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Anticoagulant activities of oleanolic acid via inhibition of tissue factor expressions

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Oleanolic acid (OA), a triterpenoid known for its anti-inflammatory and anti-cancer properties, is commonly present in several medicinal plants but its anticoagulant activities have not been studied. Here, the anticoagulant properties of OA were determined by monitoring activated partial thromboplastin time (aPTT), prothrombin time (PT), fibrin polymerization as well as cell-based thrombin and activated factor X (FXa) generation activities. Data showed OA prolonged aPTT and PT significantly and inhibited thrombin catalyzed fibrin polymerization. In addition, OA inhibited the activities of thrombin and FXa and inhibited the generation of thrombin or FXa in human endothelial cells. OA also inhibited TNF-α-induced tissue factor expression on human endothelial cells. In accordance with these anticoagulant activities, OA showed an anticoagulant effect *in vivo*. These results indicate that OA possesses antithrombotic activities and suggest that daily consumption of a herb containing OA may be preventing thrombosis in pathological states. [BMB Reports 2012; 45(7): 390-395]

INTRODUCTION

The vascular endothelium performs a number of important functions that maintain an adequate blood supply to vital organs. These functions include the prevention of coagulation, the regulation of vascular tonus, the orchestration of blood cell migration via the expression of adhesion molecules, and the regulation of vasopermeability (1). Of these functions, hemostatic activity is regulated by balancing pro- and anticoagulant properties (2). On the other hand, impaired endothelial function causes thrombus-related complications, such as, myocardial infarction, stroke, and thromboembolism (3). Thrombin is a pivotal enzyme for all major thrombotic processes, including physiologic hemostasis and pathologic thrombosis, and is required for the conversion of fibrinogen to fibrin (2). Thrombin resides in cells in an inactive form, called prothrombin, and is activated by coagulation cascade via formation of a complex called prothrombin activator complex (2, 4, 5). The formation of this complex occurs via two different pathways-the intrinsic and extrinsic prothrombin activation pathways. Clotting time assays measures the lag time of thrombin generation (6) and activated partial thromboplastin time (aPTT) is a performance indicator that measures the efficacy of the contact activation pathway and the common coagulation pathway (6). On the other hand, prothrombin time (PT) is measure of the extrinsic coagulation pathway (7, 8).

Mistletoe (*Viscum album*) is a semi-parasitic plant with a worldwide distribution in all over the world that grows on many kinds of trees, and its aqueous extracts have been used as anti-cancer agents for almost a century (9). Korean mistletoe (KM) is also used in traditional medicine to treat cancer, cardiovascular disease, and arthrosis (10), and studies of the biological effects of KM and of its chemical composition have demonstrated that the lectins of KM induce the apoptosis of cancer cells (11). Other studies have shown that they have immunomodulatory activity and augment antigen-specific cellular and humoral immune responses (12). Previously, we found the lectins of KM inhibit the experimental lung metastasis of tumor cells in mice and that this activity is partly due to the activations of macrophages and native killer cells (13). Even though the lectins, alkaloids, and viscotoxins isolated from KM have been shown to have anti-cancer and immunomodulating activities, the anticoagulant activities of oleanolic acid (OA) extracted from the mistletoe (*Viscum album*) have not been well studied. Here, we report on the generation of FXa and thrombin by OA and on its regulatory effect on clotting time (PT and aPTT).

RESULTS AND DISCUSSION

In recent years, naturally occurring chemical substances derived from plants have attracted interest as possible treatments for coagulation disorders and as template molecules for the development of new drugs (14). These molecules interfere with

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two key processes involved in the blood coagulation process, namely, PT and aPTT (14). Oleanolic acid (OA), is a natural pentacyclic triterpene widely found in a variety of plants, and has been shown to display numerous biological properties with therapeutic potential (15, 16). In the present study, the anticoagulant properties of OA were examined.

The effects of OA on anticoagulant activities were examined using aPTT and PT assays and human plasma; results are summarized in Table 1. Although the anticoagulant activities of OA were found to be weaker than those of heparin, aPTT and PT were significantly prolonged by OA at concentrations of $\geq$ 10 μM. The prolongation of aPTT suggests inhibition of the intrinsic and/or the common coagulation pathways, whereas prolongation of PT suggests inhibition of the extrinsic coagulation pathway. To confirm our *in vitro* results, *in vivo* tail bleeding times were measured. As shown in Table 1, tail bleeding times were significantly prolonged by OA versus untreated controls.

