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Panaxadiol from *Panax ginseng* C.A. Meyer Inhibits Synthesis of Thromboxane A_2 in Platelet Aggregation Induced by Thrombin

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Abstract□Panaxadiol (PD) fr· .n korean red ginseng C.A. Meyer did not control the concentration of cytosolic free Ca^{2+} influxed by thrombin (5 u/m*l*). However, PD strongly inhibited the synthesis of thromboxane A_2 (TXA_2) in the aggregation of human platelets induced by thrombin (5 u/m*l*). These results suggest that PD blocks the any pathway transforming to TXA_2 from arachidonic acid (AA) which release out of plasma membrane phospholipids by Ca^{2+}-dependent phospholipase C or phospholipase A_2. It may be also concluded that PD has the antiplatelet function by inhibiting the synthesis of TXA_2, which known to be the potent stimulator of the aggregation of human platelet.

Key words□Panaxadiol, inhibition of platelet aggregation, inhibition of thromboxane A_2.

Introduction

Ca^{2+}, a second messenger for agonists, has various physiological functions. When platelets are stimulated by thrombin, collagen, Ca^{2+}-ionophore (A_{23187}), ADP and so on, Ca^{2+} is mobilized from endoplasmic reticulum (ER) or influxed through Ca^{2+}-channel in plasma membrane, and accumulated into the cytosol.[1-3]

Accumulated cytosolic Ca^{2+} allow the AA to release from phosphoinositides of plasma membrane by activating the Ca^{2+}-dependent phospholipase C.[4,5]

Phospholipase A_2 also requires Ca^{2+} to release the AA from phospholipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidic acid and phosphatidylserine) in plasma membrane.[6-9] Consequently, the synthesis of TXA_2 and the liberation of AA are closely related with the concentration of cytosolic Ca^{2+}. Thrombus was mainly resulted from the excessive production of TXA_2.[10] Aspirin and imidazole are known as the antiplatelet drugs.[11,12] We examined that how panaxadiol saponin from Korean red ginseng exerts an influence on the concentration of cytosolic Ca^{2+} and the synthesis of TXA_2, and discussed its possibility of functioning as an antiplatelet drug.

Materials and Methods

1. Materials

Thromboxane A_2[^{3}H] assay kit was purchased from Amersham Life Science Co. Thrombin (from bovine plasma), Quin-II/AM and other chemicals were from Sigma Chemical Co. Panaxadiol was the gift from Dr. Kang Ju Choi, Analytical center, Korea Ginseng & Tobacco Research Institute.

2. Preparaton of washed platelets

Platelet-rich plasma (PRP) obtained from the antecubital vein of normal human volunteers, was purchased from Taejon Red Cross Blood Center, Korea. The blood was anticoagulated 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 min to remove red blood cells and washed twice in Tris-citrate-bicarbonate buffer (pH 6.5,[13] containing 2 mM EDTA) by centrifugation at 1,100 x g for 10 min. Because EDTA has an inhibitory action on platelet aggregation, the washed platelets were recentrifuged twice with suspending buffer (pH 6.9,[13] without EDTA) to remove

EDTA. Finally, platelet number was adjusted to 5×10^8 cells/ml in suspending buffer. All of the above procedures were carried out at 25℃ to avoid platelet aggregation by its cooling.

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

Platelet-rich plasma (PRP) was incubated with 25 μM Quin II/AM at 37℃ for 1 hour. Because Quin II/AM is light-sensitive, the tube containing PRP was covered with an aluminum foil during loading. The Quin II-loaded platelets were prepared according to the same procedure as described above. EDTA, a Ca^{2+} chelator, was removed by washing the platelets twice with suspending buffer (pH 6.9). Ca^{2+} was measured with gentle stirring at 37℃ according to Tsien's method[14] on fluorescence spectrophotometer (Perkin Elmer, LS-50). Because the panaxadiol was dissolved in DMSO, determination of Ca^{2+} concentration was calculated by substracting the effect of DMSO.

4. Measurement of thromboxane B_2

TXB_2, a stable metabolite of thromboxane A_2 (TXA_2), was measured with thromboxane B_2 radioimmunoassay kit as indicated by manufacturer. The TXB_2 of platelets was calculated by substracting the effects of solvents such as DMSO.

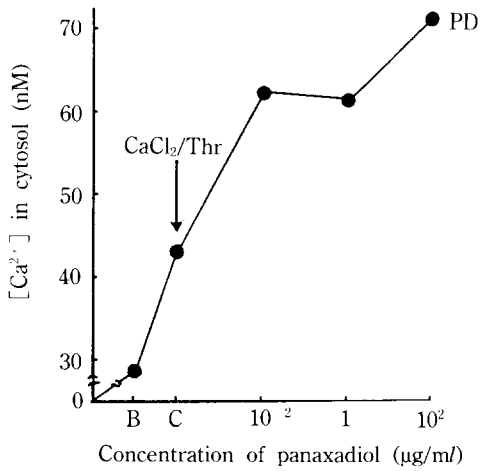

Fig. 1. Effects of panaxadiol on calcium influx in thrombin-induced human platelet. The quantitation of Ca^{2+} was assayed as described in "Methods". Human platelet containing 1 mM Ca^{2+} was preincubated with or without panaxadiol for 2 min, and the reaction was continued for another 5 min with the addition of thrombin (5 u/ml). B : basal Ca^{2+} concentration of intact platelet, C : concentration of platelet cytosolic Ca^{2+} followed by stimulated of thrombin.

