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Effects of Brazilin on the Phospholipase A_2 Activity and Changes of Intracellular Free Calcium Concentration in Rat Platelets

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Brazilin {7,11b-dihydrobenz[*b*]indeno[1,2-*d*]pyran-3,6a,9,10(6*H*)-tetrol} inhibited thrombin-, collagen- and ADP-induced aggregation of washed rat platelets. Thrombin- and collagen-induced ATP release were also inhibited by brazilin in a concentration-dependent manner. Brazilin inhibited the formation of platelet thromboxane A_2 caused by thrombin, whereas it had no effect on the prostaglandin D_2 formation. Brazilin inhibited [^{3}H]-arachidonic acid liberation from membrane phospholipids of thrombin-stimulated platelets. Brazilin inhibited the rise of intracellular free calcium caused by thrombin. These results indicate that the inhibition of phospholipase (PLA_2) activity and $[Ca^{2+}]_i$ elevation might be at least a part of antiplatelet mechanism of brazilin.

Key words : Brazilin, *Caesalpinia sappan*, Aggregation, Phospholipase A_2, Platelet, Intracellular free calcium

INTRODUCTION

Brazilin, the main component of Sappan wood (*Caesalpinia sappan* L.), has been previously reported to have hypoglycemic action in experimental diabetic animals without any significant change in plasma insulin level (Moon *et al.*, 1993). Additional studies revealed that brazilin modulated immune functions mainly by augmenting T-cell activity in halothane administered mice (Choi and Moon, 1997a, Choi *et al.*, 1997b) and decreased the PKC activity in 3T3-L1 fibroblasts and adipocytes (Kim *et al.*, 1995). Recently we found that calmodulin and intracellular calcium concentration may be essential for the stimulating effects of brazilin on glucose transport (Khil *et al.*, 1997). In addition to these various biological activities, brazilin was supposed to have antithrombotic activities, considering the fact that Sappan wood has been used as a remedy for thrombosis in the oriental traditional medicine. From this reason, the present study was motivated to investigate the antiplatelet effect of brazilin and its action-mechanism in the light of the PLA_2 activity and the changes of $[Ca^{2+}]_i$.

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MATERIALS AND METHODS

Materials

Brazilin (Fig. 1) was purchased from the Aldrich Chemical Co., U.S.A.. Radioimmunoassay (RIA) kits of thromboxane B_2 (TXB_2), and prostaglandin D_2 (PGD_2) were purchased from Amersham Co., England. Thrombin, propyl gallate, bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), EDTA, and EGTA were purchased from the Sigma Chemical Co., U.S.A. Collagen, ADP, Triton X-100 and luciferin-luciferase reagent were purchased from Chrono-Log. Co. U.S.A. [5, 6, 8, 9, 11, 12, 14, 15-^{3}H]-arachidonic acid (AA) (219 Ci/mmol) was purchased from Amersham Co., England. Sepharose 2B gel was purchased from Pharmacia Fine Chemical Co., U.S.A. Other reagents were purchased from Sigma Chemical Co., U.S.A. They were of the highest quality available.

Preparation of platelets

Sprague-Dawley female rats (Laboratory Animal Center, Seoul National University, Seoul, Korea) weighing 200~250 g were used. Blood was collected from the abdominal aorta and was anticoagulated with sodium citrate (3.8%; 1:9, v/v). Blood was centrifuged at 120×g for 15 min. The supernatants were pooled and

