Korean J. Ginseng Sci.
Vol. 18, No. 1, 44~48(1994)

Panaxadiol and Panaxatriol from *Panax ginseng* C.A. Meyer Inhibit the Synthesis of Thromboxane A_2 in Adrenaline-Stimulated Human Platelet Aggregations

Kyeong-Mee Park, Man-Whee Rhee and Hwa-Jin Park*
Department of Biochemical Pharmacology, Korea Ginseng & Tobacco Research Institute, Taejon 305-345, Korea
(Received November 20, 1993)

Abstract□In adrenaline-stimulated human platelets, panaxadiol (PD) and panaxatriol (PT) from *Panax ginseng* C.A. Meyer did not inhibit the Ca^{2+}-influx, but inhibited the formation of thromboxane A_2 and the platelet aggregations. It seems that PD and PT block a pathyway interconverting arachidonic acids (20 : 4) to thromboxane A_2 (TXA_2), because the amount of Ca^{2+} which phospholipase C or phospholipase A_2 requires to liberate 20 : 4 from membrane phospholipids was increased by PD and PT. These results mean that PD and PT have an antiplatelet effect by inhibiting the formation of TXA_2.

Key words□*Panax ginseng* C.A. Meyer, panaxadiol and panaxatriol, inhibition on aggregation, thromboxane A_2, cytosolic Ca^{2+}.

Introduction

Ca^{2+} is a second messenger which exhibits various biological activities for extracellular signals. When platelets are stimulated by thrombin, collagen, Ca^{2+}-ionophore or ADP, Ca^{2+} is accumulated in the cytosol[1] and activates Ca^{2+}-dependent phospholipase C or phospholipase A_2 to liberate arachidonic acid (20 : 4) which is a precursor of thromboxane A_2 from phosphoinositides or phosphatidylcholine.[1-5] Because the production of TXA_2 is closely related to the concentration of cytosolic Ca^{2+} and results in a thrombosis,[4] antiplatelet drugs such as aspirin and imidazole exert their effects by blocking the production of TXA_2.[6,7] In this experiment, we examined how the cytosolic Ca^{2+} concentration and the production of thromboxane A_2 are affected by panaxadiol and panaxatriol, ones of tetracyclic triterpenoids in dammarane family of *Panax ginseng* C.A. Meyer (Fig. 1).

Materials and Methods

1. Materials

Panaxadiol and panaxatriol were offered from the analysis center, Korea Ginseng & Tobacco Research Institute. Thromboxane B_2 [^3H] assay kit was purchased from Amersham Life Science Co. Adrenaline, Quin II/AM, and other chemicals were from Sigma Chemical Co.

2. Preparation of washed platelets

Platelet-rich plasma (PRP) obtained from the antecubital vein of normal human voluteers, was purchased from Taejon Red Cross Blood Center, Korea. During PRP preparation, blood had been preserved against coagulation with CPD sol. (sodium citrate, NaH_2PO_4, glucose, adenine mixture; Korea Green Cross Pharm. Co.). PRP was centrifuged at 125 x g for 10 minutes to remove red blood cells and was washed twice with Tris-citrate-bicarbonate buffer (pH 6.5[8], containing 2 mM EDTA), by centrifuging at 1,100 x g for 10 minutes. The washed platelets were recentrifuged twice with suspending buffer (pH 6.9[8], without EDTA) to remove EDTA. The platelet number was finally adjusted to 5×10^8 cells/m*l* with suspending buffer. All the procedure above were carried out at 25℃ to avoid platelet

*To whom correspondence should be made.

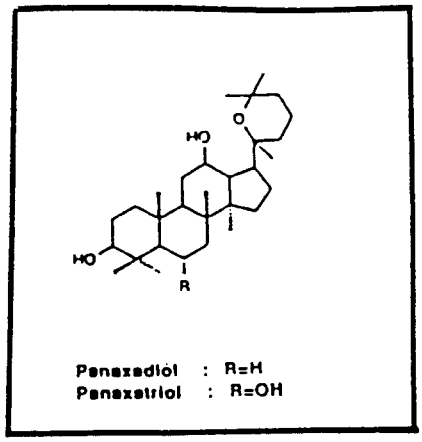

Fig. 1. Structures of panaxadiol and panaxatriol from *Panax ginseng* C.A. Meyer.

aggregation by its cooling.

