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, anti-proliferative 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 cascadia 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 phospholipid (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 ailments (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 bisde-methoxycurcumin (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...
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

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dose</th>
<th>aPTT (s)</th>
<th>PT (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Saline</td>
<td>36.2 ± 1.2</td>
<td>17.5 ± 0.4</td>
</tr>
<tr>
<td>Curcumin</td>
<td>0.1 μM</td>
<td>37.2 ± 1.3</td>
<td>17.4 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>0.5 μM</td>
<td>48.3 ± 1.4</td>
<td>18.2 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>1 μM</td>
<td>52.6 ± 1.8</td>
<td>19.8 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>5 μM</td>
<td>65.3 ± 1.5</td>
<td>21.6 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>10 μM</td>
<td>77.5 ± 2.1</td>
<td>27.5 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>20 μM</td>
<td>91.8 ± 1.5</td>
<td>31.8 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>50 μM</td>
<td>119.8 ± 0.9</td>
<td>35.2 ± 0.4</td>
</tr>
<tr>
<td>Heparin</td>
<td>1.5 (μg/ml)</td>
<td>15 (μg/ml)</td>
<td>61.5 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>&gt;300</td>
<td>18.2 ± 0.5</td>
<td>17.4 ± 0.3</td>
</tr>
</tbody>
</table>

In vitro coagulant assay

Table 2. Anticoagulant activity of BDMC

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dose</th>
<th>aPTT (s)</th>
<th>PT (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Saline</td>
<td>35.8 ± 1.3</td>
<td>17.5 ± 0.4</td>
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<tr>
<td>BDMC</td>
<td>0.1 μM</td>
<td>38.9 ± 0.8</td>
<td>17.5 ± 0.5</td>
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<tr>
<td></td>
<td>0.5 μM</td>
<td>41.6 ± 1.5</td>
<td>17.9 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>1 μM</td>
<td>48.5 ± 2.1</td>
<td>18.2 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>5 μM</td>
<td>68.5 ± 1.2</td>
<td>19.2 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>10 μM</td>
<td>70.5 ± 1.6</td>
<td>20.9 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>20 μM</td>
<td>87.6 ± 1.5</td>
<td>25.4 ± 0.3</td>
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<td></td>
<td>50 μM</td>
<td>98.6 ± 1.4</td>
<td>29.8 ± 0.5</td>
</tr>
<tr>
<td>Heparin</td>
<td>1.5 (μg/ml)</td>
<td>15 (μg/ml)</td>
<td>61.5 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>&gt;300</td>
<td>17.9 ± 0.6</td>
<td>17.5 ± 0.5</td>
</tr>
</tbody>
</table>

In vitro coagulant assay

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dose</th>
<th>Tail Bleeding time (s)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Saline</td>
<td>54.2 ± 8</td>
<td>3</td>
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<tr>
<td>Curcumin</td>
<td>100 mg/kg</td>
<td>702 ± 2</td>
<td>3</td>
</tr>
<tr>
<td>Heparin</td>
<td>50 mg/kg</td>
<td>158.6 ± 4</td>
<td>3</td>
</tr>
</tbody>
</table>

In vivo bleeding time

*Each value represents the means ± SD (n = 5). \(^{\text{a}}\)P < 0.05 as compared to control. \(^{\text{b}}\)P < 0.01 as compared to control.
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 CaCl2 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-
loration of vasopermeability (31). Among these, regulation of he- 
mostatic 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 inhibiting 
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 ef-
fects 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 com-
plex, it triggers enormous amounts of thrombin (2, 5). Throm-
bin is the final enzyme in the blood clotting cascade respon-
sible 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 in-
hibitory effects of curcumin on TNF-α induced PAI-1 secretion. 
These results suggest that the NF-κB and JNK pathway are in-

duced in curcumin mediated inhibition of TNF-α induced 
PAI-1 expression in HUVECs. Thus, these results seem to in-
dicate that curcumin decreases PAI-1 levels via inhibition of the 
NF-κB and JNK pathways.

Noting the effects of curcumin on the anticoagulant ac-
tivity 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-met-
hoxy group in position 3 of the phenyl moiety in curcumin. 
(36) While curcumin has two symmetric ortho-methoxy phe-
nols linked through the a,b-unsaturated b-diketone moiety, 
BDMC, which is also symmetric, is deficient in the two or-
tho-methoxy substitutions. Although curcumin and bisdemeth-
xyoxycurcumin differ in their chemical structures only with re-
gard to the ortho-methoxy substitution, they exhibit signifi-
cantly different antioxidant, antitumor, and antiinflammatory 
activities. The hydrogen bonding interaction between the phe-
nolic 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-defi-
cient BDMC was a more potent ROS inducer and the ortho-met-
hoxy substituted curcumin was a more potent suppressor of 
NF-κB activation (38). According to our results, the ortho-mete-
hoxy 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 inhib-
iting FXa and thrombin generation in HUVECs. These re-

sults add 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 bisdemethoxycurcu-
min (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, VII, VIIIa, FX, FXa, antithrombin III (AT III), prothrombin 
and thrombin were obtained from Haemalogistic Technologies 
(Essex Junction, VT, USA). aPTT assay reagent and PT reagents 
were purchased from Fisher Diagnostics (Middletown, Virgini-
a, USA). Chromogenic substrates S-2222, and S-2238 were 
purchased 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 incu-
bated 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 CaCl2 (100 μl) was added to the mixture 
and incubated for 1 min at 37°C. Then, aPTT assay reagent (200 μl), 
preincubated for 10 min at 37°C, 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% CO2 
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) and thrombin activation 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 generation on the surface of HUVECs

HUVECs were preincubated 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.

Acknowledgements

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REFERENCES

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