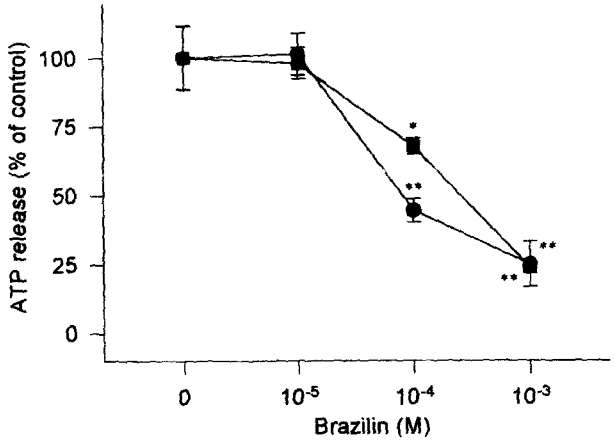

Fig. 1. Effects of brazilin on ATP release induced by thrombin or collagen. Platelets were incubated with brazilin or saline at for 5 min prior to the addition of thrombin (0.5 U/ml, ●), or collagen (1 mg/ml, ■). The released ATP level was obtained from luminescence peak using ATP (0.04 μM) 5 min after the addition of stimulator. The percentage release of ATP was calculated assuming the value of the control (without brazilin) to be 100%. Each datum expressed as mean±SEM (N=6). Key: (*) P<0.05, and (**) P<0.01 as compared with the control.

centrifuged at 600×g for 15 min at room temperature. The platelet pellets were washed with modified Tyrode-HEPES buffer (129 mM NaCl, 2.8 mM KCl, 8.9 mM $NaHCO_3$, 0.8 mM $MgCl_2$, 0.8 mM KH_2PO_4, 2 mM EGTA, 5.6 mM glucose, 10 mM HEPES, 0.35% BSA, pH 7.4) and were centrifuged at 600×g for 15 min. This washing procedure was repeated twice and platelets were gently resuspended in Tyrode-HEPES buffer (129 mM NaCl, 2.8 mM KCl, 8.9 mM $NaHCO_3$, 0.8 mM $MgCl_2$, 0.8 mM KH_2PO_4, 1 mM $CaCl_2$, 5.6 mM glucose, 10 mM HEPES, 0.35% BSA, pH 7.4). Platelet counts were determined with a Coulter Counter model ZM (Coulter Electronics, Hialeah, FL).

Platelet aggregation and ATP release assay

Platelet aggregation studies were conducted according to the turbidimetric method (Mustard *et al.*, 1972). ATP released from platelets was detected by the bioluminescence method (DeLuca and McElroy, 1978). Both aggregation and ATP release were measured simultaneously in a Lumi-aggregometer (Model 450, Chrono-Log Co.) connected to dual channel recorder. Platelet preparations were stirred at 12,000 rpm. 0.04 μM ATP was used to calibrate the amount of ATP released from platelets.

TXB_2 and PGD_2 assay

EDTA (2 mM) and indomethacin (50 μM) were added to platelet suspension 5 min after the addition of the agonist. TXB_2 and PGD_2 in the supernatants were obtained after centrifugation in an Eppendorf centrifuge (Model 5413) for 2 min and assayed by RIA.

Measurement of [^{3}H]AA release from platelets

The liberated [^{3}H]AA was determined according to the method of Smith *et al* (Smith *et al.*, 1985). Platelet-rich plasma (PRP) was incubated for 2 hrs with 12.5 μCi of [5,6,8,9,11,12,14,15-^{3}H]-AA (219 Ci/mmol). After labelling, platelets were diluted to 6 vol. of platelet poor plasma, centrifuged at 800×g for 15 min, and resuspended in the buffer (138 mM NaCl, 2.9 mM KCl, 20 mM HEPES, 3.3 mM NaH_2PO_4, 1.0 mM $MgCl_2$, 1.0 mM Glucose, 20 mM EDTA, pH 7.4). This washing procedure was repeated and the platelets were finally resuspended in buffer without EDTA. Aliquots (0.5 ml) of platelets were preincubated for 1 min with propyl gallate, a cyclooxygenase/lipoxygenase dual inhibitor. Brazilin or buffer was added before stimulation with thrombin (1 U/ml). Samples were taken after 5 min for the determination of liberated AA. For lipid extraction, EDTA was added (pH 7.4, 20 mM final concentration) plus 1 drop of formic acid (90%, w/v) and 3.75 vol. of ice-cold chloroform/methanol (1:2, v/v). The extract was partitioned into two phases by addition of 1.25 vol. of chloroform and 1.25 vol. of 2 M KCl. The lower phase was removed and the upper phase was washed with 2.5 vol. of chloroform. The pooled organic extracts were evaporated under nitrogen at 37°C and stored in chloroform/methanol at -18°C. All of the organic solvents contained butylated hydroxytoluene (50 μg/ml) as an antioxidant. Radioactive AA was resolved by thin layer chromatography on silica-gel plates (Merck, Darmstadt) using the upper phase of the solvent system ethylacetate/iso-octane/acetic acid/water (90:50:20:100, v/v). Appropriate zone corresponding to AA was scraped into counting vials and the radioactivity determined using toluene based cocktail.

