Characterization of the Intracellular Mechanisms Involved in the Antiaggregant Properties of Cinnamtannin B-1 from Bay Wood in Human Platelets

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Cinnamtannin B-1, a natural A-type proanthocyanidin recently identified as a radical scavenger component of *Laurus nobilis* L., exerts antiaggregant and antiapoptotic effects in human platelets. Here, we have investigated the intracellular mechanisms involved in the antiaggregant effects of cinnamtannin B-1. Cinnamtannin B-1 showed a greater free radical scavenging activity than vitamin C, vitamin E, or Trolox, among other antioxidants and reduced thrombin-evoked tubulin reorganization and platelet aggregation. Thrombin-evoked activation of Btk and pp60^{src} was also inhibited by cinnamtannin B-1. In conclusion, we show that cinnamtannin B-1 is a powerful oxygen radical scavenger that reduces thrombin-evoked microtubular remodeling and activation of the tyrosine kinases Btk and pp60^{src}, which leads to inhibition of platelet aggregation. These observations suggest that cinnamtannin B-1 may prevent thrombotic complications associated to platelet hyperaggregability and hyperactivity, although further studies are necessary to establish appropriate therapeutic strategies.

Introduction

Proanthocyanidins belong to a class of polyphenols that are widely distributed throughout the plant kingdom. These compounds have long been investigated because of the antioxidant functions, which have been shown to involve radical scavenging, quenching, and enzyme-inhibiting actions.[1] The antioxidant activity of proanthocyanidins is stronger than that of vitamin C or vitamin E in aqueous systems, and their protective effects on diseases related to reactive oxygen species (ROS) have been demonstrated in a number of tissues.[2,3]

Cinnamtannin B-1 is a naturally occurring A-type proanthocyanidin composed of tree monomeric unit flavan-3-ol ((−)-epicatechin) that has been recently identified as a radical scavenging constituent of *Laurus nobilis*[4] and *Cinnamomum zeylanicum* fruits.[5] We have recently demonstrated that *in vitro* platelet treatment with the antioxidant cinnamtannin B-1 reverses the enhanced oxidant production, the altered Ca^{2+} mobilization, and the hyperaggregability described in platelets from diabetic patients, supporting that natural antioxidants may prove useful for therapeutic procedures to reduce cardiovascular complications in type 2 diabetes mellitus.[6] In addition, our previous results indicate that thrombin, a physiological platelet agonist, stimulates caspase-3 and -9 activation and its association with the cytoskeleton in human platelets, which leads to the development of an apoptosis-like process and, therefore, phosphatidylserine (PS) exposure. We have found that cinnamtannin B-1 induces a significant inhibitory effect on thrombin-induced activation and cytoskeletal association of caspases-3 and -9. The effect of cinnamtannin B-1 might be mediated by its antioxidant effects since it inhibits H_2O_2-induced caspase-3 and -9 activation and PS externalization.[7] In spite of that, the molecular mechanisms that underlie the biological effects of cinnamtannin B-1 in human platelets remain unclear.

Platelet aggregation by physiological agonists involves the activation of a number of intracellular mechanisms that includes protein tyrosine phosphorylation and reorganization of the cytoskeleton.[8−12] ROS have been shown to modulate protein tyrosine phosphorylation in different cell types,[13−14] including human platelets, where H_2O_2 induces Btk activation and plays an important role in the activation of Btk by thrombin.[15] ROS also have profound effects on the kinetics of microtubule polymerization through the oxidation of microtubule-associated proteins.[16]

Here we show that cinnamtannin B-1, in the micromolar range, significantly reduces the percentage and rate of platelet aggregation stimulated by thrombin. This action might be due to the impairment of thrombin-induced microtubular reorganization and tyrosine phosphorylation, which, in turn, are likely mediated by inhibition of the Btk and pp60^{src} activation that the physiological agonist thrombin promotes in human platelets.

Materials and Methods

Materials. Cinnamtannin B-1 has been isolated from *Laurus nobilis* L. wood (bay wood).[7] Hydroxytyrosol was from Cayman Chemical (Ann Arbor, MI). 2,2-Diphenyl-1-picryhydrazyl radical (DPPH•), rosmarinic acid, 2,6-di-*tert*-butyl-hydroxytoluene (BHT), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxilic acid (Trolox), L-ascorbic acid (vitamin C), α-tocopherol (vitamin E), apyrase (grade VII), EGTA, aspirin, bovine serum albumin (BSA), thrombin, catalase, paraformaldehyde, and Nonidet P40 were from Sigma (Madrid, Spain). Oregon Green 488 paclitaxel, fura-2 AM, calcein, and colchicine were from Molecular Probes (Leiden, The Netherlands). LFM-A13 (α-cyano-*β*-hydroxy-*β*-methyl-*N*-(2,5-dibro-

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[a] Abbreviations: Btk, Bruton's tyrosine kinase; ROS, reactive oxygen species; PS, phosphatidylserine; DPPH•, 2,2-diphenyl-1-picryhydrazyl radical; BHT, 2,6-di-*tert*-butyl-hydroxytoluene; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxilic acid; BSA, bovine serum albumin; LFM-A13, α-cyano-*β*-hydroxy-*β*-methyl-*N*-(2,5-dibromophenyl)propenamide; PP1, 4-amino-5-(4-methylphenyl)-7-(*tert*-butyl)pyrazolo[3,4-*d*]pyrimidine; HBS, HEPES-buffered saline; TMS, tetramethylsilane; PBS, phosphate-buffered saline; TBST, Tween 20; DMSO, dimethyl sulfoxide; PKC, protein kinase C.

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mophenyl)propenamide) was from Calbiochem (Madrid, Spain). 4-Amino-5-(4-methylphenyl)-7-(*tert*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP1) was from Alexis Corporation (Nottingham, UK). Anti-phosphotyrosine monoclonal antibody (4G10) and anti-p60^{src} monoclonal antibody GD11 were from Upstate Biotechnology (Madrid, Spain). Horseradish peroxidase-conjugated ovine anti-mouse IgG antibody (NA931) was from Amersham (Buckinghamshire, U.K.). Anti-phospho-Btk (Y-223) and anti-Btk antibodies were from Cell Signaling Technology (Beverly, MA). Anti-phospho-c-Src (Y-416) antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG antibody were from Santa Cruz (Santa Cruz, CA). Enhanced chemiluminescence detection reagents were from Pierce (Cheshire, U. K.). Hyperfilm ECL was from Amersham (Arlington Heights, IL). All other reagents were purchased from Panreac (Barcelona, Spain).

Platelet Preparation. Blood was obtained from healthy volunteers, according to the rules of the Declaration of Helsinki, and mixed with one-sixth volume of acid/citrate dextrose anticoagulant containing (in mM): 85 sodium citrate, 78 citric acid, and 111 D-glucose. Platelet-rich plasma was then prepared by centrifugation for 5 min at 700*g*, and aspirin (100 μM) and apyrase (40 μg/mL) were added. Cells were then collected by centrifugation at 350*g* for 20 min and resuspended in HEPES-buffered saline (HBS) containing (in mM): 145 NaCl, 10 HEPES, 10 D-glucose, 5 KCl, 1 $MgSO_4$, pH 7.45, and supplemented with 0.1% w/v bovine serum albumin and 40 μg/mL apyrase.

Cell Viability. Cell viability was assessed using calcein and trypan blue. For calcein loading, cells were incubated for 30 min with 5 μM calcein-AM at 37 °C and centrifuged, and the pellet was resuspended in fresh HBS. Cells were treated with the different inhibitors, centrifuged and resuspended in HBS. Fluorescence was recorded from 2 mL aliquots using a spectrophotometer (Varian Ltd., Madrid, Spain). Samples were excited at 494 nm, and the resulting fluorescence was measured at 535 nm. The results obtained with calcein were confirmed using the trypan blue exclusion technique. Ninety five percent of cells were viable in our platelet suspensions, and no effect was observed after treatment with inhibitors.

