-9756 Acta Pharmacologica Sinica 中国药理学报 1999 Feb; 20 (2)
E-mail aps@server.shcnc.ac.cn Phn/Fax 86-21-6474-2629

The present study attempted to further define the platelet events that were regulated by tyrosine phosphorylation and to specify the possible molecular interactions that coupled PTK with receptor-triggered signaling pathways.

MATERIALS AND METHODS

Materials Genistein (Gen), platelet activating factor (PAF), indometacin (Ind), staurosporine (Sta), serotonin (5-HT), BAPTA [5,5'-dimethyl-bis-(*o*-aminophenoxy)-ethane-*N*,*N*,*N'*,*N'*-tetracetic acid], egtazic acid, Me_2SO, phenylmethylsulfonylfluoride (PMSF), *o*-phthaldialdehyde (OPA), Na_3VO_4, Triton X-100, HEPES, and antiphosphotyrosine monoclonal antibody were purchased from Sigma Co. Rainbow Molecular Weight Markers (43 - 200 kDa), horseradish peroxidase-conjugated goat anti-mouse IgG, ECL detection reagents, and nitrocellulose membranes were from Huamei Biotechnology Inc, Shanghai. Western blot chemiluminescence reagent was from NEN™ Life Science. Fura 2-AM and BCECF-AM were from Molecular Probes Co.

Platelet preparation and activation Washed rabbit platelets were prepared[6], and resuspended in HEPES-buffered solution (HBS) (NaCl 145, KCl 5, $MgCl_2$ 1, NaH_2PO_4 0.5, glucose 10, HEPES 10 mmol·L^{-1}, and BSA 0.1 %, pH 7.4, apyrase 1 mg·L^{-1}, Ind 0.1 μmol·L^{-1}). Platelets (1×10^{12}·L^{-1}) were activated with PAF 20 nmol·L^{-1} in the presence of Ca^{2+} 1 mmol·L^{-1} under stirring condition. Platelets were incubated with Gen or Gen vehicle (< 1 % Me_2SO), BAPTA 200 μmol·L^{-1}, Sta 20 nmol·L^{-1}, and egtazic acid 2 mmol·L^{-1} for 30 min before PAF stimulation. Activation was terminated by the addition of an equal volume of ice-cold lysis buffer (Tris·Cl 10, NaCl 150, egtazic acid 1, Na_3VO_4 1, PMSF 1 mmol·L^{-1}, 1 % Triton X-100, pH 7.2). The lysates were centrifuged at 12 000 × *g* at 4 ℃ for 10 min. Protein concentration was estimated[7]. In other experiments, platelets (4×10^{11}·L^{-1}) were used to test the effects of Gen on platelet activation.

Platelet aggregation and 5-HT release Platelet aggregation was monitored photometrically using a dual channel aggregometer (Type DAM-1) at 37 ℃. Platelet 5-HT secretion was quantitated by a fluorimetric method according to the principle that 5-HT forms a fluorephore with OPA, which was determined with a spectrofluorimeter (RF-5000, Shimadzu, Japan)[8].

Measurement of $[Ca^{2+}]_i$ and pH_i Platelets were preincubated at 37 ℃ with either Gen or Me_2SO control for 30 min and washed once to remove Gen or Me_2SO for avoiding the interruption of Gen on fluorescence signals, then loaded with Fura 2-AM 1 μmol·L^{-1} and BCECF-AM 2 μmol·L^{-1} for 30 min. Fluorescence was monitored with a spectrofluorimeter. The $[Ca^{2+}]_i$ and pH_i were assayed[6,9].

Determination of tyrosine phosphorylation by Western blotting Platelet lysates (40 μg/track) were boiled at 95 ℃ for 5 min in 4 times Lammli's sample buffer, and submitted to SDS-PAGE in 7.5 % SDS gel. Proteins were transferred to nitrocellulose membrane, which was blocked for 1 h with 5 % BSA in PBST (Na_2HPO_4 80, NaH_2PO_4 20, NaCl 100 mmol·L^{-1} containing 0.05 % Tween-20). The blots were incubated with the primary anti-phosphotyrosine monoclonal antibody at a 1 : 2000 dilution for 1 h and washed four times in PBST. Antibody binding was detected using 1:500 horseradish peroxidase-conjugated goat anti-mouse IgG and visualized with enhanced chemiluminescence reaction reagents on Konica X-ray film and quantitated by densitometry (CS-930, Japan)[12].

Statistical analysis Data were expressed as $\bar{x} \pm s$ and assessed by ANOVA and *t*-test.

