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Anticoagulant activities of curcumin and its derivative

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Curcumin, a polyphenol responsible for the yellow color of the curry spice turmeric, possesses antiinflammatory, antiproliferative and antiangiogenic activities. However, anticoagulant activities of curcumin have not been studied. Here, the anticoagulant properties of curcumin and its derivative (bisdemethoxycurcumin, BDMC) were determined by monitoring activated partial thromboplastin time (aPTT), prothrombin time (PT) as well as cell-based thrombin and activated factor X (FXa) generation activities. Data showed that curcumin and BDMC prolonged aPTT and PT significantly and inhibited thrombin and FXa activities. They inhibited the generation of thrombin or FXa. In accordance with these anticoagulant activities, curcumin and BDMC showed anticoagulant effect *in vivo*. Surprisingly, these anticoagulant effects of curcumin were better than those of BDMC indicating that methoxy group in curcumin positively regulated anticoagulant function of curcumin. Therefore, these results suggest that curcumin and BDMC possess antithrombotic activities and daily consumption of the curry spice turmeric might help maintain anticoagulant status. [BMB reports 2012; 45(4): 221-226]

INTRODUCTION

The key of the blood clotting pathway is the production of thrombin which is required for the conversion of fibrinogen to fibrin (1, 2). Thrombin resides in the cell in an inactive form, called prothrombin, and is activated by the coagulation cascadevia formation of a complex called the prothrombin activator complex (1-5). The formation of the prothrombin activator complex occurs by two different pathways: the intrinsic prothrombin activation pathway and the extrinsic prothrombin activation pathway. Though the ultimate goal of both the pathways is the generation of the prothrombin activator complex, alternate routes are used, each giving rise to a different form of the prothrombin activator (1-5). In the extrinsic pathway, prothrombin activator complex consists of activated factor X (FXa), tissue factor (TF), activated factor VII (FVIIa) and the cofactor activated factor V (FVa) (1-5). This complex, specifically FXa, along with the cofactor FVa, then converts prothrombin to active thrombin. Fibrin forms a mesh within the platelet aggregate to stabilize clots (1-5). In contrast, in the intrinsic pathway, prothrombin activator complex consists of FXa, FVa, activated factor VIII (FVIIIa) and phopspholipid (PL) (1-5). The clotting time assay measures the lag time of thrombin generation (6) and the activated partial thromboplastin time (aPTT) is a performance indicator measuring the efficacy of both the contact activation pathway and the common coagulation pathways (6). Further, the prothrombin time (PT) is measure of the extrinsic pathway of coagulation (7, 8).

The rhizome of *Curcuma longa* has been used in indigenous medicine for the treatment of inflammatory disorders and its medicinal activity has been known since ancient times. Turmeric derived from the rhizome has been widely used by the people in the Middle East for centuries as a food component (9, 10). The use of turmeric extract or turmeric oil as a spice and household remedy has been known to be safe for centuries. Bhide, et al. also revealed the safety and tolerance of turmeric through human clinical trials (11). In many previous studies, extracts prepared from *Curcuma longa* have been used as antiinflammatory agents to treat gas, colic, toothaches, chest pains, menstrual difficulties, stomach and liver ailmetns (9, 12, 13). Polyphenolic phytochemicals are common in the diet and have been suggested to have a wide range of beneficial health effects and the polyphenolic compounds in turmeric are responsible for a number of its beneficial health effects (14, 15). Turmeric contains three major polyphenolic analogues. The majority is curcumin and the compounds in smaller amounts are demethoxycurcumin, and bisdemethoxycurcumin (BDMC) (16, 17). Recent studies indicate that dietary administration of curcumin may have beneficial effects in conditions such as cancer (18), Alzheimer's disease (19) and cystic fibrosis (20). With regard to mode of action, curcumin exhibits a diverse array of metabolic, cellular and molecular activities. Although curcumin analogues exhibit activities very similar to curcumin, their potencies compared to curcumin have not been clearly established. In most systems, curcumin is found to be most potent (21, 22) and in some systems, BDMC was found

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to exhibit different (in some cases, more potent) activities (23-25). There is an increasing demand for comparison study between curcumin and BDMC, due to the discovery of their new biological activities (21, 26, 27). Identification of novel biological activities of curcumin and its analogues is of interest both preclinically and clinically. Additionally, anticoagulant activities of curcumin have not been well studied. Herein, the anticoagulant properties of curcumin and its derivative, BDMC on the generation of FXa and thrombin as well as the regulation of clotting time (PT and aPTT) were determined.

