Inhibitory effects of lycopene on in vitro platelet activation and in vivo prevention of thrombus formation

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Lycopene is a natural carotenoid antioxidant that is present in tomatoes and tomato products. The pharmacologic function of lycopene in platelets is not yet understood. Therefore, in this study we sought to systematically examine the effects of lycopene in the prevention of platelet aggregation and thrombus formation. We found that lycopene concentration-dependently (2–12 μmol/L) inhibited platelet aggregation in human platelets stimulated by agonists. Lycopene (6 and 12 μmol/L) inhibited phosphoinositide breakdown in platelets labeled with tritiated inositol, intracellular Ca^{+2} mobilization in Fura-2 AM–loaded platelets, and thromboxane B_2 formation stimulated by collagen. In addition, lycopene (6 and 12 μmol/L) significantly increased the formations of cyclic GMP and nitrate but not cyclic AMP in human platelets. Rapid phosphorylation of a protein of 47,000 Da (P47), a marker of protein kinase C activation, was triggered by PDBu (60 nmol/L). This phosphorylation was markedly inhibited by lycopene (12 μmol/L) in phosphorus-32–labeled platelets. In an in vivo study, thrombus formation was induced by irradiation of mesenteric venules in mice pretreated with fluorescein sodium. Lycopene (5, 10, and 20 mg/kg) significantly prolonged the latency period for the induction of platelet-plug formation in mesenteric venules. These results indicate that the antiplatelet activity of lycopene may involve the following pathways: (1) Lycopene may inhibit the activation of phospholipase C, followed by inhibition of phosphoinositide breakdown and thromboxane B_2 formation, thereby leading to inhibition of intracellular Ca^{+2} mobilization. (2) Lycopene also activated the formations of cyclic GMP/nitrate in human platelets, resulting in the inhibition of platelet aggregation. The results may imply that tomato-based foods are especially beneficial in the prevention of platelet aggregation and thrombosis. (J Lab Clin Med 2005;146:216–226)

Abbreviations: DMSO = dimethylsulfoxide; EDTA = ethylenediaminetetraacetate; FITC = fluorescein isothiocyanate; Fura-2-AM = 1-(2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy)-2-(2′-amino-5′-methyl-phenoxy)ethane-N,N,N′,N′-tetraacetic acid; IP = inositol monophosphate; IP_2 = inositol-4,5-biphosphate; IP_3 = inositol-1,4,5-trisphosphate; GMP = guanosine monophosphate; NO = nitric oxide; PGE_1 = prostaglandin E_1; PRP = platelet-rich plasma; PSL = photostimulated luminescence

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Dietary intakes of tomatoes and tomato products containing lycopene have been shown to be associated in many studies with a decreased risk of illnesses such as cancer and cardiovascular disease.[1] Lycopene is a potent antioxidant among various common carotenoids.[2] The findings of several epidemiologic studies have suggested that fruits and vegetables are good sources of dietary carotenoids, including lycopene.[3,4] Tomatoes are rich in carotenoids, especially lycopene. This product is a long-chain polyunsaturated aliphatic compound similar to carotene. The antioxidant properties of lycopene have been suggested to be responsible for the beneficial effects of these food products.

Epidemiologic evidence indicates that consumers of tomatoes have a lower risk of many types of chronic illnesses, including heart disease (eg, coronary heart disease) and key types of cancer (eg, lung, breast, ovary, intestinal tract, and prostate gland).[1] The risks of myocardial infarction may be reduced in people with higher adipose tissue concentrations of lycopene.[1] Furthermore, lycopene has been shown to be the only antioxidant that occurred at significantly lower levels in men in whom prostate cancer later developed than in status-matched controls.[5]

Intravascular thrombosis is a factor in the generation of a wide variety of cardiovascular diseases (eg, coronary heart disease). The initiation of intraluminal thrombosis is believed to involve platelet adherence and aggregation. Therefore, platelet aggregation may play a crucial role in atherothrombotic processes. Indeed, antiplatelet agents (eg, ticlopidine and aspirin) have been shown to reduce the incidence of stroke in high-risk patients.[6]

Although the epidemiologic studies conducted so far provide convincing evidence of the role of lycopene in the prevention of cardiovascular disease, this evidence is at best only suggestive and not proof of a causal relationship between lycopene intake and the risk of cardiovascular disease. We therefore systematically examined the influence of lycopene in washed human platelets and utilized the findings to characterize the mechanisms involved in this influence. We have previously reported that platelet thrombi are induced by irradiation with filtered light in the microvasculature of mice pretreated with fluorescein sodium.[7] We therefore used this model to further evaluate the inhibitory effect of thrombus formation by lycopene in vivo.

METHODS

Materials. Collagen (type I, bovine Achilles tendon), lycopene, sodium citrate, luciferin-luciferase, indomethacin, fluorescein sodium, Dowex-1 (100–200-μm mesh; X_8, chloride form), ADP, myoinositol, prostaglandin E_1 (PGE_1), arachidonic acid, phorbol-12, PDBu, apyrase, and heparin were purchased from Sigma-Aldrich (St Louis, Mo). Fura 2-AM and FITC were purchased from Molecular Probes, Inc (Eugene, Ore).*Trimeresurus flavoviridis* venom was purchased from Latoxan (Rosans, France). Myo-2-[^{3}H] inositol was purchased from Amersham (Buckinghamshire, UK). Thromboxane B_2, cyclic AMP, and cyclic GMP enzyme immunoassay kits were purchased from Cayman (Ann Arbor, Mich). Lycopene was dissolved in 0.5% DMSO for in vitro platelet-aggregation study and dissolved in 20% Tween-80 with normal saline solution for in vivo study. In this study, a vehicle solvent control was always included.

Preparation of human PRP and washed platelet suspensions. Human platelet suspensions were prepared as previously described.[8] In this study, human volunteers gave informed consent. In brief, blood was collected from healthy volunteers, who had taken no medicine during the preceding 2 weeks, and was mixed with either 3.8% sodium citrate (9:1, vol/vol) for the preparation of PRP or acid/citrate/glucose (9:1, vol/vol) for the preparation of washed platelets. After centrifugation at 120*g* for 10 minutes, the supernatant (PRP) was supplemented with PGE_1 (0.5 μmol/L) and heparin (6.4 IU/mL), then incubated for 10 minutes at 30°C and centrifuged at 500*g* for another 10 minutes. The platelet pellets were suspended in 5 mL of Tyrode's solution (pH 7.3); then apyrase (1 U/mL) and heparin (6.4 IU/mL) were added and the mixture was incubated for 10 minutes at 30°C. After centrifugation of the suspension at 500*g* for 10 min, the washing procedure was repeated. The washed platelets were finally suspended in Tyrode's solution containing bovine serum albumin (3.5 mg/mL) and adjusted to a concentration of 4.5×10^8 platelets/mL. The final concentration of Ca^{+2} in Tyrode's solution was 1 mmol/L.

