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Anticoagulant activities of piperlonguminine *in vitro* and *in vivo*

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Piperlonguminine (PL), an important component of *Piper longum* fruits, is known to exhibit anti-hyperlipidemic, anti-platelet and anti-melanogenic activities. Here, the anticoagulant activities of PL were examined by monitoring activated-partial-thromboplastin-time (aPTT), prothrombin-time (PT), and the activities of thrombin and activated factor X (FXa). The effects of PL on the expressions of plasminogen activator inhibitor type 1 (PAI-1) and tissue-type plasminogen activator (t-PA) were also tested in tumor necrosis factor-α (TNF-α) activated HUVECs. The results showed that PL prolonged aPTT and PT significantly and inhibited the activities of thrombin and FXa. PL inhibited the generation of thrombin and FXa in HUVECs. In accordance with these anticoagulant activities, PL prolonged in vivo bleeding time and inhibited TNF-α induced PAI-1 production. Furthermore, PAI-1/t-PA ratio was significantly decreased by PL. Collectively, our results suggest that PL possesses antithrombotic activities and that the current study could provide bases for the development of new anticoagulant agents. [BMB Reports 2013; 46(10): 484-489]

INTRODUCTION

Coagulation is a major defense mechanism against bleeding. The coagulation system consists of a series of stepwise, coordinated reactions involving specific plasma proteins and blood cells that culminate in the formation of an insoluble clot (1). The coagulation system can be divided into the extrinsic and intrinsic pathway. Activation of the extrinsic pathway is generally considered to initiate both haemostasis and thrombosis. Haemostasis is initiated when blood is exposed to tissue factor located in the adventitia of blood vessels, and thrombosis is initiated when blood is exposed to tissue factor in the necrotic core of the ruptured atherosclerotic plaques, in the subendothelium of injured vessels and on the surface of activated leucocytes attracted to the damaged vessel (2). The final common mediator of both the intrinsic and extrinsic coagulation pathways is thrombin. Thrombin triggers platelet activation, as well as the production of factor V, VIII, and IX, and mediates the proteolytic cleavage of fibrinogen to fibrin and then it binds to fibrin where it remains active (3). The physiological function of the coagulation system is to prevent the loss of blood after injury. Given the central role of thrombin in the development of a thrombus, many strategies for preventing and treating thromboembolic events have focused on inhibiting thrombin generation or blocking its activity (2, 3).

The search for anticoagulant agents from natural herbal medicines is an area of considerable interest (4). *Piper longum* L. (Piperaceae) is a slender aromatic climber with perennial woody roots, occurring primarily in tropical regions. The fruits are used as a spice and also as a preservative in pickles. In traditional medicines, *P. longum* fruits have been attributed with the treatment of bronchial diseases, menstrual pain, tuberculosis, muscular pain, sleeping disorders, and certain forms of arthritis (5-7). The anticoagulant effects of extracts of the *P. longum* fruits have not been previously determined. During our continued search for natural products that modulate coagulation cascade, we found that the methanol extract of the *P. longum* fruits exhibited potent anticoagulant activity in both cell and animal models. The fruits of the *P. longum* have been shown to contain alkaloids and amides; pipericide, piperine, piperlonguminine, piplartine, and aristolactams (8-11). Activity-guided purification of the extract of *P. longum* fruits resulted in isolation of one active principle, which was subsequently identified as piperlonguminine (PL). Piperlonguminine showed various biological properties including anti-hyperlipidemic, anti-platelet and anti-melanogenesis activities (12-15). However, to our knowledge, the anticoagulant functions of piperlonguminine have not been studied yet. Therefore, we sought to test the anticoagulant activities of PL by examining the inhibitory effects of PL on the generation of factor Xa (FXa) and thrombin and clotting time changes (PT and aPTT), and their fibrinolytic activities.

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RESULTS AND DISCUSSION

The present study for the first time reports the anticoagulant effects of piperlonguminine (compound 1, PL), isolated from *P. longum* fruits, and the mechanisms responsible for these effects.

