JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2008,** *56,* 9429–9433 **9429**

Involvement of Ca^{2+} in the Inhibition by Crocetin of Platelet Activity and Thrombosis Formation

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Crocetin, a unique carotenoid with potent antioxidative and anti-inflammatory activities, is a major ingredient of saffron used as an important spice and food colorant in various parts of the world. In the present study, the effects of crocetin on platelet activity and thrombosis formation were systematically investigated. Crocetin showed a dose-dependent inhibition of platelet aggregation induced by ADP, collagen, but not by arachidonic acid (AA). Crocetin significantly attenuated dense granule release, while neither platelets adhesion to collagen nor cyclic AMP level was altered by crocetin. Pretreatment with crocetin was confirmed to partially inhibit Ca^{2+} mobilization via reducing both intracellular Ca^{2+} release and extracellular Ca^{2+} influx. Besides that, crocetin prolonged the occlusive time in electrical stimulation-induced carotid arterial thrombosis. These findings suggest that the favorable impacts of crocetin on platelet activity and thrombosis formation may be related to the inhibition of Ca^{2+} elevation in stimulated platelets.

KEYWORDS: Crocetin; platelet aggregation; dense granule release; thrombosis formation; cyclic AMP; Ca^{2+}

INTRODUCTION

Intravascular thrombosis leads to sudden death in about 23% of patients all over the world, while the estimated direct and indirect cost of atherothrombotic diseases for 2007 is 130.1 billion RMB in China only (*1*). It has been reported that the platelet-rich thrombi are the indispensable sources of thromboembolic complications, such as atherosclerosis, heart attacks, strokes, and peripheral vascular diseases (*2*−*4*). Therefore, inhibition of platelet functions represents a promising approach for the prevention and treatment of cardiovascular diseases, such as thrombosis. Indeed, antiplatelet agents (e.g., ticlopidine and Aspirin) have been shown to reduce the incidence of stroke in high-risk patients (*5*). Today, many drugs have been used in the clinical therapy of thrombosis, but limited effectiveness, unfavorable adverse effect profile, and cost to benefit ratio emphasize the need for better and efficacious antithrombic agents.

Functional plant-based foods that contain bioactive components may provide desirable health benefits beyond basic nutrition and are practically useful for the prevention of chronic diseases such as cardiovascular diseases and cancer. *Crocus sativus* (saffron) has been cultivated in the region from Greece to Persia for at least 35 centuries. Nowadays, the fruits and flowers of *Crocus sativus* are still important dietary ingredients in various parts of the world (*6*). There are many uses for saffron in Ayurveda, Unani, and Chinese medicine, for instance in asthma, arthritis, cancer, and kidney problems. Crocetin, one of the major components of *Crocus sativus*, has multiple pharmacological effects including inhibiting tumor cell proliferation (*7*), protecting against hepatotoxicity (*8*), and preventing atherosclerosis (*9*). Besides that, saffron was described in the Chinese compendium Bencao Gangmu in 1596, mentioning that it was used to benefit blood (vitalize blood and stop bleeding). As an oxygen free radical scavenger (*10*), crocetin has been reported to inhibit platelet aggregation in rats with a hypercoagulative state (*11*), but the mechanism involved is unclear. Therefore, in the present study, we systematically investigated the effect of crocetin on thrombosis formation and platelet functions to reveal the possible mechanism.

MATERIALS AND METHODS

Materials. Collagen, TritonX-100, AA, adenosine diphosphate (ADP), DMSO, ethylenediamine tetraacetic acid (EDTA), ethyleneglycol bis(2-aminoethyl ether)tetraacetic acid (EGTA), and hydroxyethyl piperazine ethanesulfonic acid (HEPES) were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fluo-3-AM was obtained from Molecular Probes Inc. (Eugene, Oregon, USA). Newborn calf serum was from Invitrogen Corp. (Carlsbad, CA, Biosciences (Piscataway, NJ, USA). Cyclic AMP Kit was from Shanghai University of Traditional Chinese Medicine (Shang hai, China).

In the experiments in vitro, crocetin (China Pharmaceutical University, HPLC > 98%) was dissolved in dimethyl sulfoxide (DMSO) and diluted to the indicated concentration with PBS before the experiment, and the final concentration of DMSO did not exceed 0.01% (v/v). In

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10.1021/jf802027a CCC: $40.75
Published on Web 09/26/2008

the studies in vivo, crocetin was suspended in 0.5% sodium carboxymethyl cellulose (CMC-Na). All other chemicals used were analytically pure.

