LIPOPHILIC FRACTION FROM KOREAN RED GINSENG REGULATES THE PHOSPHORYLATION OF PLATELET PROTEIN(50KD) BY ELEVATING CYCLIC-GMP *IN VIVO* AND *IN VITRO*

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ABSTRACT

Lipophilic fraction(LF) from *Panax ginseng C.A.* Meyer inhibited the aggregation of human platelets induced by th rombin(0.1u/mℓ). LF and Molsidomine(vasodilator) induced the stimulation of cGMP-elevation and 50KD-Phosphorylation, and then the inhibition of 20KD-Phosphorylation in human platelets activated by thrombin. LF also inhibited the Ca^{2+}-influx into platelets. When rat(SD : male) was fed with LF, the level of cGMP was increased in rat platelets stimulated by collagen and thrombin.

On the other hand, verapamil, Ca^{2+}-antagonist increased cAMP level in platelet stimulated by thrombin, but LF does not affected. However LF potently inhibited the thromboxane A_2(TXA_2) production. The results suggest that the inhibitory effects of LF are mediated by regulation the phosphorylation of 50KD via cGMP-elevation and depend upon the decrease of TXA_2 level.

INTRODUCTION

The aggregation of human platelets is dependent on TXA_2 level(1-3). TXA_2 is a potent platelet-aggregating agent and is generated from arachidonic acid released by phospho lipid breakdown(1-3). It is known that TXA_2 interacts with the membrane receptors and activated platelets by increasing the intracellular Ca^{2+} concentration(4-5). Antiplatelet-drug such as verapamil, elevates the level of cAMP and decreases the concentration of cytosolic free Ca^{2+}(6, 7). Vasodilators(i.e. molsidomine, nitroprusside, nitroglycerin) inhibit the platelet activation such as an aggregatory reaction by increasing the phosphorylation of 50KD(8-10). Phosphorylation of this protein is mediated by vasodilator-evoked cGMP elevation(8-11). cGMP-elevating antiplatelet drugs also inhibit the thrombin-induced calcium increase in the presence or absence of external calcium(6, 12). Elevated cytosolic free Ca^{2+} is associated with both Ca^{2+}/calmodulin-dependent phosphorylation of myosin light chains(20KD) and 1, 2-diacylglycerol-dependent phosphorylation of cytosolic protein(40-47KD) resulting in platelet aggregation(13).

In the present studies, we found that lipophilic fraction (LF) from Korean red ginseng inhibits the aggregation of human platelets induced by thrombin, and then examined how the inhibition is related to the production of TXA_2, cAMP and cGMP. When rat was fed with LF, the level of cGMP was also increased in rat platelets stimulated by collagen and thrombin. We report presently that the inhibition by LF on the thrombin-induced platelet aggregation is dependent not on the increase of cAMP but on the decrease of TXA_2 production and on the elevation of cGMP that phosphorylates the 50KD by activating the cGMP-dependent kinase.

MATERIALS AND METHODS

Materials

Assay kits of [^{3}H] cAMP, [^{3}H]cGMP, [^{3}H]thromboxane B_2 were purchased from Amersham Life Science Co. Thrombin(from bovine plasma), Verapamil(HCl), molsidomine and other chemical reagents were from Sigma Chemical Co.

Preparation of Lipophilic Fraction from Korean Red Ginseng

500g of dry Korean red ginseng were ground into fine powder by a cut mill and then has been deposited in 2500 mℓ of petroleum ether for 7 days. Then, it was extracted 3 times in petroleum ether at room temperature and concentrated with vacuum evaporator. Put to use the following experiments. This non-saponin fraction is lipophilic complex.

Experiment I. Effects of Lipophilic Fraction on Human Platelet Aggregation

Prepration of washed human platelets

Platelet-rich plasma(PRP) obtained from the antecubital vein of normal human volunteers, was purchased from Taejon Red Cross Blood Center. The blood was anticoagulated with CPD sol.(sodium citrate, NaH_2PO_4, glucose, adenine mixture ; Korea Green Cross Pharm.), PRP was centrifuged at 125×g for 10min to remove red blood cells and was washed twice in Tris-citrate-bicarbonate buffer(pH 6.5[14], containing 2mM EDTA) by centrifugation at 1,100×g for 10min. Because EDTA has an inhibitory action on platelet aggregation, the washed platelets were recentrifuged twice with suspending buffer(pH 6.9[14], without EDTA) to remove EDTA. Finally. platelet mumber was adjusted to 5×10^8cells/ml in suspending buffer. All the above procedures were carried out at 25℃ to avoid platelet aggregation by its cooling.