Platelet aggregation. We applied the turbidimetric method to measure platelet aggregation,[8] using a Lumi-Aggregometer (Payton, Canada). Both platelet suspensions (4.5×10^8 platelets/mL, 0.4 mL) and PRP were prewarmed to 37° for 2 minutes (with stirring at 1200 rpm) in a silicone-treated glass cuvette. Lycopene (2–12 μmol/L) was added 3 minutes before the addition of platelet-aggregation inducers. The reaction was allowed to proceed for at least 6 minutes, and the extent of aggregation was expressed in light-transmission units. While measuring ATP release, we added 20 μL of a luciferin/luciferase mixture 1 minute before adding the agonists and compared ATP release with that of the control.

Flow-cytometric analysis of the glycoprotein IIb/IIIa complex. Triflavin, a specific fibrinogen receptor (glycoprotein IIb/IIIa complex) antagonist, was prepared as previously described.[9] Fluorescence-conjugated triflavin was also prepared as previously described.[9] 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 lycopene (6 and 12 μmol/L) for 3 minutes, followed by the addition of 2 μL of FITC-triflavin. The suspensions were then incubated for another 5 minutes, and the volume was adjusted to 1 mL/tube with Tyrode's solution. The suspensions were then assayed for fluorescein-labeled

platelets with the use of a flow cytometer (FACScan System, Becton Dickinson, San Jose, Calif). We collected data from 50,000 platelets per experimental group. All experiments were repeated at least 5 times as a means of ensuring reproducibility.

Measurement of the production of tritiated-inositol–labeled phosphates. To measure the production of phosphates labeled with tritiated inositol, we used a method described previously.[10] In brief, citrated human PRP was centrifuged and the pellets suspended in Tyrode's solution containing tritiated inositol (75 μCi/mL). Platelets were incubated for 2 hours, then centrifuged, and finally resuspended in Ca^{+2}-free Tyrode's solution (5×10^8/mL). Lycopene (6 and 12 μmol/L) was preincubated with 1 mL of loaded platelets at room temperature for 3 minutes, after which collagen (1 μg/mL) was added to trigger aggregation for 6 minutes. The reaction was stopped with the addition of 1 mL trichloroacetic acid (10% wt/vol), and samples were centrifuged at 1000*g* for 4 minutes. The inositol phosphates of the supernatants were separated in a Dowex-1 anion-exchange column. Only [^{3}H]-IP was measured as an index of total inositol phosphate formation.

Measurement of platelet (Ca^{+2})i mobilization. Citrated whole blood was centrifuged at 120*g* for 10 minutes. The supernatant was protected from light and incubated with Fura 2-AM (5 μmol/L) at 37°C for 1 hour. Human platelets were then prepared as described above. Finally the external Ca^{+2} concentration of the platelet suspensions was adjusted to 1 mmol/L. The increase in $[Ca^{+2}]$i was measured with the use of a fluorescence spectrophotometer (CAF 110; Jasco, Tokyo, Japan) at excitation wavelengths of 340 and 380 nm and an emission wavelength of 500 nm. We calculated $[Ca^{+2}]$i from the fluorescence, using 224 nmol/L as the Ca^{+2}–Fura-2 dissociation constant.[11]

Measurement of thromboxane B_2 formation. PRP was preincubated for 3 minutes in the presence or absence of lycopene (6 and 12 μmol/L) before the addition of collagen (1 μg/mL). Six minutes after the addition of agonists, 2 mmol/L EDTA and 50 μmol/L indomethacin were added to the reaction suspensions. The vials were then centrifuged for 3 minutes at 15,000*g*. Thromboxane B_2 levels in the supernatants were measured with the use of an enzyme immunoassay kit in accordance with the instructions of the manufacturer.

Estimation of platelet cyclic AMP, cyclic GMP, and nitrate formations. In brief, PRP was warmed to 37°C for 1 minute, after which PGE_1 (10 μmol/L), nitroglycerin (10 μmol/L), or lycopene (6 and 12 μmol/L was added and the mixture was incubated for 6 minutes. The incubation was stopped, and the solution was immediately boiled for 5 minutes. After cooling to 4°C, the precipitated protein was collected as sediment after centrifugation. Fifty microliters of supernatant was used to determine the cyclic AMP and cyclic GMP contents with the use of enzyme immunoassay kits after acetylation of the samples as described by the manufacturer.

NO was assayed in PRP as previously described.[12] In brief, PRP was preincubated with collagen (1 μg/mL) or lycopene (6 and 12 μmol/L for 6 min), followed by centrifugation. We measured the amount of nitrate in the supernatants (10 μL) by adding a reducing agent (0.8% VCl_3 in 1 mol/L HCl) to the purge vessel to convert nitrate to NO, which was stripped from the supernatants with the use of a helium purge gas. The NO was then drawn into a NO analyzer (Sievers 280 NOA; Sievers, Boulder, Colo). We calculated nitrate concentrations by comparing them with those of standard solutions of sodium nitrate.

Measurement of protein kinase C activity. Washed human platelets (2×10^9/mL) were incubated for 60 minutes at 37°C with phosphorus-32 (0.5 mCi/mL). Platelet suspensions were next washed twice with Tris-saline buffer. The phosphorus-32–labeled platelets were preincubated with lycopene (6 and 12 μmol/L in an aggregometer at 37°C for 3 minutes, then PDBu (60 nmol/L) was added for 1 minute to trigger protein kinase C activation. Activation was terminated with the addition of Laemmli sample buffer, and the results were analyzed with the use of electrophoresis (12.5%, wt/vol) as described previously.[13] The gels were dried, and the relative intensities of the radioactive bands were analyzed with the use of a bioimaging analyzer system (FAL2000; Fuji, Tokyo, Japan), and expressed as PSL per mm^2.