3. Measurement of platelet aggregation

It is general that platelet aggregations are measured by the change of light transmission at 660 nm with aggregometer (Lumiaggregometer, Model; 400, Chrono-Log, USA),[9] but we used a UV/visible spectrophotometer instead because aggregation also induced a fall down of light absorbance on a spectrophotometer.[10] The washed platelets were preincubated in a cuvette of UV/visible spectrophotometer (Beckman DU-6) with gentle stirring for 3 min at 37℃. Each reaction cuvette contained 2 mM $CaCl_2$ with either testing material or not. Platelets were stimulated by adrenaline for 5 min with gentle stirring. An aggregation degree was determined by following calcualtion.

$$T=\frac{1}{10^{\Delta A}}$$

$\Delta A = 5A - 3A$

5A = absorbance after aggregation reaction occurred for 5 min

3A = absorbance after preincubation had been carried out for 3 min

Suspending buffer was used as reference (absorbance 0). PD and PT were dissolved in the 3rd distilled water (D.W.).

4. Determination of cytosolic Ca^{2+} concentration

Platelet-rich plasma (PRP) was incubated with 25 μM Quin II/AM at 37℃ for 60 min. Because Quin II/AM is light-sensitive, the tube containing PRP was covered with an aluminium foil throughout the loading process. The Quin II-loaded platelets were also prepared same as the normal preparation. EDTA, a Ca^{2+} chelator, was removed from platelets by washing twice with suspending buffer (pH 6.9).

The concentration of Ca^{2+} was determined by Tsien's method[11] with gentle stirring for 5 min at 37℃ in a fluorescence spectrophotometer (Perkin Elmer, LS-50).

5. Measurement of thromboxane B_2

TXB_2, a stable metabolite of thromboxane A_2 (TXA_2), was measured by a thromboxane B_2 radioimmunoassay kit. The radioimmunoassay was perfomred to the instruction manual given by a manufacturer.

Results and Discussions

The concentration of Ca^{2+} in the platelets was increased from 27 nM to 149 nM when the platelets were stimulated by adrenaline (40 μM) (Fig. 2), and this is well consistent with the fact that adrenaline increases the level of cytosolic Ca^{2+} and decreases the level of cAMP in platelets.[12,13] When platelet suspensions containing PD and PT were aggregated by adrenaline, the concentration of Ca^{2+} was increased to 353 nM and 359 nM, respectively (Fig. 2), but the level of TXA_2 was decreased (Table 1).

As mentioned in the "Introduction", the elevated free Ca^{2+} in the cytosol activates Ca^{2+}-dependent phospholipase C or phospholipase A_2, to liberate arachidonic acid which is a precursor of TXA_2 from phosphoinositides or phosphatidylcholine.[14,15] The platelet aggregation caused by TXA_2 is the first order aggregation and that by secreted substances (ADP, serotonin, platelet-activating factor, etc) is the second order aggregation.[14] Explaining in detail, TXA_2 releases Ca^{2+} out of endoplasmic reticulum to stimulate the Ca^{2+}/diacylglycerol-dependent kinase and Ca^{2+}/calmoduline-dependent kinase which phosphorylate 40 KD- and 20 KD-polypeptides in platelets, and successively 40 KD- and 20 KD-polypeptides are associated with the release of ADP,

Table 1. Effects on TXA_2 formation in adrenaline-stimulated human platelet aggregation

	None	Adr. 40 μM	Adr. 40 μM+ PT (100 μg/ml)	Adr. 40 μM+ PD (100 μg/ml)
TXA_2 ng/10^8 plateles	1.44± 0.5	18.6± 0.04	3.2± 0.3	2.7± 0.1

The platelet aggregations were performed by what as described in the "Method", and terminated with indomethacin (100 μM). Human platelets (10^8/ml) were aggregated by adrenaline (40 μM) with either 100 μg of PD, PT/ml or none. TXA_2 was measured with TXB_2 radioimmunoassay kit from Amersham Life Sciences. The data are given as Mean± S.D. (n=6).
None : Intact platelets, Adr : Adrenaline, PT : Adrenaline+Panaxatriol, Adrenaline+Panaxadiol.

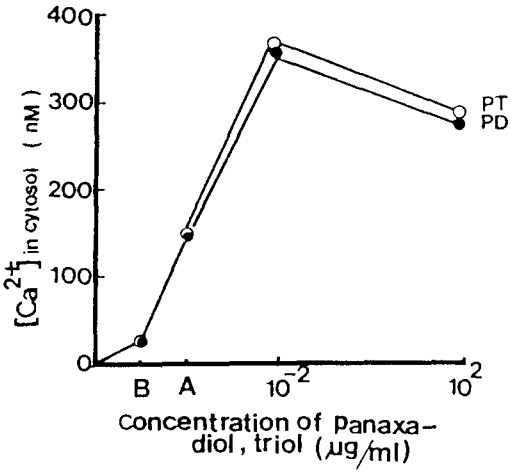

Fig. 2. Effects on concentrations of cytosolic Ca^{2+} in adrenaline-stimulated human platelets. The concentration of cytosolic Ca^{2+} was determined by what as described in the "Method". Platelet suspension (10^8 platelets/ml) including either PD, PT or none were preincubated with 1 mM of $CaCl_2$ for 2 min at 37℃, and then aggregated by adrenaline (40 μM) for 5 min.
B : Cytosolic basal concentration
A : Adrenaline, PD : Panaxadiol
PT : Panaxatriol