$[Ca^{2+}]_i$ measurement in aequorin-loaded platelets

Rat platelets were loaded with aequorin according to the method of Johnson *et al.* (Johnson *et al.*, 1985). In brief, 1 μM PGE_1 was added to 15 ml PRP. After centrifugation, the platelets were resuspended in HEPES-Tyrode's buffer containing 10 mM EGTA and PGE_1 1 μM. After centrifugation, the platelets were resuspended in solution A (150 mM NaCl, 5 mM Hepes, 5 mM ATP, 2 mM $MgCl_2$, 10 mM EGTA, PGE_1 1 μM, aequorin 0.2 mg/ml). This suspension was incubated at 0°C for 1 hr, and then recentrifuged. The platelets were resuspended in solution B (150 mM NaCl, 5 mM Hepes, 5 mM ATP, 10 mM $MgCl_2$, 0.1 mM EGTA, PGE^1 1 μM) and were reincubated for 1 hr. $CaCl_2$ was added to the suspension (300 μM, final concentration); the platelets were rewarmed at room temperature and then passed through a column of Sepharose 2B equilibrated and eluted with Hepes-Tyrode's buffer containing 1 mM

Ca^{2+}. The platelet count was then adjusted to 2×10^8/ml with the same buffer. The aequorin response and aggregation were simultaneously monitored with Platelet Ionized Calcium Aggregometer (Chrono-Log Co.). $[Ca^{2+}]_i$ was measured according to Johnson *et al.* (Johnson *et al.*, 1985).

Statistical analysis

The data shown are the mean±S.E.M. Significance of difference was calculated by Student's t-test. ($P<0.05$ or $P<0.01$).

RESULTS AND DISCUSSION

Thrombin (0.5 U/ml), collagen (1 mg/ml) and ADP (2 μM) caused 75-85% aggregation of washed rat platelets. Brazilin showed antiplatelet effects at higher concentrations (10^{-4} M~10^{-5} M), which might be almost impossible to be displayed *in vivo* (Table I). But brazilin was found to inhibit the thrombosis induced by agonists *in vivo* at the dose of 10 mg/kg. To understand the difference between the effects of *in vivo* and *in vitro* experiments, our first trial was directed to examine the antiplatelet mechanism *in vitro*. Brazilin was examined on its effects on thrombin- and collagen-induced ATP release and the ATP release was significantly inhibited (Fig. 1). It is well known that dense granular release from platelets mediates irreversible platelet aggregation, since dense granules contain several mediators of platelet aggregation such as ADP, ATP and calcium.

TXA_2 is an important mediator of granule release reaction and aggregation of platelets (Hornby and Skidmore, 1982). TXA_2 formation by stimulated platelets represents an potent amplifying signal for platelet activation (FitzGerald, 1991). To investigate the effect of brazilin on TXA_2 synthesis in stimulated platelets, we measured the formation of TXB_2, the stable metabolite of TXA_2, by RIA. TXB_2 level of resting platelets was less than 0.5 ng/3×10^8 platelets. Thrombin (0.5 U/ml) and collagen (0.1 mg/ml) caused the marked elevation of TXB_2 formation. As shown in Table II, brazilin significantly inhibited thrombin- and collagen-induced TXB_2 formation at the concentration where it inhibited platelet aggregation and granule release. These results indicate that the antiplatelet effect of brazilin was due, at least partly, to the inhibition of TXA_2 formation. Levels of TXA_2 are increased in several thrombotic disorders (FitzGerald *et al.*, 1987). Therefore, agents that inhibit the formation of TXA_2 would be greatly useful in treatment of thrombotic disorders.

