Gallic Acid Attenuates Platelet Activation and Platelet-Leukocyte Aggregation: Involving Pathways of Akt and GSK3β

Running Title: Gallic Acid Inhibits Platelet Activation

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Abstract

Platelet activation and its interaction with leukocytes play an important role in atherothrombosis. Cardiovascular diseases resulted from atherothrombosis remain the major causes of death worldwide. Gallic acid, a major constituent of red wine and tea, had been shown to have properties of cardiovascular protection, which was possibly associated with its anti-oxidant effects. Nonetheless, there were few and inconsistent data regarding the effects of gallic acid on platelet function. Therefore, we designed this *in vitro* study to determine whether gallic acid could inhibit platelet activation and the possible mechanisms. From our results, gallic acid could concentration-dependently inhibit platelet aggregation, P-selectin expression and theplatelet-leukocyte aggregation. Gallic acid prevented the elevation of intracellular calcium and attenuated phosphorylation of PKCα/p38 MAPK and Akt/GSK3β on platelets stimulated with the stimulants (ADP or U46619). This is the first mechanistic explanation of the inhibitory effects on platelets from gallic acid.

KEYWORDS: Gallic acid, platelet, P-selectin, platelet-leukocyte aggregate, calcium, MAPK, GSK

Introduction

Platelets are essential for primary hemostasis and the repairof endothelium, but they also play a key role in the development of acute coronary syndromes and contribute to cerebrovascular events.The activation of platelets by inflammatory triggers is a critical component of atherothrombosis[1]. In addition, platelets participate in the process of forming and extending atherosclerotic plaques[2]. When activated, platelets co-aggregate with circulating leukocytes via P-selectin glycoprotein ligand-1 (PSGL-1) and P-selectin interactions. These interactions trigger autocrine and paracrine activation processes that lead to the recruitment of the leukocytes into the vascular wall, which is important in the formation of atherothrombosis[3]. In a large-scale prospective human study, the risk of future cardiovascular events increased with increasing level of plasma platelet-leukocyte aggregation[4].

Gallic acid (3,4,5-trihydroxybenzoic acid), a naturally occurring plant phenol, is abundant in natural plants, tea and red-wines[5] and has been demonstrated to have various biological properties, including anti-oxidant[6], anti-cancer[7] and anti-inflammatory activities[8]. Epidemiological studies have suggested that the consumption of red wine is associated with a reduction in overall mortality[9]. Although the exact nature of the protective effect of red wine is unclear, it might be partially attributable to its ability to reduce the progression of atherosclerotic lesions[10]. Green tea had also been reported to have protective effects on cardiovascular diseases[11]. Gallic acid itself had been shown to protect the myocardium against isoproterenol-induced oxidative stress in rats[12]. Previous reports on the favorable effects of gallic acid focused on its anti-oxidant and anti-inflammatory properties[8, 13]. Nonetheless, it is not known whether gallic acid may be atheroprotective through non-antioxidant mechanisms, e.g., through inhibiting platelets activation. Till now, there were only scanty and inconsistent data reporting the effects of gallic acid on platelet function. Therefore, the purpose of our study was to determine whether gallic acid could inhibit platelet function in

vitro and to elucidate the underlying molecular mechanisms.

Materials and Methods

Antibodiesand Reagents

The following antibodies were used: anti-CD42a-PE antibody (Becton Dickinson, San Jose, CA, USA), a platelet-specific monoclonal antibody (mAb) conjugated with phycoerythrin (PE) which recognizes platelet Glycoprotein IX complex independent of activation; anti-CD62P-PE antibody (Becton Dickinson), a mAb conjugated with PE that is directed against P-selectin expressed on the platelet surface; and anti-CD14-allophycocyanin (APC) antibody (Becton Dickinson), a mAb recognizes a myelo-monocytic differentiation antigen expressed by monocytes. Polyclonalantibodies against p38 mitogen-activated protein kinase (MAPK), protein kinase C-alpha (PKCα) and Aktwere obtained from Cell Signaling (Boston, MA, USA). Polyclonalantibodies against glycogen synthase kinase-3β (GSK3β) were purchased from R&D Systems (Minneapolis, MN, USA).2′7′-dichlorodihydrofluorescein diacetate (DCFH-DA) and fluo-3 acetoxymethyl ester (fluo-3 AM) were obtained from Molecular Probes (Eugene, OR). Adenosine 5'-diphosphate (ADP), gallic acid and paraformaldehyde were purchased from Sigma Chemicals (St. Louis, MO, USA). U46619, a thromboxane A_2(TxA_2) mimetic, was obtained from Cayman Chemical (Ann, Arbor, Michigan, USA). Gallic acid was dissolved in dimethylsulfoxide (DMSO). Steps were taken to ensure that the concentration of DMSO was always the same (0.1%).

