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Potent antiplatelet activity of sesamol in an *in vitro* and *in vivo* model: pivotal roles of cyclic AMP and p38 mitogen-activated protein kinase☆

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Abstract

Sesamol is a potent phenolic antioxidant which possesses antimutagenic, antihepatotoxic and antiaging properties. Platelet activation is relevant to a variety of acute thrombotic events and coronary heart diseases. There have been few studies on the effect of sesamol on platelets. Therefore, the aim of this study was to systematically examine the detailed mechanisms of sesamol in preventing platelet activation *in vitro* and *in vivo*. Sesamol (2.5−5 μM) exhibited more potent activity of inhibiting platelet aggregation stimulated by collagen than other agonists. Sesamol inhibited collagen-stimulated platelet activation accompanied by $[Ca^{2+}]_i$ mobilization, thromboxane A_2 (TxA_2) formation, and phospholipase C (PLC)γ2, protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) phosphorylation in washed platelets. Sesamol markedly increased cAMP and cGMP levels, endothelial nitric oxide synthase (eNOS) expression and NO release, as well as vasodilator-stimulated phosphoprotein (VASP) phosphorylation. SQ22536, an inhibitor of adenylate cyclase, markedly reversed the sesamol-mediated inhibitory effects on platelet aggregation and p38 MAPK phosphorylation, and sesamol-mediated stimulatory effects on VASP and eNOS phosphorylation, and NO release. Sesamol also reduced hydroxyl radical ($OH^{\bullet}$) formation in platelets. In an *in vivo* study, sesamol (5 mg/kg) significantly prolonged platelet plug formation in mice. The most important findings of this study demonstrate for the first time that sesamol possesses potent antiplatelet activity, which may involve activation of the cAMP-eNOS/NO-cGMP pathway, resulting in inhibition of the PLCγ2-PKC-p38 MAPK-TxA_2 cascade, and, finally, inhibition of platelet aggregation. Sesamol treatment may represent a novel approach to lowering the risk of or improving function in thromboembolism-related disorders.
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Keywords: Cyclic AMP; Nitric oxide; PLCγ2; p38 MAPK; Platelet activation; Sesamol

1. Introduction

The health-promoting properties of sesame oil have been observed for millennia. Sesame oil contains sesamolin, sesamin and sesamol [1]. After roasting sesame seeds, the sesamolin content is lost, but the content of the derivative compound, sesamol, increases [2]. Sesamol is a potent phenolic antioxidant contained only in processed sesame oil. Recent studies showed that sesamol can act as a metabolic regulator and possesses chemopreventic, antimutagenic, antihepatotoxic and antiaging properties [1,3–5]. Sesamol was found to induce growth arrest and apoptosis in cancer and cardiovascular cells [6]. In addition, sesamol was found to enhance both vascular fibrinolytic capacity through regulating gene expression of a plasminogen activator and nitric oxide (NO) release in endothelial cells [7,8].

Intravascular thrombosis is one of the generators of a wide variety of cardiovascular diseases. Initiation of intraluminal thrombosis is believed to involve platelet adherence and aggregation. Thus, platelet aggregation may play a crucial role in the atherothrombotic process [9]. Indeed, antiplatelet agents (e.g., ticlopidine and aspirin) were shown to reduce the incidence of stroke in high-risk patients [10].

Despite the very important roles of platelets in the development of acute thrombosis, coronary heart diseases and atherosclerosis, no data are available concerning the effect of sesamol in platelet activation. The detailed mechanisms underlying sesamol's signaling pathways remain obscure. Platelets are anucleate, do not differentiate or proliferate, and thus are a good model for studying signal transductions of sesamol and its functions. We therefore for the first time examined the cellular signal events associated with sesamol-inhibited platelet activation *in vitro* and platelet plug formation *in vivo* in the present study.

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2. Materials and methods

2.1. Chemicals and reagents

Sesamol, collagen (type I), luciferin-luciferase, fluorescein sodium, arachidonic acid (AA), phorbol-12,13-dibutyrate (PDBu), 5,5-dimethyl-1 pyrroline N-oxide (DMPO), SQ22536, ODQ and thrombin were purchased from Sigma (St Louis, MO, USA); Fura 2-AM and fluorescein iso-thiocyanate (FITC) were from Molecular Probe (Eugene, OR, USA); the thromboxane B_2, cAMP and cGMP enzyme immunoassay (EIA) kits were from Cayman (Ann Arbor, MI, USA); the anti-vasodilator-stimulated phosphoprotein (VASP Ser^{157}) monoclonal antibody (mAb) was from Calbiochem (San Diego, CA, USA); the anti-phospho-p38 mitogen-activated protein kinase (MAPK) Ser^{182} mAb was from Santa Cruz Biotechnology (Santa Cruz, CA, USA); the anti-p38 MAPK and anti-phospho-c-Jun N-terminal kinase (JNK) (Thr^{183}/Tyr^{185}) mAbs, anti-phospholipase Cγ2 (PLCγ2), anti-phospho (Tyr^{759}) PLCγ2 mAbs and the anti-phospho-p44/p42 extracellular signal-regulated kinase (ERK) (Thr^{202}/Tyr^{204}) polyclonal antibody were from Cell Signaling (Beverly, MA, USA); the anti-α-tubulin mAb was from NeoMarkers (Fremont, CA, USA); and the Hybond-P PVDF membrane, ECL Western blotting detection reagent and analysis system, horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG and sheep anti-mouse IgG were from Amersham (Buckinghamshire, UK). Sesamol was dissolved in 0.5% dimethyl sulfoxide (DMSO) and stored at 4°C until used.

2.2. Platelet aggregation

Human platelet suspensions were prepared as previously described [9]. This study was approved by the Institutional Review Board of Taipei Medical University (no. P960313) and conformed to the principles outlined in the Helsinki Declaration, and all human volunteers provided 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 (9:1:1, v/v). After centrifugation, the supernatant (platelet-rich plasma) was supplemented with prostaglandin E_1 (PGE_1) (0.5 μM) and heparin (6.4 IU/ml). The washed platelets were finally suspended in Tyrode's solution containing bovine serum albumin (BSA) (3.5 mg/ml). The final concentration of Ca^{2+} in Tyrode's solution was 1 mM.