Table I. Effects of brazilin on thrombin-, collagen or ADP-induced aggregation of washed rat platelets

		Aggregation (%)		
		Thrombin (0.5 U/ml)	Collagen (1 mg/ml)	ADP (2 μM)
Control		68.7±4.2	65.0±3.2	48.5±4.5
Brazilin,	10^{-5} M	60.0±3.1	65.0±3.8	
	10^{-4} M	47.5±3.5*	59.0±2.8	
	10^{-3} M	13.7±4.5**	19.0±3.3**	8.5 ± 1.8**

Platelets were incubated with brazilin or saline at 37°C for 5 min; thrombin (0.5 U/ml), collagen (1 mg/ml), or ADP (2 μM) was then added to trigger aggregation. The peak level of aggregation was measured 5 min after the addition of stimulator. Percent inhibitions produced by brazilin were calculated from the reduction of the maximal level of the aggregation tracings in relation to the values obtained in the absence of brazilin. Each datum is expressed as the mean±SEM (N=7).
*, **Significantly different from the control: *$p<0.05$, and **$p<0.01$.

Table II. Effects of brazilin on thromboxane B2 formation in washed rat platelets caused by thrombin or collagen

	Thromboxane B_2 (ng/3×10^8 platelets)	
	Thrombin (0.5 U/ml)	Collagen (0.1 mg/ml)
Control	69.87±5.73	36.42±3.99
Brazilin, 10^{-5} M	67.35±6.42	31.38±3.02
10^{-4} M	57.54±4.34*	30.24±3.21*
10^{-3} M	39.51±4.77**	22.92±2.22**

Saline (control) or brazilin was preincubated with platelets at 37°C for 5 min; then the inducer was added. TXB_2 formation was terminated by EDTA (2 mM) and indomethacin (50 μM) 5 min after the addition of inducer. Values are presented as mean±SEM (N=6).
*, ** Significantly different from the control: *$p<0.05$, and **$p<0.01$.

TXA_2 is produced from AA cleaved from the *sn*-2-position of phospholipids through the activity of PLA_2 in stimulated platelets. AA is metabolized by cyclooxygenase (COX) to PG endoperoxide intermediates, such as PGG_2 and PGH_2. In platelets, endoperoxides are further metabolized to TXA_2 by TXA_2 synthase. Under normal conditions, minor amounts of PGE_2, PGD_2 and $PGF_{2\alpha}$ are also produced in stimulated platelets (Gresele *et al.*, 1991). Since a selective TXA_2 synthase inhibitors, in addition to suppressing the formation of TXA_2 selectively, cause a redirection of PG endoperoxide metabolism toward other PGs, such as PGD_2, PGE_2, $PGF_{2\alpha}$ in platelets (Gresele *et al.*, 1991). If the inhibition of TXA_2 synthase activity is a critical step for the inhibitory effect of brazilin on TXA_2 synthesis, brazilin could increase the formation of PGD_2 in stimulated platelets. To test this possibility, we examined the effect of brazilin on the PGD_2 formation in stimulated platelets. The PGD_2 level of resting platelets was less than 0.07 ng/3×10^8 platelets. PGD_2 formation was increased by thrombin (0.5 U/ml) and collagen (0.1 mg/ml). This PGD_2 formation was significantly inhibited by brazilin (Table III). This indicates that the site of action of brazilin may be upstream from TXA_2 synthase step.

Since the intracellular concentration of free AA is low, the liberation of AA by PLA_2 is thought to be

Table III. Effects of brazilin on prostaglandin D_2 formation in washed rat platelets caused by thrombin or collagen

	Prostaglandin D_2 (pg/3×10^8 platelets)	
	Thrombin (0.5 U/ml)	Collagen (0.1 mg/ml)
Control	6.95±0.50	4.17±0.80
Brazilin, 10^{-5} M	6.45±0.53	3.96±0.88
10^{-4} M	4.44±1.30*	3.39±0.65*
10^{-3} M	1.69±0.48**	1.83±0.74**