Preparation of Platelet Suspension

Human platelets were purified as previously described[14]. Whole blood for this in vitro study was sampled from six healthy volunteers with age ranging from 27 to 53 years, who had not taken any medication for at least 15 days. Blood was collected from the antecubital vein into

acid-citrate-dextrose (9:1) and centrifuged at 200 × g for 20 min at 25 °C to prepare platelet-rich plasma (PRP). PRP then was firstly washed with modified Tyrode's solution (NaH_2PO_4, 0.42 mM,NaCl136.9 mM, KCl2.68mM; $NaHCO_3$, 11.9 mM; $CaCl_2$, 1.85mM; $MgCl_2$, 1.0mM; 0.35% BSA and 0.1% glucose) containing heparin (7 U/ml) and PGE_1 (0.6 μM), and centrifuged at 600 × g for 15 min at 25 °C. After descanting the supernatant, pellet was then washed twice with modified Tyrode's solution containing heparin and PGE_1. Finally, washed platelet were resuspended to a final concentration of 3×10^8 platelets/ml in Tyrode's solution containing 0.35% BSA and incubated at 37 °C.

Platelet Aggregation

Platelet aggregation was measured with an aggregometer (Payton Scientific, Buffalo, NY, USA) as previously described[15]. Briefly, platelet suspension was applied to the aggregometer and stirring was initiated at 900 rpm for 1 min at 37 °C with a small magnetic bar. Then various concentrations of indicated gallic acid were added and incubated for 3 min followed by adding proaggregatory substance ADP (2.5 μM) and TxA_2 analog U46619 (1.5 μM). We used PowerLab 8/SP (ADInsturments, Sydney, Australia) to analyze the extent of platelet aggregation that was continuously monitored for 8 min by turbidimetry and expressed as increase of light transmission.

Assess Platelet-Leukocyte Aggregates and P-selectin Expression by Flow Cytometry

The amount of platelet-leukocyte aggregates (PLA) and P-selectinexpression on platelets was determined by cytofluorimetric analysis.Anti-coagulated whole blood and PRP were preincubated with the indicated concentration of gallic acid for 15 minutes at 37^{o}C. The blood samples were treated for 15 minutes stimulation at room temperature with ADP and U46619 at a concentration of 2 μM in whole blood and 5 μM in PRP. To determine PLA, whole blood was

mixed with saturated concentrations of anti-CD42a-PE mAb and anti-CD14-APC mAb. To determine platelet P-selectin expression, PRP samples were mixed with saturated concentrations of anti-CD62p-PE mAb and anti-CD42a-PE mAb. Both samples were then fixed with 1% paraformaldehyde and maintained at 4^{o}C. After fixation, blood samples were immediately processed for flow cytometric analysis in a FACSCanto (Becton Dickinson). Granulocytes were recognized by size (forward scatter) and granularity (side scatter). Anti-CD14-APC fluorescence was used to further differentiate monocytes. The amount of platelets attached to granulocytes and monocytes were further measured by the anti-CD42a fluorescence. To determine platelet CD62P expression in PRP, individual platelets were identified by size (forward scatter) and anti-CD42a-PE immunofluorescence. P-selectin expression on the surface of platelets was defined as positive for anti-CD62P-PE. Results are expressed as mean fluorescence intensity (MFI) and percentage of positive CD62P cells.

