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α-Naphthoflavone, a Potent Antiplatelet Flavonoid, Is Mediated through Inhibition of Phospholipase C Activity and Stimulation of Cyclic GMP Formation

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The aim of this study was to systematically examine the inhibitory mechanisms of the flavonoid α-naphthoflavone (α-NF) in platelet activation. In this study, α-NF concentration dependently (5−20 μM) inhibited platelet aggregation stimulated by agonists. α-NF (5 and 10 μM) inhibited intracellular Ca^{2+} mobilization, phosphoinositide breakdown, and thromboxane A_2 formation stimulated by collagen (1 μg/mL) in human platelets. In addition, α-NF (5 and 10 μM) markedly increased levels of cyclic GMP and cyclic GMP-induced vasodilator-stimulated phosphoprotein (VASP) Ser^{157} phosphorylation. Rapid phosphorylation of a platelet protein of *Mr* 47 000 (P47), a marker of protein kinase C activation, was triggered by phorbol-12,13-dibutyrate (60 nM). This phosphorylation was markedly inhibited by α-NF (5 and 10 μM). However, α-NF (5 and 10 μM) did not reduce the electron spin resonance (ESR) signal intensity of hydroxyl radicals in collagen (1 μg/mL)-activated platelets. These results indicate that the antiplatelet activity of α-NF may be involved in the following pathways. (1) α-NF may inhibit the activation of phospholipase C, followed by inhibition of phosphoinositide breakdown, protein kinase C activation, and thromboxane A_2 formation, thereby leading to inhibition of intracellular Ca^{2+} mobilization. (2) α-NF also activated the formation of cyclic GMP, resulting in inhibition of platelet aggregation. These results strongly indicate that α-NF appears to represent a novel and potent antiplatelet agent for treatment of arterial thromboembolism.

KEYWORDS: α-Naphthoflavone; platelet aggregation; protein kinase C; cyclic GMP; vasodilator-stimulated phosphoprotein

INTRODUCTION

Flavonoids are substances of low molecular weight occurring naturally in fruit, vegetables, grains, and other plants (*1*). These natural products are known for their beneficial effects on health, especially for protection against cardiovascular diseases and cancer (*2*, *3*). However, the structural complexity of flavonoids in foods and beverages (over 4000 flavonoids have been identified) makes it difficult to extrapolate from studies on individual flavonoids the effects of consumption of flavonoids in foods, beverages, and dietary supplements. Recent studies in vitro and in vivo show that some flavonoids exhibit antioxidant and free radical-scavenging properties (*4*). Therefore, dietary flavonoids, which possess antioxidant activity, may play a role in human health, particularly in diseases believed to involve, in part, oxidation, such as coronary heart disease, inflammation, and mutagenesis leading to carcinogenesis (*4*, *5*). α-Naphthoflavone (α-NF) is a prototype flavone that belongs to a group of phytochemicals and is a normal component of human diets (*6*). Most studies have demonstrated the effect of α-NF on cytochrome P450-mediated hydroxylation of benzopyrene, an environmental carcinogenic pollutant in experimental animals (*7*). Furthermore, α-NF is also an antagonist at the aromatic hydrocarbon receptor, the cellular receptor of benzopyrene and other polycyclic aromatic hydrocarbons (PAHs) (*8*, *9*). Recently, Cheng et al. (*5*) reported that α-NF also induces vasorelaxation in rat thoracic aorta.

Intravascular thrombosis is a generator of a wide variety of cardiovascular diseases. The 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 adherent platelets release some biologically active constituents and aggregate (*10*). Thus, platelet aggregation may play a crucial role in the atherothrombotic process. Indeed, antiplatelet agents (e.g., ticlopidine and aspirin) have been shown to reduce the incidence of stroke in high-risk patients (*11*). Flavonoids have been shown to inhibit platelet aggregation (*12*, *13*). However, the pharmacological function

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of α-NF in platelets has not yet been studied, and no data are available concerning the detailed effect of α-NF on platelet aggregation. We therefore systematically examined the influence of α-NF in washed human platelets and utilized the findings to characterize the mechanisms involved in this influence.

MATERIALS AND METHODS

Materials. Collagen (type I, bovine achilles tendon), α-NF, ADP, luciferin-luciferase, fluorescein sodium, Dowex-1 (100−200 mesh; X_8, chloride form), myoinositol, prostaglandin E_1 (PGE_1), arachidonic acid, phorbol-12, 13-dibutyrate (PDBu), apyrase, osmium tetroxide, bovine serum albumin, heparin (porcine intestinal mucosa), and thrombin were purchased from Sigma Chem. (St. Louis, MO). Fura 2-AM was purchased from Molecular Probe (Eugene, OR). Myo-2-[^{3}H] inositol was purchased from Amersham (Buckinghamshire, HP, U.K.). Thromboxane B_2, cyclic AMP, and cyclic GMP EIA kits were purchased from Cayman (Ann Arbor, MI). α-NF was dissolved in dimethyl sulfoxide (0.5% DMSO) and stored at −4 °C until use.

Preparation of Human Platelet Suspensions. Human platelet suspensions were prepared as previously described (*14*). 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 the blood was mixed with acid/citrate/glucose (ACD). After centrifugation at 120*g* for 10 min at room temperature, the supernatant (platelet-rich plasma; PRP) was supplemented with PGE_1 (0.5 μM) and heparin (6.4 IU/mL) and then incubated for 10 min at 37 °C and centrifuged at 500*g* for 10 min. The washed platelets were finally suspended in Tyrode's solution containing bovine serum albumin (3.5 mg/mL) and adjusted to about 4.5×10^8 platelets/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 (*15*) using a Lumi-Aggregometer (Payton, Canada). Platelet suspensions (4.5×10^8 platelets/mL, 0.4 mL) were prewarmed to 37 °C for 2 min, and α-NF (5−20 μM) and a solvent control (0.5% DMSO) were added 3 min before the addition of agonists. 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 agonists, and ATP release was compared with that of the control.

Analysis of the Platelet Surface Glycoprotein IIb/IIIa Complex by Flow Cytometry. Triflavin, a specific fibrinogen receptor (glycoprotein IIb/IIIa complex) antagonist, was prepared as previously described (*16*, *17*). Fluorescence-conjugated triflavin was also prepared as previously described (*18*). The final concentration of FITC-conjugated triflavin was adjusted to 1 mg/mL. Human platelet suspensions were prepared as described above. Aliquots of platelet suspensions (4.5×10^8/mL) were preincubated with α-NF (5 and 10 μM) or isovolumetric solvent control (0.5% DMSO) for 3 min, followed by the addition of 2 μL of FITC-triflavin. 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 with 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.

