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Characterization of a novel and potent collagen antagonist, caffeic acid phenethyl ester, in human platelets: In vitro and in vivo studies

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Abstract

Objective: Caffeic acid phenethyl ester (CAPE), which is derived from the propolis of honeybee hives, has been demonstrated to possess multiple pharmacological activities. In the present study, CAPE (6–25 μM) specifically inhibited collagen-induced platelet aggregation and the ATP release reaction in platelet suspensions.
Methods: Platelet aggregation, flow cytometric analysis, immunoblotting, and electron spin resonance (ESR) were used to assess the anti-platelet activity of CAPE. Fluorescein sodium-induced platelet thrombi in mesenteric microvessels of mice were used for an in vivo study.
Results: CAPE (15–100 μM) produced a concentration-related rightward displacement of the collagen concentration–response curve, and the Schild plot gave pA_2 and pA_{10} values of 4.28±0.07 and 3.14±0.73, respectively, with a slope of −0.83±0.16, indicating specific antagonism. CAPE (25 μM) also inhibited platelet aggregation stimulated by the glycoprotein VI agonist, convulxin, and the $\alpha_2\beta_1$ integrin agonist, aggretin. CAPE (25 μM) also markedly interfered with FITC-collagen binding to platelet membranes. CAPE (15 and 25 μM) concentration-dependently inhibited collagen-induced platelet activation accompanied by $[Ca^{+2}]_i$ mobilization, phosphoinositide breakdown, activation of protein kinase C and mitogen-activated protein kinases (i.e., ERK2, JNK, and p38 MAPK), Akt phosphorylation, and thromboxane A_2 formation. In the ESR study, CAPE (15 and 25 μM) markedly reduced hydroxyl radical ($OH^{\bullet}$) formation in collagen-activated platelets. In an in vivo study, CAPE (5 mg/kg) significantly prolonged the latency in inducing platelet plug formation in mesenteric venules of mice.
Conclusions: The most important findings of this study suggest that CAPE specifically inhibits collagen-induced platelet activation. Thus, CAPE treatment may represent a novel approach to lowering the risk of or improving function in thromboembolism-related disorders.

Keywords: CAPE; Collagen antagonist; Hydroxyl radical; MAPKs; Platelet activation

1. Introduction

Caffeic acid phenethyl ester (CAPE) is an active component of propolis obtained from honeybee hives. It is reported to have anti-inflammatory, anti-viral, anti-mitogenic, anti-carcinogenic, and immunomodulatory effects [1,2]. CAPE has anti-oxidant activity and inhibits lipoxygenase, tyrosine kinase, and NFκB activations [1,3]. CAPE has been shown to inhibit the growth of different types of transformed cells [2]. In transformed cells, CAPE alters the redox state and induces apoptosis [2]. CAPE also inhibits tumor promotion and lung fibrosis [2,4].

Abbreviations: AA, arachidonic acid; ATP, adenosine triphosphate; CAPE, caffeic acid phenethyl ester; $cPLA_2$, cytosolic phospholipase A_2; ERK, extracellular signal-regulated kinase; ESR, electron spin resonance; GP, glycoprotein; IP, inositol monophosphate; IP_3, inositol 1,4,5-trisphosphate; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; mAb, monoclonal antibody; PI3-kinase, phosphoinositide 3-kinase; PKC, protein kinase C; PLC, phospholipase C; PRP, platelet-rich plasma; TxA_2, thromboxane A_2.

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Intravascular thrombosis is a generator of a wide variety of cardiovascular diseases. Initiation of an intraluminal thrombosis is believed to involve platelet adherence and aggregation. In normal circulation, platelets cannot aggregate by themselves. However, when a blood vessel is damaged, platelets adhere to the disrupted surface, and the adhering platelets release certain biologically active constituents which induce aggregation [5]. Platelet activation is relevant in a variety of cardiovascular and cerebrovascular disorders; however, no data are available concerning the effect of CAPE in platelet activation. Studies of the effects of CAPE on platelets are relatively rare, and the detailed mechanisms underlying the CAPE signaling pathways remain obscure. In a preliminary test, we found that CAPE specifically inhibited collagen-induced platelet aggregation. Platelets are anucleate, do not differentiate or proliferate, and thus are a good model for studying signal transductions of CAPE and its functions. We therefore for the first time systematically examined the influence of CAPE in washed human platelets and in platelet plug formation in vivo, and utilized the findings to characterize the mechanisms involved in this influence.

2. Materials and methods

2.1. Materials

CAPE, collagen (type I, bovine achilles tendon), luciferin–luciferase, fluorescein sodium, Dowex-1 (100–200 mesh; X_8, chloride form), myoinositol, prostaglandin E_1 (PGE_1), arachidonic acid (AA), ADP, U46619, bovine serum albumin (BSA), and thrombin were purchased from Sigma Chem. (St Louis, MO); Fura 2-AM from Molecular Probe (Eugene, OR); the thromboxane B_2 enzyme immunoassay (EIA) kit from Cayman (Ann Arbor, MI); the anti-phospho-p38 MAPK (Ser^{182}) monoclonal antibody (mAb) from Santa Cruz (Santa Cruz, CA); the anti-p38 MAPK, anti-SAPK/JNK, anti-phospho-SAPK/JNK (Thr^{183}/Tyr^{185}), anti-p44/p42 ERK, and anti-phospho-p44/p42 ERK (Thr^{202}/Tyr^{204}) mAbs from Cell Signaling (Beverly, MA); the anti-phospho-Akt (Ser^{473}) polyclonal antibody from Biovision (Mountain View, CA); and the anti-α-tubulin mAb from NeoMarkers (Fremont, CA). The Hybond-P PVDF membrane, myo-2-[^{3}H] inositol, ECL Western blotting detection reagent and analysis system, horseradish peroxidase-conjugated donkey anti-rabbit IgG, and sheep anti-mouse IgG were purchased from Amersham (Buckinghamshire, UK). CAPE (Mw. 284.3) was dissolved in dimethyl sulfoxide (0.5% DMSO) and stored at −4 °C until use.

2.2. Preparation of human platelet suspensions

Human platelet suspensions were prepared as previously described [6]. This study conformed to the principles outlined in the *Helsinki Declaration*, and 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 (9:1, v/v). After centrifugation, the supernatant (platelet-rich plasma; PRP) was supplemented with PGE_1 (0.5 μM) and heparin (6.4 IU/ml), then incubated for 10 min and centrifuged at 500 ×*g*. The washed platelets were suspended in Tyrode's solution containing BSA (3.5 mg/ml). The final concentration of Ca^{+2} in Tyrode's solution was 1 mM.