RESULTS

Effects of Gen on 5-HT release and platelet aggregation Gen 100 and 200 μmol·L^{-1} suppressed PAF-stimulated 5-HT release by 23.7 % ± 2.0 % and 41 % ± 8 %, respectively (Tab 1).

Tab 1. Effect of Gen on PAF-stimulated platelet 5-HT release. n = 4 independent experiments, the average of triplicate constitutes one determination. $\bar{x} \pm s$. $^cP < 0.01$ vs control ([Ca^{2+}]$_o$ 1 mmol·L^{-1}).

	PAF-stimulated platelet 5-HT release, nmol·L^{-1}/10^{11} platelet	Inhibition/%
Control	302 ± 14	
Gen 100 μmol·L^{-1}	233 ± 6^c	23.7 ± 2.0
Gen 200 μmol·L^{-1}	178 ± 28^c	41.2 ± 8.1

Effects of Gen on PAF-evoked shifts in [Ca^{2+}]$_i$ and pH$_i$ In the presence of Ca^{2+} 1 mmol·L^{-1}, PAF increased [Ca^{2+}]$_i$ [Δ[Ca^{2+}]$_i$ = (548 ± 81) nmol·L^{-1}] and intracellular alkalization (ΔpH$_i$ = 0.13 ± 0.01). Pretreatment with Gen 100 and 200 μmol·L^{-1} inhibited the increase in [Ca^{2+}]$_i$ and pH$_i$ significantly (Tab 2, Fig 1).

Tab 2. Effect of Gen on PAF-evoked shifts in [Ca^{2+}]$_i$ and pH$_i$. n = 5 independent experiments, the average of triplicate constitutes one determination. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ vs control ([Ca^{2+}]$_o$ 1 mmol·L^{-1}).

	Δ[Ca^{2+}]$_i$/nmol·L^{-1}	ΔpH$_i$
Control	548 ± 81	0.130 ± 0.014
Gen vehicle (< 1 % Me_2SO)	521 ± 38^a	0.129 ± 0.012^a
Gen 100 μmol·L^{-1}	374 ± 34^c	0.070 ± 0.020^c
Gen 200 μmol·L^{-1}	213 ± 43^c	0.020 ± 0.010^c

Western blotting for PTP Up stimulation by PAF 20 nmol·L^{-1}, there was a pronounced increase in PTP of several bands with M_r 70 000, 60 000, 50 000, 42 000/40 000, and 34 000. Pretreatment with Gen 200 and 400 μmol·L^{-1} markedly decreased the levels of PTP relative to its control. The most noticeable inhibition by Gen was for 50, 60, 70 kDa protein. Sta 20 nmol·L^{-1} showed a similar inhibition on PTP. Pretreatment with BAPTA 200 μmol·L^{-1} and egtazic acid 2 mmol·L^{-1}, which blocked [Ca^{2+}]$_i$ elevation and Ca^{2+} influx respectively, also reduced the formation of PTP (Fig 2).

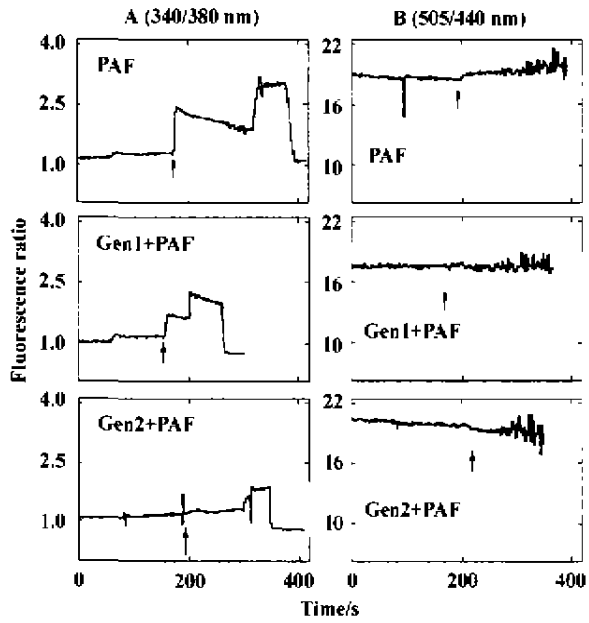

Fig 1. Fura (A) and BCECF (B) fluorescence tracings in response to PAF (20 nmol·L^{-1}, arrow) in the presence of Gen 100 and 200 μmol·L^{-1}.