Results

1. Effects of PD on the concentration of cytosolic Ca^{2+}

As shown in Fig. 1, when the human platelets were stimulated by the high dose (5 u/ml) of thrombin, the concentration of cytosolic Ca^{2+} was increased to 46 nM from 27 nM which was the concentration of intact platelet. This indicates 19 nM of Ca^{2+} is influxed into the cytosol by thrombin. When platelets were stimulated by thrombin in the presence of panaxadiol, the concentration of cytosolic Ca^{2+} was increased to 71 nM. These results suggest that both thrombin and PD exert synergistic on the influx of Ca^{2+}.

2. Effects on TXA_2 production

Thrombin induced TXA_2 production to 1300 nM (Table 1). But when the platelets were preincubated in the presence of PD before stimulation with thrombin, TXA_2 level was significantly decreased. These results mean that PD (100 μg/ml) inhibits the production of TXA_2 by thrombin.

Discussion

From the above results, it is supposed that PD

Table 1. The effect of panaxadiol on the production of TXA_2

	None	Thrombin (5 u/ml)	Thrombin (5 u/ml)+ Panaxadiol (100 μg/ml)
TXA_2 ng /10^8 platelets	4.9± 0.89 (n=3)	$13\times 10^2\pm 140$ (n=3)	64.0± 6.3 (n=3)

Human platelet containing 1 mM Ca^2 was preincubated with or without panaxadiol for 2 min, and the reaction was continued for another 5 min with the addition of thrombin (5 u/ml). The aggregatory reaction was terminated with 100 μM indomethacin. TXA_2 assay was described in "Methods".

influxes Ca^{2+} into cytosol to liberate AA from phospholipids, but inhibits TXA_2 production by affecting a certain enzyme involving in AA cascades (i.e.; AA →cyclooxygenase→PGH_2/PGG_2→TXA_2 synthase →TXA_2). The enzymes involving in TXA_2 production are known to phospholipase A_2, phospholipase C, cyclooxygenase and thromboxane synthase.[4, 9, 15]

In our experiment, since cytosolic Ca^{2+} was increased by PD, it could be ruled out the possibility that Ca^{2+}-dependent phospholipase C and phospholipase A_2 would be inhibited by PD. It is reported that the inhibitory effect of PD on TXA_2 production is not contributed to the inhibition of cyclooxygenase.[16]

Cytosolic Ca^{2+} was increased by thrombin and PD (Fig. 1), which means that the activation of phospholipases and the elevation of AA were induced by PD and thrombin. To elucidate the inhibitory effect of PD on TXA_2 production, further study may be required. AA is a substrate of cyclooxygenase and lipoxygenase.

Prostaglandins such as PGH_2, PGG_2, PGE_2, PGD_2 and TXA_2 are produced via cyclooxygenase, and leukotrienes are produced from hydroxy fatty acids generated by lipoxygenase in platelets. These metabolites are released from platelets and play the role as an autacoid for platelets or other cells. Leukotrienes do not act to the platelet.[17] But prostaglandins react to the receptor of platelets to activate platelets.[17] Consequently platelets are aggregated by PGH_2, PGG_2, TXA_2.

PGD_2 and PGE_2 inhibit platelet aggregation. A drug which inhibit the production of prostglandins such as TXA_2, PGG_2 and PGH_2 is known to antiplatelet drug. The representative antiplatelet drugs which inhibit cyclooxygenase and thromboxane synthase are said to aspirin, indomethacin and imidazole.

Efficacious concentration of antiplatelet drugs such as aspirin, indomethacin, and imidazole on human platelet is 1～100 μM *in vitro*.[18] The concentration of panaxadiol (100 μg/ml, av. MW=1,045) used in our study corresponds to 96 μM.

Considering the fact which this concentration of panaxadiol inhibits the TXA_2 formation, it is concluded that panaxadiol saponin may be a useful antiplatelet drug. TXA_2 constrict the blood vessel potently as well as stimulates the platelet aggregation.[10] Hence, TXA_2 is the principal factor of heart attack and cardiac infraction.[10] The effects of panxadiol on the aggregation of platelet exhibit dual nature which increase the influx of Ca^{2+} and inhibits the synthesis of TXA_2. Also, the production of Ca^{2+}-dependent cGMP may affect the feed back system, which inhibits the aggregation of platelets. It is known that Ca^{2+} closely relating to the synthesis of TXA_2 accelerates the producton of cGMP.[19]

요 약

고려홍삼(Korean red ginseng)으로부터 얻은 panaxadiol(PD)는 thrombin(5 u/ml)이 혈소판 내부로 유입(influx)시킨 Ca^{2+}의 농도를 억제시키지 않았다. 그러나, thrombin(5 u/ml)유인 혈소판 응집반응에서 thromboxane A_2(TXA_2)의 생성을 현저하게 억제시키고 있었다. 이 결과는 PD가 혈소판 막의 phospholipids로부터 TXA_2의 전구체인 arachidonic acid(AA)를 유리시키는데 phospholipase C가 필요로 하는 Ca^{2+}의 유입(influx)을 억제시키지 않고 AA로부터 TXA_2의 어떤 생성단계를 억제시키고 있음을 시사하는 것이다. 따라서 PD는 혈소판 응집반응을 강하게 촉진하는 것으로 알려져 있는 TXA_2의 생성을 억제시켜 항혈소판 작용(antiplatelet function)을 하고 있는 것으로 결론지을 수 있다.

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