RESULTS

Effects of curcumin and BDMC on aPTT and PT

The anticoagulant properties of curcumin and BDMC were tested in aPTT and PT assays using human plasma and are summarized in Table 1 and 2. Although the anticoagulant activities of curcumin and BDMC were weaker than those of heparin, aPTT and PT were significantly prolonged by curcumin or BDMC at concentrations at or greater than 5 μM. Prolongation of aPTT suggests inhibition of the intrinsic and/or the common pathway while prolonged PT indicates that curcumin and BDMC could also inhibit the extrinsic pathway of coagulation. To confirm these *in vitro* data, *in vivo* tail bleeding time was determined. As shown in Table 1 and 2, tail bleeding time was significantly prolonged by curcumin or BDMC with respect to the control. Surprisingly, effects of curcumin on the clotting time were better than that of BDMC suggesting that methoxy group in curcumin positively regulates the anticoagulant function of curcumin.

Effects of curcumin and BDMC on inactivation of thrombin or FXa

To elucidate the inhibitory mechanism of curcumin and BDMC on coagulation time, their inhibitory effect on thrombin and FXa activities was 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 inhibited by curcumin and BDMC in a dose-dependent manner, showing that the anticoagulant directly inhibited thrombin activity. However, in the presence of AT III, thrombin activity was essentially unchanged (Fig. 1A, B). Thus, AT III was unable to potentiate the activity of curcumin or BDMC. Further, the effects of curcumin and BDMC on FXa activity in the absence or presence of AT III were also investigated. The anticoagulant showed direct inhibitory effects on FXa activities at high concentrations, and the inhibitory effect of AT III was not changed by curcumin or BDMC (Fig. 1C, D). These results were consistent with the antithrombin assay. Therefore, these results suggested that the antithrombotic mechanism of curcumin and BDMC appears to be due to inhibition of fibrin polymerization and/or the intrinsic/extrinsic pathway without potentiation by AT III. Furthermore, the methoxy group in curcumin positively regulates the anticoagulant effects on the inhibition of thrombin or FXa activity because the anticoagulant effects of curcumin were better than those of BDMC.

Table 1. Anticoagulant activity of curcumin[a]

In vitro coagulant assay			
Sample	Dose	aPTT (s)	PT (s)
Control	Saline	36.2 ± 1.2	17.5 ± 0.4
Curcumin	0.1 μM	37.2 ± 1.3	17.4 ± 0.3
	0.5 μM	48.5 ± 1.4[b]	18.2 ± 0.7
	1 μM	52.6 ± 1.8[c]	19.8 ± 0.5[b]
	5 μM	65.3 ± 1.5[c]	21.6 ± 0.6[b]
	10 μM	77.5 ± 2.1[c]	27.5 ± 0.5[c]
	20 μM	91.8 ± 1.5[c]	31.8 ± 0.4[c]
	50 μM	119.8 ± 0.9[c]	35.2 ± 0.4[c]
Heparin		1.5 (μg/ml) >300[c]	15 (μg/ml) 61.5 ± 0.5[c]
In vivo bleeding time			
Sample	Dose	Tail Bleeding time (s)	n
Control	Saline	54.2 ± 8	3
Curcumin	100 mg/kg	102 ± 2[c]	3
Heparin	50 mg/kg	158.6 ± 4[c]	3

[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

Table 2. Anticoagulant activity of BDMC[a]

In vitro coagulant assay			
Sample	Dose	aPTT (s)	PT (s)
Control	Saline	35.8 ± 1.3	17.5 ± 0.4
BDMC	0.1 μM	38.9 ± 0.8	17.5 ± 0.5
	0.5 μM	41.6 ± 1.5[b]	17.9 ± 0.6
	1 μM	48.5 ± 2.14[b]	18.2 ± 0.5[b]
	5 μM	68.5 ± 1.2[c]	19.2 ± 0.7[c]
	10 μM	70.5 ± 1.8[c]	20.9 ± 0.4[c]
	20 μM	87.6 ± 1.5[c]	25.4 ± 0.3[c]
	50 μM	98.6 ± 1.4[c]	29.8 ± 0.5[c]
Heparin		1.5 (μg/ml) >300[c]	15 (μg/ml) 61.5 ± 0.5[c]
In vivo bleeding time			
Sample	Dose	Tail Bleeding time (s)	n
Control	Saline	54.2 ± 8	3
BDMC	100 mg/kg	82 ± 2[b]	3
Heparin	50 mg/kg	158.6 ± 4[c]	3