Fluorescein sodium–induced platelet thrombi in mesenteric microvessels of mice. As we previously described,[7] mice were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg). After a tracheotomy, an external jugular vein was cannulated with polyethylene tubing (PE-10) for the administration of the dye and drug (by an intravenous bolus); additional tubing was threaded through the femoral artery as a means of monitoring blood pressure. A segment of the small intestine with its mesentery attached was loosely brought outside the body through a midline incision in the abdominal wall and placed on a transparent culture dish for microscopic observation. Microvessels in the mesentery were observed under transillumination from a halogen lamp. Venules ranging in diameter from 30 to 40 μm were selected for irradiation to produce a microthrombus. In the epiillumination system, light from a 100-W mercury lamp was passed through a filter (B-2A; Nikon, Tokyo, Japan) with a dichromic mirror (DM 510; Nikon). Filtering the light eliminated wavelengths below 520 nm, and this light was used to irradiate a microvessel (the area of irradiation was about 100 μm in diameter on the focal plane) through an objective lens (20×). The dose of fluorescein sodium used was 5 mg/kg. Various doses of lycopene (5, 10, and 20 mg/kg), solvent control (20% Tween-80 normal saline solution), or isovolumetric normal saline solution was administered 1 minute after the addition of fluorescein sodium. The injected volume of the lycopene and solvent control was less than 50 μL. We measured the time it took for a thrombus to form and cause cessation of blood flow. The elapsed time for the induction of platelet-plug formation was measured every 5 minutes during the irradiation of the venules.

Statistical analysis. The experimental results are expressed as the mean ± SEM and are accompanied by the number of observations. Student's paired *t* test was used to determine significant differences between the solvent control and lycopene-treated groups in the study of fluorescein sodium–induced platelet thrombi in mice. We used analysis

of variance to assess the findings of the other experiments. If this analysis indicated significant differences among the group means, each group was compared using the Newman-Keuls method. *P* values of less than .05 were considered statistically significant.

RESULTS

Effect of lycopene on platelet aggregation. Lycopene (2–12 μmol/L) concentration-dependently inhibited platelet aggregation and the ATP-release reaction stimulated by collagen (1 μg/mL), ADP (20 μmol/L), and arachidonic acid (60 μmol/L) in both washed human platelets (Fig 1, *A* and *C*) and PRP (Fig 1, *B* and *C*). The inhibitory effect of lycopene in ADP (20 μmol/L)–induced platelet aggregation was present in fibrinogen (300 μg/mL) in washed-platelet suspensions. Furthermore, lycopene inhibited the ATP-release reaction when stimulated by agonists (eg, collagen; Fig 1, *A*). The 50% inhibitory concentration values of lycopene with regard to platelet aggregation induced by collagen, ADP, and arachidonic acid were estimated to be approximately 5.8, 6.2, and 6.8 μmol/L, respectively, in washed-platelet suspensions and approximately 6.5, 6.7, and 7.7 μmol/L, respectively, in PRP. Lycopene is approximately 170 times more potent than α-tocopherol in inhibiting platelet aggregation compared with the 50% inhibitory concentrations on a molar basis.[14] Furthermore, when platelets were preincubated with a higher concentration of lycopene (50 μmol/L) and normal saline solution for 10 minutes, respectively, followed by 2 washes with Tyrode's solution, we found no significant differences between the 2 aggregation curves stimulated by collagen (1 μg/mL) in both platelet suspensions, indicating that the effect of lycopene on inhibition of platelet aggregation occurs in a reversible manner (data not shown). In contrast to lycopene, solvent control (0.5% DMSO) did not significantly inhibit collagen (1 μg/mL)– or arachidonic acid (60 μmol/L)–induced platelet aggregation (data not shown). In the following experiments, we used collagen as an agonist to explore the inhibitory mechanisms of lycopene in platelet aggregation.

Effect of lycopene on the glycoprotein IIb/IIIa complex in human platelets. Triflavin is an arginine-glycine-Asp–containing antiplatelet peptide purified from *T flavoviridis* snake venom.[9,15] Triflavin inhibits platelet aggregation through direct interference with fibrinogen binding to the glycoprotein IIb/IIIa complex ($\alpha_{IIb}\beta_3$ integrin).[9] A wealth of evidence suggests that the binding of fibrinogen to the glycoprotein IIb/IIIa complex is the final common pathway for agonist-induced platelet aggregation. We therefore decided to further evaluate whether lycopene 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 about 998.9 ± 19.8 (Fig 2, *A*), and it was markedly reduced in the presence of 5 mmol/L EDTA (negative control) (Fig 2, *B*). Lycopene (6 and 12 μmol/L) did not significantly inhibit FITC-triflavin binding to the glycoprotein IIb/IIIa complex in platelet suspensions (Fig 2, *C* and *D*), indicating that the mechanism of lycopene's inhibitory effect on platelet aggregation does not involve binding to the glycoprotein IIb/IIIa complex.

Effect of lycopene on phosphoinositide breakdown. Phosphoinositide breakdown occurs in platelets activated by many different agonists.[16] In this study, we found that collagen (1μg/mL) induced the rapid formation of radioactive IP, inositol-4,5-biphosphate (IP_2), and inositol-1,4,5-trisphosphate (IP_3) in human platelets loaded with [^{3}H]-inositol. We only measured [^{3}H]-IP formation as an index of total inositol phosphate formation. As shown in Fig 3A, the addition of collagen (1 μg/mL) resulted in a marked increase in IP formation compared with that in resting platelets ([9.1 ± 0.1 vs 37.5 ± 1.9]×10^3 cpm). In the presence of lycopene (6 and 12 μmol/L), the radioactivity of IP formation in collagen-stimulated human platelets markedly decreased. We also measured free cytoplasmic Ca^{+2} concentrations in human platelets using the Fura 2-AM–loading method. As shown in Fig 3, *B*, collagen (1 μg/mL) evoked an increase in [Ca^{+2}]i from 47.9 ± 6.8 to 576.2 ± 45.9 nmol/L. This collagen-evoked increase in [Ca^{+2}]i was markedly inhibited in the presence of lycopene (6 μmol/L, 50.3% ± 8.5%; 12 μmol/L, 82.3% ± 3.3%) (Fig 3, *B*). This finding suggests that lycopene exerts an inhibitory effect on phosphoinositide breakdown and [Ca^{+2}]i mobilization in human platelets stimulated by collagen.

Effects of lycopene on thromboxane B_2, cyclic AMP, cyclic GMP, and nitrate formation. As shown in Table I, resting platelets produced less thromboxane B_2 than did collagen-activated platelets. PGE_1 (10 μmol/L) inhibited thromboxane B_2 formation in collagen-activated platelets by 82% (data not shown). Furthermore, results obtained with the use of various concentrations of lycopene indicated that lycopene (6 and 12 μmol/L) concentration-dependently inhibited thromboxane B_2 formation in PRP stimulated by collagen (1 μg/mL). In addition, the level of cyclic AMP in unstimulated platelets was about 18.6 ± 2.5 pmol/mL. The addition of PGE_1 (10 μmol/L) markedly increased the level of cyclic AMP (Table I). Lycopene (6 and 12 μmol/L) did not significantly increase cyclic AMP levels in human PRP (Table I). We also performed a similar study