A turbidimetric method was applied to measure platelet aggregation [9], using the Lumi-Aggregometer (Payton, Scarborough, Ontario, Canada). Platelet suspensions (0.4 ml) were preincubated with various concentrations of sesamol or an isovolumetric solvent control (0.5% DMSO) for 3 min before the addition of agonists. The reaction was allowed to proceed for 6 min, and the extent of aggregation was expressed in light-transmission units. When measuring ATP release, 20 μl of a luciferin/luciferase mixture was added 1 min before the addition of agonists, and ATP release was compared to that of the control.

2.3. Flow cytometric analysis

Triflavin, an $\alpha_{IIb}\beta_3$ integrin antagonist, was prepared as previously described [11]. Fluorescence-conjugated triflavin was prepared as previously described [11]. Platelet suspensions (3.6×10^8/ml) were preincubated with sesamol (2.5 and 5 μM) or a solvent control for 3 min, followed by the addition of 2 μl of FITC-triflavin (2 μg/ml). The suspensions were then assayed for fluorescein-labeled platelets using a flow cytometer (Beckman Coulter, Miami, FL, USA). Data were collected from 50,000 platelets per experimental group, and the platelets were identified on the basis of their characteristic forward and orthogonal light-scattering profile. All experiments were repeated at least four times to ensure reproducibility.

2.4. Measurement of platelet $[Ca^{2+}]_i$ 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

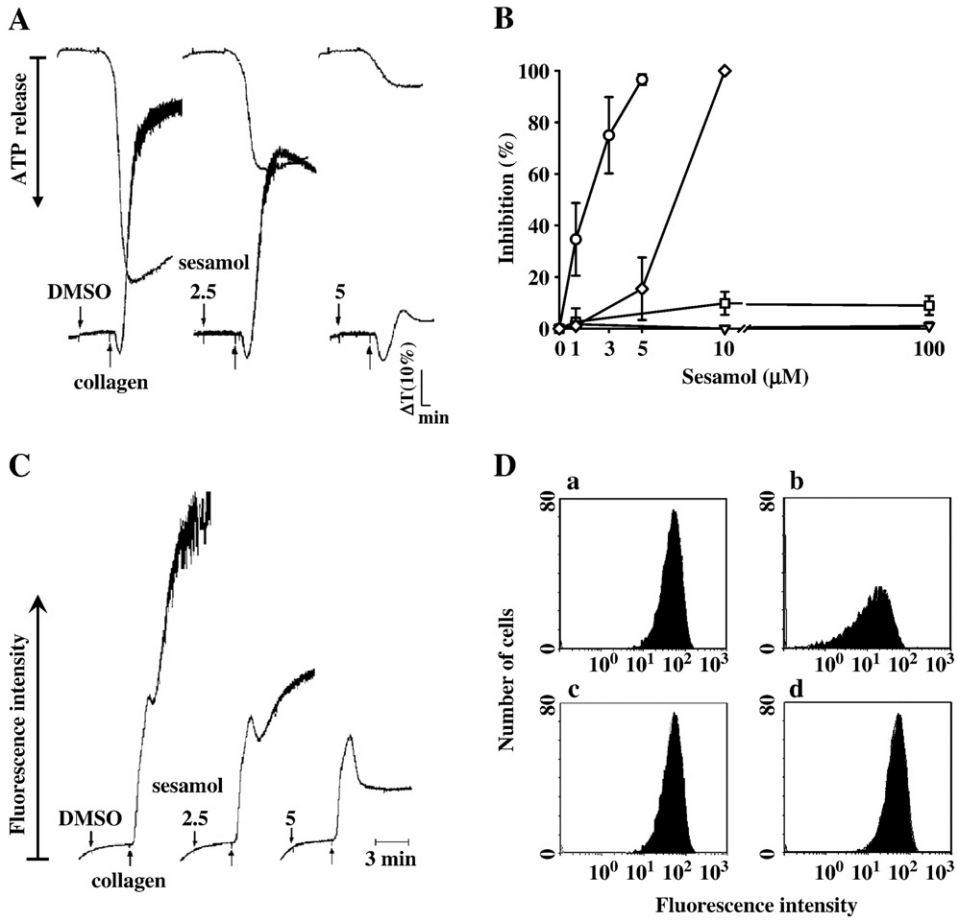

Fig. 1. Effects of sesamol on the inhibition of (A and B) platelet aggregation, (C) $[Ca^{2+}]_i$ mobilization and (D) FITC-triflavin binding to the $\alpha_{IIb}\beta_3$ integrin in activated platelets. Washed platelets (3.6×10^8/ml) were preincubated with sesamol (1–100 μM) or 0.5% DMSO, followed by the addition of collagen (1 μg/ml, ○), U46619 (1 μM, ▽), thrombin (0.01 U/ml, □) or arachidonic acid (60 μM, ◊) to trigger platelet aggregation (A and B) and ATP-release reaction (A, upper tracings) or (C) $[Ca^{2+}]_i$ mobilization. (D) The solid line represents the fluorescence profiles of (a) FITC-triflavin (2 μg/ml) in the absence of sesamol as a positive control; (b) in the presence of EDTA (5 mM) as a negative control; or in the presence of sesamol [(c) 2.5 μM and (d) 5 μM], followed by the addition of FITC-triflavin (2 μg/ml). The profiles (A and C–D) are representative examples of four similar experiments. Data in (B) are presented as the means±S.E.M. (n=4).

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 [9].

2.5. Measurement of thromboxane B_2 and cyclic nucleotide formation

Platelet suspensions (3.6×10^8/ml) were preincubated with sesamol (2.5 and 5 μM) or solvent control for 3 min 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 suspensions. The thromboxane B_2 (TxB_2) levels of the supernatants were measured using an EIA kit. In addition, platelet suspensions were incubated with nitroglycerin (NTG; 10 μM), PGE_1 (10 μM) or sesamol (2.5 and 5 μM) in the presence of IBMX (100 μM) for 6 min. The incubation was stopped, and the solution was immediately boiled for 5 min. Fifty microliters of the supernatant was used to determine the cAMP and cGMP contents with EIA kits.