The effects of OA on thrombin-catalyzed fibrin polymerization in human plasma were monitored as the changes in absorbance at 360 nm as described in Materials and methods. The results, presented in Fig. 1, demonstrated that incubation of human plasma with OA significantly decreased the maximal velocity of fibrin polymerization in human plasma. To eliminate the effect of different pH in the samples all dilutions were in 50mM TBS, pH7.4. We also checked the effect of DMSO on human plasma at the same volume as in the case of OA. We did not observe any differences in coagulation properties. To examine the possibility that the anticoagulant activities of purified OA were different with commercially available OA, single compound OA from Sigma Aldrich (St. Louis, MO, USA) was used. As shown in Fig. 1 and Table 1, there was no difference in fibrin polymerization and *in vivo* tail bleeding time between purified OA and single chemical OA.

To elucidate the inhibitory mechanism responsible for the effect of OA on coagulation time, its inhibitory effects on thrombin and FXa activities were measured using chromogenic substrates in the absence or presence of antithrombin III (AT III). In the absence of AT III, the amidolytic activity of thrombin was dose-dependently inhibited by OA, showing that its anticoagulant effect involved the direct inhibition of thrombin activity. However, in the presence of AT III, thrombin activity was essentially unchanged (Fig. 2A), indicating that AT III was unable to potentiate the activity of OA. In addition, we also investigated the effects of OA on FXa activity in the absence or presence of AT III. Data showed direct inhibitory effects OA on FXa activities at high concentrations, and the inhibitory effect of AT III was not changed by OA (Fig. 2B). These results are consistent with our antithrombin assay findings, and suggest that the antithrombotic mechanism underlying the effects of OA appears to be due to the inhibition of fibrin polymerization and/or of the intrinsic/extrinsic pathway without potentiation by AT III.

Previously, Sugo *et al*. reported that endothelial cells are able to support prothrombin activation by FXa (17). 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. 3A). Furthermore, OA inhibited the thrombin activation of prothrombin dose-dependently (Fig. 3A). Rao *et al*. showed endothelium provides the functional equivalent of pro-

Table 1. Anticoagulant activity of OA[a]

In vitro coagulant assay				
Sample	Dose	aPTT (s)	PT (s)	PT (INR)
Control	Saline	37.2 ± 0.8	15.9 ± 0.8	1.00
OA	0.5 μM	37.3 ± 0.9	16.1 ± 0.6	1.03
	1 μM	38.5 ± 1.2	16.5 ± 0.3	1.08
	5 μM	39.7 ± 2.7	17.1 ± 1.2	1.17
	10 μM	45.3 ± 1.5[b]	20.3 ± 0.8[b]	1.71
	20 μM	52.8 ± 2.3[c]	23.8 ± 1.1[c]	2.43
	30 μM	64.8 ± 0.9[c]	27.9 ± 1.3[c]	3.45
	40 μM	65.3 ± 1.5[c]	28.3 ± 1.2[c]	3.56
Heparin		0.5 (μg / ml) 113.8 ± 0.5[c]	10 (μg / ml) 40.9 ± 0.8[c]	7.99

In vivo bleeding time			
Sample	Dose	Tail bleeding time (s)	n
Control	Saline	51.5 ± 2.5	5
Purified OA	100 mg/kg	82 ± 1.8[c]	5
Chemical OA	100 mg/kg	85 ± 2.3[c]	5
Heparin	50 mg/kg	147.3 ± 3.2[b]	5

[a]Each value represents the means ± SD (n = 5). [b]$P < 0.05$ as compared to control. [c]$P < 0.01$ as compared to control.

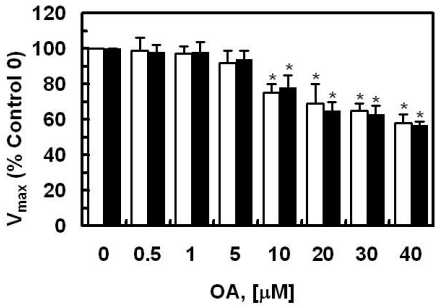

Fig. 1. Effect of OA on of fibrin polymerization in human plasma. Thrombin-catalyzed fibrin polymerization by indicated concentrations of purified OA (white box) or single chemical OA (black box) was monitored by catalytic assay as described in "Materials and Methods". Data represent means ± SD of 3 independent experiments done in triplicate. The results are measured as Vmax, and expressed as % versus control (0 μM). *$P < 0.05$ as compared to 0.