Structural Data of Cinnamtannin B-1. The structure of the compound isolated from *L. nobilis* wood, with a 90% of purity established by ^{1}H NMR,[7] was elucidated by the following spectroscopic methods: optical rotation ($[\alpha]_D$) was recorded in MeOH on a Perkin-Elmer 241 automatic polarimeter using quartz cells of 1-dm path length; concentration (*c*, expressed in g/100 mL) is given in parentheses. The ultraviolet (UV) spectrum was recorded in MeOH on a Perkin-Elmer UV/vis Lambda 19 spectrophotometer using quartz cells of 1-dm path length. The infrared (IR) spectrum was recorded on a Perkin-Elmer FT-IR 1760X spectrometer using a thin film between KBr plates (neat), and only characteristic absorptions (ν, cm^{-1}) are reported. ^{1}H NMR spectrum was recorded at 400 MHz on a Bruker Avance 400 spectrometer using CD_3OD as solvent and tetramethylsilane (TMS) as internal reference; chemical shift values are reported in parts per million (ppm, δ scale) and coupling constants (*J*) in hertz (Hz); multiplicity of signals is expressed as s: singlet, d: doublet, t: triplet, m: multiplet, br: broad, dd: double doublet. ^{13}C NMR spectrum was recorded at 100 MHz on the same instrument; chemical shifts are reported in ppm relative to TMS (CD_3OD = 49.00); carbon substitution was established by a DEPT multipulse sequence. 2D NMR experiments (DQF-COSY, HSQC, HMBC, TOCSY, NOESY) were carried out on the same instrument. Mass spectrum was obtained from an Esquire 6000 Electrospray mass spectrometer using the negative-ion mode (ESIMS). $[\alpha]_D$ +95.5° (*c* 1.02); UV λ_{max} (log ϵ) 231 (4.60), 279 (4.21); IR (neat) ν 3332, 1599, 1514, 1433, 1384, 1059; ^{1}H NMR δ 2.84 [2H, m, H-4(I)], 3.29 [1H, d, *J*=3.5 Hz, H-3(C)], 3.86 [1H, br t, *J* = 3.1 Hz, H-3(I)], 4.13 [1H, br d, *J* = 1.2 Hz, H-3(F)], 4.15 [1H, d, *J* = 3.5 Hz, H-4(C)], 4.38 [1H, br s, H-2(I)], 4.56 [1H, br s, H-4(F)], 5.70 [1H, br s, H-2(F)], 5.81 [1H, s, H-6(D)], 5.97 [1H, d, *J* = 2.3 Hz, H-6(A)], 6.02 [1H, d, *J* = 2.3 Hz, H-8(A)], 6.10 [1H, s, H-6(G)], 6.72 [1H, dd, *J* = 8.2, 1.8 Hz, H-6′(H)], 6.75 [1H, d, *J* = 8.2 Hz, H-5′(H)], 6.82 [1H, d, *J* = 8.2 Hz, H-5′(E)], 6.83 [1H, d, *J* = 1.8 Hz, H-2′(H)], 6.84 [1H, d, *J* = 7.8 Hz, H-5′(B)], 6.86 [1H, dd, *J* = 7.8, 2.1 Hz, H-6′(B)], 7.04 [1H, d, *J* = 2.1 Hz, H-2′(B)], 7.19 [1H, dd, *J* = 8.2, 2.1 Hz, H-6′-(E)], 7.32 [1H, d, *J* = 2.1 Hz, H-2′(E)]; ^{13}C NMR δ 28.82 [C-4(C)], 29.79 [C-4(I)], 38.23 [C-4(F)], 67.14 [C-3(C)], 67.48 [C-3(I)], 72.52 [C-3(F)], 78.81 [C-2(F)], 80.24 [C-2(I)], 96.06 [C-6(D)], 96.48 [C-6(G)], 96.54 [C-8(A)], 98.28 [C-6(A)], 99.91 [C-2(C)], 100.05 [C-10(G)], 104.96 [C-10(A)], 106.39 [C-8(D)], 106.69 [C-10(D)], 108.80 [C-8(G)], 115.44 [C-2′(H)], 115.71 [C-2′(B)], 115.73 [C-5′-(E)], 115.99 [C-5′(H)], 116.13 [C-5′(B)], 116.68 [C-2′(E)], 119.40 [C-6′(H)], 119.84 [C-6′(B)], 121.31 [C-6′(E)], 131.72 [C-1′(E)], 132.41 [C-1′(B)], 133.12 [C-1′(H)], 145.28 [C-3′(H)], 145.42 [C-3′-(B)], 145.71 [C-4′(H)], 145.85 [C-3′(E)], 146.22 [C-4′(E)], 146.56 [C-4′(B)], 151.04 [C-7(D)], 151.74 [C-9(D)], 154.11 [C-9(A)], 155.50 [C-7(G)], 155.72 [C-5(D)], 155.75 [C-9(G)], 155.97 [C-5(G)], 156.71 [C-5(A)], 157.77 [C-7(A)]; ESIMS *m/z* 863 [M − H]$^-$.

Free Radical Scavenging Activity. The antioxidant activity of cinnamtannin B-1 and reference compounds (BHT, rosmarinic acid, Trolox, L-ascorbic acid, α-tocopherol, and hydroxytyrosol) was determined by spectrophotometric measurements according to the ability of the tested samples to scavenge the free radical DPPH$^•$. This activity was determined using the method employed by Brand-Williams et al.[17] and modified as described below. Methanolic solutions (1.2 mL) of varying sample concentration (2, 4, 6, 8, 10, 20, 30, 50, 100, and 200 μg/mL) were mixed in a 1-cm path length disposable plastic cuvette with methanolic solutions (2.4 mL) of DPPH$^•$ (4.7×10^{-5} M) with an absorbance at 515 nm of 0.80 ± 0.03 AU. The exact DPPH$^•$ concentration was calculated from a calibration curve. Triplicate samples were shaken and allowed to stand for 15 min in the dark at room temperature, and the decrease of absorbance at 515 nm was measured using a Perkin-Elmer UV/vis spectrophotometer Lambda 19 (Perkin-Elmer Instruments, Norwalk, CT).

Platelet Aggregation. Aggregation of washed platelets was monitored in a Chronolog (Havertown, PA) aggregometer at 37 °C under stirring at 1200 rpm.[18] Measurement of intracellular free calcium concentration ($[Ca^{2+}]_i$). Human platelets were loaded with fura-2 by incubation with 2 μM fura-2/AM for 45 min at 37 °C. Fluorescence was recorded from 2 mL aliquots of magnetically stirred cellular suspension (2×10^8 cells/mL) at 37 °C using a Cary Eclipse spectrophotometer (Varian Ltd, Madrid, Spain) with excitation wavelengths of 340 and 380 nm and emission at 505 nm. Changes in $[Ca^{2+}]_i$ were monitored using the fura-2 340/380 fluorescence ratio and calibrated as described previously.[19]

Measurement of Microtubule Content. The microtubule content of resting and activated platelets was determined modifying a previously published procedure.[19] Briefly, washed platelets (2×10^7 cells/mL) were activated in HBS. Samples of platelet suspensions (200 μL) were transferred to 200 μL of ice-cold 3% (w/v) formaldehyde in phosphate-buffered saline (PBS) for 10 min. Fixed platelets were permeabilized by incubation for 10 min with 0.025% (v/v) Nonidet P40 detergent dissolved in PBS. Platelets were then incubated for 30 min with Oregon Green 488 paclitaxel (1 μM) in PBS supplemented with 0.5% (w/v) BSA. Platelets were then collected by centrifugation in a Galaxy 7D centrifuge (VWR International, Fontenay sous Bois, France) for 60 s at 3000*g* and resuspended in PBS. Staining of 2×10^7 cells/mL was measured using a fluorescence spectrophotometer (Shimadzu, Japan). Samples were excited at 488 nm and emission was at 522 nm.

Protein Tyrosine Phosphorylation. Protein tyrosine phosphorylation was detected by gel electrophoresis and Western blotting.[20] Platelet stimulation (2×10^8 cells/mL) was terminated by mixing with an equal volume of 2 × Laemmli's buffer with 10% dithiothreitol followed by heating for 5 min at 95 °C. One-dimensional SDS-electrophoresis was performed with 10% polyacrylamide minigels (50 μg total protein loaded/sample), and separated proteins were electrophoretically transferred, for 2 h at 0.8 mA/cm^2, in a semidry blotter (Hoefer Scientific, Newcastle, Staffs., U.K.) onto nitrocellulose for subsequent probing. Blots were incubated overnight with 10% (w/v) BSA in Tris-buffered saline with 0.1% Tween 20 (TBST) to block residual protein binding sites.

Immunodetection of tyrosine phosphorylation was achieved using the anti-phosphotyrosine antibody 4G10 diluted 1:1500 in TBST for 1 h. The primary antibody was removed, and blots washed six times for 5 min each with TBST. To detect the primary antibody, blots were incubated with horseradish peroxidase-conjugated ovine anti-mouse IgG antibody diluted 1:10000 in TBST, washed six times in TBST, and exposed to enhanced chemiluminescence reagents for 5 min. Blots were then exposed to photographic films, and the integrated optical density of the blots was estimated using scanning densitometry.