[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

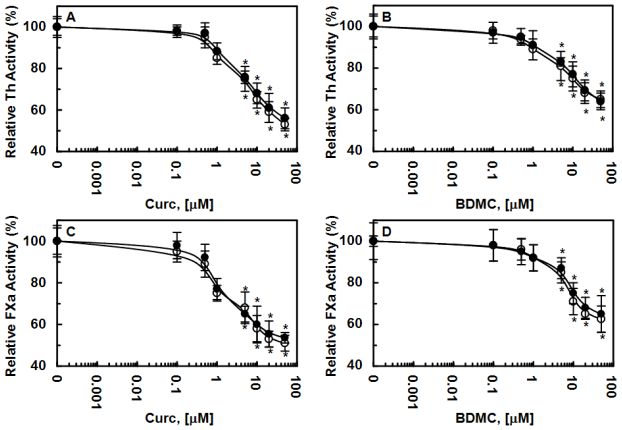

Fig. 1. Effect of curcumin and BDMC on the inactivation of thrombin and factor Xa. Inhibition of thrombin (Th) in the absence of antithrombin III (○) or in the presence of antithrombin III (●) by curcumin (A) or DBMC (B) was monitored by a chromogenic assay as described in "Materials and Methods". Inhibition of factor Xa (FXa) in the absence of antithrombin III (○) or in the presence of antithrombin III (●) by curcumin (C) or DBMC (D) was monitored by chromogenic assay as described in "Materials and Methods". *P < 0.01 as compared to 0.

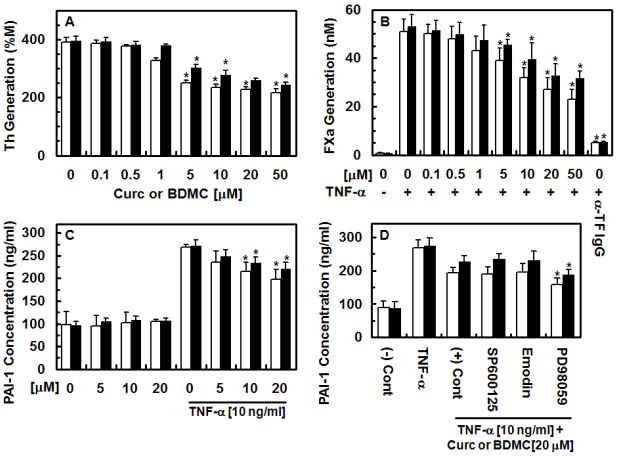

Fig. 2. Inhibition of thrombin and FXa generation by curcumin and BDMC in HUVECs. (A) FVa (100 pM) and FXa (1 nM) were preincubated on HUVECs monolayers for 10 min with indicated concentrations of curcumin (□) or BCMC (■). Prothrombin was added to a final concentration of 1 μM and the amounts of prothrombin activated were determined at 30 min as described in "Materials and Methods". (B) HUVECs were preincubated with indicated concentrations of curcumin (□) or BCMC (■) 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 cultured with curcumin (□) or BCMC (■) in the absence or presence of TNF-α (10 ng/ml) for 18 h and PAI-1 concentration in the culture mediums was examined as described in "Materials and Methods". (D) the same as C except that cells were preincubated with curcumin (□) or BCMC (■) and cultured with SP600125 (2 μM), emodin (2 μg/ml), and PD98059 (10 μM) in presence of TNF-α (10 ng/ml) for 18 h. *P < 0.05 as compared to 0 (A) or TNF-α alone (B, C) or to (+) control (D).