2.6. Immunoblotting study

Washed platelets (1.2×10^9/ml) were preincubated with sesamol (2.5 and 5 μM) or a solvent control for 3 min followed by the addition of agonists to trigger platelet activation. The reaction was stopped, and platelets were immediately resuspended in 200 μl of lysis buffer. Samples containing 80 μg of protein were separated by SDS-PAGE (12%); the proteins were electrotransferred by semidry transfer (Bio-Rad, Hercules, CA, USA). Blots were blocked with TBST (10 mM Tris-base, 100 mM NaCl and 0.01% Tween 20) containing 5% BSA for 1 h and then probed with various primary antibodies. Membranes were incubated with HRP-linked anti-mouse IgG or anti-rabbit IgG (diluted 1:3000 in TBST) for 1 h. Immunoreactive bands were detected by an enhanced chemiluminescence (ECL) system. The bar graph depicts the ratios of semiquantitative results obtained by scanning reactive bands and quantifying the optical density using videodensitometry (Bio-profil; Biolight Windows Application v. 2000.01; Vilber Lourmat, France).

2.7. Estimation of nitrate formation

NO was assayed in platelet suspensions as previously described [11]. In brief, platelet suspensions (1×10^9/ml) were preincubated with PGE_1 (10 μM) or sesamol (2.5 and 5 μM) for 3 min, followed by centrifugation. The amount of nitrate in the platelet suspensions (10 μl) was measured by adding a reducing agent to the purge vessel to convert nitrate to NO which was stripped from the suspensions by purging with helium gas. The NO was then drawn into a Sievers Nitric Oxide Analyzer (Sievers 280 NOA, Sievers, Boulder, CO, USA). Nitrate concentrations were calculated by comparison with standard solutions of sodium nitrate.

2.8. Measurement of free radicals by electron spin resonance spectrometry

The electron spin resonance (ESR) method used a Bruker EMX ESR spectrometer as described previously [12]. In brief, platelet suspensions (3.6×10^8/ml) were preincubated with sesamol (2.5 and 5 μM) or solvent control for 3 min before the addition of collagen (1 μg/ml). The reaction was allowed to proceed for 5 min, followed by the addition of DMPO (100 μM) for the ESR study. The rate of free radical-scavenging activity is defined by the following equation: inhibition rate=1−[signal height (sesamol)/signal height (control)] [12].

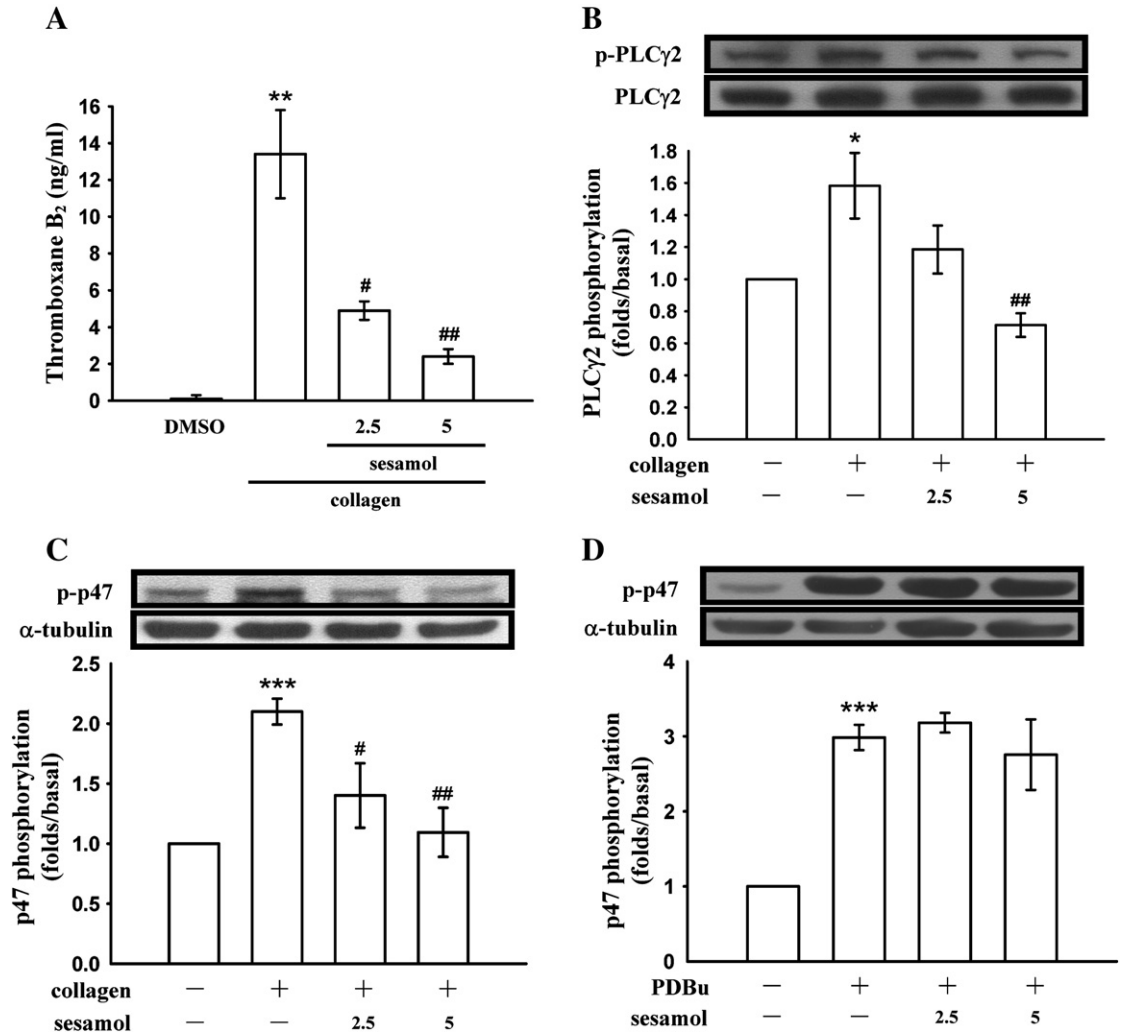

Fig. 2. Effects of sesamol on (A) thromboxane B_2 formation and (B) phospholipase Cγ2 and (C and D) PKC phosphorylation in activated platelets. Washed platelets were preincubated with sesamol (2.5 and 5 μM) or 0.5% DMSO, followed by the addition of collagen (1 μg/ml) or PDBu (0.15 μM) to trigger platelet activation. Cells were collected, and subcellular extracts were analyzed for (A) thromboxane A_2 formation, (B) phospholipase Cγ2 phosphorylation and (C and D) phospho-PKC substrates (p-p47) as described in Materials and Methods. Data are presented as the means±S.E.M. (n=4). $^{*}P<.05$, $^{**}P<.01$, and $^{***}P<.001$, compared to the control group; $^{\#}P<.05$ and $^{\#\#}P<.01$, compared to the collagen group.