Animals. Male Sprague−Dawley rats were purchased from Slac Laboratory Animal Co. (Shanghai, China) with a body weight of 250−300 g. The rats had free access to a standard rat chow and water. Animal care procedures were approved by the Animal Care and Use Committee in China Pharmaceutical University prior to initiation of the experiment.

In the studies in vivo, rats were randomly divided into each group (8 animals each) and treated with 0.5% CMC-Na [control], 25 mg/kg or 50 mg/kg crocetin, and 30 mg/kg Aspirin. Drugs and CMC-Na were given for two days, twice a day. On the third day, one hour after the last administration, rats were anesthetized with ethyl ether, and blood was collected from the abdominal aorta.

Preparation of Platelets. Blood was collected from the abdominal aorta and anticoagulated with 3.8% sodium citrate (9:1, v/v), then centrifuged at 120 g for 5 min to get platelet-rich plasma (PRP). The supernatants were centrifuged at 600*g* for 15 min at room temperature to obtain platelet-poor plasma (PPP) and platelets. In the in vitro studies, the platelet pellets were washed with modified Tyrode-HEPES buffer (129 mM NaCl, 2.8 mM KCl, 8.9 mM $NaHCO_3$, 0.8 mM $MgCl_2$, 0.8 mM KH_2PO_4, 1 mM EGTA, 5.6 mM glucose, 10 mM HEPES, and 0.35% BSA, pH 7.4) and centrifuged at 600*g* for 15 min. After washing twice, platelets were then gently resuspended in Tyrode-HEPES buffer (129 mM NaCl, 2.8 mM KCl, 8.9 mM $NaHCO_3$, 0.8 mM $MgCl_2$, 0.8 mM KH_2PO_4, 1 mM $CaCl_2$, 5.6 mM glucose, 10 mM HEPES, and 0.35% BSA, pH 7.4) (*12*).

In Vivo Platelet Aggregation Assay. PRP and PPP were obtained as previously described. The platelet aggregation study was evaluated with the turbidimetric method described by Born and Cross (*13*). Aggregation was measured by the use of an aggregometer (Model LBY-NJ4, Beijing). Briefly, the amount of platelets in PRP was adjusted to 3×10^8/mL with PPP. Platelet suspension was incubated at 37 °C for 5 min in the aggregometer before aggregation induced by ADP (10 μM), collagen (50 μg/mL), or AA (0.3 mM), respectively. The aggregation was recorded in 5 min.

Assay of Platelet Adhesion to Collagen. Platelet adhesion to collagen was studied as previously described (*14*). Briefly, blood was collected from the abdominal aorta and anticoagulated with 2% EDTA (9:1, v/v). PPP and PRP were obtained as previously described. Platelet count in PRP was adjusted to $(3-4) \times 10^8$ mL^{-1} with PPP. PRP (0.3 mL) and collagen (50 $\mu g \cdot mL^{-1}$) were added to preheated tubes for comparative tests in the aggregometer at 37 °C, stirring for 10 min. Then, platelets in this suspension were counted. Comparison of the platelet count before the addition of collagen with that after contacting with collagen for 10 min with stirring was used to calculate the adhesion ratio.

Assay of Serotonin Secretion. The secretion of the dense granules was measured by the method of Mustard et al. using [^{14}C]-labeled serotonin-incorporated platelets (*15*). Washed platelets (3×10^8 platelets/mL) were incubated with [^{14}C]serotonin (0.2 μCi/mL) for 30 min at 37 °C, washed twice, and then resuspended in modified Tyrode's solution (pH 7.4), including imipramine (2 μM) to prevent reuptake of [^{14}C]serotonin. Subaliquots (450 μL) were withdrawn at 5 min after the addition of ADP and placed in ice-cold tubes, which contained 90 μL of 630 mM formaldehyde and 50 mM EDTA, followed by centrifuging at 12,000*g* for 2 min at room temperature. In order to determine the secretion of [^{14}C] serotonin from platelets, 100 μL aliquot of the supernatant was assayed by liquid-scintillation counting (tricarb(R), PerkinElmer). Percent of [^{14}C] serotonin secretion was determined as described by Holmsen et al. (*16*).