Measurement of platelet aggregation

Platelet aggregation was measured by aggregometer(Lumiaggregometer, Model : 400, Chronolog USA) as the change of transmission at 660nm(15). Therefore we utilized the spectrophotometer in consideration of lowering the absorbance when aggregation is occured(16). The washed platelets were preincubated in cuvette with gentle stirring for 3 min at 37℃ in the presence of 2mM $CaCl_2$ and testing materials or none, and then stimulated with 0.1 units of thrombin/mℓ for 5 min with gentle stirring. It was measuresd absorbance with uv/visible spectrophotometer(Beckman DU-6) at 660nm and calculated transmission(T) as following formula.

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

$$\triangle A = 5A - 3A$$

5A = absorbance when aggregation reaction has occurred for 5 min

3A = absorbance when preincubation has carried out for 3 min

Suspending buffer was used as reference(absorbance 0). The LF was dissolved in dimethyl sulfoxide(DMSO), therefore, its pure activity was calculated by subtracting that of DMSO.

Measurement of cAMP, cGMP and thromboxane B_2

An aggregation reaction was terminated with 80% ethanol and 100μM indomethacin to assay cAMP and cGMP, and thromboxane B_2(TXB_2) respectively.

cAMP and cGMP were measured with radioimmunoassay kit of [^{3}H]cAMP and [^{3}H]cGMP. TXB_2, a stable metabolite of thromboxane A_2, was measured with thromboxane B_2 radioimmunoassay kit as indicated by manufacturer. The levels of cAMP, cGMP and TXB_2 in platelets were calculated by subtracting the effects of solvents such as DMSO and ethanol.

Determination of cytosolic Ca^{2+} concentration

Platelet-rich plasma(PRP) was incubated with 25 μM Quin II/AM at 37℃ for 60min. 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 the same 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(17) on fluorescence spectrophotometer(Perkin Elmer, LS-50). Because the LF was dissolved in DMSO, Ca^{2+} was calculated by substracting the effect of DMSO.

Labelling of platelets with ^{32}Pi

PRP added ^{32}Pi(1 mCi, carrier free) was incubated for 60 min at 37℃. The washed platelets were prepared as the above method.

Phosphorylation reaction of proteins

The protein-phosphorylations were carried out according to the method of Laemmli(18). Platelet suspension(10^8 cells/mℓ) containing 1.7mg of proteins were preincubatied at 37℃ for 2min with an addition of 2mM $CaCl_2$ or LF, and then stimulated with thrombin(01.μ/ml) for 5 min. The stop solution(0.125M Tris, 4% SDS, 20% glycerol, 5% 2-mercaptoethanol, 0.01% bromophenol blue, pH 6.8) was added to stop the phosphorylation reaction with the same volume of the reaction. 50μg of proteins from the reaction tubes were taken out and loaded onto SDS-PAGE(1.5㎜, 10%). Separated proteins were stained, destained, dried, and autoradiographed onto the x-ray film(Fuji Medical x-ray Film) in -70℃ for 15 days. Phosphorylated proteins were assayed in CAMAG TLC scanner at 554nm.

Experiment II. Effects of Lipophilic Fraction on Rat Platelet Aggregation

Animals and diets

Male Sprague-Dawley rat(200g) were used. The basal diet was prepared with the following weight percentage composition : vitamin free casein 20, corn starch 45, sucrose 10, DL-methionine 0.3, mineral mixture 3.5, vitamin mixture 1.0, choline bitartrate 0.2 and cellulose powder 5. Corn oil was added at 15%(w/w) to the basal diet. The diets were prepared daily and fed *ad libitum* to groups of 5 rats.

In the experiment to modify the lipophilic fraction from Korean red ginseng, rats were first fed the basal diet containing 15% corn oil for 3 weeks and then the basal diet containing 15% corn oil and lipophilic fraction 25mg(mg/kg-diet) for 3 weeks.

Preparation of washed rat platelets

Rats were anesthetized with diethylether and blood was collected from the heart in the presence of 10% ACD solution(0.8% citric acid, 2.2% sodium citrate , 2.45% glucose). Platelet-rich plasma(PRP) was obtained by centrifugation at 340×g for 10min.

