

Mechanisms involved in the antiplatelet effect of C-phycoyanin

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C-phycoyanin (cpc), a biliprotein isolated from *Spirulina platensis*, has been reported to exert many therapeutic and nutritional values. In the present study, we examined whether cpc has an antiplatelet activity *in vitro* and further investigated the possible anti-aggregatory mechanisms involved. Our results showed that preincubation of cpc (1–50 µg/ml) with rabbit washed platelets dose-dependently inhibited the platelet aggregation induced by collagen (10 µg/ml) or arachidonic acid (100 µM), with an IC₅₀ of about 10 µg/ml. Furthermore, the thromboxane B₂ formation caused by collagen or arachidonic acid was significantly inhibited by cpc due to suppression of cyclooxygenase and thromboxane synthase activity. Similarly, the rise of platelet intracellular calcium level stimulated by arachidonic acid and collagen-induced platelet membrane surface glycoprotein IIb/IIIa expression were also attenuated by cpc. In addition, cpc itself significantly increased the platelet membrane fluidity and the cyclic AMP level through inhibiting cyclic AMP phosphodiesterase activity. These findings strongly demonstrate that cpc is an inhibitor of platelet aggregation, which may be associated with mechanisms including inhibition of thromboxane A₂ formation, intracellular calcium mobilization and platelet surface glycoprotein IIb/IIIa expression accompanied by increasing cyclic AMP formation and platelet membrane fluidity.

C-phycoyanin: Platelet aggregation: Thromboxane B₂: Cyclic AMP

C-phycoyanin (cpc), a biliprotein found in *Spirulina platensis*, is often used as a dietary nutritional supplement in many countries due to its therapeutic values including hepatoprotective, neuroprotective and reactive oxygen species-scavenging actions (Kay, 1991; Vadiraja *et al.* 1998; Bhat & Madyastha, 2000; Romay *et al.* 2003). It has been demonstrated that oral administration of cpc exhibits an anti-inflammatory effect in several animal models, such as in mice with arthritis or sepsis (Romay *et al.* 1998, 2001; Remirez *et al.* 1999). The cpc is composed of two dissimilar α and β protein subunits of 17 000 and 19 500 Da, respectively, with one bilin chromophore attached to the α subunit (α 84) and two to the β subunit (β 84, β 155) (Romay *et al.* 2003). The cpc exists as a complex interacting mixture of trimer, hexamer and decamer aggregates. The relative amount of each species has been reported to be a function of pH, ionic strength, temperature and protein concentration (Berns, 1970).

It is well known that enhanced platelet activation induced by various inducers such as platelet activating factor (1-*O*-alkyl-2-acetyl-sn-glycero-3-phosphocholine) plays an important role in the initiation and development of atherothrombosis and CVD (Antiplatelet Trialists, 1994; Ruggeri, 2002; Demopoulos *et al.* 2003). Therefore, to prevent and treat thrombosis and vascular

diseases, antiplatelet therapy may be a beneficial strategy. Platelet activation is a result of a complex signal transduction cascade reaction mediated by various agonists. One of the important inducers for platelet aggregation and release action is thromboxane A₂ (TXA₂). TXA₂ is formed by the conversion of arachidonic acid (AA) by the enzymes cyclooxygenase (COX) and thromboxane synthase (Herman, 1998). Given the fact that cpc significantly inhibits the activity of COX and phospholipase A₂ (Reddy *et al.* 2000; Romay *et al.* 2000), we propose that cpc may suppress platelet aggregation through attenuating the AA–TXA₂ pathway. However, whether cpc inhibits platelet aggregation is still unknown. Thus, in this *in vitro* study, we studied the effect of cpc on platelet aggregation and further investigated the possible mechanisms involved.

