

Antiplatelet Activity of Caffeic Acid Phenethyl Ester Is Mediated through a Cyclic GMP-Dependent Pathway in Human Platelets

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Abstract

The aim of this study was to examine the inhibitory mechanisms of caffeic acid phenethyl ester (CAPE), which is derived from the propolis of honeybee, in platelet activation. In this study, CAPE (15 and 25 μM) markedly inhibited platelet aggregation stimulated by collagen (2 $\mu\text{g}/\text{ml}$). CAPE (15 and 25 μM) increased cyclic GMP level, and cyclic GMP-induced vasodilator-stimulated phosphoprotein (VASP) Ser¹⁵⁷ phosphorylation, but did not increase cyclic AMP in washed human platelets. Rapid phosphorylation of a platelet protein of *Mw.* 47,000 (P47), a marker of protein kinase C activation, was triggered by phorbol-12, 13-dibutyrate (150 nM). This phosphorylation was markedly inhibited by CAPE (15 and 25 μM). The present study reports a novel and potent antiplatelet agent, CAPE, which involved in the following inhibitory pathways: CAPE increases cyclic GMP/VASP Ser¹⁵⁷ phosphorylation, and subsequently inhibits protein kinase C activity, resulting in inhibition of P47 phosphorylation, which ultimately inhibits platelet aggregation. These results strongly indicate that CAPE appears to represent a novel and potent antiplatelet agent for treatment of arterial thromboembolism.

Key Words: caffeic acid phenethyl ester, cyclic GMP, vasodilator-stimulated phosphoprotein, protein kinase C

Introduction

Caffeic acid phenethyl ester (CAPE) (Fig. 1) is an active component of propolis obtained from honeybee hives. It has anti-inflammatory, anti-viral, anti-mitogenic, anti-carcinogenic, and immunomodulatory effects (10, 18). It has also been reported that CAPE exhibits antioxidant activity and inhibits lipoxigenase activities, protein tyrosine kinase, and NF κ B activation (9, 18). The possibility that CAPE displays pharmacological activity by inhibiting the release of arachidonic acid and the enzyme activities of cyclooxygenase (COX)-I

and COX-II was also suggested (18).

Intravascular thrombosis is one of the generators of a wide variety of cardiovascular diseases. The initiation of an intraluminal thrombosis is believed to involve platelet adherence and aggregation (14, 16). In normal circulation, platelets cannot aggregate by themselves. However, when a blood vessel is damaged, platelets adhere to the disrupted surface, and the adherent platelets release some biologically active constituents which induce aggregation (12). Thus, platelet aggregation may play a crucial role in the atherothrombotic process. Indeed, antiplatelet

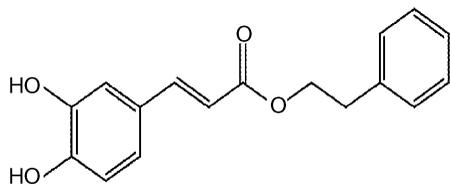


Fig. 1. Chemical structure of caffeic acid phenethyl ester (CAPE).

agents (*e.g.*, ticlopidine and aspirin) have been shown to reduce the incidence of stroke in high-risk patients (11). However, the pharmacological function of CAPE in platelets has not yet been studied, and no data are available concerning the detailed effects of CAPE in platelet aggregation. In the present study, we first report that CAPE (15 μM) possesses potent inhibitory activity against platelet aggregation in washed human platelets.

Materials and Methods

Materials

CAPE, collagen (type I, bovine achilles tendon), EDTA, prostaglandin E_1 (PGE_1), arachidonic acid, nitroglycerin, phorbol-12, 13-dibutyrate (PDBu), apyrase, heparin, phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin, NaF, and bovine serum albumin (BSA) were purchased from Sigma Chem. (St. Louis, MO, USA). Cyclic AMP and cyclic GMP EIA kits were purchased from Cayman (Ann Arbor, MI, USA). The Hybond-P PVDF membrane, ECL Western blotting detection reagent and analysis system, horseradish peroxidase-conjugated goat anti-rabbit Ig, and goat anti-mouse Ig were purchased from Amersham (Buckinghamshire, UK). CAPE was dissolved in dimethyl sulfoxide (DMSO) and stored at -4°C until use.