2.3. Platelet aggregation

A turbidimetric method was applied to measure platelet aggregation [6], using a Lumi-Aggregometer (Payton, Canada). Platelet suspensions (3.6×10^8 platelets/ml, 0.4 ml) were pre-warmed to 37 °C for 2 min, then CAPE (6–25 μM) or an isovolumetric solvent control (0.5% DMSO) was added 3 min before addition of the agonists (i.e., 1 μg/ml collagen, 0.01 U/ml thrombin). The reaction was allowed to proceed for at least 6 min, and the extent of aggregation was expressed in light-transmission units. For measuring ATP release, 20 μl of a luciferin/luciferase mixture was added to the platelet suspensions (3.6×10^8/ml, 0.4 ml) 1 min before the addition of agonists (i.e., 1 μg/ml collagen), and ATP release was compared with that of the control. Whole-blood aggregation was measured by the impedance method with citrated whole blood and monitored by a whole-blood aggregometer (Chrono-Log, Havertown, PA).

2.4. Labeling of membrane phospholipids and measurement of the production of [^{3}H]-inositol phosphates

The method was carried out as previously described [7]. Briefly, citrated PRP was centrifuged, and the pellets were suspended in Tyrode's solution containing [^{3}H]-inositol (75 μCi/ml). Platelets were incubated for 2 h followed by centrifugation, and were finally resuspended in Ca^{+2}-free Tyrode's solution (5×10^8/ml). CAPE (15 and 25 μM) or the solvent control (0.5% DMSO) was pre-incubated with 1 ml of loaded platelets for 3 min, and then collagen (1 μg/ml) was added to trigger activation. The reaction was stopped, and samples were centrifuged for 4 min. The inositol phosphates of the supernatants were separated in a Dowex-1 column. Only [^{3}H]-inositol monophosphate (IP) was measured as an index of total inositol phosphate formation.

2.5. Measurement of platelet $[Ca^{+2}]_i$ mobilization by Fura 2-AM fluorescence

Citrated whole blood was centrifuged at 120 ×*g* for 10 min. The supernatant was incubated with Fura 2-AM (5 μM) for 1 h. Human platelets were then prepared as described above. Finally, the external Ca^{+2} concentration of the platelet suspensions was adjusted to 1 mM. The rise in $[Ca^{+2}]_i$ was measured using a fluorescence spectrophotometer (CAF 110, Jasco, Tokyo, Japan) at excitation wavelengths of 340 and 380 nm, and an emission wavelength of 500 nm.

2.6. Measurement of thromboxane B_2 formation

Washed human platelets (3.6×10^8/ml) were pre-incubated with CAPE (15 and 25 μM) or the solvent control (0.5% DMSO) for 3 min before the addition of collagen (1 μg/ml). Six minutes after the addition of collagen, EDTA (2 mM) and indomethacin (50 μM) were added to the reaction suspensions. The vials were then centrifuged for 3 min. The TxB_2 levels of the supernatants were measured using an EIA kit according to the manufacturer's protocol.

2.7. Flow cytometric analysis

Triflavin, an $\alpha_{IIb}\beta_3$ integrin antagonist, was prepared as previously described [5]. Fluorescence-conjugated triflavin and collagen were prepared as previously described [7]. The final concentrations of FITC-conjugated triflavin and collagen were adjusted to 1 mg/ml. Washed platelets were prepared as described above. Aliquots of platelet suspensions (3.6×10^8/ml) were pre-incubated with CAPE (15 and 25 μM), collagen (100 μg/ml), or the solvent control (0.5% DMSO) for 3 min, followed by the addition of 2 μl of FITC-triflavin (2 μg/ml) or FITC-collagen (1 μg/ml), respectively. The suspensions were then incubated for another 5 min, and the volume was adjusted to 1 ml/tube with Tyrode's solution. The suspensions were then assayed for fluorescein-labeled platelets using a flow cytometer (Becton Dickinson, FACScan Syst., San Jose, CA). Data were collected from 50,000 platelets per experimental group. All experiments were repeated at least four times to ensure reproducibility.

2.8. Quantitative immunoblotting

Washed platelets (1.2×10^9/ml) were pre-incubated with CAPE (15 and 25 μM) or the solvent control (0.5% DMSO) for 3 min, followed by the addition of collagen or thrombin to trigger platelet activation. The reaction was stopped by the addition of EDTA (10 mM), and the suspensions were centrifuged at 3000 ×*g* for 5 min and then immediately resuspended in 200 ml of lysis buffer (50 mM Hepes, 5 mM EDTA, 50 mM NaCl, 1% Triton X-100, 10 μg/ml aprotinin, 1 mM PMSF, 10 μg/ml leupeptin, 10 mM NaF, 1 mM sodium orthovanadate, and 5 mM sodium pyrophosphate). Collected lysates were centrifuged at 10,000 ×*g* for 5 min, after which the supernatants were dissolved in β-mercaptoethanol (5%) and bromophenol blue (0.1%). Samples containing 80 μg of protein were separated by SDS-PAGE (12%); the proteins were electrotransferred to a Hybond-P PVDF membrane by a semidry method (Bio-Rad, Hercules, CA). Blots were blocked with TBST (10 mM Tris-base, 100 mM NaCl, and 0.01% Tween 20) containing 5% BSA for 1 h, then probed with the following primary antibodies for 2 h: anti-p-p38 MAPK, anti-p38 MAPK, anti-p-ERKs (p42/p44), anti-ERKs (p42/p44), anti-p-JNK, anti-JNK, anti-p-Akt, and anti-p-P47 (diluted 1:1000 in TBST). Membranes were washed and then incubated with horseradish peroxidase-linked anti-mouse IgG or anti-rabbit IgG (diluted 1:3000 in TBST) for 1 h. Immunoreactive bands were detected by chemiluminescence using the ECL-enhanced chemiluminescence system. 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).

2.9. Measurement of hydroxyl radicals in platelet suspensions by ESR spectrometry

The ESR method used a Bruker EMX ESR spectrometer as described previously [8]. In brief, platelet suspensions (3.6×10^8 platelets/ml) were pre-treated with CAPE (15 and 25 μM) or the solvent control (0.5% DMSO) for 3 min, followed by the addition of collagen (1 μg/ml). DMPO (100 mM) was added to the reaction for the ESR analysis. The rate of free radical-scavenging activity is defined by the following equation: inhibition rate = 1 − [signal height (CAPE)/signal height (control)] [8].

2.10. Fluorescein sodium-induced platelet thrombi in mesenteric microvessels of mice

As described previously [6], mice were anesthetized, and an external jugular vein was cannulated with PE-10 for administration of the dye and drug (by an i.v. bolus). A segment of the small intestine was placed onto a transparent culture dish for microscopic observation. Venules (30–40 μm) were selected for irradiation to produce a microthrombus. In the epi-illumination system, light from a 100-W mercury lamp was passed through a B-2A filter (Nikon, Tokyo, Japan) with a DM 510 dichromic mirror (Nikon). Filtered light for which wavelengths below 520 nm had been eliminated was used to irradiate a microvessel (the area of irradiation was about 100 μm in diameter on the focal plane). Various doses of CAPE (5 and 10 mg/kg) or an isovolumetric solvent control (0.5% DMSO) were administered 1 min after fluorescein sodium (15 μg/kg) administration. Five minutes after administration of the fluorescein sodium, irradiation by filtered light and the video timer were simultaneously begun, and platelet aggregation was observed on a TV monitor. The time lapse for inducing thrombus formation leading to the cessation of blood flow was measured.