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 [Ca^{2+}]i rise was measured using a fluorescence spectrophotometer (CAF 110, Jasco, Tokyo, Japan) with excitation wavelengths of 340 and 380 nm and an emission wavelength of 500 nm. The [Ca^{2+}]i was calculated from the fluorescence measured using 224 nM as the Ca^{2+}-Fura 2 dissociation constant (*19*).

Labeling of Membrane Phospholipids and Measurement of the Production of [^{3}H]-Inositol Phosphates. The method was carried out as previously described (*14*). Briefly, citrated human platelet-rich plasma 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 Tyrode's solution (5×10^8/mL). α-NF (5 and 10 μM) or isovolumetric solvent control (0.5% DMSO) was preincubated with 1 mL of loaded platelets for 3 min, and collagen (1 μg/mL) was then added to trigger aggregation. The reaction was stopped, and samples were centrifuged for 4 min. The inositol phosphates of the supernatants were separated in a Dowex-1 anion exchange column. Only [^{3}H]-inositol monophosphate (IP) was measured as an index of total inositol phosphate formation.

Measurement of TxB_2, Cyclic AMP, and Cyclic GMP Formation. Platelet suspensions (4.5×10^8/mL) were preincubated for 3 min in the presence or absence of α-NF (5 and 10 μM) before the addition of collagen (1 μg/mL). Six minutes after the addition of agonists, 2 mM EDTA and 50 μM indomethacin were added to the reaction suspensions. The vials were then centrifuged, and TxB_2 levels of the supernatants were measured using an EIA kit. In addition, platelet suspensions were incubated with nitroglycerin (10 μM), PGE_1 (10 μM), and α-NF (5 and 10 μM) for 6 min. The incubation was stopped, and the solution was immediately boiled for 5 min. Fifty microliters of 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.

Measurement of Protein Kinase C (PKC) Activity. Washed platelets (2×10^9/mL) were incubated for 60 min with phosphorus-32 (0.5 mCi/mL). The [^{32}P]-labeled platelets were incubated with α-NF (5 and 10 μM) for 3 min, and then PDBu (60 nM) was added for 1 min to trigger protein kinase C activation. Activation was terminated by the addition of Laemmli sample buffer and was analyzed by electrophoresis (12.5%; wt/vol) as described previously (*20*). The gels were dried, and the relative intensities of the radioactive bands were analyzed using a Bio-imaging analyzer system (FAL2000, Fuji, Tokyo, Japan) and were expressed as PSL/mm^2 (PSL, photostimulated luminescence).

Western Blot Analysis of Vasodilator-Stimulated Phosphoprotein (VASP) Phosphorylation. The method of Li et al. (*21*) was followed. In brief, 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, pH7.5), incubated with the mAb 5C6 (CALBIOCHEM), specific for the phosphorylated Ser^{157} site of VASP (0.1 μg/mL). After three washes in TBS containing 0.05% Tween 20, the membrane was incubated with peroxidase-conjugated goat antimouse IgG (Amersham) for 2 h. The band with peroxidase activity was detected by enhanced chemiluminescence detection reagents (ECL^+ system; Amersham). Densitometric analysis of specific bands was performed with a Photo-Print Digital Imaging System (IP-008-SD) with analytical software (Bio-1Dlight, V 2000).

Measurement of Free Radicals in Platelet Suspensions by Electron Spin Resonance (ESR) Spectrometry. The ESR method used a Bruker EMX ESR spectrometer as described previously (*22*). In brief, platelet suspensions (4.5×10^8 platelets/mL, 0.4 mL) were prewarmed to 37 °C for 2 min, and then α-NF (5 and 10 μM) was added for 3 min before the addition of collagen (1 μg/mL). The reaction was allowed to proceed for 1 min, followed by the addition of 100 mM 5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide (DEPMPO) for the ESR study. ESR spectra were recorded on a Bruker EMX ESR spectrometer using a quartz flat cell designed for aqueous solutions. Conditions of ESR spectrometry were as follows: 20 mW power at 9.78 GHz, 1 G modulation, and 100 G scanning for 42 s, with 10 scans accumulated.

Statistical Analysis. The experimental results are expressed as the means ± SEM 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

Effect of α-NF on Platelet Aggregation in Human Platelets. α-NF (5−20 μM) concentration dependently inhibited