Materials and methods

Materials

Collagen (type 1, equine tendon), AA, indomethacin, 3-isobutyl-1-methylxanthine (IBMX), prostaglandin E₁ (PGE₁), prostaglandin H₂ (PGH₂) and diphenylhexatriene (DPH) were purchased from Sigma Chemical Company (St. Louis, MO,

Abbreviations: AA, arachidonic acid; [Ca²⁺]_i, intracellular Ca²⁺ concentration; COX, cyclooxygenase; cpc, C-phycoyanin; DPH, diphenylhexatriene; EIA, enzyme immunoassay; Fura-2/AM, Fura-2 acetoxymethyl ester; GPIIb/IIIa, glycoprotein IIb/IIIa; IBMX, 3-isobutyl-1-methylxanthine; PGE₁, prostaglandin E₁; PGE₂, prostaglandin E₂; PGH₂, prostaglandin H₂; TXA₂, thromboxane A₂; TXB₂, thromboxane B₂.

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USA). Thromboxane B₂ (TXB₂), cyclic AMP, cyclic GMP and prostaglandin E₂ (PGE₂) enzyme immunoassay (EIA) kits were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). The cpc purchased from Sigma Chemical Company was dissolved in normal saline.

Preparation of washed platelet suspension and assay of platelet aggregation

Blood was withdrawn from rabbit marginal ear vein, mixed with EDTA anticoagulant (100 mM, 14:1, v/v) and centrifuged at 160 g at 25°C for 10 min to obtain platelet-rich plasma. The platelet suspension was prepared from platelet-rich plasma by washing procedures described previously (Chou *et al.* 2000) and finally suspended in Tyrode solution of the following composition (mM): CaCl₂ (1.0), NaCl (136.8), KCl (2.7), NaHCO₃ (11.9), MgCl₂ (2.1), NaH₂PO₄ (0.4) and glucose (10.0) containing bovine serum albumin (0.35%). Following a 3 min equilibration at 37°C, the platelet suspension (3.0 × 10⁸ platelets/ml) was incubated with cpc (1–50 µg/ml) for 3 min before the addition of collagen (10 µg/ml) or AA (100 µM) to induce platelet aggregation. Platelet aggregation was measured turbidimetrically by an aggregometer (Chrono-Log, Havertown, PA, USA) and was recorded for 6 min. The extent of aggregation was evaluated by measuring the maximum height reached by the aggregation curves. Data are shown as the percentage of maximal aggregation.

Measurement of thromboxane B₂

After incubation with cpc (1–50 µg/ml) or Tyrode solution for 3 min followed by adding collagen or AA for 6 min, the platelet suspension was immediately boiled for 5 min. Then, after centrifugation at 10 000 g for 5 min, the amount of TXB₂ in the supernatant was measured using an EIA kit.

Thromboxane synthase activity assay

After incubation of platelets with cpc (1–50 µg/ml) for 3 min, 5 µM-PGH₂ was added for 6 min. Then, the reaction was stopped by boiling for 5 min. Following centrifugation for 5 min at 10 000 g, the amount of TXB₂, reflecting thromboxane synthase activity, in the supernatant was measured.

Platelet cyclic AMP and cyclic GMP determination

Platelet suspension was incubated with Tyrode solution, cpc, PGE₁ (1 µM), an adenylyl cyclase activator, IBMX (50 µM), a cyclic AMP phosphodiesterase inhibitor, or combination of IBMX + cpc or PGE₁ + cpc for 3 min at 37°C with stirring. Then the incubation was stopped by adding 10 mM-EDTA and immediately boiling for 5 min. After centrifugation at 10 000 g for 5 min, the supernatants were used for the determination of cyclic AMP and cyclic GMP content using EIA kits.

Measurement of platelet intracellular calcium mobilization by Fura-2/AM fluorescence

The intracellular calcium level ([Ca²⁺]_i) of platelets was measured by using a Fura-2 acetoxyethyl ester (Fura-2/AM) as described previously (Chou *et al.* 2000). The fluorescence

(excitation 340 nm, emission 500 nm) was measured with a fluorescence spectrophotometer (CAF-100; Jasco, Tokyo, Japan). The [Ca²⁺]_i was calculated from the fluorescence measured using 224 nm as the Ca²⁺-Fura-2 dissociation constant in the presence of external calcium (1 mM) or in Ca²⁺-free solution with EGTA (5 mM).