Preparation of Human Platelet Suspensions

Human platelet suspensions were prepared as previously described (15) with some modifications. In this study, human volunteers gave informed consent. In brief, blood was collected from healthy human volunteers who had taken no medicine during the preceding 2 weeks, and was mixed with acid/citrate/glucose. After centrifugation at $120 \times g$ for 10 min at room temperature, the supernatant (platelet-rich plasma) was supplemented with heparin (6.4 IU/ml), EDTA (2 mM) and apyrase (1 U/ml), then incubated for 10 min at 37°C and centrifuged at $500 \times g$. The washed platelets were finally suspended in Tyrode's solution containing BSA (3.5 mg/ml). The final concentration of Ca^{2+} in Tyrode's solution was 1 mM.

Platelet Aggregation

The turbidimetric method was applied to measure platelet aggregation, using a Lumi-Aggregometer (Payton, Scarborough, Ontario, Canada). Platelet suspensions (3.6×10^8 platelets/ml, 0.4 ml) were pre-warmed to 37°C for 2 min, and then CAPE (15 and 25 μM) was added 3 min before the addition of the collagen (2 $\mu\text{g}/\text{ml}$). The reaction was allowed to proceed for at least 6 min, and the extent of aggregation was expressed in light-transmission units. While measuring ATP release, 20 μl of a luciferin/luciferase mixture was added 1 min before the addition of collagen, and ATP release was compared with that of the control.

Measurement of Cyclic AMP and Cyclic GMP Formations

Platelet suspensions ($3.6 \times 10^8/\text{ml}$) were incubated with isovolumetric solvent control (0.5% DMSO), nitroglycerin (10 μM), PGE_1 (10 μM), or CAPE (15 and 25 μM) for 6 min. The incubation was stopped by the addition of EDTA (5 mM), and the solution was immediately boiled for 5 min. Fifty microliters of the supernatant was used to determine the cyclic AMP and cyclic GMP contents with EIA kits following acetylation of the samples as described by the manufacturer.

Western Blot Analysis of Vasodilator-Stimulated Phosphoprotein (VASP) Phosphorylation

The method of Hsiao *et al.* (2) was followed. In brief, washed platelets ($1.2 \times 10^9/\text{ml}$) were incubated with solvent control (0.5% DMSO), nitroglycerin (10 μM) or CAPE (15 and 25 μM) for 3 min in the presence or absence of ODQ (20 μM) (20). The reaction was terminated by the addition of EDTA (10 mM), platelet lysates were analyzed by SDS-PAGE gel (10%) and electrotransferred to PVDF membranes. The membranes were blocked with 5% nonfat dry milk in Tris (tris(hydroxymethyl)-aminomethane)-buffered saline (TBS, pH 7.5), incubated with the monoclonal antibody 5C6 (Calbiochem, Merck KGaA, Darmstadt, Germany), specific for the phosphorylated Ser¹⁵⁷ site of VASP (0.1 $\mu\text{g}/\text{ml}$). After three washes in TBS containing 0.05% Tween 20, the membrane was incubated with peroxidase-conjugated goat anti-mouse IgG (Amersham) for 2 h. The band with peroxidase activity was detected by enhanced chemiluminescence detection reagents (ECL⁺ system; Amersham). The bar graph depicts the ratios of quantitative results obtained by scanning reactive bands and quantifying the optical density using Videodensitometry (Bio-1D version 99 image software).

Measurement of Protein Kinase C (PKC) Activity

Washed platelets ($1.2 \times 10^9/\text{ml}$) were preincubated

with solvent control (0.5% DMSO) or CAPE (15 and 25 μM) for 3 min, then PDBu (150 nM) was added for 1 min to trigger protein kinase C (PKC) activation. Activation was terminated by the addition of Laemmli sample buffer, and analyzed by electrophoresis (12.5%) and electrotransferred to PVDF membranes. The membranes were blocked with 5% nonfat dry milk in Tris (tris(hydroxymethyl)-aminomethane)-buffered saline (TBS, pH 7.5), incubated with the polyclonal antibody raised against phospho-PKC (S1) (BD, Transduction Lab. San Diego, CA, USA) (1: 200 in TBS). After three washes in TBS containing 0.05% Tween 20, the membrane was incubated with peroxidase-conjugated goat anti-rabbit IgG (Amersham) for 2 h. The band with peroxidase activity was detected by enhanced chemiluminescence detection reagents (ECL⁺ system; Amersham). The bar graph depicts the ratios of quantitative results obtained by scanning reactive bands and quantifying the optical density using Videodensitometry (Bio-1D version 99 image software).