Measurements of Intracellular Ca^{2+}Concentration

Intracellular Ca^{2+} levels were determined with the Ca^{2+}-sensitive fluorochrome fluo-3 AM using flow cytometry as previously described[16]. Briefly, washed human platelets (3×10^{8} platelets/ml) were loaded with 8 μM fluo-3 AM for 30 min at 37°C in the dark. After being washed once, platelets were re-suspended and the external Ca^{2+} was adjusted to 1 mMand then the dyed platelets were incubated with ADP (10 μM) or U46619 (2 μM) and different concentrations of gallic acid (100 μM, 500 μM), or control vehicles at 37 °C for 3 min in the dark, and analyzed by flow cytometry.

Determination of Reactive Oxygen Species Formation

The influence of gallic acid on reactive oxygen species (ROS) production of stimulated platelets were tested by 2′7′-dichlorodihydrofluorescein diacetate (DCFH-DA)(Molecular

Probes, Eugene, OR) and flow cytometry as previously described [17]. In brief, PRP was preloaded with 10 μM DCFH-DAfor 30 min at 37°C followed by stimulation of U46619 (2μM). Oxidation was quantified by measuring the increase in fluorescence of 2′7′-dichlorodihydrofluorescein (DCF) with a flow cytometer. The effect of gallic acid on ROS production of platelets was decided by pre-treatment of gallic acid of indicated concentration for 15 minutes before adding U46619.

Western Blot Analysis

The method of western blotting was performed as described previously[18]. Various concentrations of indicated gallic acid were added to PRP and incubated for 3 min followed by adding proaggregatory substance ADP (2.5 μM).The reaction was stopped with 2 μM EDTA and 50 μM Indomethacin.Proteins were extracted with lysis buffer for 30 min. Lysates were centrifuged, and the supernatant (46.4 μg protein) was subjected to SDS PAGE (10%) and blotted on PVDF membrane. Immunodetection was carried out by using antibodies directed against phosphorylated and origin forms of p38 MAPK, PKCα, Akt and GSK3β. The immunoreactive band was detected by enhanced chemiluminescence.

Statistical Analysis

All data are expressed as the mean ± standard error of the mean. Each sample was compared with the corresponding control sample. Analysis of statistical significance was performed using one-way analysis of variance (ANOVA) combined with the Turkey test. For comparison between two groups Student's *t* test was used. $P < 0.05$ was considered to be significant for a difference.

Results

Gallic Acid Inhibits Platelet Aggregation

To test the influence of gallic acid on platelets, we performed *in vitro* platelet aggregation studies. The PRP was incubated with different concentrations of gallic acid for 3 minutes before the addition of ADP or TxA_2 analog U46619. Gallic acid significantly inhibited platelet aggregation induced by ADP (2.5 μM) and U46619 (1.5 μM) in a concentration dependent manner (Fig. 1A, 1B). The aggregation of platelets cannot be fully inhibited by gallic acid. Gallic acid exerted no effects on the initial phase of platelet aggregation induced by ADPand U46619. The solvent control (0.5% DMSO) did not affect platelet aggregation stimulated by ADP or U46619 in either washed platelets or PRP (data not shown).

Gallic Acid Inhibits Platelet-Leukocyte Aggregates (PLA)

The influence of gallic acid on PLA was determined by flow cytometry on whole blood stimulated with ADP (Fig. 2A) and U46619 (Fig. 2B). The population of the granulocytes and monocytes was defined by size and the granularity. Monocytes were further probed by anti-CD14. The amount of platelets attached to the leukocytes was determined by the fluorescence of CD42a on granulocytes and monocytes. PLA increased significantly after adding ADP and U46619. Gallic acid (100 and 500 μM) concentration-dependently inhibited ADP- and U46619-induced PLA in wholeblood.

Gallic Acid Inhibits P-selectin Expression of Platelets

The influence of gallic acid on CD62P surface expression after stimulation of ADP(Fig. 3A) and U46619 (Fig. 3B) on PRP was measured by flow cytometry. The percentage of CD62P positive platelets and MFI of CD62P on platelets was quantitatively assessed. Pre-incubation with increasing gallic acid concentrations (50, 100, 500 μM) had inhibitory effectsof the P-

selectin expression on platelets in response to ADP or U46619 and the inhibitory influence of gallic acid on platelets was concentration dependent.