serotonin, platelet-activating factor, etc.[14-16] In the adrenaline-induced platelet aggregation, PD and PT inhibited the synthesis of TXA_2, but the level of TXA_2 in that aggregation reaction was much more than in intact platelets by two folds (Table 1).

As shown in Fig. 2, cytosolic Ca^{2+} was increased by adrenaline, PD and PT, meaning that the phospholipases are activated and that arachidonic acids (20 : 4) were elevated in the cytosol by adrenaline, PD and PT. The Ca^{2+} increased by PD and PT are not abundunt enough to activate the Ca^{2+}/diacylglycerol-dependent kinase and Ca^{2+}/calmodulin-dependent kinase. Because the cytosolic free Ca^{2+} concentration which is necessary for activating those kinases is above 1 μM.[15,16] To elucidate the inhibitory mechanism on TXA_2-production by PD and PT, studies on the effects on Ca^{2+}/diacylglycerol-dependent kinase and Ca^{2+}/calmodulin-dependent kinase are still remained. However, it is not at all disputable that the inhibition of PD and PT on the adrenaline-induced platelet aggregation is due to the inhibited TXA_2-production. Also that 20 % of aggregation was maintained even with PD and PT in the adrenaline-induced aggregation (Table 2), is well explained by the fact that this aggregation is independent on the secrtion but dependent on the TXA_2 which is produced by two folds in that situation compared to the level of TXA_2 in the intact platelets (Table 1).

20 : 4 is a starting point of prostaglandin metabolism. It is a substrate of both cyclooxygenase and lipoxygenase. Eicosanoids such as PGH_2, PGG_2,

Table 2. Effects on adrenaline-stimulated human platelet aggregations

	Adr. 40 μM	Adr. 40 μM +PD (100 μg/ml)	Adr. 40 μM +PD (100 μg/ml)
Degree aggregations (100%)	100	21.8± 1.2	24.8± 1.5

Aggregations were carried out by what as described in the "Method", and the degree of aggregations was calculated as percentage of that of adrenaline by which 65% light transmission is produced in platelet suspensions. The data are given as Mean± S.D. (n=4).
Adr : Adrenaline, PD : Panaxadiol, PT : Panaxatriol.

PGE_2, PGD_2 and TXA_2 are produced via cyclooxygenase pathway, and leukotrienes are produced from hydroxy fatty acids generated by lipoxygenase pathway in platelets. These metabolites are released out from cells and play as an autacoid in the producing cells and neighboring other cells. Eicosanoids react to the receptor of platelets to activate platelets, but leukotrienes do not.[18] Among eicosanoids, PGH_2, PGG_2 and TXA_2 cause to aggregate the platelets, but PGE_2 and PGD_2 inhibit the aggregation. Of particular, because TXA_2 constricts the blood vessel potently as well as stimulates the platelet aggregation,[5] TXA_2 is also a principal factor of heart attack and cardiac infraction.[5] Drugs such as aspirin, indomethacin and imidazole inhibit cyclooxygenase and thromboxane A_2 synthase and thus inhibit the production of eicosanoids; TXA_2, PGG_2 and PGH_2. While slightly different from the above drugs in that the inhibitory effect on TXA_2-production by PD is not contributed to by the inhibition of cyclooxygenase,[17] PD and PT are potential candidates for antiplatelet drugs. The Ca^{2+} closely related to the synthesis of TXA_2 is reported to accelerate the production of cGMP as well, in a recent paper.[19] Additionally the Ca^{2+}-dependent cGMP-production may affect the aggregation of platelets, inhibiting in a feed-back mechanism. Our results show that PD and PT increase the influx of Ca^{2+} and inhibit the synthesis of TXA_2, and this is well consistent with the above finding.