Assay of Cyclic AMP in Platelets. After a 5 min of platelet aggregation induced by ADP, the platelet suspension was immediately transferred to an ice-cold tube to end the aggregation, followed by centrifugation at 2000*g* for 15 min at 4 °C to obtain aggregated platelets.

Cyclic AMP in platelets was extracted as previously described with a slight modification (*17*). Briefly, rested and aggregated platelets were resuspended in PBS (pH 7.4) softly, and then 10% trichloroacetic acid was added to split the platelet membrane thoroughly. After centrifugation at 2000*g* for 15 min, the supernatant was transferred to another eppendorf tube and extracted twice with water-saturated diethyl ether to eliminate trichloroacetic acid. Finally, the solution was dried at 60 °C for 24 h to obtain cyclic AMP. The contents of cyclic AMP in rested and stimulated platelets were tested according to the instructions of the RIA kit (Shanghai, China).

Table 1. Inhibition of ADP, Collagen, AA- Induced Platelet Aggregation by Crocetin in Vivo[a]

	ADP-induced		collagen-induced		AA-induced	
group	aggregation (%)	inhibition (%)	aggregation (%)	inhibition (%)	aggregation (%)	inhibition (%)
control	56.0 ± 9.3		56.3 ± 7.7		67.0 ± 4.2	
crocetin						
25	40.4 ± 7.4[b]	28.2	39.0 ± 3.9[b]	30.4	65.0 ± 5.5	
50	35.7 ± 9.4[b]	36.6	37.4 ± 9.0[b]	33.3	67.1 ± 5.6	
Aspirin	30.1 ± 5.6[b]	46.6	29.9 ± 9.6[b]	46.5	11.3 ± 3.1[b]	83.2

[a] Values are the mean ± S.D. [b] $P < 0.01$, vs control.

Assay of Intracellular Ca^{2+} Mobilization in Platelets. The $[Ca^{2+}]_i$ of Fluo-3-AM-loaded platelets was determined by the method previously described with a slight modification (*18*). Briefly, platelets (8×10^8 /mL) were incubated with Fluo-3-AM (3 μM) for 45 min at 37 °C in the dark. Excess Fluo-3-AM was removed by centrifuging at 2000*g* for 15 min at room temperature and washing with EGTA (1 mM)-containing Tyrode-HEPES buffer. After centrifugation, platelets were gently resuspended in Tyrode-HEPES buffer containing 1 mM $CaCl_2$ or 1 mM EGTA to make a concentration of 3×10^8 /mL and were used within one hour. Fluorescence was measured at 37 °C in a spectrofluorometer (tecan safire2, Switzerland) at 505 nm excitation and 530 nm emission. In order to prevent leakage of dye, probenecid (2 mM) was added to platelet-rich plasma and all of the buffers throughout the experiments. $[Ca^{2+}]_i$ was calibrated by lysing the cells with 0.1% (v/v) Triton X-100 in the presence of 1 mM $CaCl_2$ or 10 mM EGTA (pH 9.0).

Assay of Electrical Stimulation-Induced Carotid Arterial Thrombosis. Thrombosis was induced by an electric stimulation according to Schumacher with a slight modification (*19*). Briefly, rats were anesthetized (pentobarbital, 40 mg/kg i.p.), fixed on an operation table, and kept warm by radiant heat from a light bulb. The left common carotid artery was exposed at the minimal length of 20 mm and carefully freed from the surrounding tissue. A piece of parafilm was placed under the exposed vessel for electrical isolation. A double-stainless-steel L-shape wire was placed on the adventitial surface of the artery proximal to the temperature inductor (BT87-3, Baotou, China). Electrical current of 1.5 mA was delivered by a constant current stimulator for 7 min. When the blood flow of the carotid artery decreased to zero, the potentiometer sounded an alarm. The occlusive time was from the beginning of current irritation to the occurrence of zero blood flow. Sham operation is the same as others, except the current was not applied. An occlusive time that is more than one hour is recorded as one hour.

Statistical Analysis. The data are expressed as the mean ± SD. Differences among groups were tested by one-way analysis of variance (ANOVA) with SPSS, and a P value less than 0.05 was considered as an appropriate level of significance.