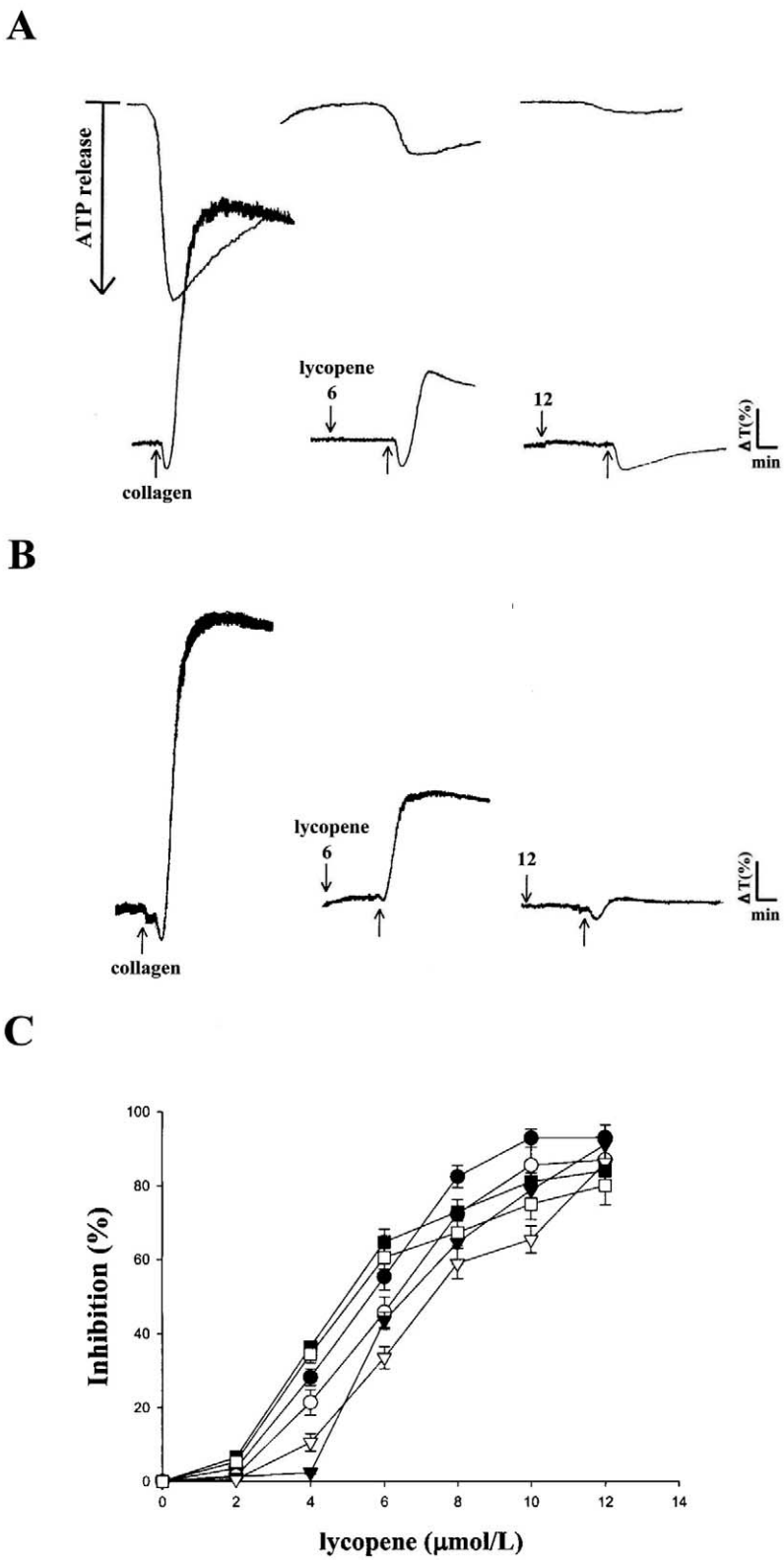

Fig 1. (**A**, **B**) Aggregation curves and (**C**) concentration-inhibition curves of lycopene on collagen (1 μg/mL, *circles*)–induced, arachidonic acid (60 μmol/L, *triangles*)–induced, and ADP (20 μmol/L, squares)–induced platelet aggregation in both washed human-platelet suspensions (*black symbols*) and PRP (*white symbols*). Platelets were preincubated with lycopene (2–12 μmol/L) for 3 minutes; 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, $n = 6$) (**C**).

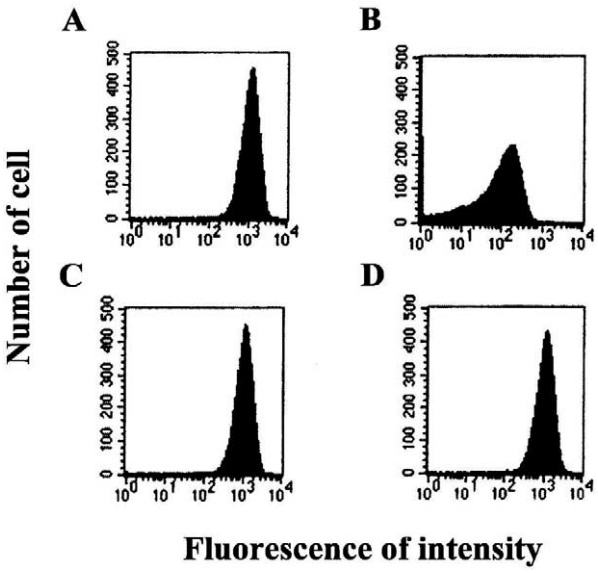

Fig 2. Flow-cytometric analysis of FITC-triflavin binding to human platelets in the absence or presence of various concentrations of lycopene (6 and 12 μmol/L). The *solid line* represents the fluorescence profiles of FITC-triflavin (2 μg/mL) (**A**) in the absence of lycopene as a positive control, (**B**) in the presence of EDTA (5 mmol/L) as the negative control, or in the presence of lycopene (**C**) 6 μmol/L or (**D**) 12 μmol/L. The profiles are representative examples of 4 similar experiments.

measuring the cyclic GMP response. The level of cyclic GMP in unstimulated platelets was less, but when nitroglycerin (10 μmol/L) was added to the PRP, the cyclic GMP level markedly increased from the resting level to 39.5 ± 4.1 pmol/mL (Table I). The addition of lycopene (6 and 12 μmol/L) resulted in significant increases in platelet cyclic GMP levels (Table I).

We quantified NO using a sensitive and specific ozone redox-chemiluminescence detector. As shown in Table I, collagen (1 μg/mL) caused about a 4.5-fold increase in nitrate formation compared with that in resting platelets. In the presence of lycopene (6 and 12 μmol/L), nitrate production significantly increased after incubation with PRP (Table I).