Btk and pp60^{src} Activation. Autophosphorylation of Btk at Y-223 and pp60^{src} at Y-416, and thus activation was detected by gel electrophoresis and Western blotting as described above. Phosphorylation of Btk at Y-223 was detected with the anti phospho-Btk (Y-223) antibody diluted 1:1000 in TBST for 2 h.[15] Immunodetection of p60^{src} phosphorylated at Y-416 was achieved using the anti-phospho-c-Src (Y-416) antibody, diluted 1:1000, according to manufacturer's instruction, in TBST for 2 h.[21] The primary antibody was removed, and blots washed six times for 5 min each with TBST. To detect the primary antibody, blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody diluted 1:10 000 in TBST, washed six times in TBST, and exposed to enhanced chemiluminescence reagents for 5 min. Blots were exposed to photographic films, and the optical density was estimated using scanning densitometry. For protein loading control, membranes were stripped of bound antibodies by incubating in stripping buffer (0.1 M glycine, pH 2.2, 1% Tween 20, 0.1% SDS) at room temperature with agitation and reprobed with the anti-Btk or anti-pp60^{src} antibodies, as appropriate.

Statistical Analysis. Analysis of statistical significance was performed using Student's *t* test. $p < 0.05$ was considered to be significant for a difference.

Results

Identification of Cinnamtannin B-1. The ESIMS of the compound isolated from *L. nobilis* wood (Figure 1A) gave a $[M - H]^-$ peak at *m/z* 863, which provided initial evidence that it was a doubly linked procyanidin trimer of the A-type. This was also suggested from the ^{13}C NMR spectrum, since it showed 45 signals, 9 of them with chemical shifts at 29.79, 38.23, 28.82, 67.48, 72.52, 67.14, 80.24, 78.81, and 99.91, being diagnostic of the respective C-4, C-3, and C-2 of the pyran ring. The quaternary C-2 chemical shift was downfield positioned at δ 99.91 due to its A-type interflavanoid bonding. The small vicinal coupling constants of H-2 and H-3 (rings F and I), according to a *cis* relative substitution at C-2 and C-3, indicated that middle and bottom units were epicatechin-like moieties. The connectivities between the hydrogens within each heterocyclic ring were further corroborated by the TOCSY experiment. The specific position of the linkage between top, middle, and bottom units was established by examination of the long-range proton-carbon couplings (HMBC). The stereochemical assignment was accomplished by examination of the space proton−proton couplings (NOESY), and it was supported by the use of a Dreiding-type molecular model. The configuration of the interflavonoid bond at C-4 (middle unit) was denoted as β according to the nomenclature of oligoflavonoids with an A-type unit.[22] This compound was therefore identified as cinnamtannin B-1 [epicatechin-(4β→8,2β→*O*→7)-epicatechin-(4β→8)-epicatechin] by its spectroscopic data, which are in agreement with those reported in the literature.[5,23]

Antiradical Measurements. The radical scavenging activity of the tested samples was expressed as the molar amount of antioxidant necessary to decrease the initial DPPH$^\bullet$ concentration by 50% (efficient concentration = EC_{50} (moles of antioxidant/moles of DPPH$^\bullet$)) and as the antiradical power (ARP = $1/EC_{50}$), for reasons of clarity. In Table 1, cinnamtannin B-1 and reference compounds are presented in increasing order of its EC_{50} values. The results of this comparative study of the antioxidative potential showed that cinnamtannin B-1 is the most potent compound, with higher antiradical power than the natural antioxidants used as standards. It is worth noting that BHT, a commercial synthetic antioxidant, appears with the lowest antiradical power. However, it could be clearly explained since BHT is a hindered phenol and its reaction with the very stable radical DPPH has shown slow reaction kinetics.[17]

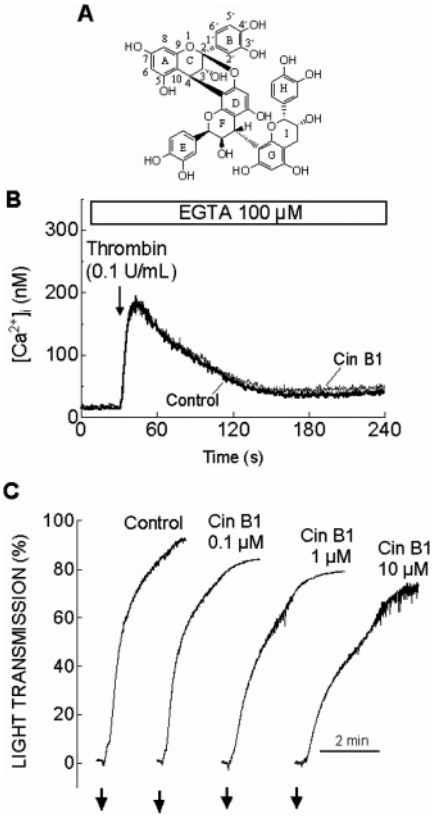

Figure 1. Effect of cinnamtannin B-1 on thrombin-induced platelet aggregation and calcium mobilization. A, The isolated compound from *Laurus nobilis* L. (cinnamtannin B-1). B, Fura-2-loaded human platelets were stimulated with 0.1 U/mL thrombin in a Ca^{2+}-free medium (100 μM EGTA was added). Elevations in $[Ca^{2+}]_i$ were monitored using the 340/380 nm ratio, and traces were calibrated in terms of $[Ca^{2+}]_i$. Traces shown are representative of four separate experiments. C, Human platelets were suspended in HBS containing 1 mM Ca^{2+} and then were treated for 30 min with increasing concentrations of cinnamtannin B-1 (0.1−10) or the vehicle as control. Cells were then stimulated with 0.1 U/mL thrombin. Aggregation of human platelets was induced at a shear rate of 1200 rpm at 37 °C as described in Material and Methods. Traces shown are representative of six separate experiments.

Table 1. Efficient Concentration (EC_{50}) and Antiradical Power (ARP) Values of Cinnamtannin B-1 and Reference Antioxidants against DPPH Radical

compound	EC_{50}	ARP
cinnamtannin B-1	0.08	12.50
rosmarinic acid	0.19	5.26
Trolox	0.30	3.33
vitamin E	0.30	3.33
hydroxytyrosol	0.38	2.63
vitamin C	0.42	2.38
BHT	5.18	0.19

[a] EC_{50} expressed as moles of compound/mol of DPPH$^\bullet$. ARP = $1/EC_{50}$.

Effect of Cinnamtannin B-1 on Thrombin-Evoked Platelet Aggregation and Calcium Mobilization. In the absence of extracellular Ca^{2+}, 100 μM EGTA was added. Treatment of human platelets with 0.1 U/mL thrombin induced a transient

Table 2. Effect of Cinnamtannin B-1 on Thrombin- or ADP-Induced Platelet Aggregation[a]

cinnamtannin B-1, μM	stimulus	lag-time (s)	% rate	% aggregation
-	Thr 0.1 U/mL	8.4 ± 0.3	54.3 ± 3.6	90.0 ± 2.7
0.1	Thr 0.1 U/mL	9.2 ± 0.3*	49.0 ± 0.8*	81.9 ± 3.7*
1	Thr 0.1 U/mL	9.8 ± 0.7*	43.7 ± 2.3*	80.6 ± 2.1*
10	Thr 0.1 U/mL	10.6 ± 0.7*	42.1 ± 3.2*	71.8 ± 3.5*
-	ADP 10 μM	9.0 ± 0.2	60.5 ± 5.3	40.2 ± 3.0
10	ADP 10 μM	15.6 ± 0.3*	9.0 ± 1.7*	9.5 ± 1.8*

[a] Human platelets were suspended in a HBS containing 1 mM Ca^{2+} and then were incubated in the absence or presence of various concentrations of cinnamtannin B-1 (0.1−10 μM) for 30 min, as indicated. Cells were then stimulated at 37 °C with 0.1 U/mL thrombin or 10 μM ADP, and platelet aggregation was determined as described under Materials and Methods. Values given are presented as mean ± SEM of six separate determinations. *$p < 0.05$ compared to thrombin- or ADP-induced response in the absence of cinnamtannin B-1.