Analysis of membrane fluidity by fluorescent probe

The platelet membrane fluidity was measured according to a previous study (Chang *et al.* 2004). Briefly, platelets were pre-incubated with various concentrations of cpc for 3 min, followed by the addition of 1 µM-DPH for 6 min. The fluorescence intensity of platelets was measured by a fluorescence spectrophotometer (RF-5301PC; Shimadzu, Kyoto, Japan). The lower fluorescence intensity indicates a higher membrane fluidity.

Analysis of platelet surface glycoprotein IIb/IIIa expression

Fluorescein isothiocyanate-labelled anti-CD41/CD61 (Serotec Ltd, Oxford, UK), which is raised against platelet glycoprotein IIb/IIIa (GPIIb/IIIa) complex, was used as our previous study (Chou *et al.* 2003). Briefly, 10 µl platelet suspension was put into polystyrene tubes containing 35 µl HEPES buffer. Then, 5 µl CD41/CD61-fluorescein isothiocyanate and various concentrations of cpc were added and incubated at 37°C for 5 min without stirring. The reaction was stopped by adding 500 µl 1% paraformaldehyde. The fluorescence intensity of 10,000 platelets per sample was analysed using a flow cytometer (FACScan, Becton Dickinson, Heidelberg, Germany).

Statistical analysis

The experimental results were expressed as means and their standard errors. Statistical analyses were performed with one-way ANOVA. Results were considered significantly different at a value of $P < 0.05$.

Results

Effect of C-phycoerythrin on platelet aggregation

Pretreatment with cpc (1–50 µg/ml) for 3 min caused a dose-dependent inhibition of rabbit platelet aggregation induced by collagen or AA, with IC₅₀ values of 13.6 (SEM 0.7) and 10.6 (SEM 0.9) µg/ml, respectively (Fig. 1).

Effect of C-phycoerythrin on thromboxane B₂ production

Addition of collagen or AA resulted in a marked increase in TXB₂ formation compared with that in resting platelets. Pretreatment with cpc dose-dependently inhibited the TXB₂ formation induced by collagen or AA (Fig. 2(A)). In resting platelets, cpc itself did not affect the TXB₂ level.

Effect of C-phycoerythrin on cyclooxygenase and thromboxane synthase activity

To investigate further whether the inhibition of TXB₂ formation resulted from attenuation of COX activity, the effect

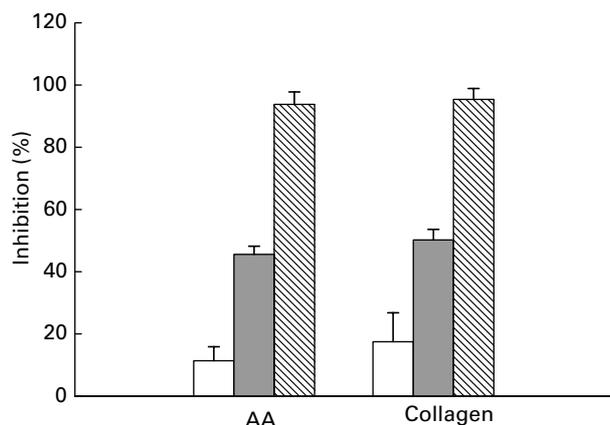


Fig. 1. Effect of C-phycoerythrin (cpc) on collagen or arachidonic acid (AA)-induced platelet aggregation. Washed rabbit platelets were preincubated with cpc (□, 1, ■, 10, ▨, 50 µg/ml) or Tyrode solution for 3 min, then collagen (10 µg/ml) or AA (100 µM) was added to trigger the platelet aggregation. Values are means with their standard errors depicted by vertical bars (*n* 6).