2.11. Statistical analysis

The experimental results are expressed as the means ± S.E.M. and are accompanied by the number of observations. Paired Student's *t*-test was used to determine significant differences in the in vivo studies of platelet plug formation. The other experiments were assessed by the method of 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 less than 0.05 was considered statistically significant.

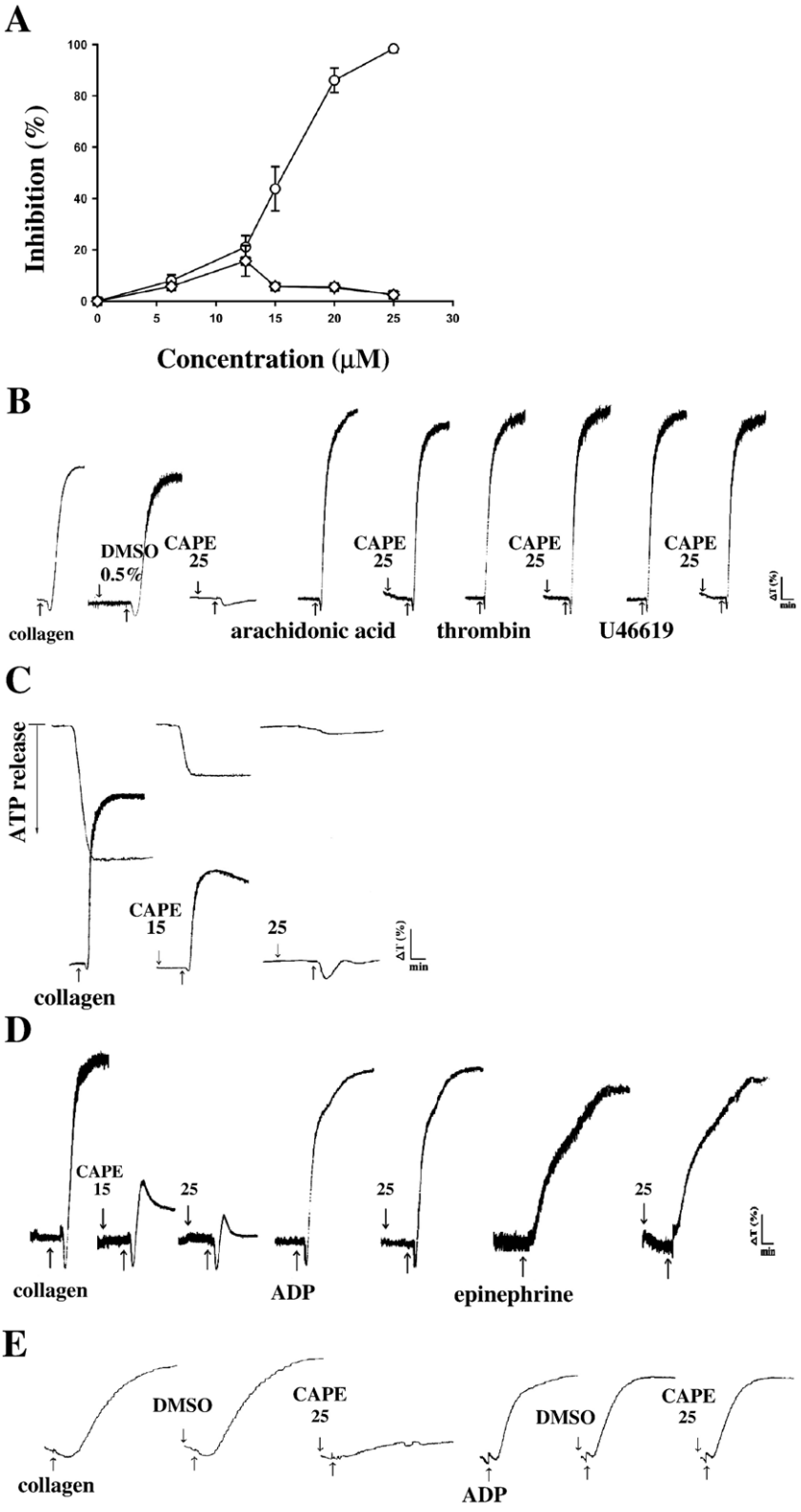

Fig. 1. Effects of CAPE inhibition of agonist-induced platelet aggregation in washed platelets, platelet-rich plasma, and whole blood. Washed platelets (3.6×10^8/ml) were pre-incubated with CAPE (6–25 μM) or a solvent control (0.5% DMSO) followed by the addition of collagen (1 μg/ml, ○), U46619 (1 μM, ▽), thrombin (0.01U/ml, □), or arachidonic acid (AA; 60 μM, ◇) to trigger platelet aggregation (A, B) and the ATP-release reaction (C, upper tracings). For other experiments, platelet-rich plasma (D) or whole blood (E) was pre-incubated with CAPE (15 and 25 μM) or a solvent control (0.5% DMSO) followed by the addition of collagen (1 μg/ml), ADP (20 μM), or epinephrine (10 μM) to trigger platelet aggregation. Data (A) are presented as the means±S.E.M. ($n=7$); and figures (B–E) are representative examples of four to seven similar experiments.

3. Results

3.1. Effect of CAPE on agonist-induced platelet aggregation in human platelets

CAPE (6–25 μM) concentration-dependently inhibited platelet aggregation and the ATP-release reaction in washed human platelets stimulated by collagen (1 μg/ml) but not by thrombin (0.01 U/ml), arachidonic acid (60 μM), or U46619 (1 μM), a prostaglandin endoperoxide analogue (Fig. 1A–C). It also inhibited collagen (1 μg/ml) — but not by ADP (20 μM) — or epinephrine (10 μM)-induced platelet aggregation in both platelet-rich plasma (PRP) (Fig. 1D). CAPE concentration-dependently and specifically inhibited collagen-induced platelet aggregation with an IC_{50} value of approximately 14 μM (Fig. 1A).

Platelet aggregation in whole blood is a more physiological condition compared with that in PRP or washed platelets. In this study, CAPE (25 μM) also specifically inhibited collagen (1 μg/ml) — but not by ADP (20 μM)-