Effects of PL on aPTT and PT

Incubation of human plasma with PL changed coagulation properties. The anticoagulatory effects of PL were tested using aPTT and PT assays and human plasma (Table 1). Although the anticoagulant activities of PL were weaker than those of heparin, aPTT and PT were significantly prolonged by PL at concentrations 20 μM and above. A prolongation of aPTT suggests inhibition of the intrinsic and/or the common pathway, whereas PT prolongation indicates that PL could also inhibit the extrinsic coagulation pathway. To confirm these *in vitro* results, tail bleeding times were determined. Assuming an average body weight of 20 g and an average blood volume of 2 ml, the test compounds administered (8.25 μg/mouse = 0.413 mg/kg) produced a concentration of approximately 30 μM in peripheral blood. As shown in Table 1, tail bleeding times were significantly prolonged by PL in comparison to the controls.

Effects of PL on thrombin-catalyzed platelet aggregation and fibrin polymerization

As shown in Fig. 1B, PL significantly inhibited mouse platelet aggregation induced by thrombin (final concentration: 3 U/ml) in a concentration dependent manner. The effects of PL on thrombin-catalyzed fibrin polymerization in human plasma were monitored as changes in absorbance at 360 nm, as described in Materials and Methods. The results (Fig. 1C) showed that incubation of human plasma with PL significantly decreased maximal fibrin polymerization rates. To eliminate the effect of pH all dilutions were made with 50 mM TBS (pH7.4). We also examined the effect of DMSO on human plasma at the same volume as in the case of PL, but no differences in coagulation properties were observed. We further characterized the influence of calcium by performing polymerization experiments without or with 1 mM $CaCl_2$. Results showed that calcium had an influence on the fibrin polymerization by PL (data not shown). To exclude the possibility that the decrease in polymerization could be due to a direct effect on thrombin leading to decrease in fibrin production rather than polymerization of fibrin formed, reptilase-catalyzed polymerization assay was included. Results showed that PL significantly decreased repti-

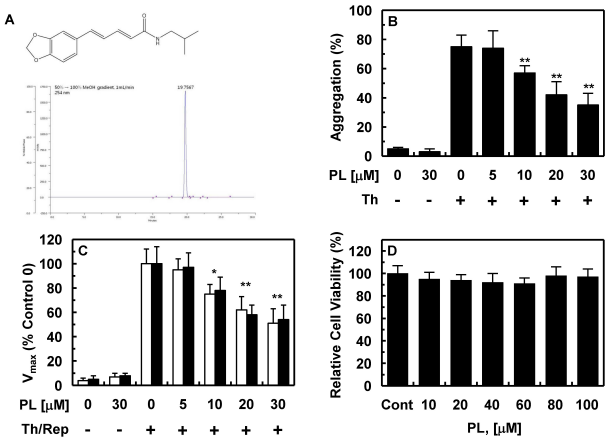

Fig. 1. Structure and characterization of piperlonguminine (PL) and effects of PL on fibrin polymerization in human plasma. (A) Structure and HPLC chromatogram of PL. (B) Effect of PL on mouse platelet aggregation induced by 3 U/ml thrombin. (C) Thrombin (Th, white box) or reptilase (Rep, black box) catalyzed fibrin polymerization by the indicated concentrations of PL was monitored using the catalytic assay described in "Materials and Methods". (D) Effect of PL on cellular viability was measured by MTT assay. Data represent the means ± SDs of 3 independent experiments performed in triplicate. The results are Vmax values expressed as percentages of thrombin alone. *P < 0.05 or **P < 0.01 as compared to Th alone (B) or Th/Rep alone (C).