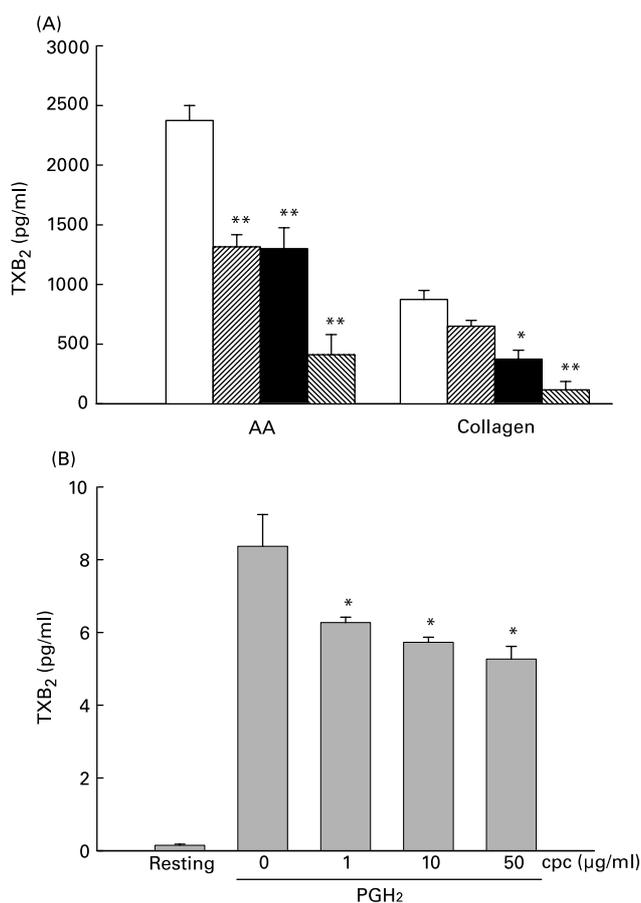


Fig. 2. Effect of C-phycoerythrin (cpc) on thromboxane A₂ (TXB₂) formation of platelets induced by AA, collagen or prostaglandin H₂ (PGH₂). Tyrode solution (control, □) or cpc (▨, 1, ■, 10, ▨, 50 µg/ml) was preincubated with platelets at 37°C for 3 min, then collagen or arachidonic acid (AA) (A) or PGH₂ (B) was added for another 6 min to trigger TXB₂ formation. Then the reaction was stopped by boiling for 5 min. The amount of TXB₂ in the samples was measured using an enzyme immunoassay kit. Values are means with their standard errors depicted by vertical bars (*n* 5). Mean values were significantly different from those of the respective control group: **P* < 0.05, ***P* < 0.01.

of cpc on AA-induced PGE₂ formation was evaluated in the presence of imidazole, a thromboxane synthase inhibitor. Our results showed that cpc significantly inhibited the AA-induced PGE₂ formation compared with that in the AA + imidazole group (Table 1), suggesting that cpc may be a COX inhibitor. Similarly, addition of indomethacin, a well-known COX inhibitor, markedly depressed the PGE₂ formation. In addition, cpc also attenuated PGH₂-induced TXB₂ formation (Fig. 2(B)) and platelet aggregation (data not shown), implying that cpc may also be a thromboxane synthase inhibitor.

Effect of C-phycoerythrin on platelet cyclic AMP and cyclic GMP formation

Addition of cpc, PGE₁ or IBMX alone all significantly increased the cyclic AMP formation compared with that of resting platelets. Combination of PGE₁ and cpc further enhanced the ability of cpc to increase cyclic AMP formation but showed no enhanced effect in the IBMX + cpc group (Table 2). These data suggest that cpc may be a cyclic AMP phosphodiesterase inhibitor. However, pretreatment with cpc (1–50 µg/ml) alone did not affect cyclic GMP production (data not shown).

Effect of C-phycoerythrin on intracellular calcium mobilization in platelets

In Fura-2/AM-loaded platelets, cpc dose-dependently depressed the rise of [Ca²⁺]_i of platelets evoked by AA in the presence of external Ca²⁺ (1 mM) (Fig. 3). In addition, in the Ca²⁺-free solution, the inhibition of cpc on the rise of [Ca²⁺]_i of platelets evoked by AA was similar to that in the presence of external Ca²⁺ (1 mM) (data not shown).