DISCUSSION

In the present study, we demonstrated that genistein, a PTK inhibitor, produced significant inhibition of platelet aggregation, serotonin secretion, intracellular Ca^{2+} mobilization and Na^+/H^+ exchange activation-induced by PAF, suggesting that PTP played an important role in all of

ISSN 0253-9756 Acta Pharmacologica Sinica 中国药理学报 1999 Feb; 20 (2)
E-mail aps@server.shcnc.ac.cn Phn/Fax 86-21-6474-2629

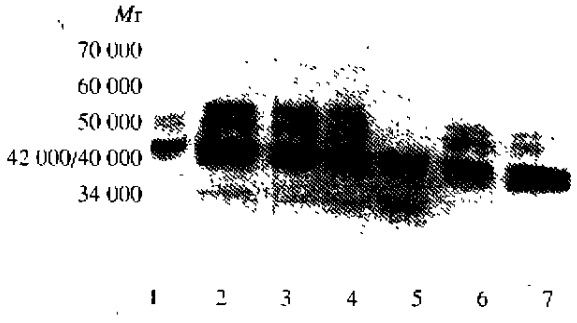

Fig 2. Effects of different treatments on PAF-induced tyrosine phosphorylation by Western blotting. Lane 1, unstimulated platelets; lane 2, PAF; lane 3 - 4, Gen (200 or 400 μmol·L^{-1}) + PAF; lane 5 - 7, Sta, BAPTA, egtazic acid + PAF.

these events. Platelets contain high level of PTK, mainly due to pp60$^{c\text{-}src}$, pp72^{syk}, pp125^{FAK} [2,10]. In this study, an increase in PTP produced by PAF focused on several bands with M_r 70 000, 60 000, 50 000, 42 000/40 000, 34 000, which are mainly substrates of pp60$^{c\text{-}src}$, pp72^{syk} [5,11]. Some high-molecular mass PTP could not be detected. The discrepancies compared with other reports may be due to different isolation procedures or antibodies used and SDS gel analysis[12]. Previous studies suggested that aggregation-dependent phosphorylation might be required for events associated directly with aggregation or downstream of aggregation, pp125^{FAK} may be a candidate[10]. In our study, the substrates of pp125^{FAK}, 95/97 kDa PTP was not detected, so it remained unclear whether lower dose of Gen had the same inhibitory effect on specific protein phosphorylation in an aggregation-dependent fashion. In addition, our results also indicated that PTP could be blocked by Sta, suggesting that PTP is consecutive to PKC activation and dependent on it. Treatment with the intracellular Ca^{2+} chelator BABTA and egtazic acid also sustains the PTP, indicating that Ca^{2+} is necessary for the negative regulatory signals. The inhibitory effect of Gen on PAF-evoked Ca^{2+} increase suggested that PTK participated in the mechanisms of intracellular Ca^{2+} accumulation. Finally, our results first indicated that PTP had a role on the activation of Na^+/H^+ exchange, which was controlled by phosphorylation of the exchanger protein and regulated by some enzymes-associated with PTK[13].

In summary, this study demonstrated that PTP was involved in multiple signal transduction pathways induced by PAF. PKC activation and Ca^{2+} signals play a role in PTP regulation.

REFERENCES

1 Clark FA, Shattil SJ, Brugge JS.
Regulation of protein tyrosine kinases in platelets.
Trends Biochem Sci 1994; 19: 464 - 9.

2 Dhar A, Shukla SD.
Tyrosine kinases in platelet signalling.
Br J Haematol 1993; 84: 1 - 7.

3 Hargreaves PG, Licking EF, Sargeant P, Sage SO, Barnes MJ, Farndale RW.
The tyrosine kinase inhibitors, genistein and methyl 2, 5-dihydroxycinnamate, inhibit the release of [^{3}H]arachidonate from human platelets stimulated by thrombin or collagen.
Thromb Haemost 1994; 72: 634 - 42.

4 Izumi T, Shimizu T.
Platelet activating factor receptor: gene expression and signal transduction.
Biochim Biophys Acta 1995; 1259: 317 - 33.

5 Dhar A, Shukla SD.
Electrotransjection of pp60$^{c\text{-}src}$ monoclonal antibody inhibits activation of phospholipase C in platelets.
J Biol Chem 1994; 269: 9123 - 7.

6 Fang X, Tang XL, Guo ZG.
Measurement of intracellular free calcium concentration in Fura-2-loaded washed rabbit platelets.
Bull Hunan Med Univ 1993; 18: 209 - 11.