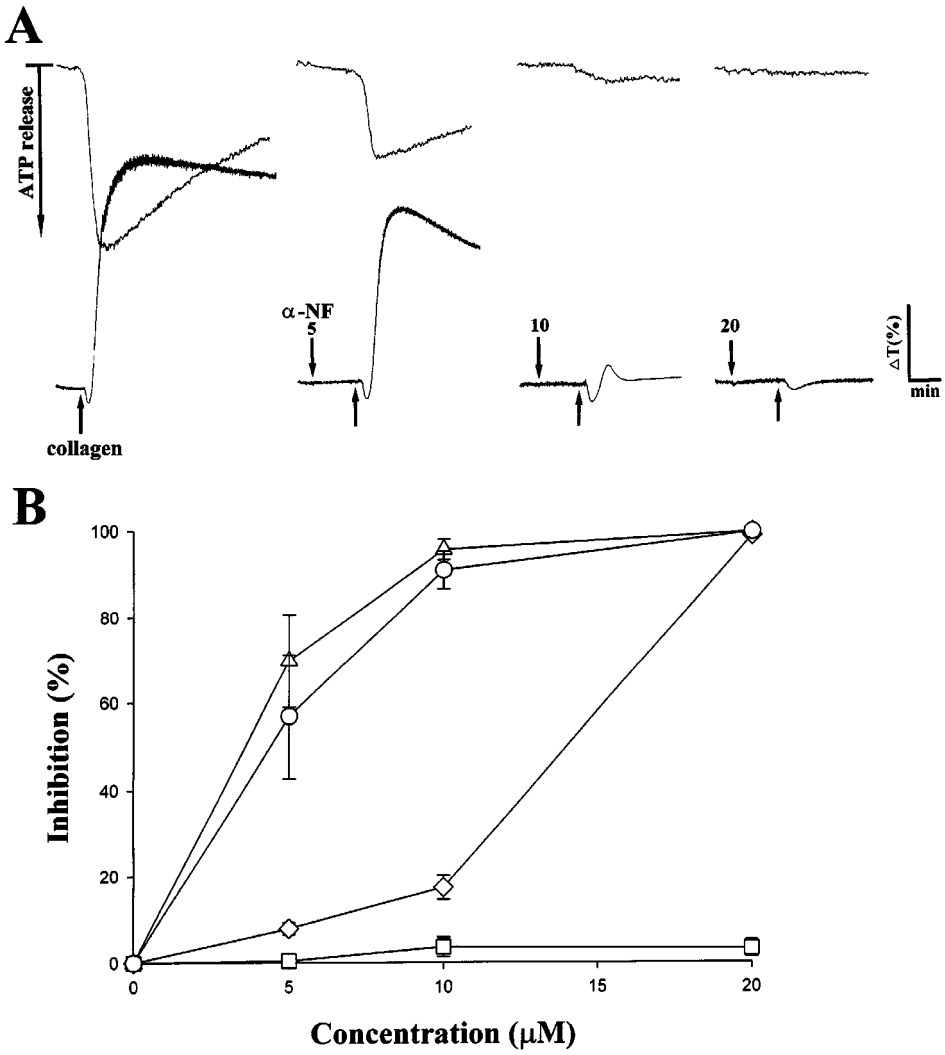

Figure 1. (**A**) Aggregation curves and (**B**) concentration-inhibition curves of α-NF on ADP (20 μM, circle), collagen (1 μg/mL, triangle), thrombin (0.01 U/mL, square), and arachidonic acid (60 μM, rhombus) induced platelet aggregation in washed human platelets. Platelets were preincubated with α-NF (5–20 μM) for 3 min; agonists were then added to trigger aggregation (lower tracings) and ATP release (upper tracings) (**A**). Data are presented as a percentage of the control (means ± SEM, $n = 4$).

platelet aggregation stimulated by ADP (20 μM), collagen (1 μg/mL), and arachidonic acid (60 μM) but not by thrombin (0.01 U/mL) in washed human platelets (**Figure 1**) and platelet-rich plasma (data not shown). Furthermore, α-NF also inhibited the ATP-release reaction when stimulated by agonists (i.e., collagen) (**Figure 1A**). IC_{50} values of α-NF for platelet aggregation induced by ADP, collagen, and arachidonic acid were estimated to be approximately 5.3, 4.8, and 11.9 μM, respectively. On the other hand, its solvent control (0.5% DMSO) did not significantly affect platelet aggregation stimulated by agonists (data not shown). When platelets were preincubated with a higher concentration of α-NF (40 μM) or 0.5% DMSO for 10 min, followed by two washes with Tyrode's solution, we found that there were no significant differences between the aggregation curves of either platelet preparations stimulated by collagen (1 μg/mL), indicating that the effect of α-NF on inhibition of platelet aggregation occurs in a reversible manner (data not shown). In subsequent experiments, we used collagen as an agonist to explore the inhibitory mechanisms of α-NF in platelet aggregation.

Effect of α-NF on Collagen-Induced Conformational Change of the Glycoprotein IIb/IIIa Complex in Human Platelets. Triflavin is an Arg-Gly-Asp-containing antiplatelet peptide purified from *Trimeresurus flavoviridis* snake venom (*16*). Triflavin inhibits platelet aggregation through direct interference with fibrinogen binding to the glycoprotein IIb/IIIa complex ($\alpha_{IIb}\beta_3$ integrin) (*16*). There is now a multitude of evidence suggesting that the binding of fibrinogen to the glycoprotein IIb/IIIa complex is the final common pathway for agonist-induced platelet aggregation. Therefore, we decided to further evaluate whether α-NF binds directly to the platelet glycoprotein IIb/IIIa complex, leading to inhibition of platelet aggregation induced by agonists.

In this study, the relative intensity of fluorescence of FITC-triflavin (2 μg/mL) bound directly to collagen (1 μg/mL)-activated platelets was 246.9 ± 15.1 ($n = 4$, data not shown), and it was markedly reduced in the presence of 5 mM EDTA (negative control, 63.0 ± 5.4, $n = 4$, $p < 0.001$) (data not shown). α-NF (5 and 10 μM) did not significantly inhibit FITC-triflavin binding to the glycoprotein IIb/IIIa complex in platelet suspensions (5 μM, 236.7 ± 12.5; 10 μM, 233.1 ± 14.4, $n = 4$) (data not shown), indicating that the inhibitory effect of α-NF on platelet aggregation does not involve binding to the glycoprotein IIb/IIIa complex.

Effect of α-NF on $[Ca^{2+}]i$ Mobilization and Phosphoinositide Breakdown in Human Platelets. Free cytoplasmic