Effect of C-phycoerythrin on platelet membrane fluidity

Platelet membrane fluidity was measured by the intensity of fluorescence in DPH-labelled platelets. Addition of cpc into

Table 1. Inhibitory effect of C-phycoerythrin (cpc) on cyclooxygenase activity†

(Mean values with their standard errors for five determinations)

Treatment	PGE ₂ (ng/ml)	
	Mean	SEM
Resting	0.35	0.09
AA (100 µM)		
+ imidazole (1 mM)	12.5	0.57
+ imidazole + cpc (1 µg/ml)	4.87***	0.32
+ imidazole + cpc (10 µg/ml)	3.22***	0.35
+ imidazole + cpc (50 µg/ml)	1.84***	0.06
+ indomethacin (2 µM)	0.32***	0.09

AA, arachidonic acid; PGE₂, prostaglandin E₂.

† Tyrode solution or cpc (1, 10, 50 µg/ml) was preincubated with platelets at 37°C for 3 min in the presence of imidazole (1 mM), then AA (100 µM) was added for another 6 min followed by boiling for 5 min. The amount of PGE₂ in the samples was measured using an enzyme immunoassay kit. The AA + indomethacin (2 µM) acted as negative control.

Mean values were significantly different from those of the AA + imidazole group: ****P* < 0.001.

Table 2. Enhanced effect of C-phycoerythrin (cpc) on cyclic AMP production ‡
(Mean values with their standard errors for five determinations)

Treatment	Cyclic AMP (pmol/ml)	
	Mean	SEM
Resting	1.53	0.12
cpc (1 µg/ml)	4.96*	1.18
cpc (10 µg/ml)	6.35*	2.22
cpc (50 µg/ml)	7.20***	0.46
IBMX (50 µM)	7.26***	0.03
PGE ₁ (1 µM)	6.10***	0.05
IBMX + cpc (10 µg/ml)	6.65*	1.31
PGE ₁ + cpc (10 µg/ml)	13.6**†	2.3

IBMX, 3-isobutyl-1-methylxanthine; PGE₁, prostaglandin E₁.
‡ Tyrode solution, cpc (1, 10, 50 µg/ml), PGE₁, IBMX or the combination of PGE₁ + cpc (10 µg/ml) or IBMX + cpc (10 µg/ml) were preincubated with platelets at 37°C for 3 min. Cyclic AMP formation was stopped by boiling for 5 min. Mean values were significantly different from those of the resting platelets group: **P*<0.05, ***P*<0.01, ****P*<0.001. Mean values were significantly different from those of the cpc (10 µg/ml) alone group: †*P*<0.05.

platelet suspension resulted in a concentration-dependent decrease in DPH-related fluorescence intensity (Fig. 4), suggesting that cpc may increase the platelet membrane fluidity.