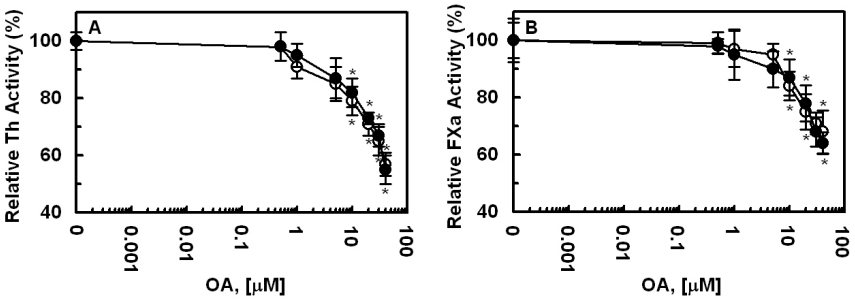

Fig. 2. Effect of oleanolic acid on the inactivation of thrombin and factor Xa. (A) Inhibition of thrombin (Th) in the absence of antithrombin III (○) or in the presence of antithrombin III (●) by OA was monitored using a chromogenic assay as described in "Materials and Methods". (B), The inhibition of factor Xa (FXa) in the absence (○) or presence of antithrombin III (●) by OA was also monitored using a chromogenic assay. *P < 0.01 as compared to 0 μM.

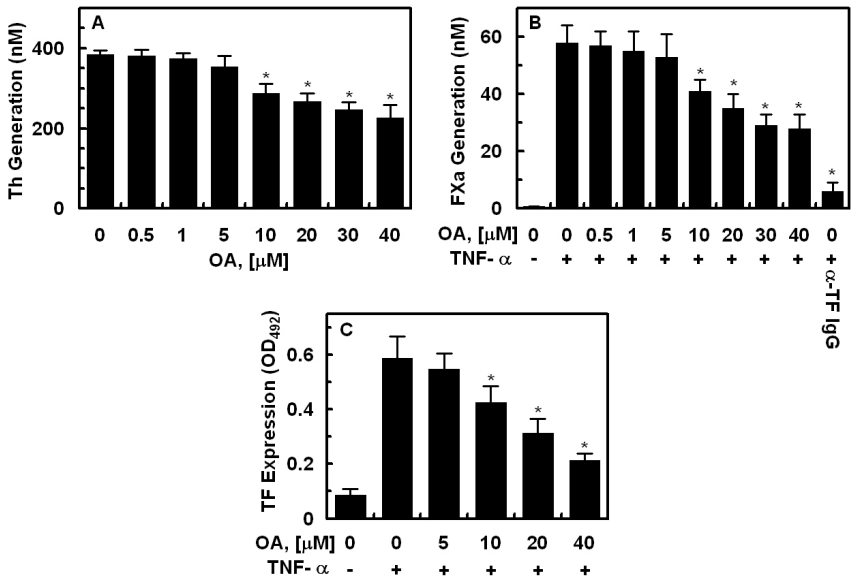

Fig. 3. nhibition of thrombin and FXa generation by oleanolic acid in HUVECs. (A) FVa (100 pM) and FXa (1 nM) were preincubated with HUVECs monolayers for 10 min with the indicated concentrations of OA. Prothrombin was added to a final concentration of 1 μM and prothrombin activation was determined at 30 min as described in "Materials and Methods". (B) HUVECs were preincubated with the indicated concentrations of OA for 10 min. TNF-α (10 ng/ml for 6 h) stimulated HUVECs were incubated with FVIIa (10 nM) and FX (175 nM) in the absence or presence of anti-TF IgG (25 μg/ml) and FXa generation was then determined as described in "Materials and Methods". (C) HUVECs were preincubated with the indicated concentrations of OA for 6 h and then TNF-α (10 ng/ml for 6 h) was added to express TF on HUVECs. Expressions of TF were determined by ELISA as described in "Materials and Methods". *P < 0.05 as compared to 0 μM (A) or TNF-α alone (B or C).

coagulant phospholipids and supports FX activation (18), and in TNF-α stimulated HUVECs, FVIIa activated FX in tissue factor (TF) expression dependent manner (19), showing that endothelium can provide support the FVIIa activation of FX. In the present study, we examined the effect of OA on the FVIIa activation of FX. HUVECs were stimulated with TNF-α to induce TF expression, and as shown in Fig. 3B, the rate of FX activation by FVIIa was found to be 100-fold higher in stimulated HUVECs (58.1 ± 6.7 nM) than in non-stimulated HUVECs (0.54 ± 0.2 nM), and this activation was prevented by anti-TF IgG (8.6 ± 0.7 nM). Moreover, preincubation with OA dose-dependently inhibited the FVIIa activation of FX (Fig. 3B). To determine whether OA inhibited TNF-α-induced TF expression on HUVECs, cells were preincubated with OA followed by TNF-α activation. As shown in Fig. 3C, OA significantly inhibited TNF-α-induced TF expressions on HUVECs. These results suggested that OA can inhibit the generation of thrombin or FXa and TNF-α-induced TF expressions on HUVECs.