Table 3. Effect of Cinnamtannin B-1, Catalase, and Colchicine on Thrombin-Induced Platelet Aggregation[a]

agent	stimulus	lag-time (s)	% rate	% aggregation
-	Thr 0.1 U/mL	8.5 ± 1.1	55.8 ± 12.6	93.0 ± 3.9
Cin B1	Thr 0.1 U/mL	10.5 ± 0.3*	45.8 ± 5.6	75.3 ± 4.1*
catalase	Thr 0.1 U/mL	7.5 ± 1.5	43.6 ± 11.1	84.1 ± 4.4*†
colchicine	Thr 0.1 U/mL	9.3 ± 1.3	59.0 ± 4.7	71.2 ± 3.6*
Cin B1 + catalase	Thr 0.1 U/mL	9.1 ± 1.2	48.1 ± 6.9	77.0 ± 3.8*
Cin B1 + colchicine	Thr 0.1 U/mL	15.0 ± 2.5*	40.3 ± 5.4	74.6 ± 4.9*

[a] Human platelets were suspended in a HBS containing 1 mM Ca^{2+} and then were incubated in the absence or presence of 10 μM cinnamtannin B-1 (Cin B1) for 30 min, 300 U/mL catalase for 10 min, 100 μM colchicine for 2 h, or a combination of either Cin B1 + catalase or Cin B1 + colchicine, as indicated. Cells were then stimulated at 37 °C with 0.1 U/mL thrombin, and platelet aggregation was determined as described under Materials and Methods. Values given are presented as mean ± SEM of six separate determinations. *$p < 0.05$ compared to thrombin-induced response in the absence of agents. †$p < 0.05$ compared to thrombin-induced response in the presence of cinnamtannin B-1.

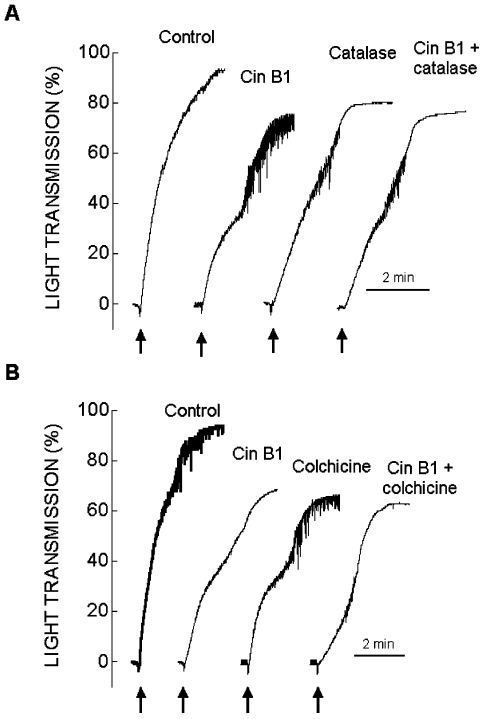

Figure 2. Effect of cinnamtannin B-1 and colchicine on thrombin-induced platelet aggregation. A, Human platelets were suspended in HBS containing 1 mM Ca^{2+} and then were treated for 30 min with 10 μM cinnamtannin B-1 (Cin B1), for 10 min with 300 U/mL catalase, both, or the vehicle as control. Cells were then stimulated with 0.1 U/mL thrombin. B, Human platelets were suspended in HBS containing 1 mM Ca^{2+} and then were treated for 30 min with 10 μM cinnamtannin B-1 (Cin B1), for 2 h with 100 μM colchicine, both, or the vehicles as control. Cells were then stimulated with 0.1 U/mL thrombin. Aggregation of human platelets was induced at a shear rate of 1200 rpm at 37 °C as described in Materials and Methods. Traces shown are representative of six separate experiments.

increase in $[Ca^{2+}]_i$ due to release of Ca^{2+} from the intracellular Ca^{2+} stores (Figure 1B). Platelet treatment for 30 min at 37 °C with 10 μM cinnamtannin B-1 (a stock solution of 10 mM was initially prepared in DMSO, and the appropriate volume was added to the platelet suspension in HBS to achieve the desired final concentration) did not modify Ca^{2+} release induced by 0.1 U/mL thrombin (the initial peak $[Ca^{2+}]_i$ elevation above basal after agonist was 192 ± 14 nM in control and 194 ± 9 nM in cinnamtanin B-1-treated cells (Figure 1B; $n = 4$)). This finding suggests that activation of thrombin receptors is not impaired by cinnamtannin B-1. However, treatment with cinnamtannin B-1 significantly reduced the percentage and rate of thrombin-induced platelet aggregation and increased the lag-time in a concentration-dependent manner (Figure 1C and Table 2; $p < 0.05$; $n = 6$). In addition, preincubation with 10 μM cinnamtannin B-1 reduced the ability of ADP to induce platelet aggregation (Table 2). Interestingly, treatment of human platelets with 10 μM cinnamtannin B-1 in combination with 300 U/mL catalase, which prevents thrombin-evoked endogenous H_2O_2 generation,[24] and reduced platelet aggregation by 10% (Table 3), did not inhibit platelet aggregation further than cinnamtannin B-1 alone (Figure 2A; Table 3).

Effect of Cinnamtannin B-1 on Microtubular Reorganization and Its Role in Platelet Aggregation. Thrombin is a physiological agonist that induces reorganization of the platelet cytoskeleton, including the microtubular network, which has been shown to support the discoid platelet shape and cellular changes during aggregation.[8,9] To further explore the effect of cinnamtannin B-1 on platelet aggregation, we tested its effect on thrombin-induced microtubular reorganization. Microtubule reorganization was analyzed by using Oregon Green 488 paclitaxel, which selectively binds to microtubules and has been successfully used to label microtubules of live cells.[25] To avoid any interference due to paclitaxel, which promotes tubulin assembly by itself, cells, once treated, were fixed and then incubated with Oregon Green 488 paclitaxel as described in Material and Methods. Platelet stimulation with thrombin (0.1 U/mL) for 1 min increases the microtubular content by 160 ± 10% (mean ± SEM). Pretreatment of platelets for 30 min at 37 °C with 10 μM cinnamtannin B-1 significantly reduced thrombin-induced increase in the microtubular content by 15 ± 4%, without having any effect on the content of microtubules of platelets at rest ($p < 0.05$; $n = 6$).

Colchicine is a microtubular disrupter that significantly reduces the microtubular content in resting platelets by 50% and abolishes thrombin-stimulated microtubular reorganization (data not shown). Treatment of human platelets for 2 h with 100 μM colchicine significantly reduce the amplitude of aggregation induced by thrombin (Figure 2B and Table 3; $p < 0.05$; $n = 6$). Similar results were obtained when platelets were preincubated with colchicine in combination with cinnamtannin B-1 (Figure 2B and Table 3; $p < 0.05$; $n = 6$), which suggests that inhibition of microtubular reorganization is involved in cinnamtannin-induced reduction of aggregation.

Cinnamtannin B-1 Inhibits Thrombin-Evoked Activation of Btk and pp60^{src}. Oxygen radicals have been reported to induce protein tyrosine phosphorylation,[26] which, in turn, plays an important role in platelet aggregation.[10−12] As previously

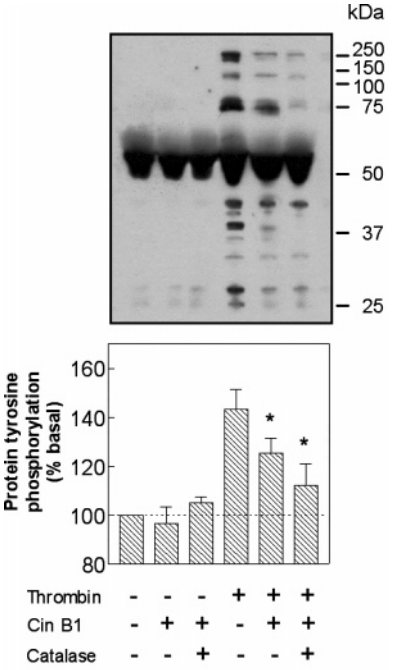

Figure 3. Effect of cinnamtannin B-1 and catalase in thrombin-induced protein tyrosine phosphorylation in human platelets. Human platelets were suspended in HBS containing 1 mM Ca^{2+} and then incubated at 37 °C for 30 min either in the presence of 10 μM cinnamtannin B-1 alone or combined with incubation for 10 min with 300 U/mL catalase or with the vehicles (control), as indicated. Cells were then stimulated with 0.1 U/mL thrombin. Samples were taken 10 s before and 60 s after stimulation with the agonist. Proteins were analyzed by SDS/PAGE and subsequent Western blotting with a specific anti-phosphotyrosine (4G10) antibody as described in Materials and Methods. Molecular size is indicated on the right. Top panel shows results from a representative experiment of three others. Histograms represent protein tyrosine phosphorylation and are presented as percentage of control (untreated resting cells) and expressed as mean ± SEM of four separate experiments. *$p < 0.05$ compared with protein tyrosine phosphorylation in thrombin-stimulated cells not treated with cinnamtannin B-1 or catalase.

reported,[27,28] treatment of platelets with thrombin 0.1 U/mL for 1 min increases the phosphotyrosine content by 143 ± 8% (Figure 3). Preincubation for 30 min with 10 μM cinnamtannin B-1 significantly reduced thrombin-induced tyrosine phosphorylation (Figure 3; $p < 0.05$; $n = 6$). In combination with catalase, cinnamtannin B-1 appeared more effective at inhibiting thrombin-evoked tyrosine phosphorylation, although the effect was not statistically different from that observed with cinnamtannin B-1 alone (Figure 3; $p > 0.05$; $n = 6$).