Statistical Analysis

The experimental results are expressed as the means \pm S.E.M. and are accompanied by the number of observations. Data were assessed using analysis of variance (ANOVA). If this analysis indicated significant differences among the group means, then each group was compared using the Newman-Keuls method. A *P* value of < 0.05 was considered statistically significant.

Results

Effects of CAPE on Platelet Aggregation in Human Platelets

CAPE (15 and 25 μM) markedly inhibited platelet aggregation stimulated by collagen (2 $\mu\text{g}/\text{ml}$) in washed human platelets (Fig. 2) and platelet-rich plasma (data not shown). The IC_{50} values of CAPE for platelet aggregation induced by collagen were estimated to be approximately 14 μM in washed platelets. CAPE (15 and 25 μM) also inhibited the ATP-release reaction when stimulated by agonists (*i.e.*, collagen) (Fig. 2). In addition, the solvent control (0.5% DMSO) did not significantly affect platelet aggregation stimulated by collagen in either washed platelets or platelet-rich plasma (data not shown). In addition, platelets were preincubated with a higher concentration of CAPE (100 μM) or normal saline for 10 min, followed by 2 washes with Tyrode's solution, we found that there were no significant differences between the aggregation curves of either platelet preparations stimulated by collagen (2 $\mu\text{g}/\text{ml}$), indicating that the effect of CAPE on inhibition

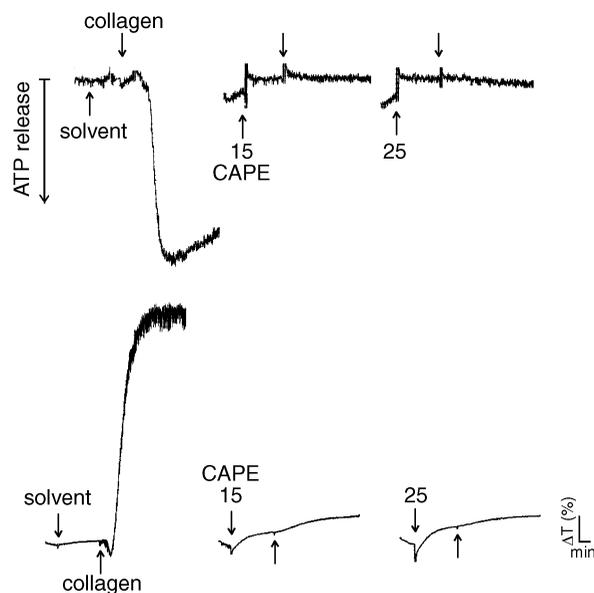


Fig. 2. Tracing curves of CAPE on collagen-induced platelet aggregation in washed human platelets. Platelets ($3.6 \times 10^8/\text{ml}$) were preincubated with isovolumetric solvent control (0.5% DMSO) or CAPE (15 and 25 μM) for 3 min, collagen (2 $\mu\text{g}/\text{ml}$) was then added to trigger platelet aggregation (lowering tracings) and ATP release (upper tracings). The curves are representative examples of four similar experiments.

of platelet aggregation occurs in a reversible manner (data not shown).

Effects of CAPE on Cyclic AMP and Cyclic GMP Formations

The level of cyclic AMP in unstimulated platelets was lower, and the addition of PGE_1 (10 μM) markedly increased approximately 15-fold of cyclic AMP level compared with the resting group (Fig. 3). CAPE (15 and 25 μM) did not significantly increase the cyclic AMP levels in human platelets (Fig. 3). We also performed a similar study measuring the cyclic GMP response. The level of cyclic GMP in unstimulated platelets was about 0.4 ± 0.1 nM, but when nitroglycerin (NTG, 10 μM) was added to the platelet suspensions, the cyclic GMP level markedly increased from the resting level to 1.0 ± 0.1 nM (Fig. 4A). The addition of CAPE (15 and 25 μM) resulted in significant increases in platelet cyclic GMP levels (15 μM , 0.9 ± 0.2 nM; 25 μM , 1.1 ± 0.3 nM; $n = 5$) (Fig. 4A). Furthermore, in the presence of ODQ (20 μM), a guanylate cyclase inhibitor (10), markedly reversed the inhibitory effect of CAPE (5 μM) in collagen (2 $\mu\text{g}/\text{ml}$)-induced platelet aggregation in washed platelets (Fig. 4B), indicating that CAPE inhibits platelet aggregation, at least in part, *via* a cyclic GMP-dependent pathway.