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

This study conformed to the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23, 1996). As described previously [13], mice were anesthetized, and an external jugular vein was cannulated with PE-10 for administration of the dye and drugs (by an intravenous 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. Filtered light from which wavelengths below 520 nm had been eliminated was used to irradiate a microvessel. Various doses of sesamol (5 and 10 mg/kg) were administered 1 min after fluorescein sodium (15 μg/kg) had been given. The time lapse for inducing thrombus formation leading to cessation of blood flow was measured.

2.10. Data 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* study of platelet plug formation. Other experiments were assessed by the method of analysis of variance. If this analysis indicated significant differences among group means, then each group was compared using the Newman–Keuls method. $P<.05$ was considered statistically significant.

3. Results

3.1. Effects of sesamol on platelet aggregation, $[Ca^{2+}]_i$ mobilization and $\alpha_{IIb}\beta_3$ integrin conformational change in human platelets

Sesamol (1–5 μM) exhibited potent activity of inhibiting platelet aggregation and the ATP-release reaction stimulated by collagen (1 μg/ml); it also significantly inhibited platelet aggregation stimulated by AA (60 μM) at higher concentrations (5–10 μM) (Fig. 1A and B). However, sesamol did not significantly inhibit platelet aggregation stimulated by other agonists such as thrombin (0.01 U/ml) or U46619 (1 μM), a prostaglandin endoperoxide analogue, even at concentrations of up to 100 μM (Fig. 1B). The IC_{50} values of sesamol for platelet aggregation induced by collagen and AA were approximately 2.5 and 6 μM, respectively. The solvent control (0.5% DMSO) did not significantly affect platelet aggregation stimulated by agonists in washed platelets (Fig. 1A). When platelets were preincubated with sesamol at a higher concentration of 100 μM or 0.5% DMSO for 10 min, followed by two washes with Tyrode's solution, there were no significant differences between the aggregation curves of either platelet preparation stimulated by collagen (1 μg/ml), indicating that the effect of sesamol 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 sesamol in platelet activation.

As shown in Fig. 1C, collagen (1 μg/ml) evoked a marked increase in $[Ca^{2+}]_i$, and this increase was markedly inhibited in the presence of sesamol. Triflavin is an Arg-Gly-Asp-containing antiplatelet peptide purified from *Trimeresurus flavoviridis* snake venom [11]. Triflavin inhibits platelet aggregation through direct interference with fibrinogen binding to the $\alpha_{IIb}\beta_3$ integrin [11]. There is now a multitude of evidence suggesting that the binding of fibrinogen to the $\alpha_{IIb}\beta_3$ integrin is the final common pathway for agonist-induced platelet aggregation. Therefore, we further evaluated whether or not sesamol directly binds to the platelet $\alpha_{IIb}\beta_3$ integrin, leading to interruption of platelet aggregation induced by collagen. In this study, the relative intensity of the fluorescence of FITC-triflavin (2 μg/ml) bound directly to collagen (1 μg/ml)-activated platelets was 130.0±14.0 ($n=4$)