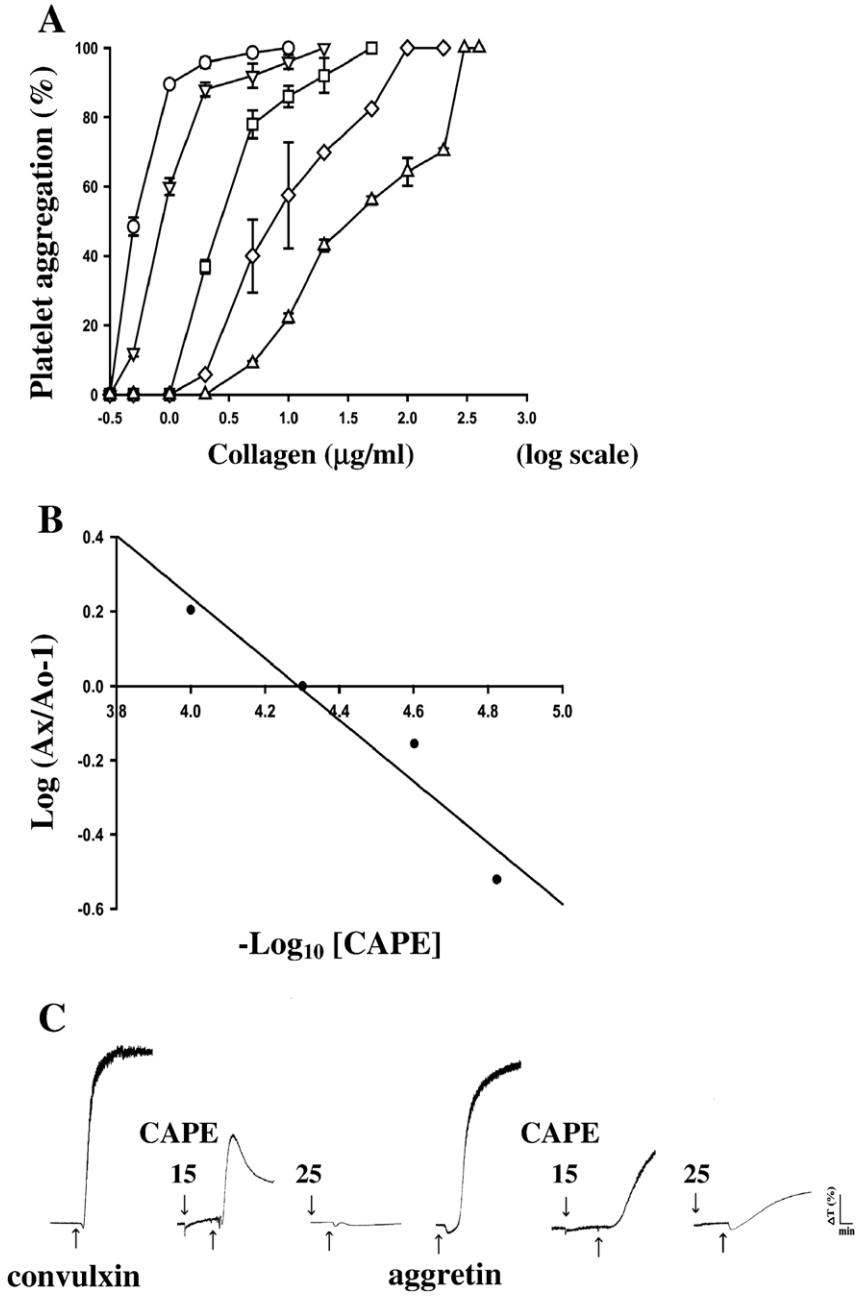

Fig. 2. Effects of CAPE on collagen receptor agonist-induced platelet aggregation and Schild plot analysis. Washed platelets (3.6×10^8/ml) were pre-incubated with a solvent control (0.5% DMSO, ○) or CAPE (15 μM, ▽; 25 μM, □; 50 μM, ◇; and 100 μM, △) followed by the addition of (A) collagen (0.5–300 μg/ml), (C) convulxin (100 ng/ml), or aggretin (3.6 μg/ml) to trigger platelet aggregation. (B) A Schild plot of the data is presented in (A). The figures in (C) are representative examples of four similar experiments.

induced platelet aggregation in whole-blood preparations (Fig. 1E). Furthermore, shear-induced platelet plug formation in whole blood was also tested using a platelet function analyzer (PFA-100 analyzer, DADE-Behring International, Miami, FL). Aliquots of citrated whole blood (900 μl) was forced under high shear stress conditions through a small aperture (150 μm in diameter) cut into a membrane coated with collagen/ADP (CADP) or collagen/epinephrine (CEPI). The time to occlusion (closure time, CT) is taken as an indicator of platelet function on the whole-blood sample. After 300 s, the process automatically terminates, inferring that the CT is longer than 300 s. When whole blood was pre-incubated with 0.5% DMSO for 2 min, the CTs of CADP and CEPI were 115±8 s and 141±23 s (n=3), respectively. CAPE (25 μM) significantly prolonged the CTs of CADP and CEPI 192±16 s and >300 s (n=3), respectively. At higher concentration (50 μM), CAPE also markedly increased both CTs as longer than 300 s (data not shown).

On the other hand, CAPE (15, 25, 50, and 100 μM) produced a concentration-related rightward, parallel shift in the collagen concentration–response curve with no alteration in the maximal responses (Fig. 2A). When the data were presented as a Schild plot, pA_2 and pA_{10} values of 4.28±0.07 and 3.14±0.73, respectively, with a slope of −0.83±0.16 were obtained (Fig. 2B). Furthermore, CAPE (15 and 25 μM) also concentration-dependently inhibited platelet aggregation stimulated by collagen receptor agonists such as the glycoprotein (GP) VI agonist, convulxin (100 ng/ml) [9], and the $\alpha_2\beta_1$ integrin agonist, aggretin (3.6 μg/ml) (Fig. 2C) [10]. In addition, pre-treatment with solvent control (0.5% DMSO) only did not significantly affect platelet aggregation in either washed platelets (Fig. 1B) or PRP (data not shown).

3.2. Effects of CAPE on FITC-collagen and FITC-triflavin binding to washed platelets

We further evaluated whether or not CAPE binds directly to the collagen receptor, leading to inhibition of platelet aggregation. In this study, the relative fluorescence intensity of FITC-collagen (1 μg/ml) bound directly to resting platelets markedly increased in comparison with the control (in the presence of unlabeled FITC only) (152.5±6.0 vs. 29.1±6.2) (Fig. 3Aa, b), and it was markedly reduced in the presence of collagen (100 μg/ml, 46.9±4.1) (Fig. 3Ac). CAPE (25 μM) markedly inhibited FITC-collagen binding to platelets (48.9±2.0) (Fig. 3Ad).

Triflavin, an $\alpha_{IIb}\beta_3$ integrin antagonist, is purified from *Trimeresurus flavoviridis* snake venom [5]. We further evaluated whether or not CAPE also binds to the $\alpha_{IIb}\beta_3$ integrin, leading to inhibition of platelet aggregation induced by collagen. In this study, the relative fluorescence intensity of FITC-triflavin (2 μg/ml) bound directly to collagen (1 μg/ml)-activated platelets was 301.9±11.2 (Fig. 3Ba), and it was markedly reduced in the presence of 5 mM EDTA (negative control, 93.4±2.0) (Fig. 3Bb). CAPE (15 and 25 μM) did not significantly interfere with FITC-triflavin binding to $\alpha_{IIb}\beta_3$ integrin in platelet suspensions (15 μM, 317.7±3.8; 25 μM, 316.6±2.3) (Fig. 3Bc, d). These results indicate that the effect of CAPE's inhibitory effect on platelet aggregation may directly antagonize the binding of collagen toward the platelet membrane, whereas it does not appear to mediate it via binding to $\alpha_{IIb}\beta_3$ integrin.