Rat platelets were prepared from PRP as described in experiment I.

Other methods

Measurements of rat platelet aggregation, cAMP and cGMP were carried out as described in experiment I.

RESULTS

Experiment I. : Effects of Lipophilic Fraction(LF) on Human Platelet Aggregation

Inhibitory effects of lipophilic fraction on platelet aggregation

Because LF was dissolved in DMSO, when platelet suspension containing DMSO corresponding to the concentration of LF(10, 20, 30μg/mℓ) was preincubated for 2 min at 37℃, thrombin-induced aggregation was inhibited to the degree of 9.52 ± 0.2% (n=5). The data of Table 1 show the compensation for the effect of this DMSO. When human platelets (10^8 cells/mℓ) were stimulated with 0.1 units of thrombin/mℓ, the extent of aggregation was 73% (Table 1). However, LF inhibited the platelet aggregation induced by thrombin, and the extent of inhibition was dependent on the concentration of LF. Verapamil, Ca^{2+} antagonist also inhibited the platelet aggregation iniduced by thrombin, and yet the inhibitory degree was weaker than that of LF. We searched for the changes of agents (cAMP, cGMP, TXA_2, Ca^{2+} influx, and protein phosphorylation) affecting on the activation or inactivation of platelets with 20μg of LF/mℓ which inhibits platelet aggregation of 80%.

Table 1. Effects of lipophilic fraction(LF) and verapamil on human platelet aggregation induced by thrombin. Washed platelets(10^8/mℓ) were preincubated with or without LF or with verapamil for 3 min with gentle stirring st 37℃. Thrombin(0.1u/mℓ) was added after preincubation, and then the reaction was continued for 5 min. Transmission of aggregation(%) was determined as ascribed under "material and methods". The data are given as the mean ± S.D.(n=4-8).

	Thrombin	Thrombin+ LF(10μg/mℓ)	Thrombin+ LF(20μg/mℓ)	Thrombin+ LF(30μg/mℓ)	Thrombin+ LF(1μM)
Transmission (%)	73.2 ± 4.8	21.7 ± 2.5[a]	15.5 ± 1.3[b]	12.3 ± 2.6[c]	31.7 ± 1.7[d]
△ %	100	29	21	17	50

LF idicates non-saponin fraction from *Panax ginseng* C. A. Meyer. [a, b, c, d] : P<0.001.

Effects on formation of cyclic nucleotides

DMSO, a solvent of LF produced 7.51 ± 2.1(n=5)p mol/10^8 platelets of cGMP. DMSO also produced 8.3 ± 0.9p mol (n=5) of cAMP and ethanol, a solvent of molsidomine produced 7.6 ± 2.0(n=5) p mol/10^8 platelets of cGMP. The data of Table 2 show the compensation for the effects of DMSO and ethanol. Verapamil increased cAMP from 4.03p mol(level in the intact platelets) to 64 p mol/10^8 platelets in the aggregation induced by thrombin(table 2). This indicates the inhibition on platelet aggregation by verapamil is dependent on the increase of cAMP level(6, 7). LF kept down cAMP content to almost intact level.

It is reported that the concentration of cGMP is 2.2p mol/10^9 cells in intact platelet(9). In our experiment, the level of cGMP in intact platelets(10^8 platelets) was less than 1.25p mol(Table 2). However, the cGMP was increased up to 30p mol/10^8 cells in thrombin-stimulated platelets(Table 2). When the plateletes are stimulated by thrombin or colla gen, cGMP level is increased and the phosphorylation of 50KD protein is accelerated(19). This fact is related with feed back system inhibiting the activation of platelets(19). Molsi domine and LF increase the cGMP level(Table 2). Because cGMP-elevating vasodilators(i. e. nitroprusside, molsidomine,

Table 2. Effects of lipophilic fraction and verapamil on the production cAMP and cGMP in platelet aggregation induced by thrombin.
The reaction of platelet aggregation was proceeded under the conditions as described in "materials and methods", the reaction was stopped with 80% ethanol. Platelets were stimulated by 0.1 units of thrombin/mℓ. The cAMP and cGMP were measured by using radioimmunoassay kit of [^{3}H] cAMP and cGMP. The data are given as the mean ± S.D(n=3-6). None indicates the level cAMP and cGMP in the intact platelets.