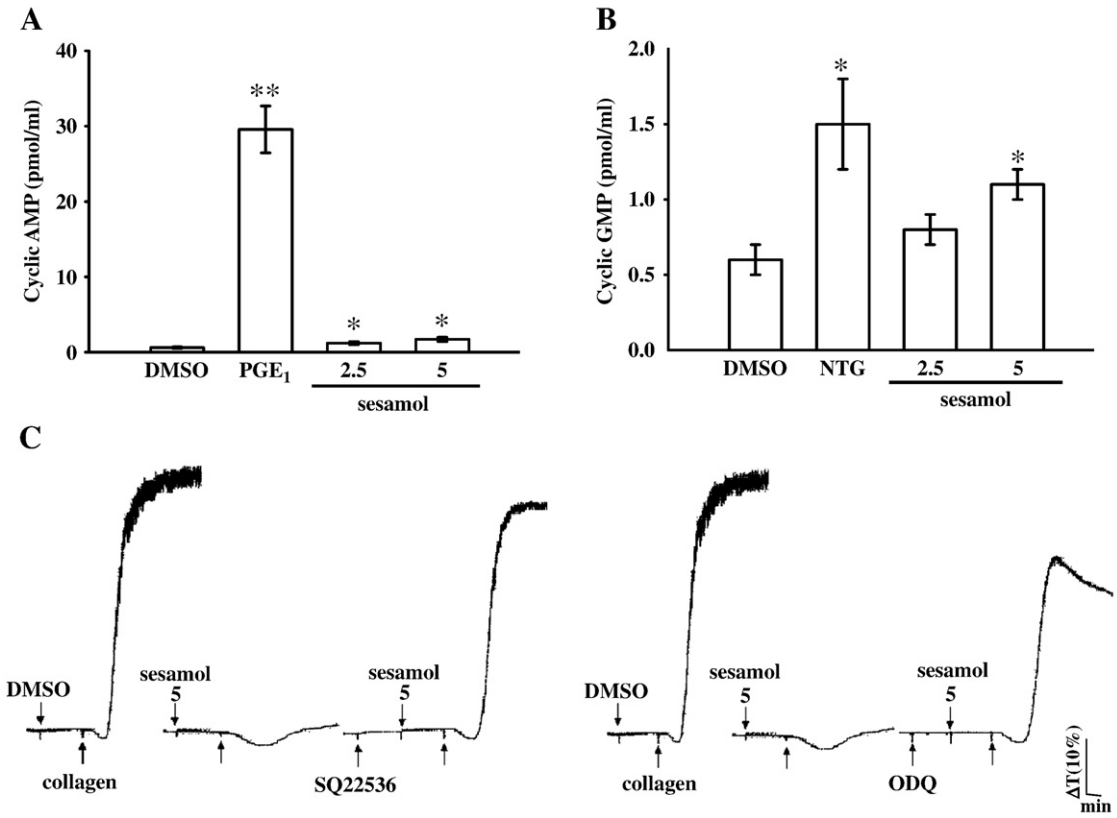

Fig. 3. Effects of sesamol on (A) cAMP, (B) cGMP and (C) platelet aggregation in the presence of inhibitors of cyclic nucleotide in washed platelets. (A and B) Platelets were incubated with prostaglandin E_1 (PGE_1, 10 μM), nitroglycerin (NTG, 10 μM), sesamol (2.5 and 5 μM) or 0.5% DMSO, and then both cAMP and cGMP levels were measured. The addition of PGE_1 and NTG to platelets served as respective positive controls of cAMP and cGMP. For platelet aggregation studies, washed platelets were preincubated with sesamol (5 μM) in the absence or presence of (C) SQ22536 (100 μM) or ODQ (20 μM), followed by the addition of collagen (1 μg/ml) to trigger platelet aggregation. Data are presented as the means±S.E.M. ($n=4$); $^{*}P<.05$ and $^{**}P<.01$, compared to the control group. The profiles (C) are representative examples of four similar experiments.

(Fig. 1D, a), and it was markedly reduced in the presence of 5 mM EDTA (negative control, 33.3±6.4, n=4, P<.01) (Fig. 1D, b). Sesamol (2.5 and 5 μM) did not significantly affect FITC-triflavin binding to the $\alpha_{IIb}\beta_3$ integrin in platelet suspensions (2.5 μM, 126.4±12.3; 5 μM, 126.6±8.8, n=4) (Fig. 1D, c and d), indicating that the inhibitory effect of sesamol on platelet aggregation does not involve binding to the platelet $\alpha_{IIb}\beta_3$ integrin.

3.2. Effects of sesamol on thromboxane A_2, PLCγ2 and PKC activation

As shown in Fig. 2A, resting platelets produced relatively little TxB_2 compared to collagen-activated platelets. Sesamol (2.5 and 5 μM) concentration-dependently inhibited TxB_2 formation in platelets stimulated by collagen (1 μg/ml).

PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate two secondary messengers: inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) [14]. DAG activates PKC, inducing protein phosphorylation (p47) and ATP release. Phosphorylation is one of the key mechanisms regulating the activity of PLC. The immunoblotting analysis revealed that treatment with sesamol (5 μM) markedly abolished the phosphorylation of PLCγ2 stimulated by collagen (1 μg/ml) (Fig. 2B). Stimulation of platelets with a number of different agonists induced activation of PKC, which then phosphorylated p47 proteins. In this study, phosphorylation experiments were performed to examine the role of sesamol in PKC activation in human platelets. When collagen (1 μg/ml) (Fig. 2C) or PDBu (150 nM) (Fig. 2D) was added to human platelets, a protein with an apparent of p47 was predominately phosphorylated compared to resting platelets. Sesamol (2.5 and 5 μM) inhibited p47 phosphorylation stimulated by collagen but not by PDBu (Fig. 2C and D).

3.3. Effects of sesamol on cyclic nucleotide formation in platelets

As shown in Fig. 3A and B, levels of cAMP and cGMP in resting platelets were relatively lower compared to those of PGE_1- (10 μM) and NTG (10 μM)-treated platelets, respectively. The addition of sesamol (2.5 and 5 μM) increased the levels of both cGMP and cAMP (Fig. 3A and B). The presence of SQ22536 (100 μM) and ODQ (20 μM), respective inhibitors of adenylate cyclase and guanylate cyclase, markedly reversed the inhibitory effect of sesamol (5 μM) on