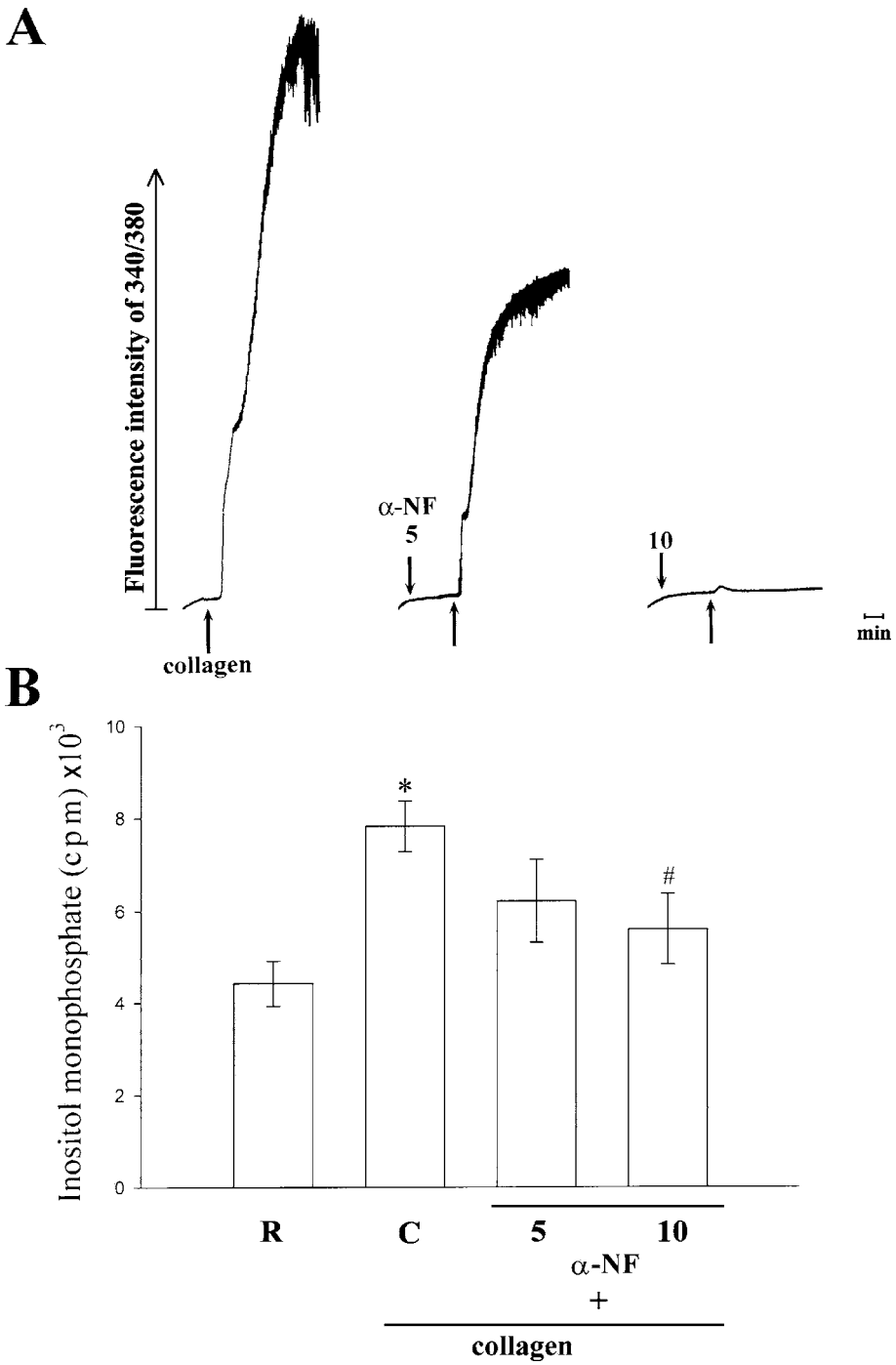

Figure 2. Effects of α-NF on (**A**) collagen-induced [Ca^{2+}]i and (**B**) collagen-induced inositol monophosphate formation in human platelets. Labeled platelets were preincubated with α-NF (5 and 10 μM) or isovolumetric solvent control (0.5% DMSO, C) followed by the addition of collagen (1 μg/mL) to trigger platelet activation as described in Materials and Methods. (**A**) Profiles are representative examples of four similar experiments; (**B**) data are presented as the means ± SEM ($n = 4$). *$p < 0.05$ as compared with the resting group (R); #$p < 0.05$ as compared with the solvent control (C) group.

Ca^{2+} concentrations in human platelets were measured by the Fura 2-AM loading method. As shown in **Figure 2A**, collagen (1 μg/mL) evoked a marked increase in [Ca^{2+}]i, and this increase was markedly inhibited in the presence of α-NF (5 μM, 56.3 ± 4.1%; 10 μM, 95.2 ± 3.6%) (**Figure 2A**). Furthermore, phosphoinositide breakdown occurs in platelets activated by many different agonists (*23*). In this study, we found that 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 **Figure 2B**, the addition of collagen (1 μg/mL) resulted in a rise of IP formation of about 1.8-fold compared to that in resting platelets ([7.8 ± 0.5 vs 4.4 ± 0.5] × 10^3 cpm). In the presence of α-NF (10 μM), the radioactivity of IP formation in collagen-stimulated human platelets was markedly decreased (**Figure 2B**).

Effect of α-NF on PDBu-Stimulated Phosphorylation of the 47-kDa Protein. Stimulation of platelets with several different agonists, PDBu in particular, induces activation of PKC, which then phosphorylates proteins of *Mr* 40 000−47 000 in addition to other proteins (*24*). In this study, phosphorylation experiments were performed to examine the role of α-NF in the activation of PKC in human platelets. When PDBu (60 nM) was added to human platelets prelabeled with $^{32}PO_4$, a protein

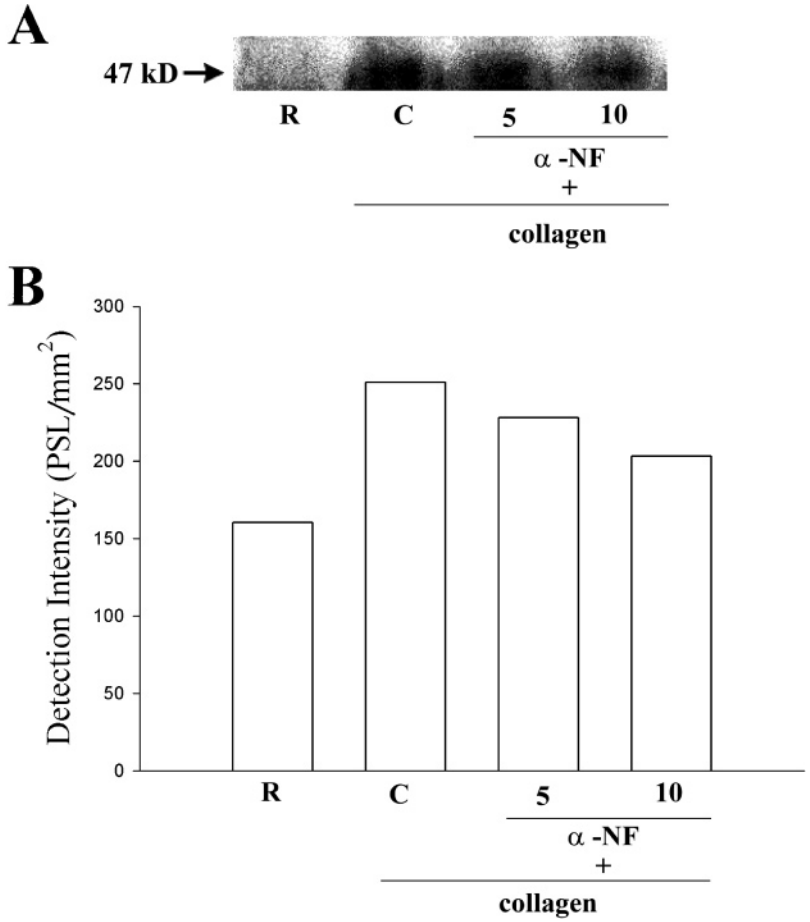

Figure 3. Effect of α-NF on phosphorylation of a protein of *Mr* 47 000 (P47) in human platelets challenged with PDBu. Platelets were preincubated with Tyrode's solution only (R), or platelets were preincubated with α-NF (5 and 10 μM) or isovolumetric solvent control (0.5% DMSO, C) followed by the addition of PDBu (60 nM) to trigger protein kinase C acivation. (**A**) The profiles are representative examples of four similar experiments. The arrow indicates a protein of *Mr* 47 000 (P47). (**B**) The relative detection densities of the radioactive bands are expressed as PSL/mm^2 (PSL, photostimulated luminescence).