Effect of lycopene on PDBu–stimulated phosphorylation of the 47-Da protein. Stimulation of platelets with several different agonists, and PDBu in particular, induces activation of protein kinase C, which then phosphorylates proteins of 40,000 to 47,000 Da in addition to other proteins.[17] In this study, we conducted phosphorylation experiments to examine the role of lycopene in the activation of protein kinase C in human platelets. When PDBu (60 nmol/L) was added to human platelets prelabeled with ^{32}P-labeled O_4 for 2 minutes, a protein with an apparent molecular weight of 47,000 Da (P47) was predominately phosphorylated compared with resting platelets (Fig 4, *A* and *B*). On the other hand, lycopene (12 μmol/L) significantly inhibited the phosphorylation of P47 in human platelets stimulated with PDBu. In this study, the extent of radioactivity in P47 was expressed as a relative detection density (PSL per mm^2) of the radioactive bands (Fig 4, *B*). Moreover, lycopene (6 and 12 μmol/L) also significantly inhibited collagen (1 μg/mL)–induced phosphorylation of P47 in human platelets (data not shown).

Effect of lycopene on thrombus formation in microvessels of mice. Lycopene inhibited platelet aggregation induced by agonists in vitro; therefore, we further examined its effect on the formation of platelet-rich thrombi in this in vivo model. Mice were treated with isovolumetric normal saline solution and solvent control (20% Tween-80 normal saline solution), respectively. We found that administration of solvent control did not significantly affect occlusion time compared with that of the group treated with normal saline solution (152.6 ± 12.8 vs. 157.1 ± 11.7 sec, $P > .05$, $n = 5$). When lycopene was administered at concentrations of 5, 10, and 20 mg/kg, the occlusion time was significantly prolonged: 1.4-fold (120.4 ± 16.7 vs 169.6 ± 21.6 seconds, $n = 7$, $P < .001$), 1.7-fold (136.3 ± 29.1 vs 215.4 ± 33.3 seconds, $n = 7$, $P < .001$), and 1.9-fold (157.8 ± 12.8 vs 289.0 ± 19.3 seconds, $n = 7$, $P < .001$) compared with the individual solvent control (Fig 5). Our data reveal that platelet aggregation usually occurred first in venules rather than in arterioles. This may be explained by the higher flow velocities found in arterioles, resulting in delayed adhesion of platelets to arteriolar endothelial cells.

DISCUSSION

In this study, platelet aggregation induced by these agonists (eg, collagen) appeared to be affected in the presence of lycopene, implying that lycopene affects Ca^{+2} release from intracellular Ca^{+2}-storage sites (eg, dense tubular systems or dense bodies) (Fig 6), and this is in accord with the concept that intracellular Ca^{+2} release is responsible for platelet aggregation. Although the action mechanisms of various platelet aggregation agonists such as collagen, ADP, and arachidonic acid differ, lycopene significantly inhibited platelet aggregation stimulated by all of them. This implies that lycopene blocks a common step shared by these inducers. These results also indicate that the site of action of lycopene 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.[9,10,15] In this study, we found that lycopene did not significantly affect FITC-triflavin binding to the glycoprotein IIb/IIIa complex,

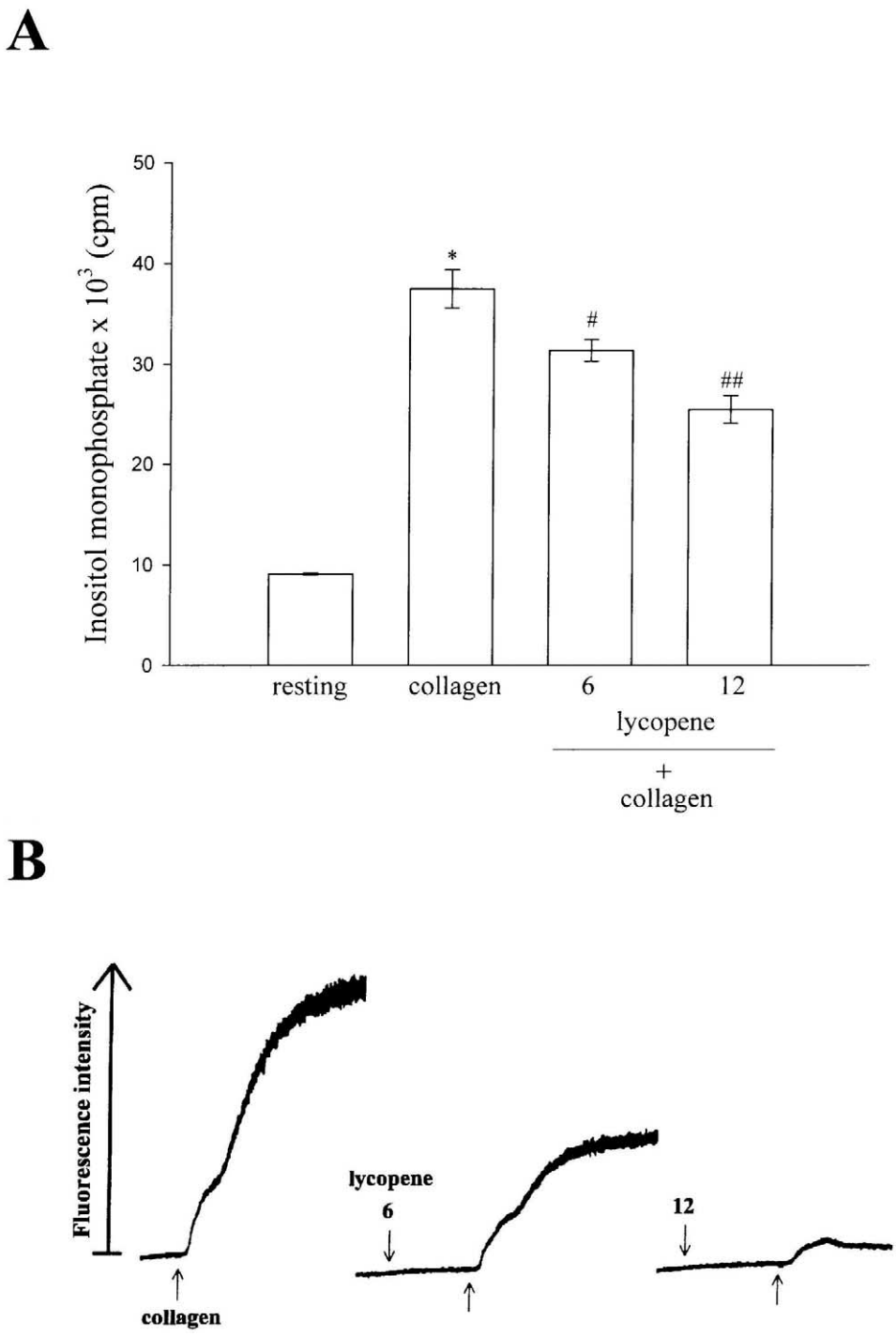

Fig 3. Effect of lycopene on collagen-induced (**A**) IP formation and (**B**) intracellular Ca^{+2} mobilization in human-platelet suspensions. Platelets were preincubated with tritiated inositol or Fura 2-AM (5 μmol/L), then exposed to collagen (1 μg/mL) in the absence or presence of lycopene (6 and 12 μmol/L), which was added 3 minutes before the addition of collagen. (**A**) Data are presented as the mean ± SEM ($n = 5$). $^{*}P < .001$ vs the resting group; $^{\#}P < .05$ and $^{\#\#}P < .001$ vs the collagen group. (**B**) The profiles are representative examples of 4 similar experiments.