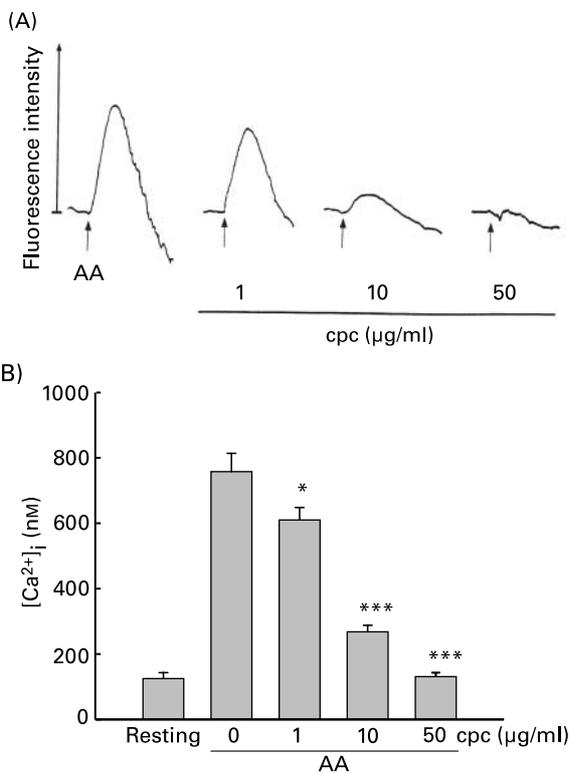


Fig. 3. Effect of C-phycoerythrin (cpc) on arachidonic acid (AA)-induced intracellular Ca²⁺ mobilization in Fura-2-loaded platelets. Fura-2-loaded platelets were preincubated with Tyrode solution or cpc (1–50 µg/ml) at 37°C for 3 min, then AA was added. The traces of fluorescence intensity are representative of five similar experiments (A). (B), The peak intracellular Ca²⁺ concentration ([Ca²⁺]_i) of each group. Values are means with their standard errors depicted by vertical bars (*n* 5). Mean values were significantly different from those of the control group: **P*<0.05, ****P*<0.001.

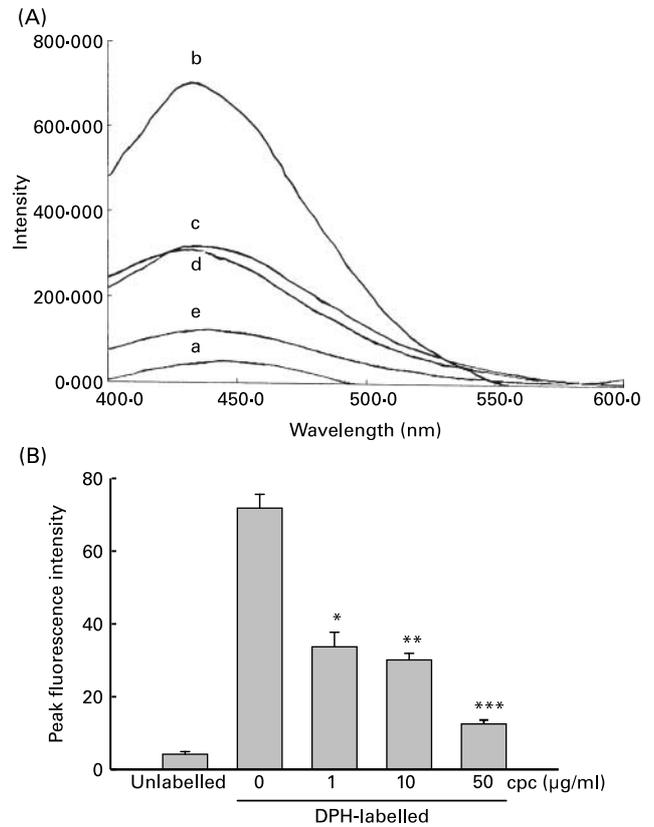


Fig. 4. Effect of C-phycoerythrin (cpc) on the fluorescence emission spectra (A) and the peak fluorescence intensity (B) in diphenylhexatriene (DPH)-labelled platelets. (A), DPH-unlabelled platelets (a); DPH-labelled platelets (b); DPH-labelled platelets in the presence of 1 (c), 10 (d) and 50 µg/ml (e) cpc. The profiles are representative examples of four similar experiments. (B), The peak fluorescence intensity of each group. Values are means with their standard errors depicted by vertical bars (*n* 4). Mean values were significantly different from those of the control group: **P*<0.05, ***P*<0.01, ****P*<0.001.

Effect of C-phycoerythrin on platelet glycoprotein IIb/IIIa expression

As shown in Fig. 5, cpc significantly decreased the collagen-induced platelet surface GPIIb/IIIa expression evaluated by the values of mean fluorescence intensity compared with that in collagen-stimulated platelets alone.

Discussion

In the present study, we first demonstrated that cpc was an inhibitor of platelet aggregation, which may be associated with the inhibition of TXA₂ formation, intracellular Ca²⁺ mobilization and platelet surface GPIIb/IIIa expression accompanied by increasing cyclic AMP formation and platelet membrane fluidity.