with an apparent *Mr* of 47 000 (P47) was predominately phosphorylated as compared with resting platelets (**Figure 3A**). α-NF (5 and 10 μM) markedly inhibited the phosphorylation of P47 stimulated by PDBu. In this study, the extent of radioactivity in P47 was expressed as a relative detection density (PSL/mm^2; PSL, photostimulated luminescence) of the radioactive bands (**Figure 3B**). Moreover, α-NF (5 and 10 μM) also significantly inhibited collagen (1 μg/mL)-induced phosphorylation of P47 in human platelets (data not shown).

Effect of α-NF on Cyclic AMP, Cyclic GMP, and TxB_2 Formation, and VASP Phosphorylation. As shown in **Figure 4A**,**B**, levels of cyclic AMP and cyclic GMP in resting platelets were relatively lower compared with those of PGE_1 (10 μM) and nitroglycerin (NTG, 10 μM) treated platelets, respectively. Addition of α-NF (10 μM) significantly increased the level of cyclic GMP (resting, 0.38 ± 0.05 pmol/mL vs 10 μM, 0.59 ± 0.05 pmol/mL; $p < 0.05$) but not cyclic AMP (**Figure 4A**,**B**). Moreover, resting platelets produced relatively little TxB_2 compared with collagen-activated platelets. α-NF (5 and 10 μM) markedly inhibited TxB_2 formation in platelets stimulated by collagen (1 μg/mL) (**Figure 4A**).

In addition, it is presumed that cyclic GMP can induce VASP Ser^{157} phosphorylation in human platelets (*21*). In this study, nitroglycerin (10 μM) markedly induced VASP Ser^{157} phosphorylation, and this phosphorylation was significantly inhibited by guanylate cyclase inhibitors, LY83583 (10 μM) (*25*) and ODQ (10 μM) (**Figure 4C**) (*26*). α-NF (5 and 10 μM) concentration dependently triggered VASP Ser^{157} phosphorylation, and this phosphorylation was potentiated by NTG (10 μM) and was inhibited by LY83583 (10 μM) or ODQ (10 μM) (**Figure 4C**).

Effect of α-NF on Free-Radical-Scavenging Activity in Collagen-Activated Platelets. The rate of free-radical-scavenging activity is defined by the following equation: inhibition rate = 1-signal height (α-NF + collagen)/signal height (collagen) (*27*). In this study, a typical ESR signal of the hydroxyl radical (OH•) (asterisks) in collagen-activated platelets was observed as shown in **Figure 5**. However, α-NF (5 and 10 μM) did not significantly suppress hydroxyl radical formation in collagen-activated platelets even at a higher concentration (20 μM) (data not shown).

DISCUSSION

This study demonstrates for the first time that α-NF possesses potent antiplatelet activity in human platelets. The principal objective of this study was also to describe the detailed inhibitory mechanisms of α-NF in platelet activation. In this study, platelet aggregation induced by these agonists (i.e., collagen) appeared to be affected in the presence of α-NF. Therefore, this partly infers that α-NF may affect Ca^{2+} release from intracellular Ca^{2+}-storage sites (i.e., dense tubular systems or dense bodies), and this is in accord with the concept that intracellular Ca^{2+} release is responsible for platelet aggregation.