Platelets incubated with thrombin is more susceptible to breakage on a gradient of glycerol-EDTA.[8] In a preparation of platelets, platelets were washed twice in buffer containing EDTA in order to remove a trace amount of Ca^{2+}.[8] Accordingly, we can not rule out the possibility that platelets are broken during washing. If platelets were broken, other intact platelets would be aggregated by stimulators of platelets such as ADP, serotonin, platelet activating factor and Ca^{2+} that are released out of granules. However, when PD and PT were added to the platelets suspended and preincubated in the buffer devoid of EDTA both platelet aggregation and synthesis of TXA_2 by adrenaline were inhibited. The report that EDTA causes a partial proteolysis of an actin-binding protein fibronectin, myosin and thrombin sensitive protein,[20] may intrigue the possibility that EDTA inactivates platelets. If the inhibition of aggregation by PD and PT were due to the inactivation of platelets resulted from EDTA, platelet aggregation, TXA_2 production and Ca^{2+}-influx would not be occurred by adrenaline. However, 65 % of platelet aggregation was occurred and the related metabolites were produced by adrenaline (Table 1, 2). The above points verify that platelets were not broken and not inactivated by EDTA, and the inhibition of aggregation and the activation of related metabolites by PD and PT are not false.

요 약

아드레날린에 의한 사람 혈소판의 응집반응에서, PD와 PT는 Ca^{2+} influx는 억제시키지 않았으나, Thromboxane A_2의 생성을 저해하고 혈소판의 응집을 억제하였다. PD와 PT는 phospholipase C나 phospholipase A_2가 막 인지질로부터 아라키돈산을 유리하는데 필요한 세포내 칼슘량의 증가를 억제하지는 않았으므로, 아라키돈산에서 트롬복산 A_2로 변하는 어떤 단계를 억제할 것으로 추측된다. 이것은 PD와 PT는 트롬복산 A_2의 생성을 억제하므로써 항혈소판 작용함을 의미하는 것이다.

Acknowledgement

We thanks Dr. Choi, Gang-Ju and Dr. Kim, Suck-Chang, for many assistances. This work was supported by a grant of Korea Tobacco and Ginseng Corporation.

References

1. Scrutton, M.C. and Athayde, C.M. : *The Platelet in Health and Disease*, Clive p.p, ed. p. 61, Blackwell Scientigic Publ., London, Edinburgh, Boston (1991).
2. Takamura, H. Narita, H., Park, H.J., Tanaka, K.I., Matsuura, T. and Kito, M. : *J. Biol. Chem.*, **262**, 2262 (1987).
3. Park, H.J., Narita, H., Tanaka, K.I., Matsuusa, T. and Kito, M. : *Agric. Biol. Chem.*, **50**, 971 (1986).
4. Bills, T.K., Smith, J.B. and Silver, M.J. : *J. Clin. Invest.*, **60**, 1 (1977).
5. Hamberg, M., Sevesson, J. and Samuelsson, B. :

Proc. Natl. Acad. Sci. USA., **72**, 2994 (1975).
6. Israel, F.C., Richard, D.F. and Thomas, C.D. : *J. Clin. Invest.*, **60**, 866 (1977).
7. Mustard, J.F. and Packham, M.A. : *Pharma. Co. Rev.*, **22**, 97 (1970).
8. Rittenhouse-Simmons, S. and Deykin, D. : *Biochim. Biophys. Acta*, **426**, 688 (1976).
9. Choung, S.Y. : *Yakhak Hoeji* (Seoul) **35**, 11 (1991).
10. Born, G.V.R. and Cross, M.J. : *J. Physiol.*, **168**, 178 (1968).
11. Tsien, R.Y., Pozzan, T. and Rink, T.J. : *J. Cell. Biol.*, **94**, 325 (1982).
12. Ware, J.A., Johnson, P.C., Smith, M. and Salzman, E.W. : *J. Clin. Invest.*, **77**, 878 (1986).
13. Marquis, N.R., Becker, J.A. and Vigdahl, R.L. : *Biochem. Biophys. Res. Commu.*, **39**, 783 (1970).
14. Charo, I.F., Feinman, R.D. and Detwiler, T.C. : *J. Clin. Invest.*, **60**, 866 (1977).
15. Kakiuchi, S. : *FEBS Lett.*, **126**, 203 (1981).
16. Hataway, D.R. : *Pro. Nat. Acad. Sci. USA.*, **76**, 1653 (1979).
17. Lee, S.H., Park, C.W., Lee, I.K. and Han, B.H. : *Korean J. Ginseng Sci.*, **13**, 202 (1989).
18. Yamamoto, S. : *Metabolism* (Japan), **18**, 307 (1981).
19. Ohsako, S. and Deguchi, T. : *Biochem. Biophys. Res. Commun.*, **122**, 333 (1984).
20. Cohen, I., Glaser, T., Veis, A. and Brunner-Lorand, J. : *Biochem. Biophys. Acta*, **676**, 137 (1981).