The significant progress made in understanding the role of FXa and thrombin in various thrombotic disease states demonstrates the potential therapeutic benefits of blocking these key enzymes in the blood coagulation cascade (20), and that potent, selective FXa or thrombin inhibitors have substantial potential therapeutic benefits (20). The findings of the present study show that OA exhibits the potency and selectivity required for such a candidate and is currently undergoing additional evaluation.

Summarizing, this study shows that OA inhibits the extrinsic and intrinsic blood coagulation pathways by inhibiting FXa and thrombin generation and TF expressions in HUVECs. These results add to previous knowledge and aid the rational design of pharmacological strategies for the treatment and prevention of vascular diseases via the regulation of thrombin generation.

MATERIALS AND METHODS

Reagents

Commercially available single chemical oleanolic acid was obtained form Sigma (St. Louis, MO, USA). TNF-α was purchased from R&D Systems (Minneapolis, MN). Anti-tissue factor antibody was purchased from Santa Cruz Biologics (Santa Cruz, CA). Factor V, Vll, and Vlla, FX, FXa, antithrombin III (AT III), prothrombin, and thrombin were obtained from Haematologic Technologies (Essex Junction, VT, USA). aPTT assay reagent and PT reagents were purchased from Fisher Diagnostics (Middletown, Virginia, USA). Chromogenic substrates S-2222, and S-2238 were from Chromogenix AB (Sweden).

Animals and husbandry

Male ICR mice (6 weeks old upon receipt, from Orient, South Korea) were used in this study after a 12-day acclimatization period. Animals were housed 5 per polycarbonate cage under controlled temperature (20-25°C) and humidity (40-45%) under a 12 : 12 hour light/dark cycle, and a normal rodent pellet diet and water were supplied during acclimatization *ad libitum*. All animals were treated in accordance with the Guidelines for Care and Use of Laboratory Animals issued by Kyungpook National University.

Plant material, extraction, and isolation

Dried mistletoe (*Viscum album*) was purchased from Daeyu Oriental Pharm Co (Daegu, Korea) -a specimen was deposited at the College of Pharmacy, Kyungpook National University, Daegu, Korea (voucher specimen number: KNUNPC-VAE-01). Dried mistletoe (2.7 kg) was refluxed with 95% EtOH for 5 h and then filtered through filter paper. The solution obtained was evaporated to dryness to yield 466 g of ethanolic extract, which was successively partitioned versus CH_2Cl_2, EtOAc, and *n*-BuOH. The active CH_2Cl_2 soluble fraction (113 g) was chromatographed on the silica gel (12 × 75 cm, CH_2Cl_2 : MeOH = 500 : 1 → 1 : 1) to give 14 fractions (Fr. 1-14). Compound 1 (3 g) was obtained from Fr. 10.

Compound 1 (Oleanolic Acid, OA)

^{1}H-NMR (400 MHz, $CDCl_3$) δ : 0.74, 0.79, 0.90, 0.92, 0.93, 0.99 and 1.12 (each 3H, s), 3.22 (1 H, dd, J = 4.0 and 9.5 Hz, H-3), 5.28 (1 H, m, H-12). ^{13}C-NMR (100 MHz, $CDCl_3$) δ : 16.0 (C-25), 16.5 (C-24), 18.0 (C-26), 19.0 (C-6), 23.7 (C-16), 23.8 (C-11 and C-30), 26.0 (C-27), 28.4 (C-2 and C-15), 28.7 (C-23), 31.1 (C-20), 33.2 (C-22 and C-29), 33.3 (C-7), 34.5 (C-21), 37.5 (C-10), 38.9 (C-1), 39.6 (C-4), 40.0 (C-8), 42.0 (C-14 and C-18), 46.5 (C-17 and C-19), 48.4 (C-9), 56.0 (C-5), 79.8 (C-3), 122.8 (C-12), 144.2 (C-13), 184.2 (C-28). ^{1}H- and ^{13}C-NMR data were consistent with previously published data (14). The chemical structure of compound 1 was identified as oleanolic acid.