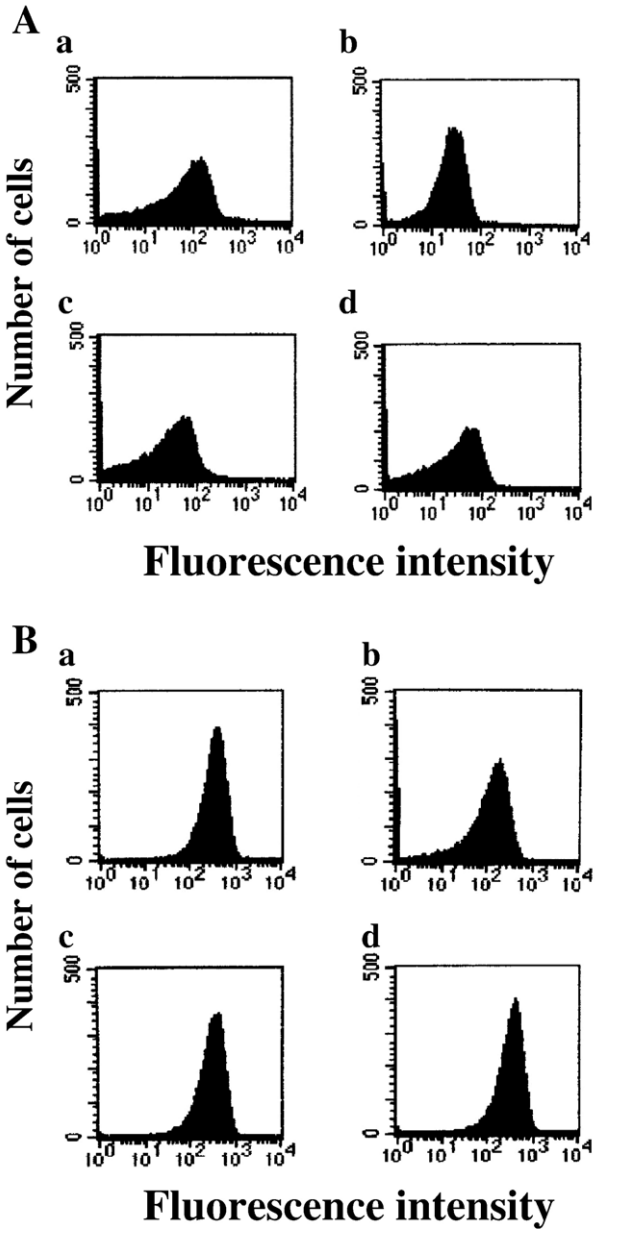

Fig. 3. Effects of flow cytometric analysis of FITC-collagen and FITC-triflavin binding to washed platelets. (A) The solid line represents the fluorescence profiles of (a) a solvent control (0.5% DMSO); or in the presence of (b) FITC only (background), (c) collagen (100 μg/ml), or (d) CAPE (25 μM), followed by the addition of FITC-collagen (1 μg/ml). (B) The solid line represents the fluorescence profiles of (a) FITC-triflavin (2 μg/ml) in the absence of CAPE as a positive control; (b) in the presence of EDTA (5 mM) as a negative control; or in the presence of CAPE (c) (15 μM) and (d) (25 μM), followed by the addition of FITC-triflavin (2 μg/ml). The profiles are representative examples of four similar experiments.

3.3. Effects of CAPE on [Ca+2]i mobilization, phosphoinositide breakdown, PKC activation, and TxA2 formation in washed platelets

As shown in Fig. 4A, collagen (1 μg/ml) evoked a marked increase in $[Ca^{+2}]_i$, and this increase was markedly inhibited in the presence of CAPE (15 μM, 67.2±3.0%; 25 μM, 87.4±7.9%). Phosphoinositide breakdown occurs in platelets activated by many agonists. In this study, collagen (1 μg/ml) induced the rapid formation of radioactive IP, IP_2, and IP_3 in human platelets loaded with [^{3}H]-inositol. We only measured [^{3}H]-IP formation as an index of total inositol phosphate formation. As shown in Fig. 4B, the addition of collagen (1 μg/ml) resulted in a rise in IP formation of approximately 1.6-fold compared to that in resting platelets ([11.9±1.7 vs. 19.1±1.5]×10^3 d.p.m.). In the presence of CAPE (25 μM), the radioactivity of IP formation in collagen-stimulated platelets was markedly inhibited (Fig. 4B).

Furthermore, stimulation of platelets with a number of different agonists induces activation of PKC, which then