Saline (control) or brazilin was preincubated with platelets at 37°C for 5 min; then the inducer was added. PGD_2 formation was terminated by EDTA (2 mM) and indomethacin (50 μM) 5 min after the addition of inducer. Values are presented as mean±SEM (N=6).
*, **Significantly different from the control: *p<0.05, and **p <0.01.

rate-limiting step in the formation of TXA_2 in platelets (Loeb and Gross, 1986). We, therefore, examined the effect of brazilin on AA liberation from plasma membrane phospholipid. [^{3}H]AA-labelled platelets were preincubated with propyl gallate, a dual inhibitor of COX/LPO, and then brazilin was added before stimulation with thrombin (1 U/ml). As shown in Fig. 2, in response to thrombin, the [^{3}H]AA release was increased from rat platelets by the action of PLA_2. Brazilin attenuated thrombin-induced augmentation of [^{3}H]AA liberation at two concentrations of 10^{-3} M and 10^{-4} M. 10^{-3} M of brazilin normalized the production of AA nearly to the resting level. Therefore, brazilin inhibited the PLA_2 enzymatic activity in the concentration ranges where it inhibited the TXA_2 production in stimulated platelets significantly. This result suggests that the antiplatelet effects of brazilin is related to PLA_2 activity.

Blood platelets contain both secretory PLA_2 and cytosolic PLA_2, which are very different enzymes, distinguished by their molecular weights of 14 and 85 kDa, respectively (Mounier *et al.*, 1993). The characteristics of cytosolic PLA_2, such as requirement for micromolar concentrations of Ca^{2+} for its activity, cytosolic localization, and specificity for phospholipids containing AA in the *sn*-2 position, are compatible with a role in AA liberation (Kim *et al.*, 1991, Takayama *et al.*, 1991). On the other hand, secretory PLA_2 appears not to be involved in AA liberation during platelet activation (Mounier *et al.*, 1993).

To investigate whether the inhibitory effect of brazilin on PLA_2 activity is related to cytosolic free calcium concentration, we examined the effect of brazilin on the rise of $[Ca^{2+}]_i$ in stimulated platelets. In aequorin-loaded platelets, brazilin inhibited the rise of $[Ca^{2+}]_i$ caused by thrombin (Fig. 3). The cytosolic PLA_2 is regulated by intracellular free calcium, which induces translocation to membranes (Clark *et al.*, 1991) through a Ca^{2+}-dependent lipid-binding motif in its N terminus (Nalefski *et al.*, 1994).

We measured $[Ca^{2+}]_i$ in resting platelets using the photoprotein aequorin (4 μM, Fig. 3). Aequorin is a protein, found in jellyfish, which emits blue light when it binds Ca^{2+}. It is thought to reflect the changes in the concentration of Ca^{2+} area directory under the plasma membrane and is thus easily affected by the extracellular Ca^{2+} influx (Alarayyed *et al.*, 1997, Kondo *et al.*, 1991).

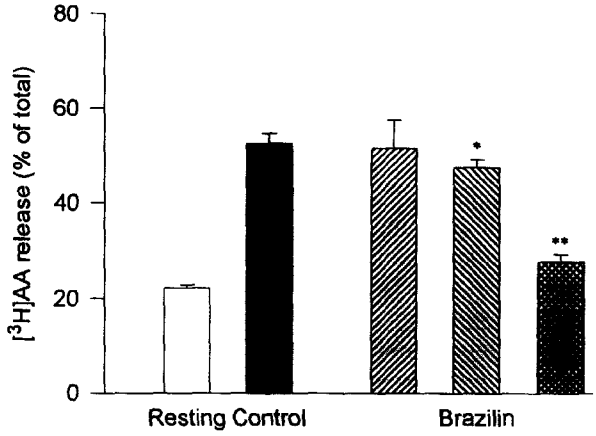

Fig. 2. Effect of brazilin on the release of [^{3}H]AA from rat platelets induced by thrombin. [^{3}H]AA-labeled platelets were treated with propyl gallate for 1 min to inhibit cyclooxygenase and lipoxygenase. Saline (□, ■) or brazilin (10^{-5} M, ▨; 10^{-4} M, ▧; 10^{-3} M, ■) was added 5 min prior to addition of saline (□) or thrombin (0.5 U/ml; ■, ▨, ▧, ■). The data are presented as the percentage of the total ^{3}H radioactivity incorporated into the platelet after subtraction of the radioactivity released from unstimulated platelets. Each datum expressed as mean±SEM (N=6). Key: *p<0.05 and, **p<0.01 as compared with the control.