Table I. Effect of lycopene on thromboxane B_2, cyclic AMP, cyclic GMP, and nitrate formation in PRP

Group	Concentration	Thromboxane B_2 (ng/mL) (n = 4)	Cyclic AMP (pmol/mL) (n = 4)	Cyclic GMP (pmol/mL) (n = 4)	Nitrate (μmol/L) (n = 4)
Resting		25.7 ± 4.8	18.6 ± 2.5	4.7 ± 0.2	1.4 ± 0.2
Collagen (μg/mL)	1	278.6 ± 23.5*	—	—	6.3 ± 0.5*
+ lycopene (μmol/L)	6	108.2 ± 15.3†	—	—	—
	12	68.6 ± 7.3†	—	—	—
PGE_1 (μmol/L)	10	—	156.4 ± 18.9*	—	—
Nitroglycerin (μmol/L)	10	—	—	39.5 ± 4.1*	—
lycopene (μmol/L)	6	—	22.6 ± 3.9	7.2 ± 0.3*	3.8 ± 0.2*
	12	—	23.7 ± 4.2	8.1 ± 0.6*	4.5 ± 0.4*

PRP was preincubated with lycopene (6 and 12 μmol/L) for 3 minutes at 37°C, and then collagen (1 μg/mL) was added to trigger thromboxane B_2 formation. Addition of PGE_1, nitroglycerin, and collagen to the PRP served as positive controls of cyclic AMP, cyclic GMP, and nitrate formation, respectively. Data are presented as the mean ± SEM.
*$P < .001$ vs resting groups.
†$P < .001$ vs the collagen group.

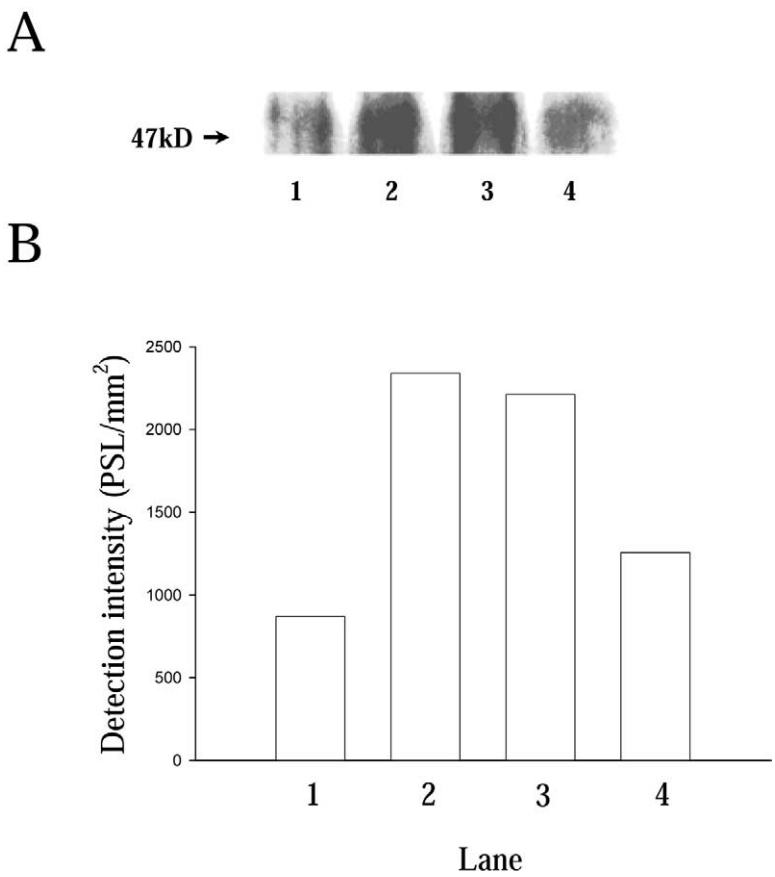

Fig 4. Effect of lycopene on phosphorylation of a protein of 47,000 Da (P47) in human platelets challenged with PDBu. Platelets were preincubated with lycopene (6 and 12 μmol/L) before challenge with PDBu (60 nmol/L). *Lane 1*, platelets with Tyrode's solution only (resting group); *lane 2*, platelets with PDBu (60 nmol/L); *lane 3*, platelets with lycopene (6 μmol/L); *lane 4*, platelets with lycopene (12 μmol/L) for 3 minutes followed by the addition of dibutyrate (60 nmol/L). (**A**) The profiles are representative examples of 4 similar experiments. The *arrow* indicates a protein of 47,000 Da (P47). (**B**) The relative detection densities of the radioactive bands are expressed as PSL per mm^2.

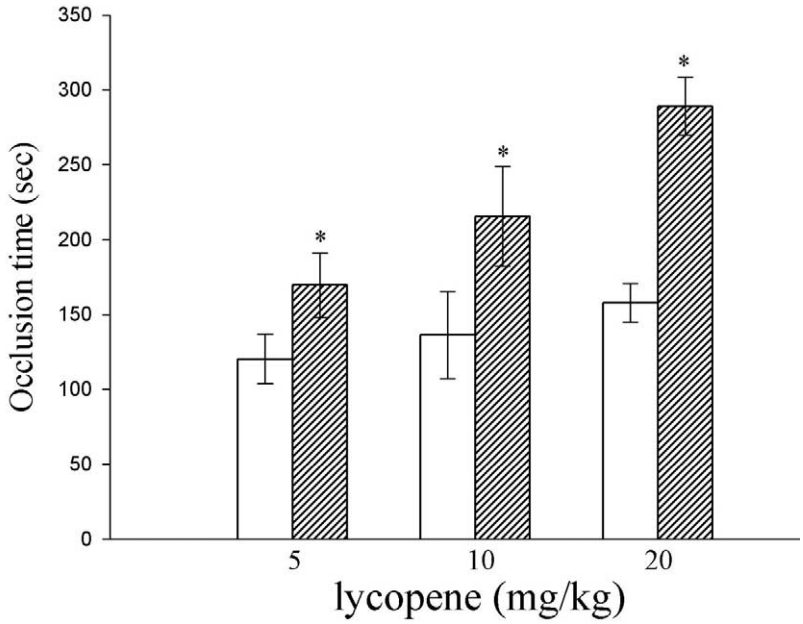

Fig 5. Effect of lycopene (5, 10 and 20 mg/kg, *hatched bars*) on occlusion times in the induction of thrombus formation during light irradiation of mesenteric venules of mice pretreated with fluorescein sodium (5 mg/kg). Data are presented as the mean ± SEM of occlusion time (seconds) of platelet-plug formation ($n = 7$). *$P <$ .001 vs the individual solvent control (*open bars*).

indicating that the antiplatelet activity of lycopene may not be directly due to interference with the binding of fibrinogen to its specific receptor on the platelet membrane.