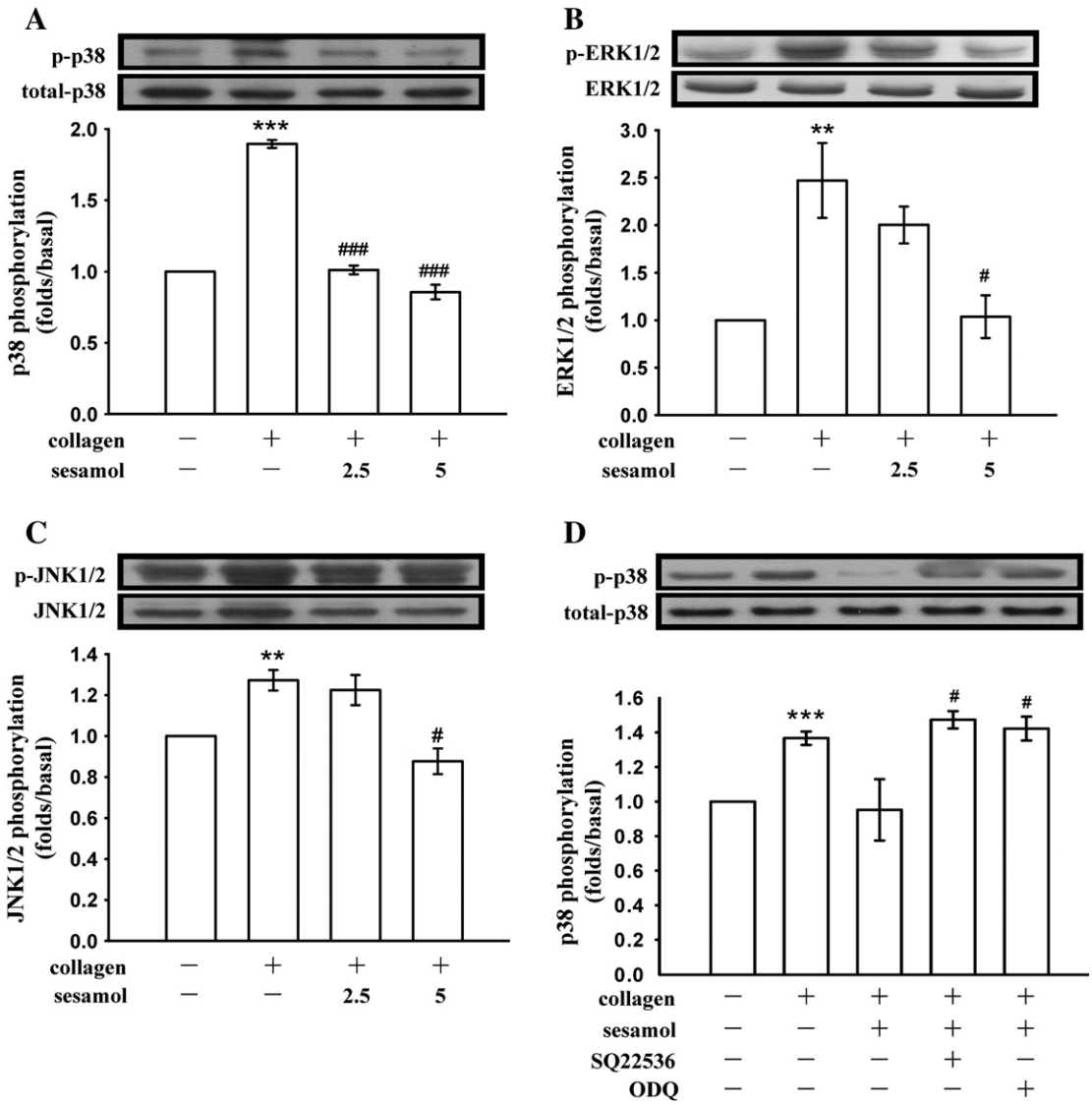

Fig. 4. Effects of sesamol on (A and D) p38 MAPK, (B) ERK1/2 and (C) JNK1/2 phosphorylation in collagen-activated platelets. Washed platelets (1.2×10^9/ml) were preincubated with sesamol (2.5 and 5 μM) or 0.5% DMSO, followed by the addition of collagen (1 μg/ml) to trigger (A and D) p38 MAPK, (B) ERK1/2 and (C) JNK1/2 phosphorylation. Data are presented as the means±S.E.M. (n=4). $^{**}P$<.01 and $^{***}P$<.001, compared to the control group; $^{\#}P$<.05 and $^{\#\#\#}P$<.001, compared to the collagen group.

collagen-stimulated platelet aggregation (Fig. 3C). This result indicates that sesamol inhibits platelet aggregation, at least in part, *via* a cyclic nucleotide-dependent pathway.

3.4. Effects of sesamol on collagen-induced MAPK phosphorylation

To further investigate the inhibitory mechanisms of sesamol in platelet activation stimulated by collagen, we further detected MAPK signaling molecules including p38 MAPK, JNK1/2 and ERK1/2. The immunoblotting analysis revealed that sesamol (2.5 and 5 μM) concentration-dependently inhibited p38 MAPK (Fig. 4A), ERK1/2 (Fig. 4B) and JNK1/2 phosphorylation (Fig. 4C) stimulated by collagen (1 μg/ml). In addition, both SQ22536 (100 μM) and ODQ (20 μM) obviously reversed the sesamol-mediated inhibitory effect of p38 MAPK phosphorylation stimulated by collagen (Fig. 4D).

3.5. Effects of sesamol on VASP and eNOS phosphorylation, and nitrate formation

It was demonstrated that cyclic nucleotides can induce VASP Ser^{157} phosphorylation in human platelets [15]. In this study, PGE_1 (10 μM) and sesamol (5 μM) markedly induced VASP Ser^{157} phosphorylation (Fig. 5A). Both SQ22536 (100 μM) and ODQ (20 μM) significantly inhibited the phosphorylation stimulated by sesamol (Fig. 5A). Furthermore, eNOS phosphorylation (Fig. 5B) and nitrate formation (Fig. 5C) were stimulated by sesamol (5 μM), and these effects were significantly inhibited in the presence of SQ22536 (100 μM) but not of ODQ (20 μM) (Fig. 5B and C).

3.6. Effects of sesamol on hydroxyl radical formation in collagen-activated platelets and thrombus formation in microvessels of fluorescein sodium-pretreated mice

In this study, a typical ESR signal of hydroxyl radical ($OH^{\bullet}$) formation was induced in collagen (1 μg/ml)-activated platelets compared to resting platelets (Fig. 6A, a and b); pretreatment with sesamol (2.5 and 5 μM) reduced hydroxyl radical formation stimulated by collagen (Fig. 6A, c and d). The antioxidant, catalase (1000 U/ml), markedly suppressed hydroxyl radical formation by about 74% (data not shown).

For the study of thrombus formation in microvessels of fluorescein sodium (15 μg/kg)-pretreated mice, the time to occlusion was approximately 170 s. When sesamol was administered at 5 and 10 mg/kg after pretreatment with fluorescein sodium, respectively, the occlusion times were dose-dependently prolonged compared to the solvent controls (0.5% DMSO, 165.6 ±2.6 s vs. 5 mg/kg, 209.1±10.1 s, n=5, P<.05; 0.5% DMSO, 160.7±8.0 s vs. 10 mg/kg, 221.6±15.6 s, n=5, P<.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 in mesenteric microvessels at 170 s but not at 5 s after irradiation in the solvent-treated group (Fig. 6C, a and b). With administration of sesamol (5 mg/kg), platelet plug formation was not observed at either 5 or 170 s after irradiation (Fig. 6C, c and d). The blood flow rate of the solvent-treated venule was slower than that of the sesamol-treated venule, because the platelet plug became apparent at 170 s (Fig. 6C, b).