Table 1. Anticoagulant activity of PL[a]

In vitro coagulant assay					*In vivo* bleeding time			
Sample	Dose	aPTT (s)	PT (s)	PT (INR)	Sample	Dose	Tail Bleeding time (s)	n
Control	Saline	30.3 ± 0.4	13.2 ± 0.3	1.00	Control	Saline	42.3 ± 1.5	10
PL	1 μM	31.8 ± 0.7	13.4 ± 0.7	1.03	PL	1.38 μg/mouse	43.8 ± 1.2	10
	2 μM	31.7 ± 0.5	13.5 ± 0.5	1.05		2.75 μg/mouse	67.4 ± 2.1[b]	10
	5 μM	31.4 ± 0.3	13.6 ± 0.4	1.07		5.50 μg/mouse	85.7 ± 2.5[b]	10
	10 μM	37.8 ± 0.4[b]	15.7 ± 0.3[b]	1.46[b]		8.25 μg/mouse	97.5 ± 2.5[b]	10
	20 μM	47.7 ± 0.7[b]	22.7 ± 0.7[b]	3.30[b]	Heparin	1 μg/mouse	132.5 ± 2.5[b]	10
	30 μM	55.3 ± 0.5[b]	24.3 ± 0.2[b]	3.83[b]				
Heparin		0.5 (mg/ml)	10 (mg/ml)	8.92[b]				
		87.6 ± 1.4[b]	35.7 ± 0.5[b]					

[a]Each value represents the means ± SD (n=5). [b]P < 0.01 as compared to control.

lase-catalyzed polymerization (Fig. 1C). To test the cytotoxicity of PL, cellular viability assays were performed in HUVECs treated with PL for 24 h. At concentrations up to 100 μM, PL did not affect cell viability (Fig. 1D).

Effects of PL on the activities of thrombin and factor Xa (FXa)

To investigate the mechanism responsible for the inhibitory effects of PL on coagulation time, we measured the inhibitory effects of PL addition on thrombin and FXa using chromogenic substrates. The amidolytic activity of thrombin was inhibited dose-dependently by PL, showing that PL directly inhibited thrombin activity (Fig. 2A). We also investigated the effects of PL on FXa. As shown in Fig. 2B, the activity of FXa was inhibited dose-dependently by PL, showing that PL directly inhibited FXa activity. These results were consistent with the results of our antithrombin assays, and suggested that the mechanism responsible for the antithrombotic effects of PL is due the inhibitions of fibrin polymerization and/or the intrinsic and extrinsic pathways.

Effects of PL on the generation of thrombin and FXa

Sugo *et al*. (16) reported that endothelial cells can support prothrombin activation by FXa. In the present study, preincubation of HUVECs with FVa and FXa in the presence of $CaCl_2$ before adding prothrombin resulted in thrombin generation (Fig. 2C). However, PL dose-dependently inhibited the thrombin activation of prothrombin (Fig. 2C). Rao *et al*. (17) showed that the endothelium provides the functional equivalent of procoagulant phospholipids and supports FX activation and that in TNF-α stimulated HUVECs, FVIIa could activate FX in a TF expression dependent manner (18). These findings suggested that the endothelium can provide support for the FVIIa activation of FX, and thus, we examined the effects of PL on the FVIIa activation of FX (Fig. 2D). When we stimulated HUVECs with TNF-α to induce TF expression, the rate of FX activation by FVIIa was 100-fold higher in stimulated HUVECs (85.3 ± 15.12 nM) compared with non-stimulated HUVECs (0.83 ± 0.25 nM), but this activation was abrogated by anti-TF IgG (7.3 ± 0.85 nM). Moreover, preincubation of HUVECs with PL dose-dependently inhibited the FVIIa activation of FX, suggesting PL can both inhibit the generation of thrombin and FXa. Collectively, Fig. 2C and D showed that the inhibitory effects of PL in thrombin production appear to be not significantly reduced in comparison with FXa reduction by PL indicating why the inhibitory effects of PL are weaker than those of heparin.