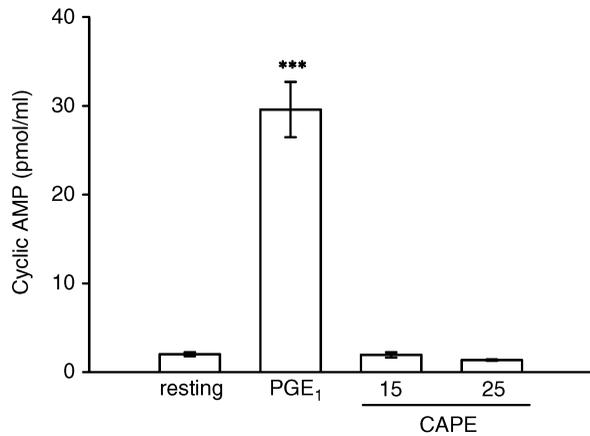


Fig. 3. Effect of CAPE on cyclic AMP formation in washed human platelets. Washed human platelets (3.6×10^8 /ml) were incubated with solvent control (0.5% DMSO), prostaglandin E₁ (PGE₁), or CAPE (15 and 25 μ M) for 3 min at 37°C, and this reaction were stopped by the addition of EDTA (5 mM). Addition of PGE₁ into the platelet suspensions served as positive control of cyclic AMP. Data are presented as the means \pm S.E.M. ($n = 5$). *** $P < 0.001$ compared with the resting group (solvent control).

Effects of CAPE on Vasodilator-Stimulated Phosphoprotein (VASP) Phosphorylation

It is presumed that cyclic GMP can induce VASP Ser¹⁵⁷ phosphorylation in human platelets (5). In this study, nitroglycerin (NTG, 10 μ M) markedly induced VASP Ser¹⁵⁷ phosphorylation, and this phosphorylation was significantly inhibited by the ODQ (20 μ M) (Fig. 5). CAPE (15 and 25 μ M) markedly triggered VASP Ser¹⁵⁷ phosphorylation, and this phosphorylation was also inhibited in the presence of ODQ (20 μ M) (Fig. 5).

Effects of CAPE on PDBu-Stimulated 47-kDa Protein Phosphorylation

Stimulation of platelets with a number of different agonists, PDBu in particular, induces activation of PKC, which then phosphorylates proteins of M_w . 40,000-47,000 in addition to other proteins (17). In this study, phosphorylation experiments were performed to examine the role of CAPE in the activation of PKC in human platelets. When PDBu (150 nM) was added to washed human platelets, a protein with an apparent M_w . of 47,000 (P47) was predominately phosphorylated as compared with resting platelets (Fig. 6). CAPE (15 and 25 μ M) markedly inhibited the phosphorylation of P47 stimulated by PDBu (Fig. 6). In addition, ODQ (20 μ M) significantly reversed the inhibitory effect of CAPE (25 μ M) in P47 phosphorylation stimulated by PDBu (150 nM) (data not shown). These results indicate