RESULTS

Effects of Crocetin on Platelet Aggregation in Vivo. In vivo, crocetin (25 and 50 mg/kg) showed a potent ($P < 0.01$), dose-dependent inhibition of platelet aggregation induced by ADP (10 μM) and collagen (50 μg/mL), with maximal suppression ratio of 36.6% and 33.3%, respectively (**Table 1**.), whereas crocetin had no influence on the aggregation induced by AA (0.3 mM). In the Aspirin-treated group, the AA-induced aggregation was inhibited by 83.2%, indicating that the method and operation here were feasible.

Effect of Crocetin on Dense Granule Release (Serotonin Secretion), Cyclic AMP and Platelet Adhesion to Collagen. Crocetin inhibited ADP-induced serotonin secretion from dense

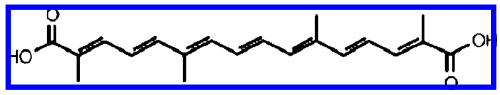

Figure 1. Chemical structure of crocetin.

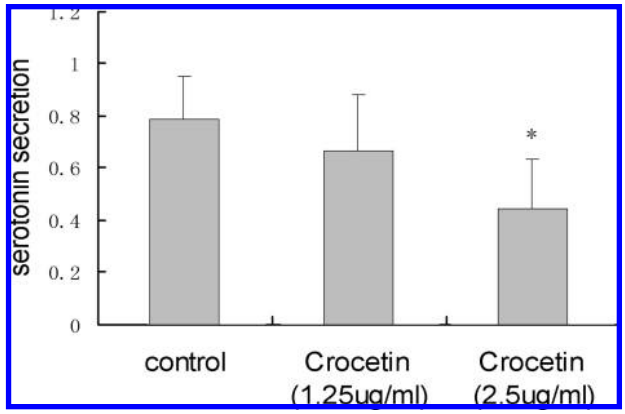

Figure 2. Effect of crocetin on ADP-induced dense granule release. The secretion of the dense granule was measured using [^{14}C] serotonin-incorporated platelets. Washed platelets were incubated with crocetin for 15 min prior to the addition of ADP (10 μM). Serotonin secretion was terminated at 5 min after the addition of ADP. *: $P < 0.05$, vs control.

granule in a concentration-dependent manner. Pretreatment with crocetin (2.5 μg/mL) could partially decrease the extent of serotonin release to 44.2 ± 19.5% from 78.8 ± 16.5% (control), with a maximal inhibition ratio of 35.3% (**Figures 1** and **2**), in accordance with the results in the aggregation studies. The content of cyclic AMP in rested platelets was not changed in the presence of crocetin, while crocetin at the concentration that exerted a great inhibition of platelet aggregation did not influence the decrease of cyclic AMP in platelets stimulated by ADP for 5 min. Also, crocetin had no effect on platelet adhesion to collagen (data not shown).

Effect of Crocetin on ADP-Induced Intracellular Ca^{2+} Release and Extracellular Ca^{2+} Influx. We examined the effect of crocetin on ADP- induced intracellular Ca^{2+} mobilization and extracellular Ca^{2+} influx in Fluo-3-AM loaded platelets. In Ca^{2+}-containing medium, ADP induced a significant rise of $[Ca^{2+}]_i$ to 660 ± 129.3 nM from 210.3 ± 40.8 nM in DMSO-pretreated platelets. Although crocetin had no effect on basal $[Ca^{2+}]_i$, crocetin (2.5 $\mu g \cdot mL^{-1}$) inhibited the maximal value of $[Ca^{2+}]_i$ induced by ADP in 5 min by approximately 31.4% (**Figure 3**).

In Ca^{2+}-free medium containing 1 mM EGTA, the basal $[Ca^{2+}]_i$ value and the first peak (intracellular Ca^{2+} release) induced by ADP for 5 min decreased significantly, compared with those in 1 mM Ca^{2+}-containing medium. If 1 mM Ca^{2+} was reintroduced into the medium, the ensuing second Ca^{2+} peak (Ca^{2+}influx) was found (**Figure 4a**). The peak of Ca^{2+} influx was a little lower than the peak appearing in **Figure 4a**, but much higher than the first peak, indicating that extracellular Ca^{2+} influx may be more important in platelet aggregation. However, pretreatment of crocetin partially attenuated both the initial Ca^{2+} mobilization and the ensuing second Ca^{2+} peak induced by ADP, with inhibitory extents of 27.2% and 32.5%, respectively (**Figure 4**).