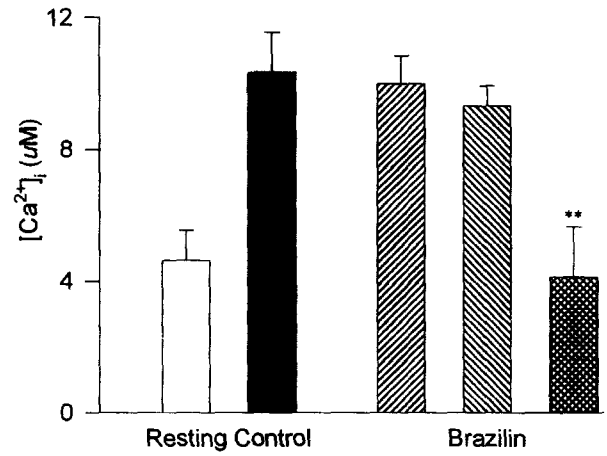

Fig. 3. Effect of brazilin on the increase of $[Ca^{2+}]_i$ induced by thrombin in aequorin-loaded platelets. Aequorin-loaded platelets were suspended in a buffer containing 1 mM $CaCl_2$, and the change in $[Ca^{2+}]_i$ was monitored continuously. Platelets were preincubated with saline (□, ■) or brazilin (10^{-5} M, ▨; 10^{-4} M, ▧; 10^{-3} M, ■) at 37°C for 5 min; then saline (□) or thrombin (0.5 U/ml; ■, ▨, ▧, ■). was added. The value of $[Ca^{2+}]_i$ was calculated from the calibration curve as suggested by the manufacturer. Each datum expressed as mean±SEM (N=5). Key: **p<0.01 as compared with the control.

Using aequorin, a Ca^{2+} level in resting platelets of 2 to 4 μM has been measured (Johnson *et al.*, 1985). Therefore, the changes of $[Ca^{2+}]_i$ level in brazilin-treated platelets represent the inhibition of the influx of Ca^{2+} from extracellular space.

The data obtained hitherto suggest that the inhibition of PLA_2 activity via the suppression of $[Ca^{2+}]_i$ elevation might be at least a part of antiplatelet mechanism of brazilin.

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REFERENCES CITED

Alarayyed, N. A., Cooper, M. B., Prichard, B. N., Betteridge, D. J. and Smith, C. C., *In vitro* adrenaline and collagen-induced mobilization of platelet calcium and its inhibition by naftopidil, doxazosin and nifedipine. *Br. J. Clin. Pharmacol.*, 43, 415-420 (1997).

Choi, S. Y. and Moon, C. K., Effects of brazilin on the altered immune functions in the early phase of halothane intoxication of C57BL/6 mice. *Planta Med.*, 63, 400-404 (1997a).

Choi, S. Y., Yang, K. M., Jeon, S. D., Kim, J. H., Khil, L. Y., Chang, T. S. and Moon, C. K., Brazilin modulates immune function mainly by augmenting T cell activity in halothane administered mice. *Planta Med.*, 63, 405-408 (1997b).

Clark, J. D., Lin, L. L., Kriz, R. W., Ramesha, C. S., Sultzman, L. A., Lin, A. Y., Milona, N. and Knopf, J. L., A novel arachidonic acid-selective cytosolic PLA2 contains a Ca(2+)-dependent translocation domain with homology to PKC and GAP. *Cell*, 65, 1043-1051 (1991).

DeLuca, M. and McElroy, W.D., Purification and properties of firefly luciferase. *Methods Enzymol.*, 57, 3-15 (1978).