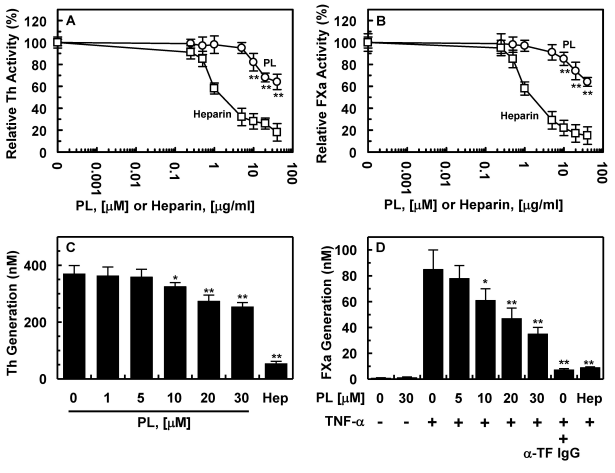

Fig. 2. Effects of PL on the inactivation and on the generation of thrombin and factor Xa. (A) Inhibitions of thrombin or (B) factor Xa by PL. (C) Generation of thrombin or (D) factor Xa in HUVECs. Heparin (40 μg/ml) was used as positive control. *P < 0.05 or **P < 0.01 as compared to 0 (A-C) or TNF-α alone (D).

Effects of PL on the secretion of PAI-1 and t-PA proteins

TNF-α appears to inhibit the fibrinolytic mechanism in HUVECs by inducing the production of PAI-I. Furthermore, the balance between t-PA and PAI-1 is known to determine procoagulant and hypofibrinolysis activities (19, 20). To examine the direct effect of PL on TNF-α stimulated PAI-1 secretion, HUVECs were cultured in media with or without PL in the presence and absence of TNF-α for 18 h. As shown in Fig. 3A, PL dose-dependently inhibited TNF-α induced PAI-1 secretion from HUVECs, and this decrease became significant at a PL dose of 10 μM.

Because TNF-α significantly did not affect t-PA production (21) and the balance between plasminogen activators and their inhibitors reflects net plasminogen-activating capacity (2, 3, 22), we sought to investigate the effects of TNF-α and PL on

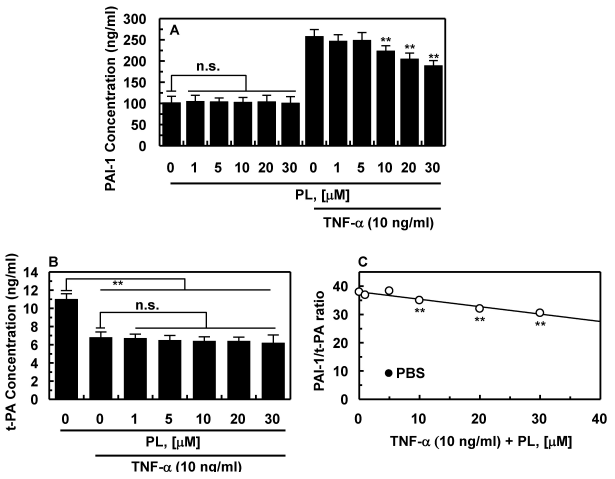

Fig. 3. Effects of PL on the secretion of PAI-1 and t-PA. (A) HUVECs were cultured with PL in the absence or presence of TNF-α (10 ng/ml) for 18 h and PAI-1 concentrations in the culture media were measured. (B) HUVECs were cultured with PL in the absence or presence of TNF-α (10 ng/ml) for 18 h and t-PA concentrations in culture media were measured. (C) PAI-1/t-PA ratio in TNF-α activated HUVECs from (A) and (B). **P < 0.01 as compared to TNF-α alone (A, B) or 0.; n.s., not significant.

t-PA production in HUVECs. Our observation further endorse a previous observation whereby TNF-α is shown to modestly decreased t-PA production in HUVECs (23) and this decrease was not significantly altered by treatment with PL during the current study (Fig. 3B). Therefore, these results collectively indicate that the PAI-1/t-PA ratio was increased by TNF-α and significantly decreased by PL compared to TNF-α treated conditions (Fig. 3C).

In conclusion, this study shows that PL inhibits the extrinsic and intrinsic pathways of blood coagulation by inhibiting FXa and thrombin generation and that both inhibit TNF-α induced PAI-1 protein secretion by HUVECs. Based on the cellular viability assays, we believe that the antithrombotic and profibrinolytic activities of PL were not due to any cytotoxic effect. We hope this study will prove helpful to those involving in pharmacological strategies for the treatment or prevention of vascular diseases via the regulation of thrombin production.