Stimulation of platelets by agonists (eg, collagen) results in phospholipase C–catalyzed hydrolysis of the minor plasma membrane phospholipid, phosphatidylinositol 4,5-bisphosphate, with concomitant formation of IP_3 and diacylglycerol (Fig 6).[18] There is strong evidence that IP_3 induces the release of Ca^{+2} from intracellular stores (Fig 6).[19] Diacylglycerol activates protein kinase C, inducing protein phosphorylation and a release reaction. In this study, phosphoinositide breakdown of collagen-activated platelets was inhibited by lycopene, suggesting that inhibition of platelet aggregation by lycopene is related to inhibition of phospholipase C activation. Moreover, thromboxane A_2 is an important mediator of the release reaction and aggregation of platelets (Fig 6).[20] The collagen-induced formation of thromboxane B_2, a stable metabolite of thromboxane A_2, was markedly inhibited by lycopene (6 and 12 μmol/L) (Table I). It has been demonstrated that phosphoinositide breakdown can induce thromboxane B_2 formation by way of free arachidonic acid release by diglyceride lipase or by endogenous phospholipase A_2 release from membrane phospholipids (Fig 6).[21] It therefore seems likely that thromboxane B_2 formation plays a role in mediating the inhibitory effect of lycopene on human platelets.

Lycopene significantly inhibited PDBu–induced activation of protein kinase C. PDBu is known to intercalate with membrane phospholipids and form a complex with protein kinase C translocated to the membrane.[22] Moreover, increased cyclic GMP formation can negatively affect agonist-induced protein kinase C activation (Fig 6).[23] Signaling by cyclic GMP somehow interferes with the agonist-stimulated phosphoinositide turnover that creates Ca^{2+}-mobilizing second messengers.[24] Lycopene increased the level of cyclic GMP in human platelets; therefore the inhibitory effect of lycopene in PDBu–induced activation of protein kinase C may be due at least in part to mediation of the increase in the formation of cyclic GMP. In this study, we found that lycopene induced NO formation in human platelets. This result is in accord with the result of a cyclic GMP study; most cellular actions of NO occur by way of stimulation of intracellular guanylate cyclase, leading to an increase in cyclic GMP.[24] The platelet-derived NO/cyclic GMP not only modulates platelet activation but also, and more importantly, markedly inhibits atherothrombotic diseases.[25]

Platelet aggregation plays a pathophysiologic role in a variety of thromboembolic disorders. Inhibition of platelet aggregation by drugs may represent an in-

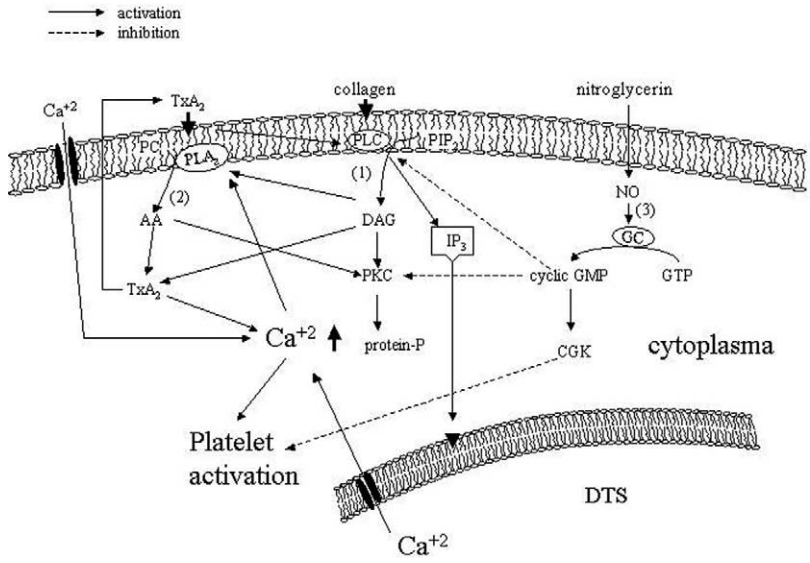

Fig 6. Signal transduction of platelet aggregation. Agonists can activate several phospholipases, including phospholipase C (*PLC*) and phospholipase A_2(*PLA_2*). (**1**) The products of phospholipase C's action on phosphatidylinositol 4,5-bisphosphate (*PIP_2*) include 1,2-diacylglycerol (*DAG*) and IP_3. DAG stimulates protein kinase C (*PKC*), followed by phosphorylation of a 47,000-Da protein. IP_3 induces the release of Ca^{+2} from the dense tubular system (*DTS*). (**2**) PLA_2 cleaves membrane phospholipids (eg, *PC*, phosphatidylcholine) and liberates arachidonic acid (*AA*), which is converted into a cyclic endoperoxide by platelet cyclooxygenase. Thromboxane synthase subsequently converts the cyclic endoperoxide into thromboxane A_2 (*TxA_2*). (**3**) NO activates guanlyate cyclase to increase the production of cyclic GMP. The cyclic GMP stimulates cyclic GMP–dependent protein kinase (*CGK*), which then inhibits platelet aggregation. Lycopene may inhibit (1) the activation of phospholipase C, followed by inhibition of phosphoinositide breakdown and thromboxane A_2 formation, thereby leading to inhibition of intracellular Ca^{+2} mobilization. Lycopene may also increase (**3**) cyclic GMP/nitrate formations and subsequently inhibits phosphoinositide breakdown and protein kinase C activation, ultimately resulting in inhibition of platelet aggregation.

creased therapeutic possibility for such diseases. In this study, we evaluated the inhibition of thrombus formation by lycopene in vivo and found that lycopene significantly prevented platelet-plug formation. Electron microscopy has shown that the thrombus formed in this experiment was mainly composed of the activated platelets adhesion to the underlying damaged endothelium.[7] It shortened the occlusion time of thrombus formation induced by irradiation of fluorescein sodium in venules or arterioles. Because the light beam covered the entire microscopic field, we were able to observe arterioles and venules simultaneously. In this system, the occlusion time was related to the blood flow rate, the diameter of the microvessel, and the dose of fluorescein dye. In this study, lycopene caused occlusion times to be significantly prolonged in mice pretreated with fluorescein sodium, mainly because of its inhibition of platelet aggregation. In the thrombotic study, the mesenteric venules were continuously irradiated with fluorescein sodium throughout the entire experimental period, leading to serious damage to endothelial cells, as described previously.[7] The dosage of lycopene employed in this model was relatively higher than that in platelet aggregation.