	None	Thrombin+	Thrombin+ LF (20μg/mℓ)	Thrombin+ Verapamil (1μM)	Thrombin+ molsidomine (1μM)
cAMP (p mol/10^8 platelets)	4.03 ± 0.27	3.16 ± 0.17	9.54 ± 0.89	64 ± 4.78	–
cGMP (p mol/10^8 platelets)	1.25<	3.00 ± 7.1	91.2 ± 13.5	–	63.8 ± 8.9

nitroglycerine) inhibit the platelet aggregation(8－10). The inhibition of thrombin－induced aggregation by LF is independent of the elevation of cAMP but is dependent upon the elevation of cGMP.

Effects on thromboxane A_2(TXA_2) production

TXA_2 level(determined as TXB_2) was increased abruptly on human platelets stimulated by thrombin. However, LF clearly depressed TXA_2 level in the platelet induced by thrombin(Fig. 1). This means that inhibition by LF on the aggregation induced by thrombin is relied on the decrease of TXA_2 formation.

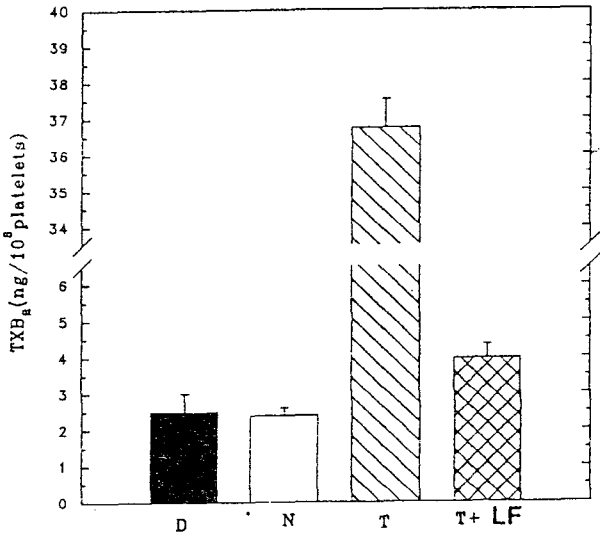

Fig. 1 Effects on TXA_2 production in human platelet aggregation induced by thrombin. After the platelets were reacted under the condition as described in Table 1, the reaction was stopped with 100μM of indomethacin. Platelet was stimulated with 0.1 unit of thrombin/mℓ, and lipophilic fraction was used as 20μg mℓ. TXA_2 was measured as TXB_2 by using a [^{3}H] thromboxane B_2 radioimmumoassay kit from Amersham Life Sciences. The data are given as the maen ± S.D(n=3－6).
N ; The level in intact platelets,
T ; Thrombin(0.1u/mℓ),
LF ; Lipophilic fraction(20μg/mℓ),
D ; Dimethyl sulfoxide

Effects on cytosolic free Ca^{2+}

Thrombin increased the concentration of cytosolic Ca^{2+} from base(64nM) to 601 nM/10^8 platelets in the presence of 2mM external calcium(Fig. 2). The elevated calcium, however, was decreased to basal concentration (64nM) when platelets were preincubated with 20μg of LF/mℓ.

Effects on protein phosphorylation

Effects of DMSO, ethanol, LF and thrombin on the pho-

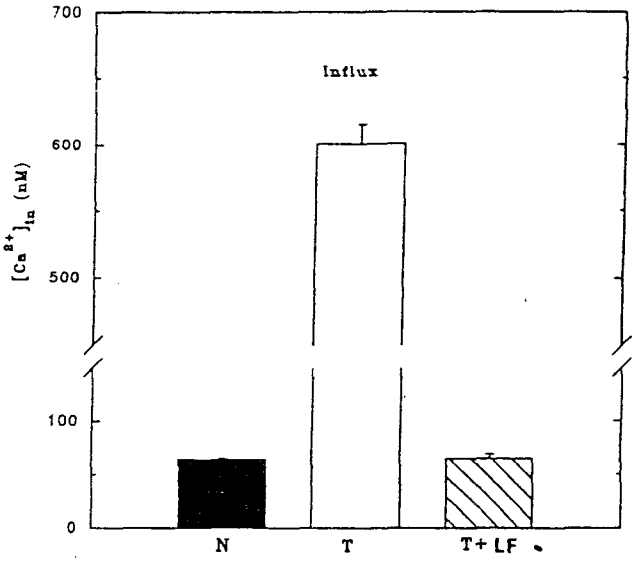

Fig. 2 Effects on Ca^{2+} influx in human platelet aggregation induced by thrombin. Quin 2－loaded platelests(10^8/mℓ) were suspended in the buffer containing 2mM $CaCl_2$, and stimulated with by 0.1 unit of thrombin mℓ. The intensity of Quin－2－fluorescence was increased up to 4.6 by DMSO, and the result of Ca^{2+}－influx was calculated by substracting the intensity of DMSO. The data are given as the mean ± S.D.(n=3).
N ; intact platelets,
T ; Thrombin(0.1 u mℓ),
LF ; Lipophilic fraction(20μg/mℓ)