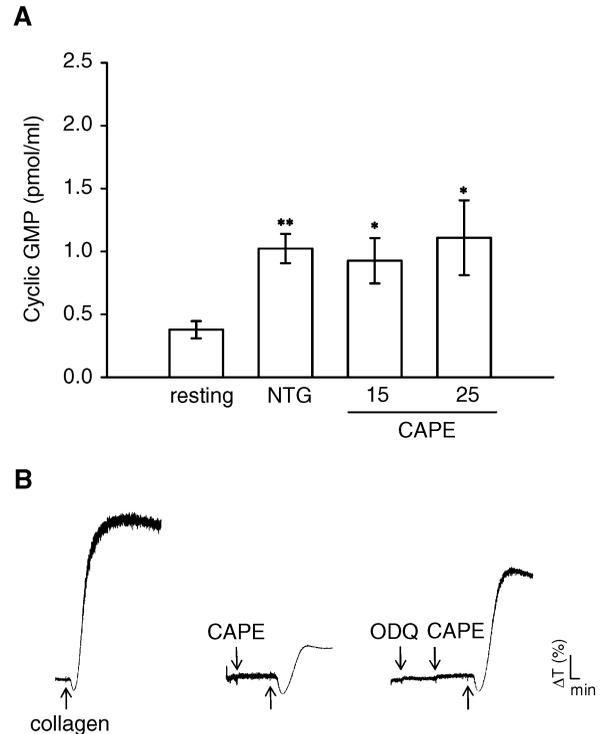


Fig. 4. Effects of (A) CAPE on cyclic GMP formation and (B) ODQ in CAPE-mediated antiplatelet activity in washed human platelets. (A) Washed human platelets (3.6×10^8 /ml) were incubated with solvent control (0.5% DMSO), nitroglycerin (NTG), or CAPE (15 and 25 μ M) for 3 min at 37°C, and this reaction were stopped by the addition of EDTA (5 mM). Addition of NTG into the platelet suspensions served as positive control of cyclic GMP. (B) For another experiment, human platelets (3.6×10^8 /ml) were incubated with CAPE (5 μ M) with or without ODQ (20 μ M) for 3 min, collagen (2 μ g/ml) was then added to trigger platelet aggregation. Data (A) are presented as the means \pm S.E.M. ($n = 5$). * $P < 0.05$ and ** $P < 0.01$ compared with the resting group (solvent control). The curves (B) are representative examples of three similar experiments.

that CAPE can directly interfere with the PKC activation in human platelets.

Discussion

This study demonstrates for the first time that CAPE possesses potent antiplatelet activity in human platelets. The principal objective of this study was to describe the inhibitory mechanisms of CAPE in platelet activation. In this study, platelet aggregation induced by collagen appeared to be affected in the presence of CAPE. The activation of human platelets is inhibited by two intracellular pathways regulated by either cyclic AMP or cyclic GMP (19). The importance of cyclic AMP in modulating platelet reactivity is well established (3). In addition to inhibiting most platelet responses,

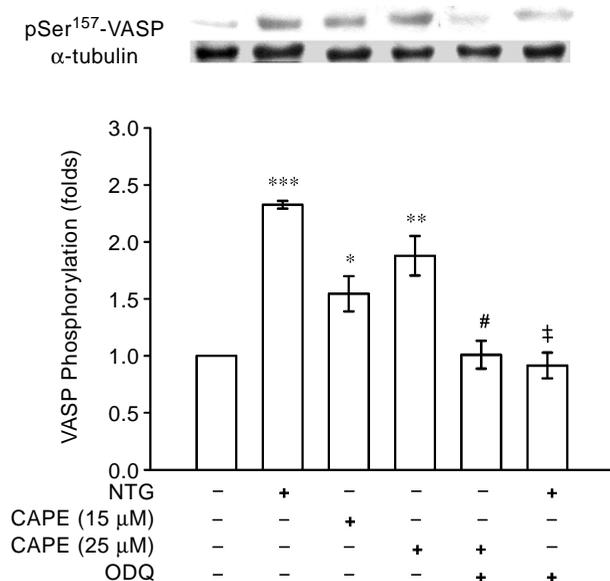


Fig. 5. Effect of CAPE on agonist-induced phosphorylation of vasodilator-stimulated phosphoprotein (VASP) at Ser¹⁵⁷ in human platelets. Platelets (1.2×10^9 /ml) were incubated with solvent control (0.5% DMSO), nitroglycerin (NTG, 10 μM) or CAPE (15 and 25 μM) in the absence or presence of ODQ (20 μM), and solubilized directly in SDS-PAGE sample buffer. Phosphorylation of VASP at Ser¹⁵⁷ was detected by immunoblotting with a monoclonal antibody specifically recognizing Ser¹⁵⁷-phosphorylated VASP. Profiles are representative examples of three similar experiments. The bar graph depicts the ratios of quantitative results obtained by scanning the reactive bands of anti-pSer¹⁵⁷-VASP against anti-α-tubulin and quantifying the optical density using Bio-1D version 99 image software. Data are presented as the means ± S.E.M. ($n = 3$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with the resting group (solvent control); # $P < 0.05$ compared with the CAPE (25 μM) group; ‡ $P < 0.001$ compared with the NTG group.

elevated levels of cyclic AMP decrease intracellular Ca²⁺ concentration by the uptake of Ca²⁺ into the dense tubular system (13) and negatively affects the action of PKC (13). The present data suggest that the antiplatelet activity of CAPE is independent of the increased level of cyclic AMP in human platelets.