Effects of curcumin and BDMC on the generation of thrombin and FXa

Sugo *et al*. reported that endothelial cells are able to support prothrombin activation by FXa (28). Preincubation of FVa and FXa in the presence of $CaCl_2$ with HUVECs before addition of prothrombin resulted in thrombin generation (Fig. 2A). The effect of curcumin and BDMC on thrombin generation showed that curcumin and BDMC inhibited thrombin activation of prothrombin dose- dependently (Fig. 2A). Rao *et al*. showed that the endothelium provides the functional equivalent of procoagulant phospholipids and supports FX activation (29) and that in TNF-α stimulated HUVECs, FVIIa could activate FX, which was completely dependent on TF expression (30). Thus, it is likely that the endothelium can provide support for FVIIa activation of FX. If so, it would be of interest to investigate the effect of curcumin and BDMC on FVIIa activation of FX. HUVECs were stimulated with TNF-α to induce TF expression. As shown in Fig. 2B, the rate of FX activation by FVIIa was 100-fold higher in stimulated HUVECs (53.3 ± 5 nM) compared with non-stimulated HUVECs (0.54 ± 0.2 nM), which was completely attenuated by anti-TF IgG (4.8 ± 0.4 nM). Moreover, preincubation with curcumin or BDMC dose-dependently inhibited FVIIa activation of FX (Fig. 2B). Therefore, these results suggested that curcumin could inhibit the generation of thrombin or FXa and the methoxy group in curcumin positively regulated these functions of curcumin.

Because plasminogen activator inhibitor type 1 (PAI-1) determines fibrinolytic activity (1), the effect of TNF-α and curcumin on PAI-1 secretion from HUVECs were investigated. As shown in Fig. 2C, curcumin dose dependently inhibited TNF-α induced PAI-1 secretion from HUVECs. To define the molecular targets of curcumin in the signal transduction pathways leading to TNF-α induced PAI-1 expression, we investigated the effects of three signal transduction inhibitors, emodin (a NF-κB inhibitor), PD98059 (an extracellular signal regulated kinase, ERK, inhibitor), and SP600125 (a c-Jun N-terminal kinase, JNK, inhibitor) on TNF-α induced PAI-1 expression in the presence or absence of curcumin. Experiments performed showed that neither SP600125 nor emodin showed any additional inhibitory effects in the presence of curcumin (Fig. 2D). However, the inhibitory effects of PD98059 were essentially additive with those of curcumin (Fig. 2D).

DISCUSSION

The vascular endothelium provides a number of important functions in order to maintain adequate blood supply to vital organs. These functions include prevention of coagulation, regulation of vascular tonus, orchestration of the migration of blood cells by the expression of adhesion molecules and regu-

lation of vasopermeability (31). Among these, regulation of hemostatic activity was regulated through a balance of pro- and anticoagulant properties (1). Impaired endothelial function causes thrombus-related complications including myocardial infarction, stroke and thromboembolism (32). In this study, we presented curcumin as a potent anticoagulant by inhibitiing thrombin or FXa. The anticoagulant activity of curcumin was evidenced by the prolongation of the clotting time in plasma-based PT and APTT assays. Additionally, the inhibitory effects by curcumin on FXa generation and further thrombin generation support the anticoagulant activities of curcumin.

It is well known that FXa has no effect on platelet activation, however, once it is assembled into the prothrombinase complex, it triggers enormous amounts of thrombin (2, 5). Thrombin is the final enzyme in the blood clotting cascade responsible for clot formation and platelet activation (2, 5). Based on the results that curcumin could inhibit generation of FXa and thrombin, the anticoagulant activity of curcumin was initiated from the inhibition of the penultimate and final enzyme in the blood clotting cascade.

TNF-α has been known to activate JNK, NF-κB, and ERK in human endothelial cells (33-35). Here, we used a JNK (SP600125), NF-κB (emodin), and ERK (PD98059) inhibitors to define the molecular target of curcumin. We observed that PD98059, but not emodin or SP600125, was additive to the inhibitory effects of curcumin on TNF-α induced PAI-1 secretion. These results suggest that the NF-κB and JNK pathway are involved in curcumin mediated inhibition of TNF-α induced PAI-1 expression in HUVECs. Thus, these results seem to indicate that curcumin decreases PAI-1 levels via inhibition of the NF-κB and JNK pathways.

Noting that the effects of curcumin on the anticoagulant activity was better than BDMC, it suggests that the *ortho*-methoxy group in curcumin positively regulates anticoagulant functions of curcumin. In a previous report, curcumin and BDMC had different redox properties due to the presence of the *ortho*-methoxy group in position 3 of the phenyl moiety in curcumin. (36) While curcumin has two symmetric *ortho*-methoxy phenols linked through the a,b-unsaturated b-diketone moiety, BDMC, which is also symmetric, is deficient in the two *ortho*-methoxy substitutions. Although curcumin and bisdemethoxycurcumin differ in their chemical structures only with regard to the *ortho*-methoxy substitution, they exhibit significantly different antioxidant, antitumor, and antiinflammatory activities. The hydrogen bonding interaction between the phenolic OH and the *ortho*-methoxy groups in curcumin markedly influences the O-H bond energy and H-atom abstraction by free radicals, thus making it a better free radical scavenger than BDMC (37). In another investigation, the *ortho*-methoxy- deficient BDMC was a more potent ROS inducer and the *ortho*-methoxy substituted curcumin was a more potent suppressor of NF-kB activation (38). According to our results, the *ortho*-methoxy group in curcumin is important for the anticoagulant effect. Thus, we can postulate that the anticoagulant activities of curcumin could be mainly caused by interaction of the target molecules with the *ortho*-methoxy group.