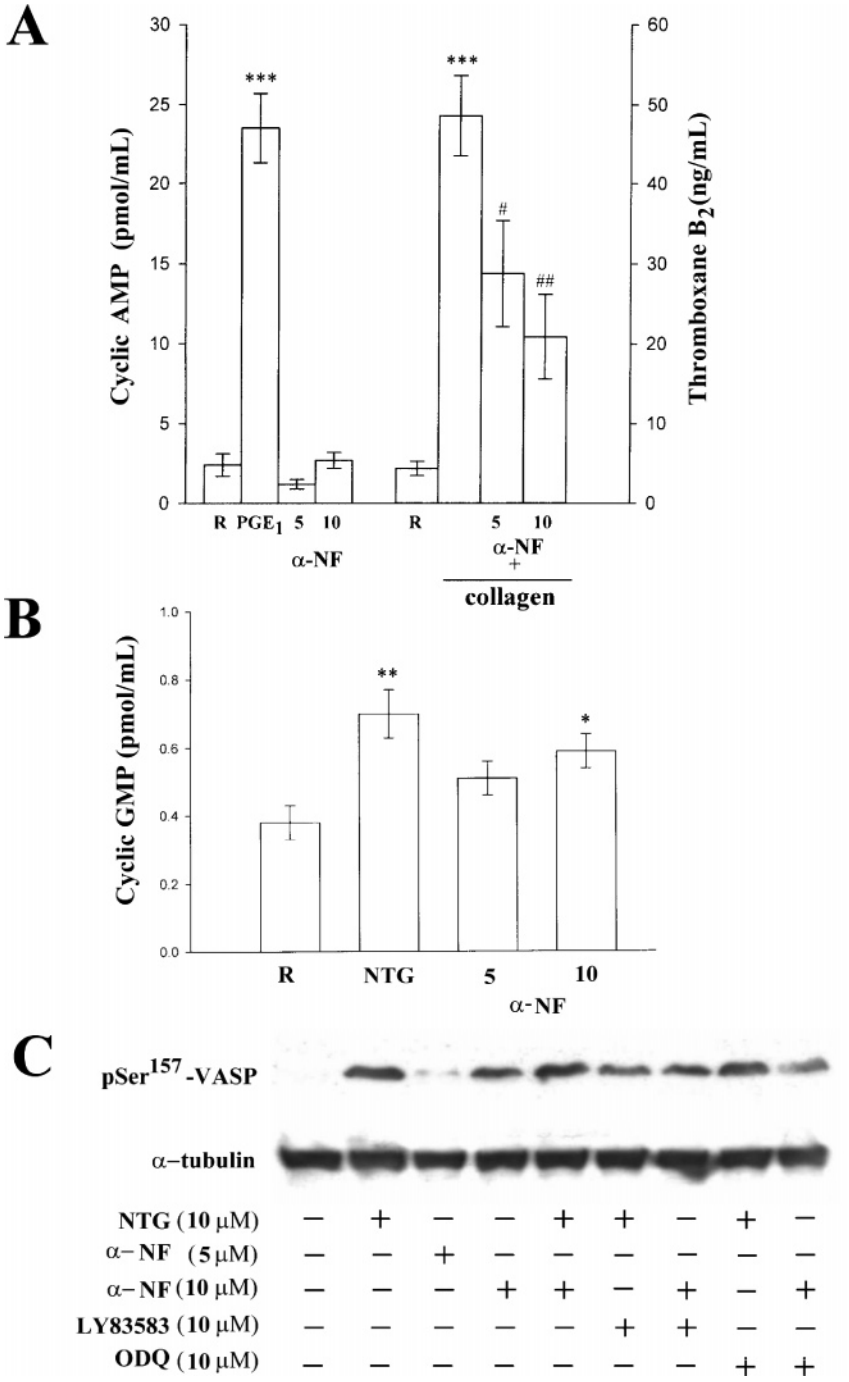

Figure 4. Effects of α-NF on (**A**) cyclic AMP and TxB_2 formation, (**B**) cyclic GMP formation, and (**C**) agonist-induced phosphorylation of VASP at Ser^{157} in human platelets. (**A**, **B**) Platelets were incubated with prostaglandin E_1 (PGE_1, 10 μM), nitroglycerin (NTG, 10 μM), and α-NF (5 and 10 μM) for measurement of cyclic AMP, cyclic GMP, and TxB_2 formation as described in Materials and Methods. The addition of PGE_1 and nitroglycerin to platelets served as a positive control of cyclic AMP and cyclic GMP, respectively. (**C**) Platelets were incubated with nitroglycerin (10 μM) and α-NF (5 and 10 μM) in the absence or presence of LY83583 (10 μM) or ODQ (20 μM) and were solubilized directly in SDS–PAGE sample buffer. Phosphorylation of VASP at Ser^{157} was detected by immunoblotting with a monoclonal antibody specifically recognizing Ser^{157}-phosphorylated VASP. (**A**, **B**) Data are presented as the means ± SEM ($n = 7–10$); $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ as compared with the resting groups (R); $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ as compared with the collagen group. (**C**) Profiles are representative examples of three similar experiments.

Although the action mechanisms of various platelet aggregation agonists, such as collagen, ADP, and arachidonic acid, differ, α-NF significantly inhibited platelet aggregation stimulated by all of them. This implies that α-NF may block a common step shared by these inducers. These results also indicate that the site of action of α-NF is not at the receptor level of individual agonists. Triflavin acts by binding to the glycoprotein IIb/IIIa complex on the platelet surface membrane, resulting in interference with the interaction of fibrinogen with its specific receptor (*16*). In this study, we found that α-NF did not significantly affect FITC-triflavin binding to the glycoprotein IIb/IIIa complex, indicating that the antiplatelet activity of α-NF is possibly not directly due to interference with the binding of fibrinogen to its specific receptor on the platelet membrane.

Stimulation of platelets by agonists (i.e., collagen) results in phospholipase C-catalyzed hydrolysis of the minor plasma membrane phospholipid, phosphatidylinositol 4,5-bisphosphate, with concomitant formation of inositol 1,4,5-trisphosphate (IP_3)

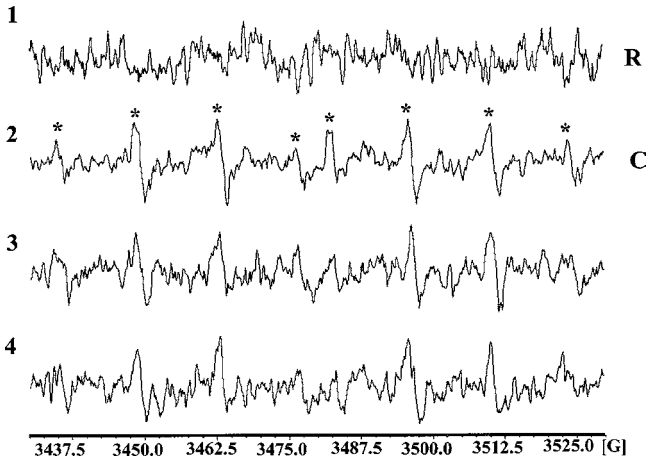

Figure 5. ESR spectra of the effect of α-NF in hydroxyl radical formation in collagen-activated platelets. (**1**) Resting platelet suspensions (R) or platelet suspensions were preincubated with (**2**) vehicle solution (0.5% DMSO) (C) or α-NF at (**3**) 5 μM and (**4**) 10 μM for 3 min, and then collagen was added (1 μg/mL) to trigger platelet activation. The reaction was allowed to proceed for 5 min, followed by the addition of DEPMPO (100 mM) for ESR experiments. The spectrum is a representative example of five similar experiments.

and diacylglycerol (*28*). There is strong evidence that IP_3 induces the release of Ca^{2+} from intracellular stores (*29*). Diacylglycerol activates PKC, inducing protein phosphorylation and a release reaction. In this study, phosphoinositide breakdown of collagen-activated platelets was inhibited by α-NF, suggesting that inhibition of platelet aggregation by α-NF is related to inhibition of phospholipase C activation. Moreover, TxA_2 is an important mediator of the release reaction and aggregation of platelets (*30*). Collagen-induced TxB_2 formation, a stable metabolite of TxA_2, was markedly inhibited by α-NF. It has been demonstrated that phosphoinositide breakdown can induce TxA_2 formation via free arachidonic acid release by diglyceride lipase or by endogenous phospholipase A_2 from membrane phospholipids (*31*). Thus, it seems likely that TxB_2 formation plays a role in mediating the inhibitory effect of α-NF on human platelets.