A number of studies have identified Btk as a cytosolic tyrosine kinase protein activated by oxidative stress, and particularly by H_2O_2 in different cell types, including platelets.[15,29] We have found that preincubation for 10 min at 37 °C with 10 μM LFM-A13, a Btk inhibitor,[30] reduced thrombin-evoked platelet aggregation (Figure 4 and Table 4; $p < 0.05$; $n = 6$), suggesting that Btk activation is important for platelet function. Hence, we have investigated whether cinnamtannin B-1 reduces thrombin-evoked Btk activation. The activation of Btk was analyzed by Western blotting using a monoclonal phosphospecific anti-Btk antibody that only detects Btk autophosphorylated at the tyrosine residue 223, which has been shown to be the full activated form of Btk.[31] Treatment of platelets with 0.1 U/mL thrombin for 30 s increases Btk activity by 134 ± 6% of basal (Figure 5, top panel and histograms). Pretreatment of platelets for 30 min at 37 °C with 10 μM

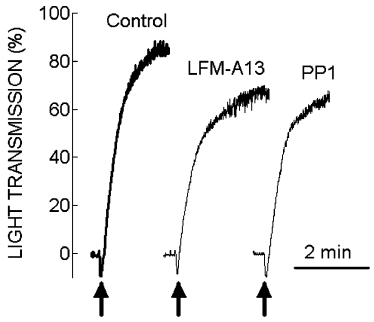

Figure 4. Role of Btk and $pp60^{src}$ on thrombin-induced platelet aggregation. Human platelets were suspended in HBS containing 1 mM Ca^{2+} and then were treated for 10 min with 10 μM PP1, 10 μM LFM-A13, or the vehicle (DMSO) as control. Cells were then stimulated with 0.1 U/mL thrombin. Aggregation of human platelets was induced at a shear rate of 1200 rpm at 37 °C as described in Materials and Methods. Traces shown are representative of six separate experiments.

Table 4. Role of the Tyrosine Kinases $pp60^{src}$ and Btk on Thrombin-Induced Platelet Aggregation[a]

agent	stimulus	lag-time (s)	% rate	% aggregation
-	Thr 0.1 U/mL	16.6 ± 1.3	68.6 ± 1.3	83.6 ± 1.7
LFM-A13	Thr 0.1 U/mL	24.3 ± 2.5*	66.3 ± 5.1	68.7 ± 1.6*
PP1	Thr 0.1 U/mL	26.5 ± 1.3*	59.7 ± 5.3*	67.1 ± 2.1*

[a] Human platelets were suspended in a HBS containing 1 mM Ca^{2+} and then were incubated for 10 min in the absence or presence of 10 μM LFM-A13 or PP1. Cells were then stimulated at 37 °C with 0.1 U/mL thrombin, and platelet aggregation was determined as described under Materials and Methods. Values given are presented as mean ± SEM of four separate determinations. *$p < 0.05$ compared to thrombin-induced response in the absence of inhibitors.

cinnamtannin B-1 significantly reduced thrombin-induced Btk activation by 50% (Figure 5; $p < 0.05$; $n = 6$). In addition, treatment with cinnamtannin B-1 in combination with catalase reduces thrombin-evoked Btk activation by 90% (Figure 5; $p < 0.05$; $n = 6$), which is consistent with the role of ROS in Btk activation.[15] Reprobing of the membranes with anti-Btk antibody revealed that a similar amount of proteins were loaded in all lanes (Figure 5, lower panel).

We have previously reported that Btk might be an upstream regulator of $pp60^{src}$, the most abundant platelet tyrosine kinase[32] that translocates to the cytoskeleton during platelet activation.[33,34] Preincubation of platelets for 10 min with 10 μM PP1, a $pp60^{src}$ inhibitor,[35] significantly reduced thrombin-stimulated platelet aggregation (Figure 4 and Table 4; $p < 0.05$; $n = 6$), which, consistent with its cytoskeletal association, suggests that $pp60^{src}$ play a role in platelet aggregation. Treatment of human platelets with 0.1 U/mL thrombin for 1 min increases $pp60^{src}$ activity by 169 ± 16% (Figure 6; top panel and histograms). Pretreatment for 30 min at 37 °C with 10 μM cinnamtannin B-1 abolished thrombin-induced response, and similar results were found after incubation with cinnamtannin B-1 in combination with catalase (Figure 6; $p < 0.05$; $n = 6$). Reprobing of the membranes with anti-$pp60^{src}$ monoclonal antibody GD11 revealed that a comparable amount of proteins were loaded in all lanes (Figure 6; lower panel).

Discussion

Plant flavonoids are a large group of naturally occurring polyphenolic compounds that show the ability to modify or modulate the activity of a variety of enzyme systems critically involved in intracellular signal transduction, including receptor signaling, immune function, cellular transformation, or tumor growth and metastasis.[36−40] These inhibitory effects of flavonoids on tumoral cells proliferation have been shown to be a

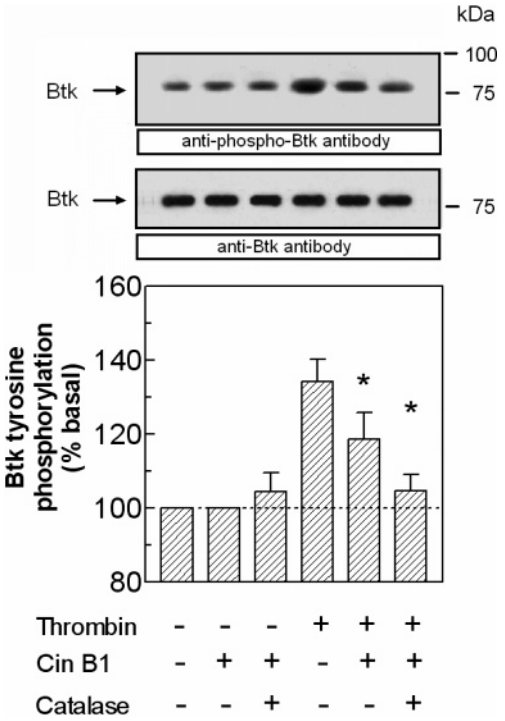

Figure 5. Effect of cinnamtannin B-1 and catalase in thrombin-induced Btk activation in human platelets. Human platelets were suspended in HBS containing 1 mM Ca^{2+} and then incubated at 37 °C for 30 min either in the presence of 10 μM cinnamtannin B-1 alone or combined with incubation for 10 min with 300 U/mL catalase or with the vehicles (control), as indicated. Cells were then stimulated with 0.1 U/mL thrombin. Samples were taken 10 s before and 30 s after stimulation with the agonist. Proteins were analyzed by SDS/PAGE and subsequent Western blotting with a specific anti-phospho-Btk (Y223) antibody or the anti-Btk antibody as described in Materials and Methods. Molecular size is indicated on the right. Top panels show results from a representative experiment of three others. Histograms represent Btk tyrosine phosphorylation at Y223 and are presented as percentage of control (untreated resting cells) and expressed as mean ± SEM of four separate experiments. *p < 0.05 compared with Btk tyrosine phosphorylation in thrombin-stimulated cells not treated with cinnamtannin B-1 or catalase.