Effect of Crocetin on Electrical Stimulation-Induced Carotid Arterial Thrombosis. Arterial thrombosis was induced by the application of 1.5 mA anodic current for 7 min. Administration of crocetin caused a marked, dose-dependent increase of occlusive time in the arterial thrombus model versus control ($p < 0.05$), from 12.8 ± 1.3 min (control) to 18.1 ± 4.7 min (50 mg/kg) (**Figure 5**). Sham operation without current application demonstrated that blood flow was maintained throughout the experiment.

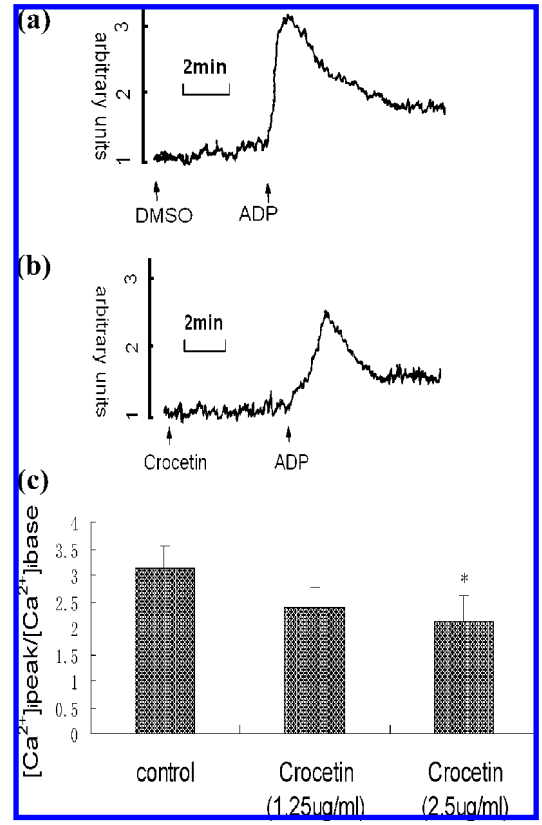

Figure 3. Effect of crocetin on cytosolic Ca^{2+} elevation in 1 mM Ca^{2+}-containing medium. (**a**) Tracing showing ADP-induced Ca^{2+} mobilization in DMSO-treated platelets in 1 mM Ca^{2+} -containing medium. (**b**) Fluo-3-AM (3 μM) loaded platelets were incubated with crocetin (1.25 and 2.5 μg/mL) for 15 min before the addition of ADP in 1 mM Ca^{2+}-containing medium. (**c**) Data were shown as the ratio value of $[Ca^{2+}]_i$ in peak with the basal level. *: $P < 0.05$, vs control.

DISCUSSION

Platelet activation is a complex process, which is mainly mediated through adhesion of platelets to the site of injury and the action of endogenous agonists such as thrombin, ADP, and collagen, followed by the release of a dense granule (*20, 21*). In this study, crocetin was found to partially reduce ADP- and collagen-induced platelet aggregation in vivo in a concentration-dependent manner, while a similar inhibitory effect of crocetin was shown in the thrombin-induced platelet aggregation (*11*). These results indicate that crocetin blocks a common step rather than on the receptor level of an individual agonist. In addition, this inhibition was not due to an influence on the use of exogenous AA and the enzymes responsible for the TXA_2 synthesis in platelet aggregation since crocetin did not affect the platelet aggregation induced by AA when compared to that in the DMSO-treated group.

After platelet activation, the dense granules released numerous endogenous agonists, including ADP and serotonin. ADP can further amplify the platelet activation stimulated by other agonists, such as thrombin and collagen (*22*). Crocetin showed similar extents of inhibition of platelet aggregation (induced by ADP, collagen or thrombin) and dense granule release (expressed by serotonin secretion), which means that the inhibitory