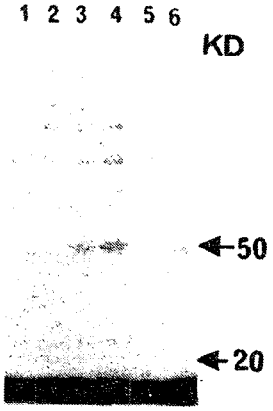

Fig. 3 Effects of LF on protein phosphorylation.
The protein phosphorylation was preformed as described in "Methods". Phosphoproteins were separated by SDS－PAGE and detected by autoradiography.
1 ; Intact platelets, 2 ; DMSO,
3 ; Thrombin(0.1 u/mℓ),
4 ; Thrombin＋NSF(20μg/mℓ), 5 ; Ethanol,
6 ; Thrombin＋Molsidomine(1μM)

sphorylation of both 50KD and 20KD are not distinguishable from the autoradiography of SDS PAGE(Fig. 3). Therefore, the bands(50KD, 20KD) of phosphoprotein were analyzed with CAMGA TLC Scanner. As the result, DMSO, a solvent of LF induced the inhibition of 50KD - phopshorylation and the stimulation of 20KD - phosphorylation to the control(Table 3). Ethanol, a solvent of molsidomine inhibited the phos phorylation of both 50KD and 20KD to the control(Table 3). These effects of solvents as DMSO and ethanol are different from the results that these solvents increased the level of cGMP as described above. These results are contrasted with the cGMP - elevating substance stimulating the phosphorylation of 50KD. Considering the effects of solvents such as DMSO and ethanol, it is recognized that LF more induces both the acceleration of 50KD - phosphorylation and the inhibition of 20KD - phosphorylation(Table 3, Fig. 3) and molsidomine also more stimulates the phosphorylation of 50KD. However, the phosphorylation of 20KD were stimulated by molsidomine (Table 3). cGMP - elevating vasodilator inhibits the phosphorylation of 20KD and of 40(47)KD, and accelerrates the phosphorylation of 50KD (6). It is likely that 47KD and 50KD proteins were overlapped each other(Fig. 3).

Table 3. Assay of protein phosphorylation.
Phosphorylated proteins which detected by autoradiography(Fig. 3) were analyzed in GAMAG TLC Scanner at 554nm. The results on the analysis were indicated as area(mm^2).

	50KD			20KD		
	Area(mm^2)	Area average	Pure change(%)	Area(mm^2)	Area average	Pure change(%)
Control	2332, 2480	2406	0	1978, 1619	1799	0
DMSO	1546, 2344	1945	−19.2[a]	2136, 2064	2100	16.7
Thrombin	4013, 4095	4054	68.5	2034, 2217	2126	18.2
Thrombin+	4676, 4153	4415	8.9[d]	2031, 2322	2182	2.6[e]
LF			(20.3)[h]			(−11.5)[i]
Ethanol	1390, 1050	1221	−49.2[b]	1203, 1953	1578	−12.3[c]
Thrombin+	3877, 3557	3717	− 8.3[f]	2243, 2347	2295	7.9[g]
Molsidomine			(20.9)[j]			(18.3)[k]

Notes : Minus signes of a, b, and c show the inhibition for the control. The pure changes(d, e, f and g) of "Thrombin+LF" and "Thrombin+Molsidomine" show the change for Thrombin. Because the phosphorylation of 50KD was inhibited by DMSO and ethanol to the control, the effects of LF and molsidomine were observed to be more decreased than the real owing to DMSO and ethanol. The leveles(h and i) of the parenthesis show the change of the level of DMSO or ethanol plus the valule of "Thrombin+LF" or "Thrombin+Molsidomine". Since the phosphorylation of 20KD was stimulated by DMSO and was inhibited by ethanol, the leveles of parenthesis(j, k) show the compensation for the value of DMSO or ethanol as well as the case of 50KD phosphorylation.