On the other hand, signaling by cyclic GMP somehow interferes with the agonist-stimulated phosphoinositide turnover that creates Ca²⁺-mobilizing second messengers (7). Furthermore, we also found that CAPE (25 μM) significantly stimulated NO production after being incubated with washed platelets (data not shown). This study suggests that antiplatelet activity of CAPE is due to its effect of increasing the level of NO/cyclic GMP, because a rise in platelet cyclic GMP blocks several stimulatory pathways in platelets. For example, PKC is a key enzyme in signal

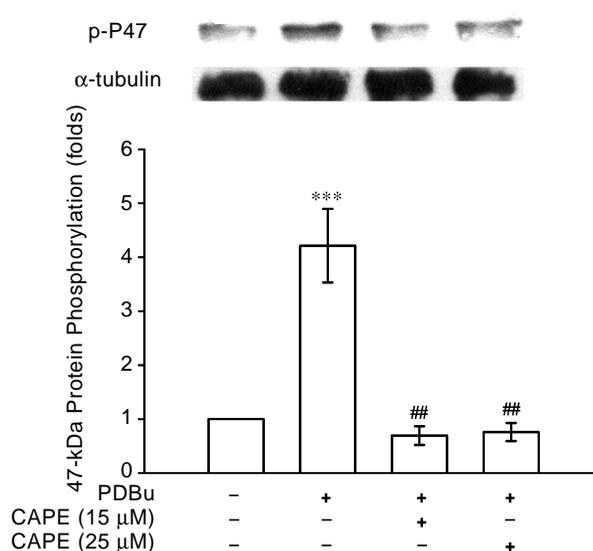


Fig. 6. Effect of CAPE on PDBu-stimulated phosphorylation of a protein of M_w . 47,000 (P47) in human platelets. Platelets (1.2×10^9 /ml) were preincubated with solvent control (0.5% DMSO) or CAPE (15 and 25 μM) before challenge with PDBu (150 nM). Profiles are representative examples of three similar experiments. The bar graph depicts the ratios of quantitative results obtained by scanning the reactive bands of anti-p-P47 against anti-α-tubulin and quantifying the optical density using Bio-1D version 99 image software. Data are presented as the means ± S.E.M. ($n = 3$). *** $P < 0.001$ compared with the resting group (solvent control); ## $P < 0.01$ compared with the PDBu group.

transduction systems involving the breakdown of phospholipids (13). In platelets, PKC plays an important role in the induction of the aggregatory and secretory responses as well as in preventing a continuous activation of the platelet excitatory signal transducing system (13, 19). Elevation of platelet cyclic GMP that leads to inhibition of platelet activation appears to interact with the excitatory pathway at the level of PKC (8). In this study, activation of PKC in human platelets occurred in response to PDBu, which is known to intercalate with membrane phospholipids and form a complex with PKC translocated to the membrane (4). Therefore, based on the above observation, we suggest that the inhibitory effect of CAPE in PDBu-induced PKC activation may be due, at least in part, to mediating the increase in cyclic GMP. On the other hand, it was recently reported that cyclic GMP-dependent protein kinase also plays a stimulatory role in mediating aggregation-dependent platelet secretion and secretion-dependent second wave platelet aggregation (6). However, its detailed mechanism still remains obscure and needs to be further study is needed.

In addition, the VASP is associated with actin filaments and focal adhesions, which form the interface between the cytoskeleton and the extracellular matrix. VASP is phosphorylated by both cyclic GMP- and cyclic AMP-dependent protein kinases in a variety of cells, including smooth muscle cells and platelets. Since both the cyclic GMP and cyclic AMP signaling cascades relax smooth muscle cells and inhibit platelet activation, it was speculated that VASP plays an important role in modulating actin filament dynamics and integrin activation (1). In this study, we found that CAPE significantly increased both cyclic GMP- and cyclic AMP-induced VASP Ser¹⁵⁷ phosphorylation in human platelets.

In conclusion, the most important findings of this study suggest that CAPE exhibits a potent activity at inhibiting collagen-induced platelet aggregation. This inhibitory effect of CAPE may possible involve the following mechanisms: CAPE increases cyclic GMP formation, which activates the cyclic GMP-dependent VASP Ser¹⁵⁷ phosphorylation, and subsequently inhibits PKC activity, ultimately resulting in inhibition of P47 phosphorylation and finally inhibition of platelet aggregation. However, we also did not rule out the possibility of other mechanisms involving in CAPE-inhibited platelet aggregation. Platelet aggregation plays a pathophysiological role in a variety of thromboembolic disorders. Therefore, inhibition of platelet aggregation by drugs may represent an increased therapeutic possibility for such diseases. This study suggests that CAPE may represent a novel and potent antiplatelet agent for treatment of arterial thromboembolism.

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