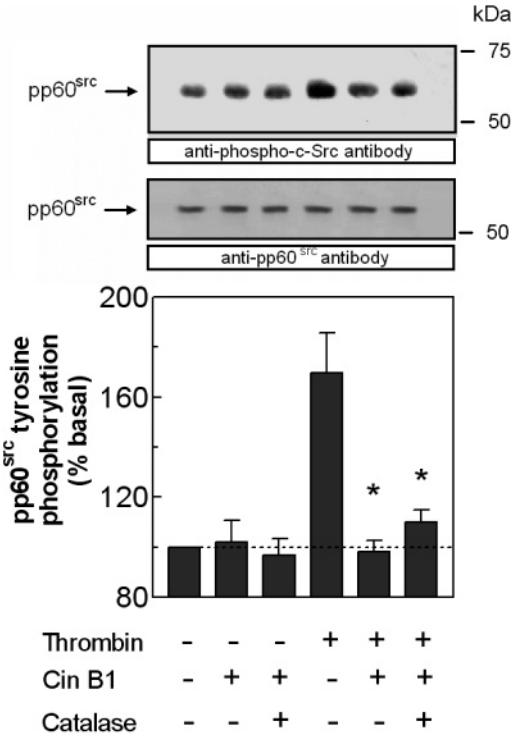

Figure 6. Effect of cinnamtannin B-1 and catalase in thrombin-induced pp60^{src} activation in human platelets. Human platelets were suspended in HBS containing 1 mM Ca^{2+} and then incubated at 37 °C for 30 min either in the presence of 10 μM cinnamtannin B-1 alone or combined with incubation for 10 min with 300 U/mL catalase or with the vehicles (control), as indicated. Cells were then stimulated with 0.1 U/mL thrombin. Samples were taken 10 s before and 60 s after stimulation with the agonist. Proteins were analyzed by SDS/PAGE and subsequent Western blotting with a specific anti-phospho-c-Src (Y-416) antibody or the anti-pp60^{src} antibody as described in Materials and Methods. Molecular size is indicated on the right. Top panels show results from a representative experiment of three others. Histograms represent pp60^{src} tyrosine phosphorylation at Y416 and are presented as percentage of control (untreated resting cells) and expressed as mean ± SEM of four separate experiments. *p < 0.05 compared with pp60^{src} tyrosine phosphorylation in thrombin-stimulated cells not treated with cinnamtannin B-1 or catalase.

consequence of the interference of flavonoids with protein kinases involved in the regulation of cellular processes;[41,42] however, the mechanisms of those effects in normal (nontumoral) cells remain unclear. They may be partially mediated by antioxidant effects of the extracts. The mechanisms of actions of polyphenols on platelets are mediated primarily through their free radical scavenging effects but they have also been shown to inhibit NADPH oxidases and the upstream regulator PKC.[43] In addition, polyphenols have also been suggested to increase the endogenous antioxidant capacity through the enhancement of the activity of superoxide dismutase, which is important for vascular superoxide production.[44]

We have identified the A-type proanthocyanidin isolated from a bay wood extract as cinnamtannin B-1 [epicatechin-(4β→8, 2β→O→7)-epicatechin-(4β→8)-epicatechin] by its spectroscopic data, which are in agreement with those reported in the literature.[5,23] The chemical assay based on the ability to scavenge a model free radical, such as DPPH$^{\bullet}$, has been chosen since it is generally accepted that the oxidative damage of cells and tissues could be initiated by a free radical attack. For screening and comparative purposes, the DPPH radical scavenging activities of cinnamtannin B-1 and other six known antioxidant compounds (rosmarinic acid, Trolox, vitamin E, hydroxytyrosol, vitamin C, and BHT) have been evaluated. The efficient concentration (EC_{50}) calculated for cinnamtannin B-1 is really low, its value being the half of that of rosmarinic acid, approximately. It means that cinnamtannin B-1 is twice more potent as a DPPH radical scavenger than rosmarinic acid, and four or five times more potent than effective antioxidants such as Trolox, vitamin E, hydroxytyrosol, or vitamin C.

Our results indicate that cinnamtannin B-1 is an effective inhibitor of thrombin (and also ADP)-evoked platelet aggregation, which is likely to be mediated by oxygen radical scavenging, including H_2O_2, since treatment with cinnamtannin B-1 in combination with catalase did not increase the ability of the proanthocyanidin to inhibit aggregation.

Previous studies by us and others have found that thrombin induces Btk activation in human platelets.[15,45] Our results indicate that cinnamtannin B-1 inhibits thrombin-induced activation of Btk and pp60^{src}. Btk and pp60^{src} activities were tested after 30 s and 1 min of thrombin stimulation, respectively, because of the delayed activation of pp60^{src} compared to that of Btk, which, in addition to the effect of Btk inhibitors on pp60^{src} activation, led us to suggest that Btk might be an upstream modulator of pp60^{src}.[15,46] Btk has been shown to be activated in response to oxygen radicals,[15] which supports the inhibitory effect of cinnamtannin B-1 on Btk activation. The

latter might provide an explanation to the antiaggregant effects of cinnamtannin B-1 since the Btk inhibitor LFM-A13 has been reported to impair platelet aggregation.[47] We have recently observed that inhibition of Btk and Src alters thrombin-induced microtubular remodeling in human platelets, preventing tubulin polymerization while maintaining intact the ability of thrombin to induce rapid depolymerization.[46] In agreement with this, we have found that cinnamtannin B-1-induced inhibition of thrombin-evoked platelet aggregation was not enhanced by simultaneous preincubation with colchicine, suggesting that the inhibition of tubulin remodeling by cinnamtannin B-1 is involved in its inhibitory effect on platelet aggregation. Btk has been shown to participate in intracellular signaling associated to the cytoskeleton in platelets. Thrombin induces translocation of Btk to the actin microfilaments,[48] which might be involved in $\alpha_{IIb}\beta_3$-dependent outside-in signals that promote actin rearrangements and cell spreading.[49] In addition, Btk is involved in cofilin activation and rapid reorganization of the actin cytoskeleton during the activation of store-operated Ca^{2+} entry.[50]

The effects of cinnamtannin B-1 on thrombin-induced platelet aggregation and activation of protein tyrosine kinases are not likely mediated by impairment of thrombin receptors activation, since cinnamtannin B-1 was unable to alter thrombin-stimulated Ca^{2+} mobilization from the intracellular Ca^{2+} stores described in human platelets, the dense tubular system, and the acidic stores.[51]

The antiaggregant effect of cinnamtannin B-1 is consistent with other studies reporting that polyphenolic compounds, including resveratrol, *p*-coumaric acid, and others, might regulate platelet function.[52−54] Previous studies have reported that the polyphenols quercetin and catechin reduce platelet recruitment and activation via inhibition of PKC-dependent NADPH oxidase activation, which provide a mechanism through which polyphenols reduce cardiovascular disease.[55] Our previous studies have reported that Btk activation in platelets is mediated by PKC and oxygen radicals,[15] which suggests that Btk might be involved in the antiaggregant activity of these polyphenols compounds.

In conclusion, the results of this study revealed the value of cinnamtannin B-1, a non commercial compound at present. It could therefore serve as a promising biochemical and pharmacological tool in the study of oxidative stress processes, with significant contributions to the health benefits. Our results indicate that cinnamtannin B-1 is a powerful free radical scavenger, with a greater antioxidant activity than other natural agents, which shows antiaggregant effects in platelets. The inhibitory effect of cinnamtannin B-1 on platelet aggregation is likely mediated by reduction of microtubular remodeling and tyrosine phosphorylation, which involves inhibition of thrombin-induced activation of Btk and $pp60^{src}$. These findings suggest that cinnamtannin B-1 might be used to protect against clinical cardiovascular events, mainly by attenuating thrombogenic responses. This antithrombogenic effect may include normalization of the abnormally high thrombogenic responsiveness in diabetics, as well as in hypertensive patients, as an alternative to the recently described effects of losartan and simvastatin.[56] The development of appropriate therapeutic strategies deserves further studies.