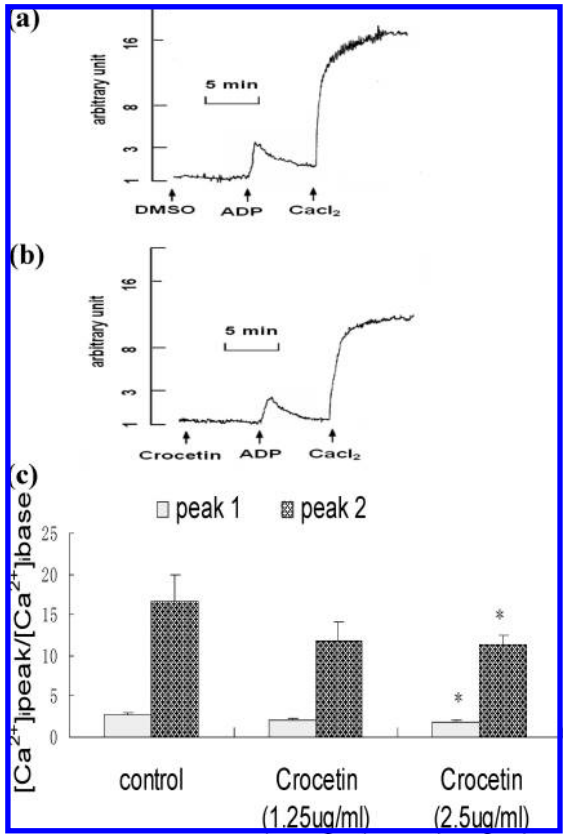

Figure 4. Effect of crocetin on intracellular Ca^{2+} mobilization and extracellular Ca^{2+} influx. (**a**) DMSO-treated platelets were stimulated with ADP in Ca^{2+} -free medium containing 1 mM EGTA. After 5 min, 1 mM Ca^{2+} was reintroduced to the medium. (**b**) Fluo-3-AM loaded platelets were incubated with crocetin (1.25 and 2.5 μg/mL) for 15 min before the addition of ADP in Ca^{2+}-free medium containing 1 mM EGTA. After 5 min of induction by ADP, 1 mM Ca^{2+} was reintroduced to the medium. (**c**) Data were shown as the ratio value of $[Ca^{2+}]_i$ in peak with the basal level. Peak 1: in Ca^{2+} -free medium containing 1 mM EGTA, ADP-induced the first rise of $[Ca^{2+}]_i$ from intracellular Ca^{2+} release. Peak 2: reintroducing 1 mM Ca^{2+} at 5 min after ADP treatment resulted in extracellular Ca^{2+} influx, which caused the second rise of $[Ca^{2+}]_i$. *: $P < 0.05$, vs control.

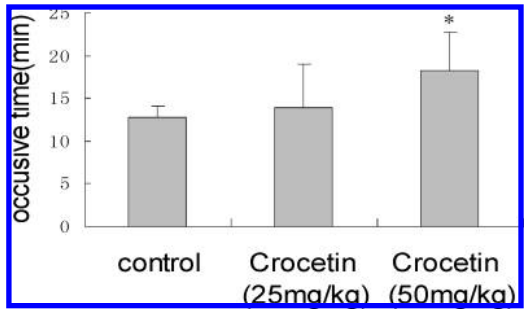

Figure 5. Effect of crocetin on electrical stimulation-induced carotid arterial thrombosis. Electrical current of 1.5 mA was delivered to the carotid artery by a constant current stimulator for 7 min. The occlusive time was from the beginning of current irritation to the occurrence of zero blood flow. Data were shown as the mean ± SD of 8 rats.*: $P < 0.05$, vs control.

effect of crocetin on platelet aggregation occurred via alleviating the release of ADP from the dense granule and ADP activity.

ADP binds to two major platelet receptors, $P2Y_1$ and $P2Y_{12}$. $P2Y_1$ is coupled to Gq, and ligation of this receptor in platelets is associated with phospholipase C-mediated cytosolic Ca^{2+} elevation and dense granule release (*23*). $P2Y_{12}$, however, is associated with the inhibition of adenylyl cyclase, activating platelets by suppression of cyclic AMP (*24*). The level of cyclic AMP in rested and stimulated platelets was unchanged in the crocetin treated group; therefore, the signal pathway beginning with G_i-coupled $P2Y_{12}$ receptor activation is not the exact site of action for crocetin. In contrast, crocetin significantly inhibited ADP-induced $[Ca^{2+}]_i$ elevation mediated by the Gq-coupled $P2Y_1$ receptor.