In conclusion, the most important observations of this study suggest that lycopene inhibits agonist-induced human platelet aggregation. This inhibitory effect may involve the following mechanisms: (1) Lycopene may inhibit the activation of phospholipase C, followed by inhibition of phosphoinositide breakdown and thromboxane B_2 formation, thereby leading to inhibition of intracellular Ca^{+2} mobilization. (2) On the other hand, lycopene increases cyclic GMP/nitrate formations and subsequently inhibits phosphoinositide breakdown and protein kinase C activation, ultimately resulting in inhibition of the phosphorylation of P47 and intracellular Ca^{+2} mobilization. Results of this study clearly indicate that lycopene may exhibit pharmacologic functions during thromboembolism formation. We therefore believe that our data represent a pharmacologically relevant function for lycopene as a potentially therapeutic agent for prevention of cardiovascular diseases. On the other hand, these findings may imply that tomato-based foods are especially ben-

eficial in the prevention of platelet aggregation and thrombosis.

REFERENCES

1. Weisburger JH. Lycopene and tomato products in human promotion. Exp Biol Med 2002;227:924–7.
2. Di Mascio P, Kaiser S, Sies H. Lycopene as the most efficient biological carotenoid singlet oxygen quencher. Arch Biochem Biophys 1989;274:532–8.
3. Kohlmeier L, Hastinhs SB. Epidemiological evidence of a role of carotenoids in cardiovascular disease prevention. Am J Clin Nutr 1995;62(suppl):1370S–6S.
4. Arab L, Steck S. Lycopene and cardiovascular disease. Am J Clin Nutr 2000;71(suppl):1691S–5S.
5. Gann PH, Ma J, Giovannucci E, Willett W, Sacks FM, Hennekens CH, Stampfer MJ. Lower prostate cancer risk in men with elevated plasma lycopene levels: results of a prospective analysis. Cancer Res 1999;59:1225–30.
6. Schleinitz MD, Weiss JP, Owens DK. Clopidogrel versus aspirin for secondary prophylaxis of vascular events: a cost-effectiveness analysis. Am J Med 2004;116:797–806.
7. Sheu JR, Chao SH, Yen MH, Huang TF. In vivo antithrombotic effect of triflavin, an Arg-Gly-Asp containing peptide on platelet plug formation in mesenteric microvessels of mice. Thromb Haemost 1994;72:617–21.
8. Sheu JR, Lee CR, Lin CC, Kan YC, Lin CH, Hung WC, Lee YM, Yen MH. The antiplatelet activity of PMC, a potent α-tocopherol analogue, is mediated through inhibition of cyclo-oxygenase. Br J Pharmacol 1999;127:1206–12.
9. Sheu JR, Lin CH, Peng CH, Huang TF. 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;213:71–9.
10. Huang TF, Sheu JR, Teng CM. Mechanism of action of a potent antiplatelet peptide, triflavin, from *Trimeresurus flavoviridis* snake venom. Thromb Haemost 1991;66:489–93.
11. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{+2} indicator with greatly improved fluorescence properties. J Biol Chem 1985;260:3440–50.
12. Sheu JR, Hung WC, Su CH, Lin CH, Lee LW, Lee YM, et al. The antiplatelet activity of *Escherichia coli* lipopolysaccharide, is mediated through nitric oxide/cyclic GMP pathway. Eur J Haematol 1999;62:317–26.
13. Grabarek J, Raychowdhury M, Ravid K, Kent KC, Newman PJ, Ware JA. Identification and functional characterization of protein kinase C isozymes in platelets and HEL cells. J Biol Chem 1992;267:10011–7.
14. Sheu JR, Lee CR, Hsiao G, Hung WC, Lee YM, Chen YC, et al. Comparison of the relative activities of α-tocopherol and PMC on platelet aggregation and antioxidative activity. Life Sci 1999; 65:197–206.
15. Sheu JR, Hung WC, Wu CH, Ma MC, Kan YC, Lin CH, et al. Reduction in lipopolysaccharide-induced thrombocytopenia by triflavin in a rat model of septicemia. Circulation 1999;99: 3056–62.
16. Broekman MJ, Ward JW, Marcus AJ. Phospholipid metabolism in phosphatidyl inositol, phosphatic acid and lysophospholipids. J Clin Invest 1980;66:275–83.
17. Siess W, Lapetina EG. Platelet aggregation induced by alpha 2-adrenoceptor and protein kinase C activation. A novel synergism. Biochem J 1989;263:377–85.
18. Kirk CJ, Creba JA, Downes CP, Michell RH. Hormone-stimulated metabolism of inositol lipids and its relationship to hepatic receptor function. Biochem Soc Transact 1981;9:377–9.
19. Berridge MJ. Rapid accumulation of inositol trisphosphate reveals that agonists hydrolyse polyphosphoinositides instead of phosphatidylinositol. Biochem J 1983;212:249–58.
20. Hornby EJ. Evidence that prostaglandin endoperoxides can induce platelet aggregation in the absence of thromboxane A_2 production. Biochem Pharmacol 1982;31:1158–60.
21. McKean ML, Smith JB, Silver WJ. Formation of lysophosphatidylcholine in human platelets in response to thrombin. J Biol Chem 1981;256:1522–4.
22. Kraft AS, Anderson WB. Phorbol esters increase the amount of Ca^{+2}, phospholipid-dependent protein kinase associated with plasma membrane. Nature 1983;301:621–3.
23. Murohara T, Parkinson SJ, Waldman SA, Lefer AM. Inhibition of nitric oxide biosynthesis promotes P-selectin expression in platelets. Role of protein kinase C. Arterioscler Thromb Vasc Biol 1995;15:2068–75.
24. McDonald LJ, Murad F. Nitric oxide and cyclic GMP signaling. Proc Soc Exp Biol Med 1996;211:1–6.
25. Loscalzo J. Nitric oxide insufficiency, platelet activation, and arterial thrombosis. Circ Res 2001;88:756–62.