Experiment II. : Effects of Lipophilic Fraction on Rat Platelet Aggregation

Inhibitory effects of lipophilic fraction on platelet aggregation

Rat platelets(10^8 cells/$m\ell$) were stimulated with 0.1 units of thrombin/$m\ell$ and 100μg of collagen/$m\ell$ respectively, and when the extent of their aggregation was regulated as 100%, the platelet aggregation of rat with LF was occurred by 8.38% and 11.6%, respectively(Table 4). This results mean that the inhibition of platelet aggregation stimulated by collagen and thrombin is due to the feed of LF.

Table 4. Effects of lipophilic fraction on rat platelet aggregation induced by thrombin.
Platelet aggregation was carried out as described in the "methods". The aggregation induced by thrombin(0.1μg/$m\ell$) and collagen (100μg/$m\ell$) is regared as control(100%). The data given as the mean(m=2).

	Thrombin (0.1μg/$m\ell$)	Collagen (100μg/$m\ell$)
Control	100%	100%
LF 25$m\ell$	8.4%	11.6%

Effects on formation of cyclic nucleotides in rat fed with lipophilic fraction

When platelets of rat fed with LF were stimulated by thrombin and collagen, the level of cGMP was increased to 19.26 and 7.28p mol/10^9 platelets, respectively(Table 5). These mean that LF stimulates the production of cGMP *in vivo*. Furthermore, the production of cGMP was more increased by the exogenous addition of LF and molsidomine(Table 5). It is almost certain that LF and molsidomine stimulate the formation of cGMP.

The above results suggest that the inhibitory effects of LF in platelet aggregation of rat by collagen and thrombin are dependent upon the elevation of cGMP as well the case of human platelets.

Table 5. Effects of lipophilic fraction on the production of cGMP in rat platelet aggregation induced by thrombin and collagen. The reaction of platelet aggregation was proceeded under the conditions as described in "materials and methods". The data are given as the mean ± SD(n=3). None indicates the levels of cGMP in the platelets of rats without feeding LF.

unit : p mol/10^9 platelets

	None	LF(25mg)
Control	6.90 ± 1.7	3.41 ± 0.8
Thrombin(0.1u/mℓ)	8.83 ± 0.8	19.26 ± 1.2
Thrombin+ LF(20μg/mℓ) *1	52.73 ± 2.2	323.41 ± 3.1
Thrombin+ molsidomine(1μM) *2	354.32 ± 8.1	
Collagen(100μg. mℓ)	48.64 ± 4.0	7.28 ± 0.7
Collagen+ LF(20μg. mℓ) *1	369.80 ± 10.1	184.30 ± 8.1
Collagen+ molsidomine(1μM) *2	107.05 ± 2.3	346.59 ± 5.6

Notes : *1 and *2 are indicated the exogenous addition of LF and molsidomine.

Table 6. Effects of lipophilic fraction on the production of cAMP in rat platelet aggregation induced by thrombin and collagen. The reaction of platelet aggregation was proceeded under the conditions as described in "materials and methods". The data are given as the mean ± SD(n=3). None indicates the levels of cAMP in the platelets of rats without feeding LF.

unit : p mol/10^9 platelets

	None	LF(25mg)
Control	3.60 ± 0.30	6.60 ± 0.90
Thrombin(0.1u/mℓ)	2.20 ± 0.36	1.20 ± 0.34
Thrombin+ LF(20μg/mℓ) *1	10.77 ± 0.59	5.78 ± 0.11
Thrombin+ molsidomine(1μM) *2	13.82 ± 0.30	5.7 ± 0.12
Collagen(100μg/mℓ)	2.38 ± 0.60	26.98 ± 0.90
Collagen+ LF(20μg/mℓ) *1	40.20 ± 2.00	48.68 ± 2.30
Collagen+ molsidomine(1μM) *2	9.60 ± 0.40	40.20 ± 0.42

Notes : *1 and *2 are indicated the exogenous addition of LF and theophylline.