The elevation of cytosolic Ca^{2+} plays a fundamental role in mediating dense granule release and platelet aggregation (*25*). Accordingly, agents with inhibition of the cytosolic Ca^{2+} mobilization in platelets may suppress platelet aggregation. In this study, our results showed that crocetin partially attenuated both the initial release of Ca^{2+} from intracellular stores and influx of extracellular Ca^{2+} in Fluo-3-AM loaded platelets induced by ADP. The similar inhibitory effect of crocetin on Ca^{2+} elevation was also found in vascular smooth muscle cells induced by angiotensin II (*26*). In different cells, the signal pathways to evoke Ca^{2+}elevation differ, but crocetin shows a similar inhibitory extent, revealing that crocetin may directly influence the function of Ca^{2+}channels. In addition, crocetin, as a carotenoid compound, is lipid-soluble and may act as a membrane-associated highly efficient free radical scavenger (*27*) to stabilize the Ca^{2+} channel. Crocetin might directly inhibit the intracellular Ca^{2+} elevation to mediate platelet aggregation and release of the dense granule. Further studies about the effects of crocetin on Ca^{2+} channels are needed to clarify this finding.

Cyclic AMP and Ca^{2+} are two of the most important second messengers in platelet activation. On the one hand, increases in cyclic AMP result in additional $InsP_3$ (inositol 1, 4, 5-triphosphate) receptor phosphorylation that inhibits Ca^{2+} release, thus contributing to the inhibition of platelet activation (*28*). In the present study, we found that crocetin partially attenuated Ca^{2+} elevation other than the cyclic AMP level, which means that the inhibitory effect of crocetin on Ca^{2+} mobilization induced by ADP is cyclic AMP independent. On the other hand, despite the fact that all classes of adenylyl cyclase (AC, which form cyclic AMP from ATP) exhibit inhibition at supramicromolar Ca^{2+} concentration, ACs can be Ca^{2+}-stimulable (AC_1, AC_8, and AC_3), Ca^{2+}-inhibitable (AC_5 and AC_6), or Ca^{2+}-insensitive (AC_2, AC_4, AC_7, and AC_9) at submicromolar concentration (*29*). In the rested and stimulated platelets in this research, all of the Ca^{2+} levels were in the submicromolar range, implying that the influence of Ca^{2+} on ACs or cyclic AMP may be uncertain. That is why the partial modification of Ca^{2+} concentration by crocetin does not lead to the alteration of cyclic AMP compared to the control group. These effects of crocetin on Ca^{2+} and cyclic AMP are in accordance with those of another carotenoid lycopene (*30*).

The abnormal platelet functions play a pathophysiologic role in a variety of thromboembolic disorders. Drugs that mediate platelet functions may represent an increased therapeutic possibility for such diseases. In the present study, we found that crocetin could partially inhibit platelet aggregation and granule release but not adhesion, which means that crocetin may act as an antithrombotic drug. Pretreatment with crocetin significantly prolonged the occlusive time in the electrical stimulation-induced carotid arterial thrombosis, which is mainly due to the inhibition of platelet aggregations and dense granule releases.

Taken together, the present study shows that crocetin has inhibitory effects on the activation of platelets; the antiplatelet activity of crocetin might be related to the inhibition of Ca^{2+} release from an internal store and extracellular Ca^{2+} influx. In

addition, results in this study clearly indicate that crocetin may exhibit pharmacological functions during thromboembolism formation.

Today, the World Health Organization estimates that 80% of the people in developing countries of the world rely on traditional medicine for their primary health care. On the basis of this research, we, therefore, believe that our data represent a pharmacologically relevant function of crocetin as a potential agent for the prevention and treatment of cardiovascular diseases.

ABBREVIATIONS USED

AA, arachidonic acid; AC, adenylyl cyclase; ADP, adenosine diphosphate; ANOVA, one-way analysis of variance; CMC-Na, carboxymethyl cellulose sodium; DMSO, dimethyl sulfoxide; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycol bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl) piperazine-1- ethanesulfonic acid; Fluo3-AM, 4-(6-Acetoxymethoxy-2,7-dichloro-3-oxo-9-xanithenyl)-4′-methyl-2,2′ (ethylenedioxy)dianiline-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxy methyl) ester; PRP, platelet-rich plasma; PPP, platelet-poor plasma.

SAFETY

[^{14}C] serotonin is radioactive; necessary protection is needed.

ACKNOWLEDGMENT

We thank Guan Yue and Yan Ming for technical assistance.

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Received for review July 3, 2008. Revised manuscript received July 29, 2008. Accepted September 1, 2008.

JF802027A