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References

(1) Ariga, T. The antioxidative function, preventive action on disease and utilization of proanthocyanidins. *Biofactors* **2004**, *21*, 197−201.
(2) Yamakoshi, J.; Kataoka, S.; Koga, T.; Ariga, T. Proanthocyanidin-rich extract from grape seeds attenuates the development of aortic atherosclerosis in cholesterol-fed rabbits. *Atherosclerosis* **1999**, *142*, 139−149.
(3) Bagchi, D.; Sen, C. K.; Ray, S. D.; Das, D. K.; Bagchi, M.; Preuss, H. G.; Vinson, J. A. Molecular mechanisms of cardioprotection by a novel grape seed proanthocyanidin extract. *Mutat. Res.* **2003**, *523*, 87−97.
(4) Romera, C.; Salido, S.; Linares-Palomino, P.; Nogueras, M.; Sánchez, A.; Altarejos, J. Radical scavenging constituents from *Laurus nobilis* wood. In *Abstract Book*, IVth Spanish−Portuguese−Japanese Organic Chemistry Symposium, Santiago de Compostela, Spain, September 8−11, 2006; University of Santiago de Compostela: Santiago de Compostela, Spain, 2006; p 140.
(5) Jayaprakasha, G. K.; Ohnishi-Kameyama, M.; Ono, H.; Yoshida, M.; Jaganmohan, RL. Phenolic constituents in the fruits of Cinnamomum zeylanicum and their antioxidant activity. *J. Agric. Food Chem.* **2006**, *54*, 1672−1679.
(6) Bouaziz, A.; Salido, S.; Linares-Palomino, P. J.; Sanchez, A.; Altarejos, J.; Bartegi, A.; Salido, G. M.; Rosado, J. A. Cinnamtannin B-1 from bay wood reduces abnormal intracellular Ca^{2+} homeostasis and platelet hyperaggregability in type 2 diabetes mellitus patients. *Arch. Biochem. Biophys.* **2007**, *457*, 235−242.
(7) Bouaziz, A.; Romera-Castillo, C.; Salido, S.; Linares-Palomino, P. J.; Altarejos, J.; Bartegi, A.; Rosado, J. A.; Salido, G. M. Cinnamtannin B-1 from bay wood exhibits antiapoptotic effects in human platelets. *Apoptosis* **2007**, *12*, 489−498.
(8) White, J. G.; Krumwiede, M.; Burris, S. M.; Heagan, B. Isolation of microtubule coils from platelets after exposure to aggregating agents. *Am. J. Pathol.* **1986**, *125*, 319−326.
(9) Canizares, C.; Vivar, N.; Herdoiza, M. Role of the microtubular system in platelet aggregation. *Braz. J. Med. Biol. Res.* **1994**, *27*, 1533−1551.
(10) Salari, H.; Duronio, V.; Howard, S. L.; Demos, M.; Jones, K.; Reany, A.; Hudson, A. T.; Pelech, S. L. Erbstatin blocks platelet activating factor-induced protein-tyrosine phosphorylation, polyphosphoinositide hydrolysis, protein kinase C activation, serotonin secretion and aggregation of rabbit platelets. *FEBS Lett.* **1990**, *263*, 104−108.
(11) Asahi, M.; Yanagi, S.; Ohta, S.; Inazu, T.; Sakai, K.; Takeuchi, F.; Taniguchi, T.; Yamamura, H. Thrombin-induced human platelet aggregation is inhibited by protein-tyrosine kinase inhibitors, ST638 and genistein. *FEBS Lett.* **1992**, *31*, 10−14.
(12) Tibbles, H. E.; Vassilev, A.; Wendorf, H.; Schonhoff, D.; Zhu, D.; Lorenz, D.; Waurzyniak, B.; Liu, X. P.; Uckun, F. M. Role of a JAK3-dependent biochemical signaling pathway in platelet activation and aggregation. *J. Biol. Chem.* **2001**, *276*, 17815−17822.
(13) Pani, G.; Colavitti, R.; Borrello, S.; Galeotti, T. Endogenous oxygen radicals modulate protein tyrosine phosphorylation and JNK-1 activation in lectin-stimulated thymocytes. *Biochem. J.* **2000**, *347*, 173−181.
(14) Meurer, S.; Pioch, S.; Gross, S.; Muller-Esterl, W. Reactive oxygen species induce tyrosine phosphorylation of and Src kinase recruitment to NO-sensitive guanylyl cyclase. *J. Biol. Chem.* **2005**, *280*, 33149−33156.
(15) Redondo, P. C.; Ben-Amor, N.; Salido, G. M.; Bartegi, A.; Pariente, J. A.; Rosado, J. A. Ca^{2+}-independent activation of Bruton's tyrosine kinase is required for store-mediated Ca^{2+} entry in human platelets. *Cell. Signal.* **2005**, *17*, 1011−1021.
(16) Landino, L. M.; Robinson, S. H.; Skreslet, T. E.; Cabral, D. M. Redox modulation of tau and microtubule-associated protein-2 by the glutathione/glutaredoxin reductase system. *Biochem. Biophys. Res. Commun.* **2004**, *323*, 112−117.
(17) Brand-Williams, W.; Cuvelier, M. E.; Berset, C. Use of a free radical method to evaluate antioxidant activity. *Lebensm.-Wiss. Technol.* **1995**, *28*, 25−30.
(18) Redondo, P. C.; Jardin, I.; Hernandez-Cruz, J. M.; Pariente, J. A.; Salido, G. M.; Rosado, J. A. Hydrogen peroxide and peroxynitrite enhance Ca^{2+} mobilization and aggregation in platelets from type 2 diabetic patients. *Biochem. Biophys. Res. Commun.* **2005**, *333*, 794−802.
(19) Rosado, J. A.; Sage, S. O. Farnesylcysteine analogues inhibit store-regulated Ca^{2+} entry in human platelets: evidence for involvement of small GTP-binding proteins and actin cytoskeleton. *Biochem. J.* **2000**, *347*, 183−192.
(20) Rosado, J. A.; Porras, T.; Conde, M.; Sage, S. O. Cyclic nucleotides modulate store-mediated calcium entry through the activation of protein-tyrosine phosphatases and altered actin polymerization in human platelets. *J. Biol. Chem.* **2001**, *276*, 15666−15675.