During platelet activation, AA can be released from membrane phospholipids by means of Ca²⁺-dependent phospholipase A₂, diglyceride lipase and phosphatidic acid-specific phospholipase A₂ (Bell *et al.* 1979; Billah *et al.* 1981). Then, the AA is subsequently converted to TXA₂, a potent stimulator of platelet aggregation. Our results showed that cpc dose-dependently inhibited formation of AA or collagen-induced TXB₂, a stable metabolite of TXA₂, suggesting that the antiplatelet effect of cpc is associated with the

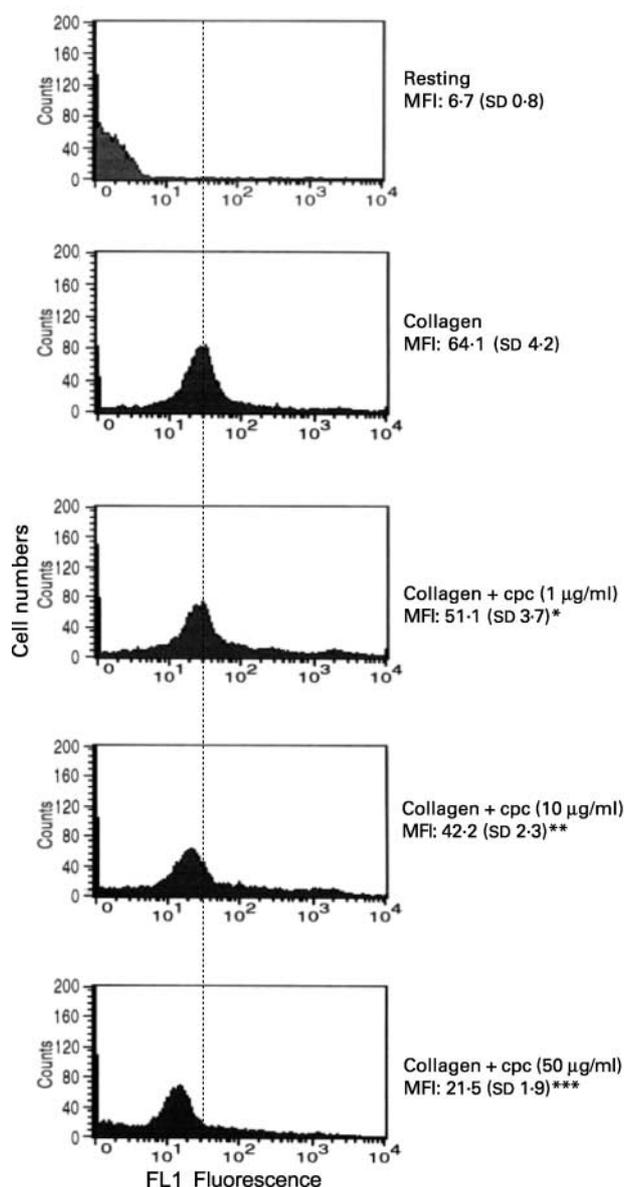


Fig. 5. Effect of C-phycoerythrin (cpc) on the platelet surface glycoprotein IIb/IIIa expression evaluated by a flow cytometric analysis. Platelets incubated with Tyrode solution alone acted as a resting group. The profiles are representative examples of five similar experiments and the mean fluorescence intensity (MFI) was expressed as means with their standard errors ($n = 5$). The dotted vertical line (:) indicates the peak cell number obtained from the curve of the collagen-treated alone group. Mean values were significantly different from those of the collagen-stimulated alone group: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

inhibition of TXA₂ formation. To investigate further the mechanisms by which cpc suppressed TXB₂ formation, the activities of COX and thromboxane synthase were evaluated. Treatment with cpc resulted in a marked inhibition of AA-induced PGE₂ formation, but only had a weaker attenuation for PGH₂-induced TXB₂ formation (Fig. 2). These findings indicate that the mechanisms by which cpc inhibits the formation of TXB₂ may be mainly through suppressing COX activity accompanied by a minor inhibition of thromboxane synthase activity. Because collagen-induced TXB₂ formation was also reduced by cpc, the possibility of cpc reducing phospholipase A₂ activity cannot be ruled out.