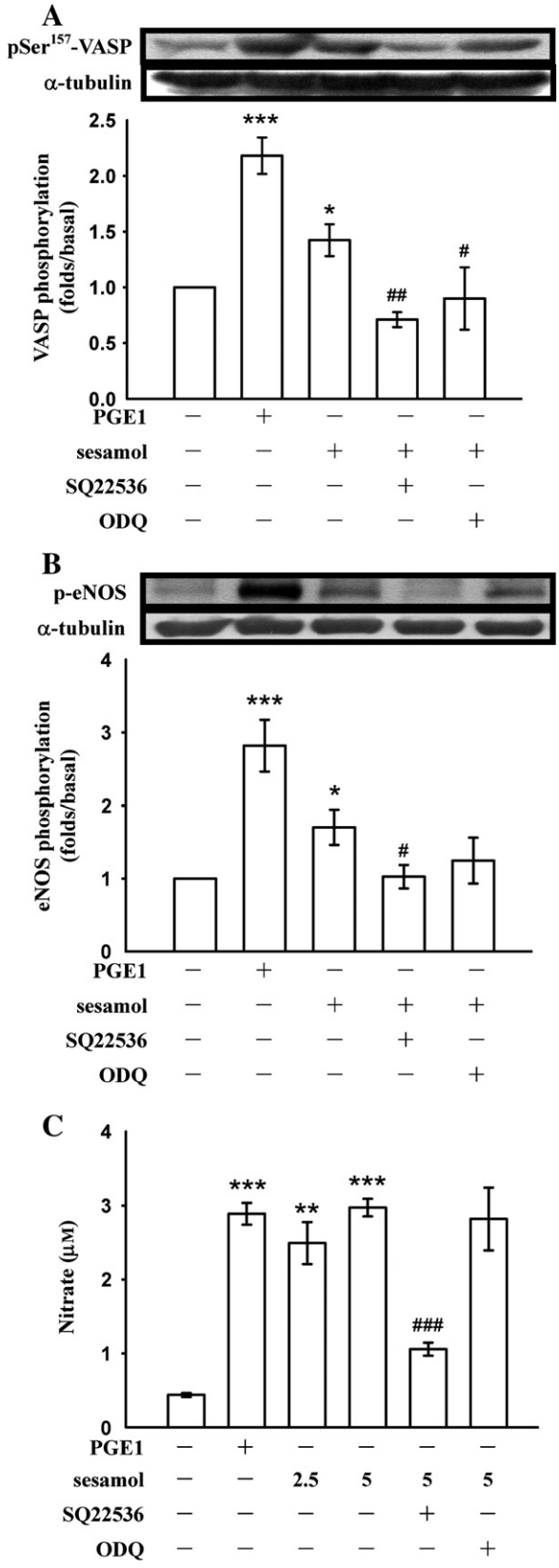

Fig. 5. Effects of sesamol on (A) Ser^{157}-vasodilator-stimulated phosphoprotein (VASP) and (B) endothelial nitric oxide synthase (eNOS) phosphorylation, and (C) nitrate formation in the presence of inhibitors of cyclic nucleotide in washed platelets. Platelets were incubated with prostaglandin E_1 (PGE_1, 10 μM), sesamol (2.5 and 5 μM) or 0.5% DMSO in the absence or presence of SQ22536 (100 μM) or ODQ (20 μM) as described in Materials and Methods. Cells were collected, and subcellular extracts were analyzed for (A) Ser^{157}-VASP and (B) eNOS phosphorylation, and (C) nitrate formation. Data are presented as the means±S.E.M. (n=4); $^{*}P$<.05 and $^{***}P$<.001, compared to the control group; $^{\#}P$<.05 and $^{\#\#}P$<.01, compared to the PGE_1 group.

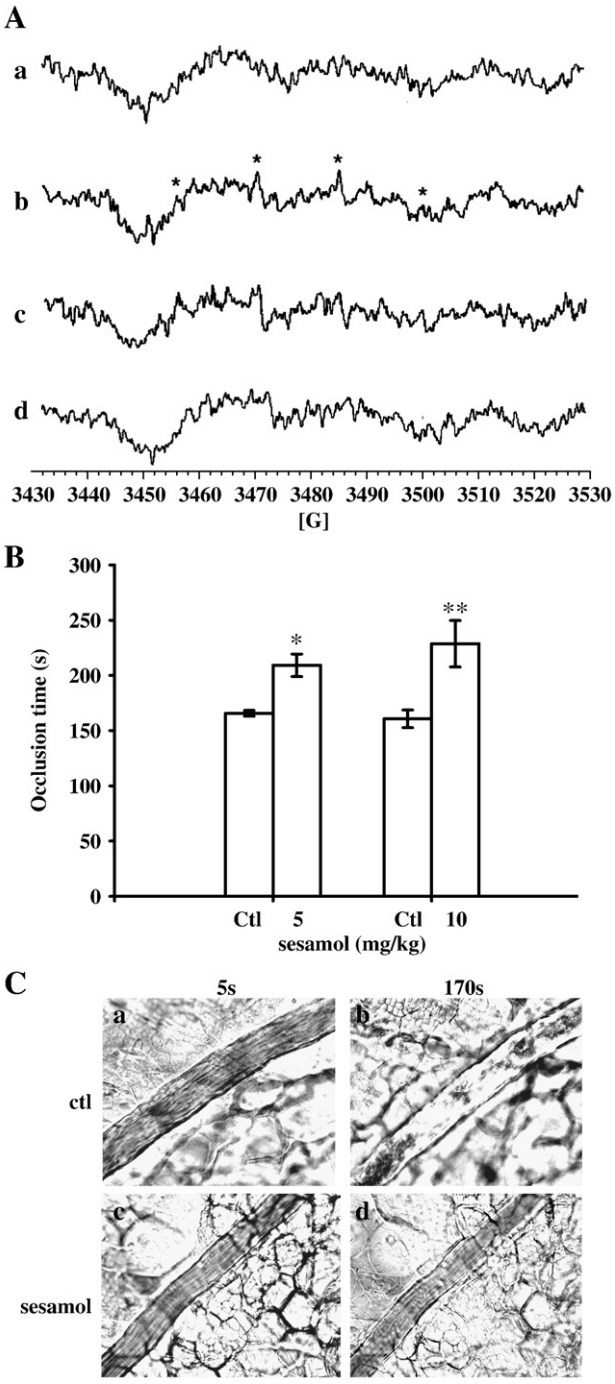

Fig. 6. Electron spin resonance spectra of sesamol-reduced hydroxyl radical ($OH^{\bullet}$) formation in collagen-activated platelets, and the effect of sesamol prolongation of the occlusion time for inducing thrombus formation in mesenteric venules of mice. (A) Washed platelets were preincubated with (a) Tyrode's solution (resting group), (b) a solvent control (0.5% DMSO) or sesamol (2.5 and 5 μM), followed by the addition of collagen (1 μg/ml). Spectra are representative examples of four similar experiments. Asterisk (*) indicates the formation of hydroxyl radical. (B and C) Mice were administered 0.5% DMSO (ctl) or sesamol (5 and 10 mg/kg), after which mesenteric venules were selected for irradiation to induce microthrombus formation. Data of the bar graphs in (B) are presented as the means±S.E.M. of the occlusion time (seconds) for inducing platelet plug formation (n=5). $^{*}P$<.05 and $^{**}P$<.01, compared to the individual solvent control group. Microscopic images in (C) were taken in solvent- (a and b) or sesamol (5 mg/kg)-treated (c and d) groups during the time courses of 5 (a and c) and 170 s (b and d) after irradiation. Photographs are representative examples of five similar experiments (×400).