7 Bradford MM.
A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.
Anal Biochem 1976; 72: 248 - 54.

8 Dangelmaier CA, Holmsen H.
Platelet dense granule and lysosome content.
In: Harker LA, Zimmerman TS, editors.
Methods in hematology: measurements of platelet function. Edinburgh: Churchill Livingstone; 1983. p 104 - 6.

9 Zeng S, Guo ZG. Effects of platelet agonists on intracellular pH in rabbit platelet.
J Chin Physiol Sci 1997; 13: 81 - 6.

10 Lipfert L, Haimovich B, Schaller MD, Cobb BS, Parsons JT, Brugge JS.
Integrin-dependent phosphorylation and activation of the protein tyrosine kinase pp125^{FAK} in platelets.
J Cell Biol 1992; 119: 905 - 12.

11 Rezaul K, Yanagi S, Sada K, Taniguchi T, Yamamura H.
Protein-tyrosine kinase p72^{syk} is activated by platelet activating factor in platelets.
Thromb Haemost 1994; 72: 937 - 41.

12 Bachelot C, Cano E, Grelac F, Saleun S, Druker BJ, Levy-Toledano S, *et al*.
Functional implications of tyrosine protein phosphorylation in platelets.
Biochem J 1992; 284: 923 - 8.

13 Aharonovitz O, Granot Y.
Stimulation of mitogen-activated protein kinase and Na^+/H^+ exchanger in human platelets.
J Biol Chem 1996; 271: 16494 - 9.

蛋白酪氨酸磷酸化在血小板激活因子诱导血小板信号传导中的调节作用[1]

曾 珊，易富贤，郭兆贵[2]（湖南医科大学分子药理研究室，长沙 410078，中国）

关键词 血小板激活因子；genistein；酪氨酸；磷酸化；血小板聚集；血清素；钙；钠/氢反向输运；血小板；蛋白质印迹

目的：研究蛋白酪氨酸磷酸化(PTP)在血小板激活因子(PAF)诱导血小板信号传导中的作用。**方法**：用水洗兔血小板考查 Gen 抑制聚集及 5-羟色胺释放，Fura 2 和 BCECF 负载测胞内钙及 pH，特异性抗酪氨酸单抗及免疫印迹法检测 PTP。**结果**：Gen 100 和 200 $\mu mol \cdot L^{-1}$ 分别抑制 PAF 诱导的 5-羟色胺释放为 23.7 % ± 2.0 % 及 41 % ± 8 %，对胞内钙增加和 Na^+/H^+ 交换也有抑制作用。PAF 增加 M_r 为70 000，60 000，50 000，42 000/40 000，34 000的 PTP。Gen 200，400 $\mu mol \cdot L^{-1}$ 明显抑制该效应。用 Sta 20 $nmol \cdot L^{-1}$，BAPTA 200 $\mu mol \cdot L^{-1}$，依他酸 2 $mmol \cdot L^{-1}$，分别阻断 PKC 及胞内钙增加和内流，也减少 PTP 形成。**结论**：PTP 参与 PAF 诱导血小板信号传导途径，PKC 活化和胞内钙动员对 PTP 有调节作用。

（责任编辑 杨如华）

1999 全国痹证学术研讨会征文通知

中医痹证包括现代医学所说之风湿性关节炎、类风湿性关节炎、风湿热、骨关节炎、纤维组织炎及神经痛等，为发掘祖国医学文化遗产，促进中西医学在痹证诊治方面的结合。中国中医药学会决定于 1999 年 7 - 8 月在安徽黄山举办"'99 全国痹证学术研讨会"，本次会议由安徽中医临床杂志社承办，现面向全国临床医药界广泛征稿。

征文主要内容 ① 痹证诊治的近现代研究成果总结及国外研究近况综述。② 痹证发生发展转归规律探讨及类风湿性关节炎的流行病学及免疫学研究。③ 现代医学对痹证所包括的各种疾病的诊断及鉴别诊断研究。④ 临床诊治痹证及其并发、后遗症经验总结及临床用药规律探讨。⑤ 痹证的临床护理经验及功能恢复方法介绍。⑥ 痹证的中医古代文献整理及近现代名医经验介绍。⑦ 民间单方验方及特种疗法(包括针灸、推拿、气功、理疗等)的介绍。⑧ 临床常用治疗痹证中成药疗效分析及新药临床验证报告。⑨ 其他相关内容。

截稿日期：1999 年 6 月 30 日(以邮戳为准)

来稿请寄：230011 安徽省合肥市大通路明光小区 5 幢 安徽中医临床杂志社 汪新安收

信封请注明"痹证"字样。