(21) Redondo, P. C.; Salido, G. M.; Pariente, J. A.; Rosado, J. A. Dual effect of hydrogen peroxide on store-mediated calcium entry in human platelets. *Biochem. Pharmacol.* **2004**, *67*, 1065−1076.
(22) Kolodziej, H.; Ferreira, D.; Lemière, G.; De Bruyne, T.; Pieters, L.; Vlietinck, A. On the nomenclature of oligoflavonoids with an A-type unit. *J. Nat. Prod.* **1993**, *56*, 1199−1200.
(23) Kamiya, K.; Watanabe, C.; Endang, H.; Umar, M.; Satake, T. Studies on the constituents of bark of *Parameria laevigata* Moldenke. *Chem. Pharm. Bull.* **2001**, *49*, 551−557.
(24) Rosado, J. A.; Redondo, P. C.; Salido, G. M.; Gomez-Arteta, E.; Sage, S. O.; Pariente, J. A. Hydrogen peroxide generation induces $pp60^{src}$ activation in human platelets: evidence for the involvement of this pathway in store-mediated calcium entry. *J. Biol. Chem.* **2004**, *279*, 1665−1675.
(25) Diaz, J. F.; Strobe, R.; Engelborghs, Y.; Souto, A. A.; Andreu, J. M. Molecular recognition of taxol by microtubules. Kinetics and thermodynamics of binding of fluorescent taxol derivatives to an exposed site. *J. Biol. Chem.* **2000**, *275*, 26265−26276.
(26) Vepa, S.; Scribner, W. M.; Natarajan, V. Activation of protein phosphorylation by oxidants in vascular endothelial cells: identification of tyrosine phosphorylation of caveolin. *Free Radic. Biol. Med.* **1997**, *22*, 25−35.
(27) Sargeant, P.; Farndale, R. W.; Sage, S. O. The tyrosine kinase inhibitors methyl 2,5-dihydroxycinnamate and genistein reduce thrombin-evoked tyrosine phosphorylation and Ca^{2+} entry in human platelets. *FEBS Lett.* **1993**, *315*, 242−246.
(28) Redondo, P. C.; Salido, G. M.; Rosado, J. A.; Pariente, J. A. Effect of hydrogen peroxide on Ca^{2+} mobilisation in human platelets through sulphydryl oxidation dependent and independent mechanisms. *Biochem. Pharmacol.* **2004**, *67*, 491−502.
(29) Qin, S.; Stadtman, E. R.; Chock, P. B. Regulation of oxidative stress-induced calcium release by phosphatidylinositol 3-kinase and Bruton's tyrosine kinase in B cells. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 7118−7123.
(30) Mahajan, S.; Ghosh, S.; Sudbeck, E. A.; Zheng, Y.; Downs, S.; Hupke, M.; Uckun, F. M. Rational design and synthesis of a novel anti-leukemic agent targeting Bruton's tyrosine kinase (BTK), LFM-A13 [alpha-cyano-beta-hydroxy-beta-methyl-N-(2,5-dibromophenyl)-propenamide]. *J. Biol. Chem.* **1999**, *274*, 9587−9599.
(31) Rawlings, D. J.; Scharenberg, A. M.; Park, H.; Wahl, M. I.; Lin, S.; Kato, R. M.; Fluckiger, A. C.; Witte, O. N.; Kinet, J. P. Activation of BTK by a phosphorylation mechanism initiated by SRC family kinases. *Science* **1996**, *271*, 822−825.
(32) Modderman, P. W.; von dem Borne, A. E.; Sonnenberg, A. Tyrosine phosphorylation of P-selectin in intact platelets and in a disulphide-linked complex with immunoprecipitated $pp60^{c-src}$. *Biochem. J.* **1994**, *299*, 613−621.
(33) Horvath, A. R.; Muszbek, L.; Kellie, S. Translocation of $pp60^{c-src}$ to the cytoskeleton during platelet aggregation. *EMBO J.* **1992**, *11*, 855−861.
(34) Rosado, J. A.; Meijer, E. M.; Hamulyak, K.; Novakova, I.; Heemskerk, J. W.; Sage, S. O. Fibrinogen binding to the integrin alpha-(IIb)beta(3) modulates store-mediated calcium entry in human platelets. *Blood* **2001**, *97*, 2648−2656.
(35) Liu, Y.; Bishop, A.; Witucki, L.; Kraybill, B.; Shimizu, E.; Tsien, J.; Ubersax, J.; Blethrow, J.; Morgan, D. O.; Shokat, K. M. Structural basis for selective inhibition of Src family kinases by PP1. *Chem. Biol.* **1999**, *6*, 671−678.
(36) Huang, Y. T.; Hwang, J. J.; Lee, P. P.; Ke, F. C.; Huang, J. H.; Huang, C. J.; Kandaswami, C.; Middleton, E., Jr.; Lee, M. T. Effects of luteolin and quercetin, inhibitors of tyrosine kinase, on growth and metastasis-associated properties in A431 cells overexpressing epidermal growth factor receptor. *Br. J. Pharmacol.* **1999**, *128*, 999−1010.
(37) Hertog, M. G. L.; Hollom, P. C. H.; Katan, M. B.; Kromhout, D. Intake of potential anticarcinogenic flavonoids and their determinants in adults in The Netherlands. *Nutr. Cancer* **1995**, *20*, 21−29.
(38) Middleton, E., Jr.; Kandaswami, C.; Theoharidis, T. C. The impact of plant flavonoids on mammalian biology: implications for inflammations. *Pharmacol. Rev.* **2000**, *52*, 673−751.
(39) Sieg, D. J.; Hauck, C. R.; Ilic, D.; Klingbeil, C. K.; Schaefer, E.; Damsky, C. H.; Schlaepfer, D. D. FAK integrates growth-factor and integrin signals to promote cell migration. *Nature Cell Biol.* **2000**, *2*, 249−256.
(40) Kappagoda, C. T.; Karim, M.; McCormic, K.; Kandaswami, C. Unraveling the French paradox. *Chem. Innov.* **2000**, *30*, 26−31.
(41) Sato, F.; Matsukawa, Y.; Matsumoto, K.; Nishino, H.; Sakai, T. Apegenin induces morphological differentiation and G2/M arrest in rat neuronal cells. *Biochem. Biophys. Res. Commun.* **1994**, *204*, 578−584.
(42) Wei, Y. Q.; Zhao, X.; Kariya, Y.; Fukata, H.; Teshigawara, K.; Uchida, A. Induction of apoptosis by quercetin: involvement of heat shock protein. *Cancer Res.* **1994**, *54*, 4952−4957.
(43) Pignatelli, P.; Di Santo, S.; Buchetti, B.; Sanguigni, V.; Brunelli, A.; Violi, F. Polyphenols enhance platelet nitric oxide by inhibiting protein kinase C-dependent NADPH oxidase activation: effect on platelet recruitment. *FASEB J.* **2006**, *20*, 1082−1089.
(44) Guzik, T. J.; Olszanecki, R.; Sadowski, J.; Kapelak, B.; Rudzinski, P.; Jopek, A.; Kawczynska, A.; Ryszawa, N.; Loster, J.; Jawien, J.; Czesnikiewicz-Guzik, M.; Channon, K. M.; Korbut, R. Superoxide dismutase activity and expression in human venous and arterial bypass graft vessels. *J. Physiol. Pharmacol.* **2005**, *56*, 313−323.
(45) Suzuki-Inoue, K.; Wilde, J. I.; Andrews, R. K.; Auger, J. M.; Siraganian, R. P.; Sekiya, F.; Rhee, S. G.; Watson, S. P. Glycoproteins VI and Ib-IX-V stimulate tyrosine phosphorylation of tyrosine kinase Syk and phospholipase Cgamma2 at distinct sites. *Biochem. J.* **2004**, *378*, 1023−1029.
(46) Bouaziz, A.; Ben Amor, N.; Woodard, G. E.; Zibidi, H.; López, J. J.; Bartegi, A.; Salido, G. M.; Rosado, J. A. Tyrosine phosphorylation/dephosphorylation balance is involved in thrombin-evoked microtubular reorganisation in human platelets. *Thromb. Haemost.* **2007**, in press.
(47) Uckun, F. M.; Vassilev, A.; Bartell, S.; Zheng, Y.; Mahajan, S.; Tibbles, H. E. The anti-leukemic Bruton's tyrosine kinase inhibitor alpha-cyano-beta-hydroxy-beta-methyl-N-(2,5-dibromophenyl) propenamide (LFM-A13) prevents fatal thromboembolism. *Leuk. Lymphoma* **2003**, *44*, 1569−1577.
(48) Mukhopadhyay, S.; Ramars, A. S.; Dash, D. Bruton's tyrosine kinase associates with the actin-based cytoskeleton in activated platelets. *J. Cell Biochem.* **2001**, *81*, 659−665.
(49) Soriani, A.; Moran, B.; de Virgilio, M.; Kawakami, T.; Altman, A.; Lowell, C.; Eto, K.; Shattil, S. J. A role for PKCtheta in outside-in alpha(IIb)beta3 signaling. *J. Thromb. Haemost.* **2006**, *4*, 648−655.
(50) Redondo, P. C.; Harper, M. T.; Rosado, J. A.; Sage, S. O. A role for cofilin in the activation of store-operated calcium entry by de novo conformational coupling in human platelets. *Blood* **2006**, *107*, 973−979.
(51) Jardin, I.; Ben Amor, N.; Bartegi, A.; Pariente, J. A.; Salido, G. M.; Rosado, J. A. Differential involvement of thrombin receptors in Ca^{2+} release from two different intracellular stores in human platelets. *Biochem. J.* **2007**, *401*, 167−174.
(52) Csiszar, A.; Smith, K.; Labinskyy, N.; Orosz, Z.; Rivera, A.; Ungvari, Z. Resveratrol attenuates TNF-alpha-induced activation of coronary arterial endothelial cells: role of NF-kappaB inhibition. *Am. J. Physiol. Heart Circ. Physiol.* **2006**, *291*, H1694−H1699.
(53) Luceri, C.; Giannini, L.; Lodovici, M.; Antonucci, E.; Abbate, R.; Masini, E.; Dolara, P. p-Coumaric acid, a common dietary phenol, inhibits platelet activity in vitro and in vivo. *Br. J. Nutr.* **2007**, *97*, 458−463.
(54) Singh, I.; Mok, M.; Christensen, A. M.; Turner, A. H.; Hawley, J. A. The effects of polyphenols in olive leaves on platelet function. *Nutr. Metab. Cardiovasc. Dis.* **2007**, in press.
(55) Pignatelli, P.; Di Santo, S.; Buchetti, B.; Sanguigni, V.; Brunelli, A.; Violi, F. Polyphenols enhance platelet nitric oxide by inhibiting protein kinase C-dependent NADPH oxidase activation: effect on platelet recruitment. *FASEB J.* **2006**, *20*, 1082−1089.
(56) Nomura, S.; Shouzu, A.; Omoto, S.; Nishikawa, M.; Fukuhara, S.; Iwasaka, T. Losartan and simvastatin inhibit platelet activation in hypertensive patients. *J. Thromb. Thrombolysis* **2004**, *18*, 177−185.

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