Anticoagulation assay

aPTT and PT were determined using a Thrombotimer (Behnk Elektronik, Germany), according to the manufacture's instructions. In brief, citrated normal human plasma (90 μl) was mixed with 10 μl of OA and incubated for 1 min at 37°C. Then, aPTT assay reagent (100 μl) was added to the mixture and incubated for 1 min at 37°C. Thereafter, 20 mM $CaCl_2$ (100 μl) was added and the clotting time was recorded. For the PT assay, citrated normal human plasma (90 μl) was mixed with 10 μl of OA and incubated for 1 min at 37°C. PT assay reagent (200 μl; preincubated for 10 min at 37°C) was then added and the clotting time was recorded. PT results expressed in seconds and INR (International Normalized Ratio) and aPTT results expressed in seconds. INR = (PT sample / PT control)^{ISI}. ISI = international sensitivity index.

Thrombin-catalyzed fibrin polymerization

Thrombin-catalyzed polymerization was monitored every 6 s for 20 min as the change of turbidity at 360 nm in spectrophotometer (TECAN, Switzerland) at ambient temperature. Control plasma and plasma incubated with OA were trebly diluted TBS (50 mM Tris-buffered physiological saline solution pH 7.4) and clotted with thrombin (final concentration -0.5 U/ml). The maximal velocity of polymerization (Vmax, ⊿mOD/min) was recorded for each absorbance curve (22). All experiments were performed 3 times.

Cell culture

Primary HUVECs were obtained from Cambrex Bio Science (Charles City, IA) and maintained as previously described (23). Briefly, cells were cultured in EBM-2 basal media supplemented with growth supplements (Cambrex Bio Science) to confluency at 37°C in 5% CO_2.

Factor Xa generation on the surfaces of HUVECs

HUVECs were preincubated with indicated concentrations of OA for 10 min. TNF-α (10 ng / ml for 6 h in serum-free medium) stimulated confluent monolayers of HUVECs in a 96-well culture plate were incubated with FVIIa (10 nM) in buffer B 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 mixture volume, 100 μl) and incubated for 15 min. The reaction was stopped by adding buffer A containing 10 mM EDTA and the amount of FXa generated in the reaction period was measured by using a chromogenic substrate, and the change in absorbance at 405 nm was monitored in a microplate reader for 2 min. The initial rate of color development was converted into FXa concentrations from a standard curve prepared with known dilutions of purified human FXa.

Thrombin generation on the surfaces of HUVECs

HUVECs were preincubated in 300 μl of OA in 50 mM Tris-HCl buffer, 100 pM FVa, and 1 nM FXa for 10 min and prothrombin was then added to a final concentration of 1 μM.

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 levels were determined by measuring the rate of hydrolysis of S2238 measured at 405 nm using standard curves prepared using known concentrations of purified thrombin.

Thrombin activity assay

A solution of OA in 50 mM Tris-HCl buffer (pH 7.4) containing 7.5 mM EDTA and 150 mM NaCl was prepared with or without 150 μl of AT III (200 nM) and incubated at 37°C for 2 min. Thrombin solution (150 μl; 10 U/ml) was the added and incubated at 37°C for 1 min. Then, substrate for thrombin (S-2238, 150 μl; 1.5 mM) solution was added and absorbance was monitored at 405 nm for 120 s using a spectrophotometer (TECAN, Switzerland).

Factor Xa (FXa) activity assay

These assays were performed in the same way as thrombin activity assays but using factor Xa (1 Uml/1) and substrate S-2222 instead of thrombin and S-2238.

Effect on bleeding time

Tail transection bleeding times were determined as described by Dejana *et al*. (24). Briefly, male ICR mice were fasted overnight and OA was administered orally. One hour later, tails were transected 2 mm from their tips. Bleeding times were defined as times taken to bleeding cessation. Bleeding times of greater than 15 min were recorded as 15 min for analysis purposes.

ELISA for TF expression on HUVECs

The expression of TF on HUVECs were determined by a whole-cell ELISA. Briefly, confluent monolayers of HUVECs were treated with OA for 6 h followed by TNF-α (10 ng/ml) for 6 h. The medium was removed, cells were washed with PBS and fixed by adding 50 μl of 1% paraformaldehyde for 15 minutes at room temperature. After washing, 100 μl of anti-TF was added. After 1 h (37°C, 5% CO_2), the cells were washed three times and then 100 μl of 1 : 2,000 peroxidase-conjugated IgG antibody (Sigma, Saint Louis, MO) was added for 1 h. The cells were washed again three times and developed using *o*-phenylenediamine substrate (Sigma, Saint Louis, MO). Colorimetric analysis was performed by measuring absorbance at 490 nm. All measurements were performed in triplicate wells.

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 of < 0.05.

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