The significant progress made in understanding the role of FXa and thrombin in various thrombotic disease states has clearly demonstrated potential therapeutic benefits of blocking these key enzymes in the blood coagulation cascade (39). A potent and selective small molecule FXa or thrombin inhibitor has the potential to offer substantial therapeutic benefits (39). Curcumin exhibits the potency and selectivity required for such a candidate and is currently undergoing additional evaluations.

In conclusion, this study showed that curcumin inhibited the extrinsic and intrinsic pathways of blood coagulation by inhibiting FXa and thrombin generation in HUVECs. These results adds to previous work and may be helpful for the rational design of pharmacological strategies for treating or preventing vascular diseases via regulation of thrombin generation.

MATERIALS AND METHODS

Reagents

Curcumin (product catalog #: C2302) and bisdemethoxycurcumin (product catalog #: B3347) were purchased from TCI Korea (Tokyo Chemical Industry Co., Ltd. Seoul, South Korea). TNF-α, JNK inhibitor (SP600125), NF-κB inhibitor (emodin), and ERK inhibitor (PD98059) were purchased from R&D Systems (Minneapolis, MN). Anti-tissue factor antibody was purchased from Santa Cruz Biologics (Santa Cruz, CA). Factor V, Vll, 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).

Anticoagulation assay

Determination of aPTT and PT were performed according to the manufacture's specifications using Thrombotimer (Behnk Elektronik, Germany). In brief, citrated normal human plasma (90 μl) was mixed with 10 μl of curcumin or BDMC 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 a curcumin or BDMC stock and incubated for 1 min at 37°C. Then, PT assay reagent (200 μl), preincubated for 10 min at 37°C, was added and the clotting time was recorded.

Cell culture

Primary HUVECs were obtained from Cambrex Bio Science (Charles City, IA) and maintained as described before (40). Briefly, cells were cultured to confluency at 37°C at 5% CO_2 in EBM-2 basal media supplemented with growth supplements (Cambrex Bio Science).

Factor Xa generation on the surface of HUVECs

HUVECs were preincubated with indicated concentrations of curcumin or BDMC 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 surface of HUVECs

HUVECs were preincubated in 300 μl containing curcumin or BDMC 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. 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 in each well to terminate prothrombin activation. Activated prothrombin was determined by measuring the rate of hydrolysis of S2238 measured at 405 nm. Dilutions with known amounts of purified thrombin were used for standard curves.

Thrombin activity assay

Curcumin or BDMC in 50 mM Tris–HCl buffer, pH 7.4 containing 7.5 mM EDTA and 150 mM NaCl was mixed in the absence or presence with 150 μl of AT III (200 nM). After the mixture was incubated at 37°C for 2 min, thrombin solution (150 μl; 10 U/ml) was 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 at 405 nm was monitored for 120 s with a spectrophotometer (TECAN, Switzerland).

Factor Xa (FXa) activity assay

These assays were performed similar to the thrombin activity assay. Instead of thrombin and S-2238, factor Xa (1 U ml/1) and substrate S-2222 were used.

ELISA for PAI-1

The concentrations of PAI-1 in HUVEC cultured supernatants were determined by ELISA methods, according to the manufacturer's recommended protocol (American Diagnostica Inc., Stamford, CT, USA).

Effect on bleeding time

The tail transection bleeding time was determined according to the method of Dejana *et al.* (41) Male C57BL/6 mice were fasted overnight and curcumin or BDMC was administered orally to mice. One hour after administration, the mouse tail was transected at 2 mm from the tip. Bleeding time was measured as time elapsed until bleeding stopped. When bleeding time lasted longer than 15 min, measurement was stopped and bleeding time was recorded as 15 min for statistical analyses.

Statistical analysis

Data are expressed as the means ± standard deviation of at least three independent experiments. Statistical significance between two groups was determined by a Student's *t*-test. The significance level was set at $P < 0.05$.

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