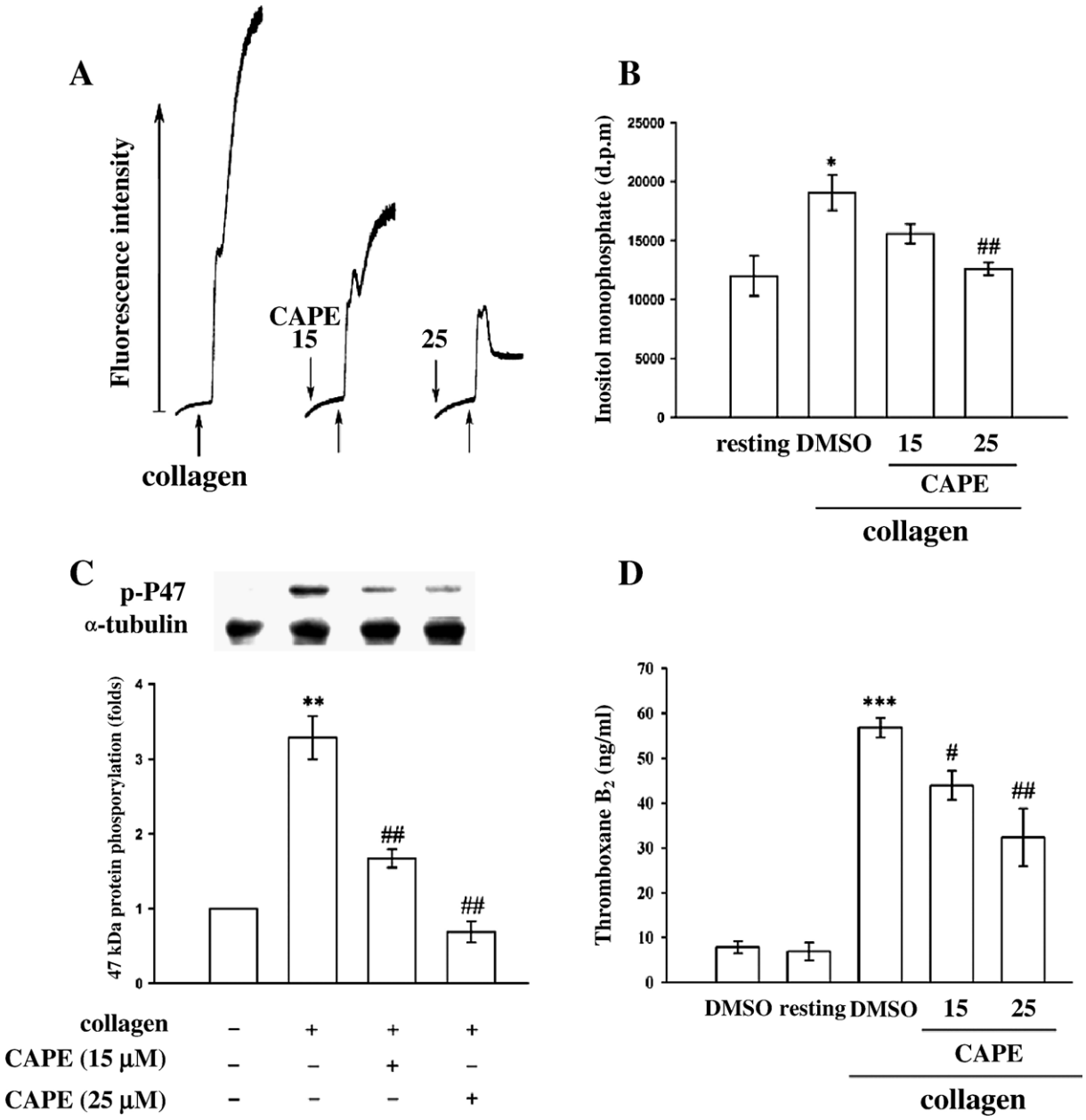

Fig. 4. Effects of CAPE on (A) $[Ca^{2+}]_i$ mobilization, (B) phosphoinositide breakdown, (C) PKC phosphorylation, and (D) thromboxane A_2 formation stimulated by collagen in washed platelets. Labeled platelets were pre-incubated with solvent control (0.5% DMSO) or CAPE (15 and 25 μM), followed by the addition of collagen (1 μg/ml) to trigger platelet activation as described in Materials and methods. The profiles in (A) are representative examples of four similar experiments. The bar graph in (C) 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 in (B–D) are presented as the means±S.E.M. (n=3–5). * P<0.05, ** P<0.01, and *** P<0.001 compared with the resting group; $^{\#}$ P<0.05 and $^{\#\#}$ P<0.01 compared with the collagen group.

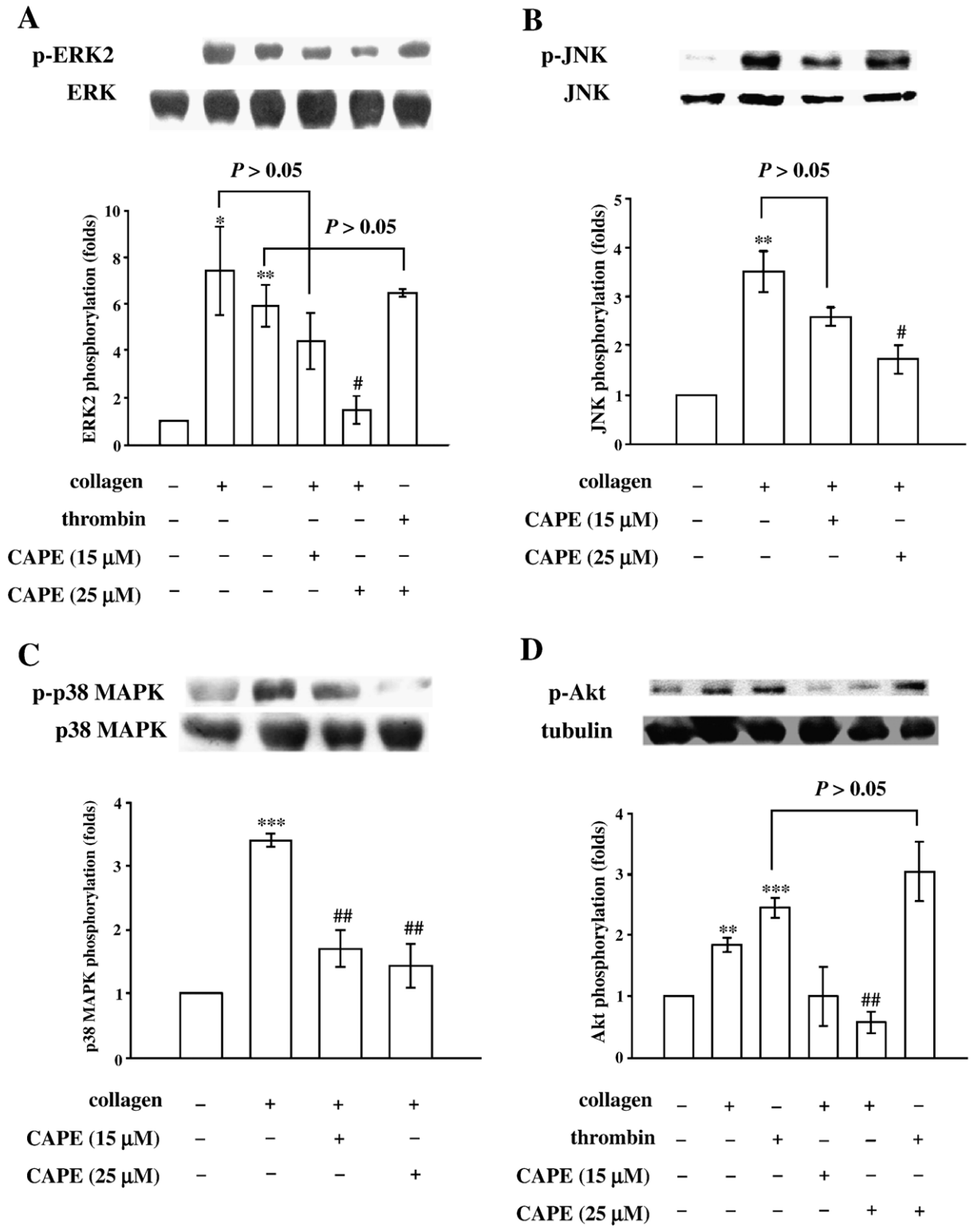

Fig. 5. Effects of CAPE on MAPK and Akt phosphorylation stimulated by collagen or thrombin in washed platelets. Washed platelets (1.2×10^9/ml) were pre-incubated with solvent control (0.5% DMSO) or CAPE (15 and 25 μM), followed by the addition of collagen (10 μg/ml) or thrombin (0.1 U/ml) to trigger platelet activation. The reactions were stopped by the addition of EDTA (10 mM), and cells were then collected, and subcellular extracts were analyzed for (A) ERK2, (B) JNK, (C) p38 MAPK, and (D) Akt phosphorylation by Western blotting as described in Materials and methods. The bar graphs in (A–D) depict the ratios of quantitative results obtained by scanning the reactive bands of anti-p-ERK2, anti-p-JNK, anti-p-p38 MAPK, and anti-p-Akt against the individual total forms or α-tubulin and quantifying the optical density. 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; $^{\#}$ $P < 0.05$ and $^{\#\#}$ $P < 0.01$ compared with the collagen group.

phosphorylates proteins of Mw 40,000–47,000. In this study, phosphorylation experiments were performed to examine the role of CAPE in PKC activation in human platelets. When collagen (1 μg/ml) was added to human platelets, a protein with an apparent Mw of 47,000 (P47) was predominately phosphorylated as compared with resting platelets (Fig. 4C). CAPE (15 and 25 μM) markedly inhibited P47 phosphorylation stimulated by collagen in human platelets (Fig. 4C). Moreover, resting platelets produced relatively little TxB_2 compared with collagen-activated platelets. CAPE (15 and 25 μM) markedly inhibited TxB_2 formation in platelets stimulated by collagen (1 μg/ml) (Fig. 4D). Furthermore, pre-treatment with solvent control (0.5% DMSO) only did not change TxB_2 formation compared to the resting platelets (Fig. 4D).