4. Discussion

This study demonstrates for the first time that sesamol possesses potent antiplatelet activity *via* an increase of cyclic nucleotides (i.e., cAMP) and inhibition of MAPK phosphorylation (i.e., p38 MAPK) in washed platelets. Jan et al. [1] studied the pharmacokinetics of sesamol (5 mg/kg iv) in rats and it was found that the maximum serum concentration of sesamol was approximated at 2.3±0.6 μg/ml (12–21 μM). The result indicates that the concentrations used in *in vitro* study (2.5 and 5 μM) are reasonable in accordance with blood level evaluated *in vivo*. Stimulation of platelets by agonists (i.e., collagen) causes marked alterations in phospholipid metabolism. The activation of PLC results in the degradation of phosphoinositides, notably, phosphatidylinositol 4,5-bisphosphate (PI4,5-P_2), resulting in the production of the second messengers, inositol 1,4,5-trisphosphate (IP_3) and DAG [16]. DAG activates PKC, inducing protein phosphorylation (p47). PKC activation represents a strategy adopted by cells to allow selected responses to specific activating signals in distinct cellular compartments [17]. Phosphoinositide-specific PLC is a key enzyme in signal transduction [18]. There are six major families of PLC enzymes which consist of at least 13 PLC isoforms [PLCβ (1–4), PLCγ (1 and 2), PLCδ (1, 3 and 4), PLCε (1), PLCζ (1) and PLCη (1 and 2)] [18]. PLCγ2 is involved in antigen-dependent signaling in B cells and collagen-dependent signaling in platelets [19]. In this study, both PLCγ2 phosphorylation and PKC activation were inhibited by sesamol, suggesting that sesamol-mediated antiplatelet activity is involved in inhibition of the PLCγ2-PKC signal pathway. Sesamol had no direct effect on PKC activation, as it did not inhibit PDBu-induced PKC activation or platelet aggregation (data not shown). In addition, collagen-induced TxB_2 formation, a stable metabolite of thromboxane A_2 (TxA_2), was markedly inhibited by sesamol. TxA_2 is important for collagen- and AA-induced platelet aggregation. This may explain the more potent activity of sesamol in inhibiting collagen- and AA-induced platelet aggregation than other agonists.

MAPKs consist of three major subgroups. The ERKs (p44 ERK1 and p42 ERK2) are involved in proliferation, adhesion and cell progression [20]. p38 MAPK and JNKs, which include the 46-kDa JNK1 and 55-kDa JNK2 isoforms, appear to be involved in apoptosis [20]. ERKs, JNKs and p38 MAPK have been identified in platelets [20]. The roles of JNKs and ERKs in physiopathology are unclear, but they have been suggested to be suppressors of $\alpha_{IIb}\beta_3$ integrin activation or negative regulators of platelet activation [21]. On the other hand, p38 MAPK provides a crucial signal as a downstream effector of PKC which is necessary for aggregation caused by collagen [22]. We also found that SB203580 (10 μM), a p38 MAPK inhibitor, markedly inhibited collagen (1 μg/ml)-induced platelet aggregation approximated at 89±7% (n=3, data not shown). Among the numerous downstream targets of p38 MAPK, the most physiologically relevant one in platelets is cytosolic phospholipase A_2 which catalyzes AA release to produce TxA_2 [23]; thus, p38 MAPK appears to provide a TxA_2-dependent platelet aggregation pathway. Sesamol significantly inhibits TxA_2 formation, at least in part, *via* inhibition of p38 MAPK phosphorylation.

Activation of human platelets is inhibited by two intracellular pathways regulated by either cAMP or cGMP. The importance of cAMP and cGMP in modulating platelet reactivity is well established [24]. In addition to inhibiting most platelet responses, elevated levels of cAMP and/or cGMP decrease intracellular Ca^{2+} concentrations by the uptake of Ca^{2+} into the dense tubular system which negatively affects the action of PKC [24]. Therefore, cAMP and cGMP act synergistically to inhibit platelet aggregation. In this study, sesamol not only increased the levels of both cAMP and cGMP, but also phosphorylated VASP in human platelets. VASP is phosphorylated by cyclic nucleotide-dependent protein kinases in a variety of cells, including smooth muscle cells and platelets.

VASP plays important roles in modulating actin filament dynamics and integrin activation [15].

Platelets produce NO in smaller amounts than do endothelial cells [25]. Most cellular actions of NO occur *via* stimulation of intracellular guanylate cyclase, leading to increases in cGMP. Both the inducible NOS (iNOS) and eNOS isoforms have been described in platelets, but eNOS is predominant [25]. Sesamol (10 μM) has been reported to induce NO release and stimulate eNOS activity in human endothelial cells [8]. In this study, sesamol was found to stimulate both NO release and eNOS phosphorylation in human platelets, and both effects were inhibited by SQ22536 but not by ODQ. This result is in accord with that of increased cAMP stimulating eNOS activity and NO biosynthesis [26].

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 [11]. Reactive oxygen species (i.e., hydrogen peroxide and hydroxyl radicals) derived from platelet activation might amplify platelet reactivity during thrombus formation. Free radical species act as secondary messengers that increase cytosolic Ca^{2+} during the initial phase of platelet activation processes, and PKC is involved in receptor-mediated free-radical production in platelets [12]. 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 [12]. Thus, sesamol's prolongation of platelet plug formation *in vivo* may be involved, at least in part, in the inhibition of free radical formation triggered by collagen. On the other hand, sesamol may induce NO-mediated endothelial vasodilation, which is probably involved in prolongation of platelet plug formation *in vivo*.

In conclusion, the most important findings of this study demonstrate for the first time that the antiplatelet activity of sesamol may involve an increase of the cAMP-eNOS/NO-cGMP pathway, followed by inhibition of the PLCγ2-PKC-p38 MAPK-TxA_2 cascade, thereby leading to inhibition of $[Ca^{2+}]_i$, and, finally, inhibition of platelet aggregation. Platelet aggregation plays important pathophysiological roles in a variety of thromboembolic disorders. Therefore, the potent antiplatelet activity of sesamol may represent an increased therapeutic potential to treat such diseases.

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