MATERIALS AND METHODS

Reagents

TNF-α was purchased from R&D Systems (Minneapolis, MN), and antitissue factor antibody from Santa Cruz Biologics (Santa Cruz, CA). Factor V, Vll, and Vlla, FX, FXa, prothrombin, and thrombin were from Haematologic Technologies (Essex Junction, VT, USA). aPTT assay reagent (APTT-XL) and PT (Thromboplastin-D) reagents were purchased from Thermo Scientific (UK), the chromogenic substrates S-2222 and S-2238 from Chromogenix AB (Sweden), and the PAI-1 and t-PA ELISA kits from American Diagnostica Inc (Stamford, CT, USA). Heparin was provided by Dr. Rezaie AR, The Edward A. Doisy Department of Biochemistry and Molecular Biology, St. Louis University (St. Louis, MO, USA).

Plant material, extraction, and isolation

Friuts of *Piper longum* L. (Piperaceae) were purchased from herbal market at Daegu, Korea, in February 2002. The plant material was identified by Dr. Seung Ho Lee at the College of Pharmacy, Yeungnam University, South Korea. A voucher specimen (SH02020) was deposited at herbarium, College of Pharmacy, Yeungnam University.

Dried friuts of *P. longum* (300 g) were crushed and extracted in MeOH at the room temperature for 7 days. After concentration the extract, the MeOH extract (23.9 g) was suspended in more than 5 volumes of water and partitioned in methylene chloride (MC) for three times. The MC-soluble fraction (9.2 g) was chromatographed on silica gel, eluting in EtOAc in n-hexatne (0-100%, step-wise), yielding seven fractions (Fr 1-Fr 7). Fr 5 (520.0 mg) was subjected to reversed phase C-18 column chromatography, subsequently eluted in a gradient of MeOH-H_2O (from 3 : 2 to 10 : 0), and fractionated (Fr 5-1-Fr 5-5). Fr 5-3 (120.1 mg) was washed in n-hexane, and insoluble part (48.3 mg) of the Fr 5-3 was purified by preparative HPLC (UV detection 254 nm) using an isocratic mixture of MeOH-H_2O (4 : 1, 1.0 ml/min) as the solvent, furnishing a piperlonguminine (t_R 27.8 min, 12.5 mg, 0.00417% w/w). The structure of compound **1** (Fig. 1A) was identified by a combination of spectroscopic methods and available literature data (9).

Anticoagulation assay

aPTT and PT were determined using a Thrombotimer (Behnk Elektronik, Germany), according to the manufacturer's instructions as described previously (24-26). In brief, citrated normal human plasma (90 μl) was mixed with 10 μl of PL and incubated for 3 min at 37°C. aPTT assay reagent (100 μl) was added and incubated for 1 min at 37°C, and then 20 mM $CaCl_2$ (100 μl) was added. Clotting times were recorded. For PT assays, citrated normal human plasma (90 μl) was mixed with 10 μl of PL stock and incubated for 3 min at 37°C. PT assay reagent (200 μl), which has been preincubated for 10 min at 37°C, was then added and clotting time was recorded. PT results are expressed in seconds and as International Normalized Ratios (INR), and aPTT results are expressed in seconds. INR = (PT sample / PT control)^{ISI}. ISI = international sensitivity index.

Cell culture

Primary HUVECs were obtained from Cambrex Bio Science (Charles City, IA) and were maintained as described previously (27).

Platelet aggregation assay

Mouse platelets from platelet-rich plasma (PRP) were washed once with HEPES buffer (5 mM HEPES, 136 mM NaCl, 2.7 mM KCl, 0.42 mM NaH_2PO_4, 2 mM $MgCl_2$, 5.6 mM glucose, 0.1% BSA (w/v), pH to 7.45). The platelet aggregation study was carried out according to a method previously reported (28). Washed platelets were incubated with indicated PL for 3min, and then stimulated by thrombin (3 U/ml, Sigma) in 0.9% saline solution at 37℃ for 5min. Platelet aggregation was recorded using a aggregometer (Chronolog, Havertown, PA, USA).