3.4. Effects of CAPE on collagen-induced MAPK and Akt phosphorylation

To further investigate the mechanisms of CAPE-inhibited collagen-induced platelet activation, we detected several signaling molecules such as Akt and MAPKs, including ERK2, JNK, and p38 MAPK. The immunoblotting analysis revealed that treatment with a higher concentration (10 μg/ml) of collagen produced more-marked MAPK phosphorylation than did a lower concentration (1 μg/ml) (data not shown). In subsequent experiments, we used a concentration of 10 μg/ml of collagen to further study MAPK and Akt phosphorylation. We found that CAPE (15 and 25 μM) concentration-dependently inhibited collagen (10 μg/ml) — but not thrombin (0.1 U/ml)-induced ERK2 (Fig. 5A), JNK, p38 MAPK (data not shown), or Akt phosphorylation (Fig. 5D).

3.5. Effects of CAPE on hydroxyl radical formation in collagen-activated platelets and thrombus formation in microvessels of fluorescein sodium-pre-treated mice

In this study, a typical ESR signal of hydroxyl radical ($OH^{\bullet}$) formation was induced by collagen (1 μg/ml) in platelets compared with resting platelets (Fig. 6Aa, b); pre-treatment with CAPE (15 and 25 μM) significantly reduced hydroxyl radical formation stimulated by collagen (1 μg/ml) (Fig. 6Ac, d). The anti-oxidant, catalase (1000 U/ml), markedly suppressed hydroxyl radical formation by about 76% (data not shown).

For the study of thrombus formation in microvessels of fluorescein sodium (15 μg/kg)-pre-treated mice, the occlusion time required was approximately 217 s. When CAPE was administered at 5 and 10 mg/kg after pre-treatment with fluorescein sodium, the occlusion times were significantly prolonged compared to the solvent controls (0.5% DMSO, 216.6±16.8 s vs. 5 mg/kg, 260.1±6.7 s, $n=5$, $P<0.01$; 0.5% DMSO, 203.5±19.5 s vs. 10 mg/kg, 343.4±41.5 s, $n=5$, $P<0.01$) (Fig. 6B). The typical microscopic image of a microthrombus formed with fluorescein sodium treatment is shown in Fig. 6C. The thrombotic platelet plug was observed

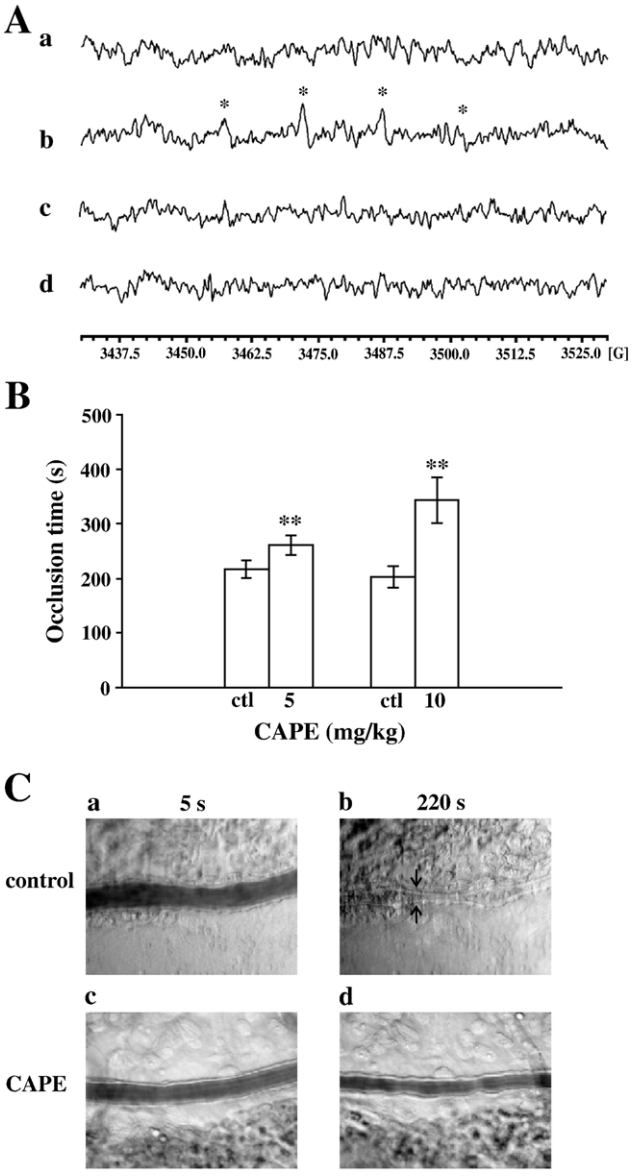

Fig. 6. Electron spin resonance (ESR) spectra of CAPE-reduced hydroxyl radical ($OH^{\bullet}$) formation in collagen-activated platelets, and the effect of CAPE prolongation of the occlusion time for inducing thrombus formation in mesenteric venules of mice. (A) Washed platelets (3.6×10^8/ml) were pre-incubated with (a) Tyrode's solution (resting group); or (b) solvent control (0.5% DMSO) and CAPE (c, 15 μM; d, 25 μM), followed by the addition of collagen (1 μg/ml) to trigger hydroxyl radical formation. DMPO (100 mM) was added to the reaction for the ESR experiments. Spectra are representative examples of four similar experiments. For the thrombotic experiments of platelet plug formation (B, C), mice were administered the solvent control (ctl, 0.5% DMSO) or CAPE (5 and 10 mg/kg), after which the mesenteric venules were then selected for irradiation to produce microthrombus formation as described in Materials and methods. Data of the bar graphs in (B) are presented as the means±S.E.M. of the occlusion time (s) for inducing platelet plug formation ($n=5$). ** $P<0.01$ compared with the individual solvent control group. Microscopic images in (C) were observed in solvent-treated (a, b) or CAPE (10 mg/kg)-treated (c, d) groups during the time courses of 5 s (a, c) and 220 s (b, d) after irradiation. (b) The arrows indicate platelet plug formation. Photographs are representative examples of five similar experiments (×400).

in mesenteric microvessels at 220 s but not at 5 s after irradiation in the solvent-treated group (Fig. 6Ca, b). With administration of CAPE (10 mg/kg), platelet plug formation was not observed at either 5 or 220 s after irradiation (Fig. 6Cc, d). The blood flow rate of the solvent-treated venule was slower than that of the CAPE-treated venule, because the platelet plug became apparent at 220 s (Fig. 6Cb). Moreover, platelet counts in mice were measured with an automatic cell counter (A^cT; Coulter, Miami, FL). Platelet counts in fluorescein sodium (15 μg/kg)-pre-treated mice were markedly lowered as compared to the isovolumetric normal saline-pre-treated mice at 220 s after irradiation (normal saline, 328±25 vs. fluorescein sodium, 189±22, $\times 10^3/mm^3$, $n=5$, $P<0.01$) (data not shown). CAPE (10 mg/kg) significantly increased platelet counts in mice compared to the solvent control (0.5% DMSO)-treated mice at 220 s after fluorescein sodium irradiation (CAPE, 276±20 vs. 0.5% DMSO, 176±29, $\times 10^3/mm^3$, $n=5$, $P<0.05$).