Furthermore, α-NF significantly inhibited PDBu-induced activation of PKC. PDBu intercalates with membrane phospholipids and forms a complex with PKC translocated to the membrane (*32*). Moreover, increased cyclic GMP can negatively affect agonist-induced PKC activation (*33*). Signaling by cyclic GMP somehow interferes with the agonist-stimulated phosphoinositide turnover that creates Ca^{2+}-mobilizing second messengers (*34*). 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 the cyclic GMP- and cyclic AMP-dependent protein kinases in a variety of cells, including smooth muscle cells and platelets (*21*). Both the cyclic GMP and cyclic AMP signaling cascades relax smooth muscle cells and inhibit platelet activation. Thus, it was speculated that VASP plays an important role in modulating actin filament dynamics and integrin activation (*35*). α-NF increased both cyclic GMP and cyclic GMP-induced VASP Ser^{157} phosphorylation in human platelets, therefore, the inhibitory effect of α-NF on PDBu-induced activation of PKC may be due, at least partly, to mediating the increase in cyclic GMP.

Reactive oxygen species (i.e., hydrogen peroxide, hydroxyl radicals) derived from platelet activation might affect cells with which they come into intimate contact, such as endothelium, and this could result in an amplification of platelet reactivity during thrombus formation. Collagen-induced platelet activation is associated with superoxide anion (O_2^-) and hydroxyl radical (OH•) formation which is dependent upon AA release and metabolism, since pretreatment arachidonyl trifluoromethyl ketone ($AACOCF_3$), a potent inhibitor of cytosolic phospholipase A_2 (*36*), inhibited collagen-induced O_2^- and OH• release in platelets. In this study, we found that α-NF (5 and 10 μM) did not effectively inhibit hydroxyl radical formation in collagen-activated platelets (**Figure 5**); this result implies that α-NF inhibited platelet aggregation and did not mediate through inhibition of free-radical formation in activated platelets.

In conclusion, the most important findings of this study suggest that α-NF could markedly inhibit agonist-induced platelet aggregation. This inhibitory effect may possibly involve the following two mechanisms. (1) α-NF may inhibit the activation of phospholipase C, followed by inhibition of phosphoinositide breakdown and thromboxane A_2 formation, thereby leading to inhibition of the activation of PKC and intracellular Ca^{2+} mobilization. (2) Moreover, α-NF increases cyclic GMP/VASP Ser^{157} phosphorylation and subsequently inhibits phosphoinositide breakdown and PKC activity, ultimately resulting in inhibition of both the phosphorylation of P47 and intracellular Ca^{2+} mobilization. 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. The important findings of this study suggest that α-NF appears to represent a novel and potent antiplatelet agent for treatment of arterial thromboembolism. The toxicity of α-NF, however, must be further assessed.

LITERATURE CITED

(1) Harborne, J. B.; Williams, C. A. Advances in flavonoid research since 1992. *Phytochemistry* **2000**, *55*, 481−504.
(2) Heo, M. Y.; Yu, K. S.; Lim, H.; Kim, H. P.; Au, W. W. Anticlastogenic effect of flavonoids against mutagen-induced micronuclei in mice. *Mutat. Res.* **1992**, *284*, 243−249.
(3) Reed, J. Cranbery flavonoids, atherosclerosis and cardiovascular health. *Crit. Rev. Food Sci. Nutr.* **2002**, *42* (suppl.), 301−316.
(4) Folts, J. D. Potential health benefits from the flavonoids in grape products on vascular disease. *Adv. Exp. Med. Biol.* **2002**, *505*, 95−111.
(5) Cheng, Y. W.; Li, C. H.; Lee, C. C.; Kang, J. J. Alpha-naphthoflavone induces vasorelaxation through the induction of extracellular calcium influx and NO formation in endothelium. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **2003**, *368*, 377−385.
(6) Das, A.; Wang, J. H.; Lien, E. J. Carcinogenicity, mutagenicity and cancer preventing activities of flavonoids structure-system-activity relation (SSAR) analysis. *Prog. Drug Res.* **1994**, *42*, 133−166.
(7) Kinoshita, N.; Gelboin, H. V. Aryl hydrocarbon hydroxylase and polycyclic hydrocarbon tumorigenesis: effect of the enzyme inhibitor 7, 8-benzoflavone on tumorigenesis and macromolecular binding. *Proc. Natl. Acad. Sci. U.S.A.* **1972**, *69*, 824−828.
(8) Dong, W.; Teraoka, H.; Kondo, S.; Hiraga, T. 2,3,7,8-Tetrachlorodibenzo-p-dioxin induces apoptosis in the dorsal midbrain of zebrafish embryos by activation of aryl hydrocarbon receptor. *Neurosci. Lett.* **2001**, *303*, 169−172.
(9) Jeon, Y. J.; Touk, E. S.; Lee, S. H.; Suh, J.; Na, Y. J.; Kim, H. M. Polychlorinated biphenyl-induced apoptosis of murine spleen cells is aryl hydrocarbon receptor independent but caspases dependent. *Toxicol. Appl. Pharmacol.* **2002**, *181*, 69−78.
(10) Sheu, J. R.; Chao, S. H.; Yen, M. H.; Huang, T. F. In vivo antithrombotic effect of triflavin, an Arg-Gly-Asp containing peptide on platelet plug formation in mesenteric microvessels of mice. *Thromb. Haemostasis* **1994**, *72*, 617−621.

(11) Hass, W. K.; Easton, J. D.; Adams, H. P.; Pryse-Philips, W.; Molony, B. A.; Anderson, S.; Kamm, B. A randomized trial comparing ticlopidine hydrochloride with aspirin for the prevention of stroke in high-risk patients. *New Engl. J. Med.* **1989**, *321*, 501−507.

(12) Gryglewski, R. J.; Korbut, R.; Robak, J.; Swies, J. On the mechanism of anti-thrombotic action of flavonoids. *Biochem. Pharmacol.* **1987**, *36*, 317−322.

(13) Lanza, F.; Bereta, A.; Stierle, A.; Corre, G.; Cazenave, J. P. Cyclic nucleotide phosphodiesterase inhibitors prevent aggregation of human platelets by raising cyclic AMP and reducing cytoplasmic free calcium mobilization. *Thromb. Res.* **1987**, *45*, 477−484.