As shown in Table 6, the level of cAMP was decreased in a weak manner on thrombin-induced platelet aggregation of rat fed with LF to the control. On the contrary, the cAMP production was increased by the exogenous addition of LF and theophylline(Table 6). cAMP-elevation by LF is the same as the case of human platelets(Table 2). Accordingly, it is suggested that LF does not affect on the production of cAMP *in vivo* and *in vitro* in thrombin-induced platelet aggregation. However, when platelets of rat fed with LF were stimulated by collagen, the level of cGMP was increased from 6.6p mol to 26.98p mol 10^{7} platelets(Table 6). This was more increased by the exogenous addition of LF. It is inferred that LF regulates the production of cAMP in the aggregation stimulated with collagen than thrombin.

DISCUSSION

It is known that thrombin-induced the platelet aggregation occurs by platelet activation of Ca^{2+}, and that the Ca^{2+} is mobilized out of intracellular Ca^{2+} pool(endoplasmic reticulum) by inositol 1, 4, 5-triphosphate produced from membrane phosphatidylinositol 4, 5-biphosphate as the result of activating phospholipase C by TXA_2(20-21). LF inhibited TXA_2 production to the control whose amounts is produced in resting platelets(Fig. 1). According to Kito's report, the concentration of TXA_2(Mw : 370.49) in the resting platelet is 8nM(5). Because 8nM is too low to activate phospholipase C(5). TXA_2 in the resting platelet does not induce platelet aggregation. It is inferred that the inhibition of TXA_2 formation by LF is owing to the inhibition of an any following step ; thrombin→plasma membrane→phospholipid breakdown→arachidonic acid →TXA_2 synthesis. Verapamil inhibits the Ca^{2+}-influx owing to stimulating the synthesis of cAMP(22, 23). LF does not stimulate the synthesis of cAMP, but inhibits the Ca^{2+}-elevation(Fig. 2, Table 2, 6). The inhibition of Ca^{2+}-elevation and the elevation of cGMP by LF(Table 2, 6) consistent with the fact that nitrovasodilator such as nitroprusside, stimulates the synthesis of cGMP and the phosphorylation of 50KD, and inhibit the Ca^{2+}-influx and mobilization induced by thrombin(6, 8). The aggregation of plateles by collagen and thrombin is due to the injury of the vessel and the tissue, respectively. When the platelet of rat fed with LF were stimulated with thrombin and collagen, the level of cGMP and cAMP was remarkably increased, respectively(Table 2, 5, 6). Accordingly, it is inferred that LF has the effect inhibiting the thrombus owing to the injury of the vessel and the tissue. LF also inhibited the release of serotonin(24). cGMP-ele vating platelet inhibitors inhibit the activity of phospholipase C and release of serotonin(6, 25). This is the cause of inhibiting the Ca^{2+}elevation by cGMP conseguently, inhibiting the phosphorylation of 20KD and 47KD(40KD) by Ca^{2+}/calmodulin-dependent kinase and 1, 2-diacylglycerol-dependent protein kinase C, respectively, results in inhibiting the platelet aggregation(6, 8). It is detected that 47KD and 50KD were overlapped each other on our SDS PAGE(Fig. 3). Vasodilators increasing the production of cGMP enhance the phosphorylation of 50KD, but inhibit the phosphorylation of 47KD(40KD) and 20KD(6, 8). Considering from inhibiting the phosphorylation of 20KD, LF also is regarded as inhibiting the phosphorylation of 47KD and stimulating the phosphorylation of 50KD. However, it is unclear that LF inhibits the phosphorylation of 47KD or not. Platelets incubated with thrombin are more susceptible to breakage on a gradient of glycerol-EDTA(14). In preparation of platelets, which were washed twice in buffer containing EDTA to remove the trace of Ca^{2+}(14). Accordingly, we can not rule out the possibility that platelets are broken during washing. If platelets were broken, the other intact platelets would be aggregated by stimulator of platelets such as platelet activating factor, serotonin, ADP and Ca^{2+} released out of granules. However, when platelets suspended in buffer without containing EDTA in the presence of LF were preincubated, platelet aggregation(Table 1) and Ca^{2+}-influx(Fig. 2) were inhibited. It is reported that EDTA cause the partial proteolysis of actin-binding protein fibronectin, myosin and thrombin sensitive protein(26). These mean that platelets inactivate owing to EDTA. If the inhibition of aggregation by LF were due to inactivation of platelets result from EDTA, our results may be false and platelet aggregation and protein phosphorylation would not be occured by thrombin. However, platelet aggregation of 73% and protein phosphorylation were occured(Table 1, Fig. 3, Table 3). The above considerations mean that platelets were not broken and were not inactivated by EDTA, and the inhibition of aggregation and its related activation by LF is not false.

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