4. Discussion

This study reveals for the first time that CAPE specifically inhibits collagen-induced platelet aggregation in both washed platelets and PRP with a Schild plot slope of −0.83±0.16 and apparent pA_2 and pA_{10} values of 4.28±0.07 and 3.14±0.73, respectively. The GP Ib/IX/V complex and $\alpha_{IIb}\beta_3$ integrin require von Willebrand factor to cross-bridge platelets to the vascular collagen [11]. Among the platelet receptors known to directly interact with collagen, recent focus has been directed toward the key roles of $\alpha_2\beta_1$ integrin and GP VI [9]. More-recent findings suggest that both $\alpha_2\beta_1$ integrin and GP VI may contribute to the overall processes of platelet adhesion, activation, and aggregation [9,12–14]. In this study, we found that CAPE at 25 μM almost completely inhibited convulxin- and aggretin-induced platelet aggregation. Convulxin, a potent platelet-aggregating protein from the venom of the snake, *Crotalus durissus terrificus*, is known to bind GP VI [9]. Aggretin is purified from *Calloselasma rhodostoma* venom, and acts as an $\alpha_2\beta_1$ integrin agonist [10]. In addition, CAPE markedly interfered with FITC-collagen binding to platelet membranes, whereas it did not affect FITC-triflavin binding to $\alpha_{IIb}\beta_3$ integrin. Therefore, the anti-platelet activity of CAPE is possibly due to direct interference with the binding of collagen to its specific receptors (i.e., $\alpha_2\beta_1$ integrin and GP VI) on the platelet membrane.

Stimulation of platelets by collagen results in PLC-catalyzed hydrolysis of the minor plasma membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2), with concomitant formation of IP_3 and diacylglycerol [15]. There is strong evidence that IP_3 induces the release of Ca^{+2} from intracellular stores [16]. Diacylglycerol activates PKC, inducing protein phosphorylation (47-kDa, P47) and a release reaction. In this study, collagen-induced phosphoinositide breakdown and P47 phosphorylation were inhibited by CAPE, whereas thrombin-induced P47 phosphorylation was not inhibited by CAPE (data not shown). Phosphoinositide breakdown can induce TxA_2 formation via free AA release by diglyceride lipase or by endogenous phospholipase A_2 from membrane phospholipids [17]. Thus, it seems likely that inhibition of the collagen–PLC–IP_3–TxA_2–Ca^{+2} pathway plays an important role in mediating the inhibitory effect of CAPE in collagen-induced platelet aggregation.

MAPKs consists of three major subgroups. The ERKs (p44 ERK1 and p42 ERK2) are involved in proliferation, adhesion, and cell progression [18]. p38 MAPK and JNKs or stress-activated protein kinases (SAPKs), which include the 46-kDa JNK1 and 55-kDa JNK2 isoforms, appear to be involved in apoptosis [18]. In platelets, ERKs (especially ERK2), JNK, and p38 MAPK, have been identified [18]. ERK2 phosphorylation seems to involve MEK 1/2 and PKC [19]. JNK is present and active in platelets and is similarly regulated like ERK2 [18]. The roles of JNK and ERK2 in physiopathology are unclear, and have been suggested as being suppressors of $\alpha_{IIb}\beta_3$ activation or negative regulators of platelet activation [20]. On the other hand, p38 MAPK provides a crucial signal which is necessary for aggregation caused by collagen or thrombin [21]. Among the numerous downstream targets of p38 MAPK, the most physiologically relevant one in platelets is $cPLA_2$ which catalyses AA release to produce TxA_2 [22]; thus, p38 MAPK appears to provide a TxA_2-dependent platelet aggregation pathway. Stimulation of platelets with various agonists results in Akt activation. It is known that Akt functions as one of several downstream effectors of PI-3 kinase [23]. However, the molecular mechanism involved in Akt activation in platelets is not well established. In this study, CAPE specifically inhibited MAPK and Akt phosphorylation stimulated by collagen but not by thrombin. These results further confirm that CAPE specifically antagonizes the collagen-induced downstream signal transduction in platelet activation.

After endothelial cell injury, exposure of subendothelial collagen is the major trigger that initiates platelet adhesion and aggregation at the site of injury, followed by arterial thrombus formation [5]. When platelets aggregate, they release a number of substances, including TxA_2 and free radicals (i.e., hydroxyl radicals), both of which can cause contraction of vascular smooth muscle cells [24]. Free radical species act as second messengers which increase the cytosolic Ca^{+2} during the initial phase of platelet activation processes [25]. It is also evident that some of the hydrogen peroxide produced by platelets is converted into hydroxyl radicals, as platelet aggregation can be inhibited by hydroxyl radical scavengers [26]. Thus, CAPE prolongation of platelet plug formation in vivo may be involved, at least in part, in the inhibition of free radical formation triggered by collagen.

The absence or pharmacological inhibition of collagen receptors may be protective against the thrombotic complications of vascular diseases. In this study, CAPE, a specific collagen antagonist, significantly prolonged thrombus formation in mice. Our result is in agreement with a previous report by He et al. [12], who found that $\alpha_2\beta_1$ integrin-deficient mice

exhibit delayed thrombus formation following carotid artery injury. This result is consistent with the previously demonstrated correlation between the high-level expression of $\alpha_2\beta_1$ integrin and an increased risk for thrombosis involving coronary and cerebral vessels [27,28]. Nieswandt et al. [29] also reported that mice depleted of GP VI were completely protected from lethal collagen-induced pulmonary thromboemboli. In the thrombotic study, the mesenteric venules were continuously irradiated by fluorescein sodium throughout the entire experimental period, thus leading to strong damage to endothelial cells as described previously [6]. Therefore, the dosage (5 mg/kg) of CAPE employed in this in vivo model was relatively higher than that used (25 μM) for the in vitro studies.

In conclusion, the most important finding of this study is, as first reported herein, that CAPE specifically inhibits collagen-induced platelet activation, which is, at least in part, mediated by binding to collagen receptors (i.e., $\alpha_2\beta_1$ integrin and GP VI). The detailed binding affinity and number of binding sites of CAPE on platelet collagen receptors, however, need to be further investigated. These findings suggest that CAPE may be a potent and effective agent in treating thromboembolic-related disorders.

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