(14) Sheu, J. R.; Lee, C. R.; Lin, C. C.; Kan, Y. C.; Lin, C. H.; Hung, W. C.; Yen, M. H. The antiplatelet activity of PMC, a potent α-tocopherol analogue, is mediated through inhibition of cyclooxygenase. *Br. J. Pharmacol.* **1999**, *127*, 1206−1212.

(15) Born, G. V. R.; Cross, M. J. The aggregation of blood platelets. *J. Physiol.* **1963**, *168*, 178−195.

(16) Sheu, J. R.; Teng, C. M.; Huang, T. F. Triflavin, an RGD-containing antiplatelet peptide, binds to GP IIIa of ADP-stimulated platelets. *Biochem. Biophys. Res. Commun.* **1992**, *189*, 1236−1242.

(17) Sheu, J. R.; Hung, W. C.; Wu, C. H.; Ma, M. C.; Kan, Y. C.; Lin, C. H.; Lin, M. S.; Luk, H. N.; Yen, M. H. Reduction in lipopolysaccharide-induced thrombocytopenia by triflavin in a rat model of septicemia. *Circulation* **1999**, *99*, 3056−3062.

(18) Sheu, J. R.; Lin, C. H.; Peng, C. H.; Huang, T. F. Triflavin, an Arg-Gly-Asp-containing peptide, inhibits the adhesion of tumor cells to matrix protein via binding to multiple integrin receptors expressed on human hepatoma cells. *Proc. Soc. Exp. Biol. Med.* **1996**, *213*, 71−79.

(19) Grynkiewicz, G.; Poenie, M.; Tsien, R. Y. A new generation of Ca^{+2} indicator with greatly improved fluorescence properties. *J. Biol. Chem.* **1985**, *260*, 3440−3450.

(20) Grabarek, J.; Raychowdhury, M.; Ravid, K.; Kent, K. C.; Newman, P. J.; Ware, J. A. Identification and functional characterization of protein kinase C isozymes in platelets and HEL cells. *J. Biol. Chem.* **1992**, *267*, 10011−10017.

(21) Li, Z.; Ajdic, J.; Eigenthaler, M.; Du, X. A predominant role for cAMP-dependent protein kinase in the cGMP-induced phosphorylation of vasodilator-stimulated phosphoprotein and platelet inhibition in humans. *Blood* **2003**, *101*, 4423−4429.

(22) Hsiao, G.; Shen, M. Y.; Lin, K. H.; Chou, C. Y.; Tzu, N. H.; Lin, C. H.; Chou, D. S.; Chen, T. F.; Sheu, J. R. Inhibitory activity of kinetin on free radical formation of activated platelets in vitro and on thrombus formation in vivo. *Eur. J. Pharmacol.* **2003**, *465*, 281−287.

(23) Broekman, M. J.; Ward, J. W.; Marcus, A. J. Phospholipid metabolism in phosphatidyl inositol, phosphatic acid and lysophospholipids. *J. Clin. Invest.* **1980**, *66*, 273−275.

(24) Siess, W.; Lapetina, E. G. Platelet aggregation induced by alpha 2-adrenoceptor and protein kinase C activation. A novel synergism. *Biochem. J.* **1989**, *263*, 377−385.

(25) Yoshioka, Y.; Yamamuro, A.; Maeda, S. Nitric oxide at a low concentration protects murine macrophage RAW264 cells against nitric oxide-induced death via cGMP signaling pathway. *Br. J. Pharmacol.* **2003**, *139*, 28−34.

(26) Zhao, Y.; Brandish, P. E.; Divalentin, M.; Schelvis, J. P.; Babcock, G. T.; Marletta, M. A. Inhibition of soluble guanylate cyclase by ODQ. *Biochemistry* **2000**, *39*, 10848−10854.

(27) Yamaguchi, F.; Yoshimura, Y.; Nakazawa, H.; Ariga, T. Free radical-scavenging activity of grape seed extract and antioxidants by electron spin resonance spectrometry in an H_2O_2/NaOH/DMSO system. *J. Agric. Food Chem.* **1999**, *47*, 2544−2548.

(28) Kirk, C. J.; Creba, J. A.; Doenes, C. P.; Michell, R. H. Hormone-stimulated metabolism of inositol lipids and its relationship to hepatic receptor function. *Biochem. Soc. Trans.* **1981**, *9*, 377−379.

(29) Berridge, M. J. Rapid accumulation of inositol trisphosphate reveals that agonists hydrolyse polyphosphoinositides instead of phosphatidylinositol. *Biochem. J.* **1983**, *212*, 249−258.

(30) Hornby, E. J. Evidence that prostaglandin endoperoxides can induce platelet aggregation in the absence of thromboxane A_2 production. *Biochem. Pharmacol.* **1982**, *31*, 1158−1160.

(31) McKean, M. L.; Smith, J. B.; Silver, W. J. Formation of lysophosphatidylcholine in human platelets in response to thrombin. *J. Biol. Chem.* **1981**, *256*, 1522−1524.

(32) Kraft, A. S.; Anderson, W. B. Phorbol esters increase the amount of Ca^{+2}, phospholipid-dependent protein kinase associated with plasma membrane. *Nature* **1983**, *301*, 621−623.

(33) Murohara, T.; Parkinson, S. J.; Waqldman, S. A.; Lefer, A. M. Inhibition of nitric oxide biosynthesis promotes P-selectin expression in platelets. Role of protein kinase C. *Arterioscler. Thromb. Vasc. Biol.* **1995**, *15*, 2068−2075.

(34) McDonald, L. J.; Murad, F. Nitric oxide and cyclic GMP signaling. *Proc. Soc. Exp. Biol. Med.* **1996**, *211*, 1−6.

(35) Aszodi, A.; Pfeifer, A.; Ahmad, M.; Glauner, M.; Zhou, X. H.; Ny, L.; Andersson, K. E.; Kegrel, B.; Offermanns, S.; Fassler, R. The vasodilator-stimulated phosphoprotein (VASP) is involved in cGMP- and cAMP-mediated inhibition of agonist-induced platelet aggregation, but is dispensable for smooth muscle function. *EMBO J.* **1999**, *18*, 37−48.

(36) Caccese, D.; Pratico, D.; Ghiselli, A.; Natoli, S.; Pignatelli, P.; Sanguigni, V.; Iuliano, L.; Violi, F. Superoxide anion and hydroxyl radical release by collagen-induced platelet aggregation--role of arachidonic acid metabolism. *Thromb. Haemostasis* **2000**, *83*, 485−490.

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