Thrombin or reptilase-catalyzed fibrin polymerization

Thrombin or reptilase-catalyzed polymerization was followed by monitoring changes in turbidity at 360 nm every 6 s for 20 min using a spectrophotometer (TECAN, Switzerland) at ambient temperature. Control plasma and plasma samples incubated with PL were three-fold diluted in TBS (50 mM Tris-buffered physiological saline solution pH 7.4) and clotted with thrombin or reptilase (final concentration - 0.5 U/ml) with or without 1 mM $CaCl_2$. Maximum polymerization rate (Vmax, ΔmOD/min) was recorded for each absorbance maximum slope from the turbidity curve (29). All experiments were performed three times.

Factor Xa generation on the surfaces of HUVECs

TNF-α (10 ng/ml for 6 h in serum-free medium) stimulated confluent monolayers of HUVECs (preincubated with the indicated

concentrations of PL for 10 min) in a 96-well culture plate were incubated with FVIIa (10 nM) in buffer B (buffer A supplemented with 5 mg/ml BSA and 5 mM $CaCl_2$) for 5 min at 37°C in presence or absence of anti-TF IgG (25 μg/ml). FX (175 nM) was then added to the cells (final reaction volume, 100 μl) and incubated for 15 min. The reaction was stopped by adding buffer A (10 mM HEPES, pH 7.45, 150 mM NaCl, 4 mM KCl, and 11 mM glucose) containing 10 mM EDTA and the amounts of FXa generated were measured by using a chromogenic substrate. Changes in absorbance at 405 nm over 2 min were monitored using a microplate reader. Initial rates of color development were converted into FXa concentrations using a standard curve prepared with known dilutions of purified human FXa.

Thrombin generation on the surface of HUVECs

HUVECs were preincubated in 300 μl of a mixture containing PL in 50 mM Tris-HCl buffer, 100 pM FVa, and 1 nM FXa for 10 min and prothrombin was added to a final concentration of 1 μM followed by calcium chloride (16.7 mM) to initiate the clotting reaction. After 10 min, duplicate samples (10 μl each) were transferred to a 96-well plate containing 40 μl of 0.5 M EDTA in Tris-buffered saline per well to terminate prothrombin activation. Activated prothrombin was determined by measuring the rate of hydrolysis of S2238 at 405 nm. Standard curves were prepared using known dilutions of purified thrombin.

Thrombin activity assay

PL was dissolved in physiological saline to the appropriate concentrations. Then, PL was mixed with 50 mM Tris–HCl buffer (pH 7.4) containing 7.5 mM EDTA and 150 mM NaCl. Heparin was used as a positive control. After incubation at 37°C for 2 min, thrombin solution (150 μl; 10 U/ml) was added to each well and incubated at 37°C for 1 min. S-2238 (150 μl; 1.5 mM) was then added and absorbance at 405 nm was monitored for 120 s using a spectrophotometer (TECAN, Switzerland).

Factor Xa (FXa) activity assay

This assay was performed in the same way as the thrombin activity assay, but factor Xa (1 U/ml) and substrate S-2222 were used instead of thrombin and S-2238, respectively.

Effect on bleeding time

Tail bleeding times were determined as described by Dejana *et al* (30). Briefly, male ICR mice were fasted overnight and PL in water was administered by oral gavage. One hour after administration, tails were transected 2 mm from their tips. Bleeding time was defined as the time elapsed until bleeding stopped. However, bleeding times that exceeded 15 min were recorded as 15 min for analyses. All animals were treated in accordance with the Guidelines for the Care and Use of Laboratory Animals issued by the Kyungpook National University.

ELISA for PAI-1 and t-PA

Concentrations of PAI-1 and t-PA in HUVEC supernatants were determined using ELISA kits (American Diagnostica Inc., CT, USA) according to the manufacturer's recommended protocol.

Statistical analysis

Data are expressed as the means ± standard deviations of at least three independent experiments. Statistical significance between two groups was determined using the Student's *t*-test. Statistical significance was accepted for P values < 0.05.

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