It has been demonstrated that elevation of intracellular cyclic AMP concentration causes inhibition of platelet responses including adhesion, aggregation, the release of granule contents and rise in intracellular Ca²⁺ mobilization (Feinstein *et al.* 1985; Sheu *et al.* 2002). The steady-state level of cyclic AMP is maintained by a balance between the rate of synthesis by adenylate cyclase and the rate of degradation by cyclic AMP phosphodiesterase. Our data showed that the increased cyclic AMP formation by cpc was markedly enhanced by addition of PGE₁ but not by IBMX. The present results suggest that the increase in cyclic AMP level by cpc is mainly mediated by inhibiting cyclic AMP phosphodiesterase activity rather than enhancing adenyl cyclase activity. However, cpc itself did not affect the cyclic GMP level, which may indicate that cpc is a more specific inhibitor of cyclic AMP phosphodiesterase.

Physiologically, the [Ca²⁺]_i of resting platelets is held at approximately 0.1 µM. When platelets are activated by stimulators, the [Ca²⁺]_i is markedly increased, which in turn causes platelet activation (Kroll & Schager, 1989). Our result indicated that the rise of [Ca²⁺]_i of platelets evoked by AA was significantly reduced by cpc in the presence of extracellular calcium (1 mM) or in the Ca²⁺-free solution cpc, implying that the inhibition of intracellular Ca²⁺ mobilization may be due to attenuation of calcium influx and calcium release from intracellular calcium stores. In addition, cyclic AMP also lowers platelet [Ca²⁺]_i by stimulating Ca²⁺ extrusion from cells and uptake of cytosolic Ca²⁺ into the dense tubular system (Feinstein *et al.* 1985; Enouf *et al.* 1987). Thus, the enhancement of cyclic AMP level by cpc may be also involved in the suppression of platelet intracellular Ca²⁺ mobilization.

Change in membrane fluidity is a critical determinant of platelet function through various pathways (Pribluda & Rotman, 1982; Tandon *et al.* 1988). Importantly, reduced platelet membrane fluidity (relative rigid membrane) often observed in patients may lead to the hyperactivity of platelet agonists *in vitro* (Winocour *et al.* 1990; Le Quan Sang *et al.* 1993). In the present study, we demonstrated that like other antiplatelet agents such as naloxone (Sheu *et al.* 1997), cpc significantly increased the platelet membrane fluidity, which may be a possible mechanism responsible for its antiplatelet effect. It has been reported that alterations in phospholipid composition of platelet membranes, the vitamin E content of platelets and membrane dynamic properties may contribute to the modulation of platelet membrane fluidity (Shattil & Cooper, 1976; Steiner, 1981; Srivastava & Dash, 2001). However, the exact mechanisms by which cpc enhances platelet membrane fluidity remain to be investigated further.

Platelet activation will finally result in the exposure and activation of the GPIIb/IIIa receptor, a binding with fibrinogen that is the final common pathway leading to platelet aggregation (Phillips *et al.* 1991). Blocking GPIIb/IIIa receptor with monoclonal antibodies will cause a broader spectrum of antiplatelet activity (Herman, 1998). As shown in Fig. 5, cpc significantly inhibited the collagen-induced platelet membrane surface GPIIb/IIIa expression, which may be a consequence of its multiple anti-aggregatory activities. Previous studies have reported that during platelet aggregation, reactive oxygen species including superoxide anion and hydrogen peroxide are released by platelets, which can induce platelet

activation (Iuliano *et al.* 1997). Furthermore, removing free radicals with superoxide dismutase or catalase resulted in an inhibition in platelet aggregation (Leo *et al.* 1997; Pignatelli *et al.* 1998). Accordingly, the antioxidant property of cpc (Romay *et al.* 2003) may, at least in part, account for its antiplatelet activity.

In conclusion, we first demonstrated that cpc is an inhibitor of platelet aggregation. Furthermore, we proposed that the antiplatelet activity of cpc may be associated with mechanisms including (1) inhibition of TXA₂ formation mainly due to suppression of COX activity; (2) elevation of cyclic AMP level through reducing cyclic AMP phosphodiesterase activity; (3) attenuation of platelet intracellular Ca²⁺ mobilization; (4) increase of platelet membrane fluidity; and (5) suppression of platelet membrane GPIIb/IIIa expression.

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