FitzGerald, G. A. Healy, C. and Daugherty, J., Thromboxane A2 biosynthesis in human disease. *Fed. Proc.*, 46, 154-158 (1987).

FitzGerald, G.A., Mechanisms of platelet activation: thromboxane A2 as an amplifying signal for other agonists. *Am. J. Cardiol.*, 68, 11B-15B (1991).

Gresele, P., Deckmyn, H., Nenci, G. G. and Vermylen, J., Thromboxane synthase inhibitors, thromboxane receptor antagonists and dual blockers in thrombotic disorders. *Trends Pharmacol. Sci.*, 12, 158-163. (1991).

Hornby, E. J. and Skidmore, I. F., Evidence that prostaglandin endoperoxides can induce platelet aggregation in the absence of thromboxane A2 production. *Biochem. Pharmacol.*, 31, 1158-60 (1982).

Johnson, P. C., Ware, J. A., Cliveden, P. B., Smith, M., Dvorak, A. M. and Salzman, E. W., Measurement of ionized calcium in blood platelets with the photoprotein aequorin. *J. Biol. Chem.*, 260, 2069-2076 (1985).

Khil, L. Y., Cheon, A. J., Chang, T. S. and Moon, C. K., Effects of calcium on brazilin-induced glucose transport in isolated rat epididymal adipocytes. *Biochem. Pharmacol.*, 54, 97-101 (1997).

Kim, D. K., Kudo, I. and Inoue, K., Purification and characterization of rabbit platelet cytosolic phospholipase A2. *Biochim. Biophys. Acta*, 1083, 80-88 (1991).

Kim, Y. M., Kim, S. G., Khil, L. Y. and Moon, C. K., Brazilin stimulates the glucose transport in 3T3-L1 cells. *Planta Med.*, 61, 297-301 (1995).

Kondo, K., Kitada, C., Yoshioka, A., Sakane, H., Kokawa, T. and Yasunaga, K., Inhibition of platelet aggregation by low concentrations of sodium fluoride. Significance of the intracellular calcium ion concentration. *Int. J. Hematol.*, 54, 411-417 (1991).

Loeb, L. A. and Gross, R. W., Identification and purification of sheep platelet phospholipase A2 isoforms. Activation by physiologic concentrations of calcium ion. *J. Biol. Chem.*, 261, 10467-10470 (1986).

Moon, C. K., Lee, S. H., Lee, M. O. and Kim, S. G., Effects of brazilin on glucose oxidation, lipogenesis and therein involved enzymes in adipose tissues from diabetic KK-mice. *Life Sci.*, 53, 1291-1297 (1993).

Mounier, C., Faili, A., Vargaftig, B. B., Bon, C. and Hatmi, M., Secretory phospholipase A2 is not required for arachidonic acid liberation during platelet activation. *Eur. J. Biochem.*, 216, 169-175 (1993).

Mustard, J. F., Perry, D. W., Ardlie, N. G. and Packham, M. A., Preparation of suspensions of washed platelets from humans. *Br. J. Haematol.*, 22, 193-204 (1972).

Nalefski, E. A., Sultzman, L. A., Martin, D. M., Kriz, R. W., Towler, P. S., Knopf, J. L. and Clark, J. D., Delineation of two functionally distinct domains of cytosolic phospholipase A2, a regulatory Ca(2+)-dependent lipid-binding domain and a Ca(2+)-independent catalytic domain. *J. Biol. Chem.*, 269, 18239-18249 (1994).

Smith, J. B., Dangelmaier, C. and Mauco, G., Measurement of arachidonic acid liberation in thrombin-stimulated human platelets. Use of agents that inhibit both the cyclooxygenase and lipoxygenase enzymes. *Biochim. Biophys. Acta*, 835, 344-351 (1985).

Takayama, K., Kudo, I., Kim, D. K., Nagata, K., Nozawa, Y. and Inoue, K., Purification and characterization of human platelet phospholipase A2 which preferentially hydrolyzes an arachidonoyl residue. *FEBS Lett.*, 282, 326-330 (1991).