Gallic Acid Inhibits Intracellular $Ca^{2+}$of Platelets

Platelet activation and aggregation was reported to be triggered by increased intracellular Ca^{2+}level[2, 19]. We further examined the effects of gallic acid on intracellular Ca^{2+} level of the platelets induced by ADP (Fig. 4A) and U46619 (Fig. 4B). As shown in Fig. 4, ADP (10μM) and U46619 (2 μM)could evoke a marked increase in Ca^{2+}concentration of platelets. Intracellular Ca^{2+}levelof platelets was concentration-dependently inhibited bypre-incubation of gallic acid (100μM and 500μM).

Effects of Gallic Acid on Activities ofPKCα, P38MAPK, Akt and GSK3β

Hydrolysis of inositol phospholipids by phospholipase C and subsequent production of the diacylglycerol (DAG) and then activating protein kinase C (PKC) is one of the important mechanisms in platelet function[2]. It had been noticed that P38 MAPK provides a crucial signal for platelet aggregation caused by collagen and U46619[20].When ADP (2.5 μM) was added to PRP, the phosphorylated PKCα and p38MAPK increased apparently as compared with resting platelets. Gallic acid (100 μM, 500 μM)concentration-dependently inhibited the phosphorylation of PKCα and p38MAPK in platelets that received ADP stimulation (Fig. 5A).

In platelet activation, Akt activity had been noticed to related to the phosphorylation of PKC and platelet Ca^{2+} concentration[21]. Recently, glycogen synthase kinase (GSK) 3β had been noticed its significant roles as an substrate for Akt in mediating platelet activation[22]. To assess the effects of gallic acid on *Akt and GSK3β,*ADP (2.5 μM) was added to PRP and the level of phosphorylated Akt and GSK3β was determined (Fig. 5B). The phosphorylation of Akt and GSK3βincreased after stimulation of ADP as compared with resting platelets. Gallic acid

(1000 μM, 500 μM and 100 μM)concentration-dependently inhibit phosphorylation of Akt and GSK3β in platelets subjected to ADP stimulation.

Effects of Gallic Acid on Reactive Oxygen Species of Platelets

Gallic acid was reported to have reactive oxygen species (ROS) scavenging effects[6]. ROS had been recognized to enhance platelet activation and thrombus formation by attenuating the function of nitric oxide[23]. We tested whether the effects of gallic acid on stimulated platelets comes from anti-oxidant ability. The effect of gallic acid on ROS production after stimulation with U46619 and ADP was determined by the fluorescence of DCF. The representative histogram (Fig. 6) showed that pre-treatment of gallic acid (500 μM) had no effects on DCF fluorescence compared to U46619 (2 μM) treatment alone. There were no influences of ROS production after incubation of gallic acid followed by stimulation with ADP (data not shown). The result was indicative of gallic acid having no interference on ROS production of the platelets stimulated with U46619 or ADP.

Discussion

Platelet aggregation and activation is a primary contributor to a variety of atherosclerotic diseases, including coronary artery disease, transplant vasculopathy, and carotid artery disease[3]. Anti-platelet therapies, including aspirin, cilostazol and clopidogrel had been the mainstay of treatment for the atherosclerotic diseases. Gallic acid, a major constituent of red wine and tea, had been widely investigated for its cardiovascular protective properties. Our present study demonstrated for the first time that gallic acid could inhibit platelet aggregation, activation and platelet-leukocyte aggregation and reduces Ca^{2+} mobilization and this involves a decrease in the phosphorylation of PKCα/p38 MAPK and Akt/GSK3β.

Platelet aggregation is known to be a result of complex signal transduction cascade reactions brought about by stimulants. Vessel wall injury triggers rolling and adhering of platelets to subendothelial matrix by their surface receptors and subsequent platelet aggregation is the principle event in thrombus formation which plays a central role in the development of acute coronary syndrome[1, 2]. A semi-synthetic anti-oxidant (hydroxy-tyrosylgallate) related to gallic acid had been demonstrated to exert an inhibitory effect on platelet aggregation stimulated by thrombin[24]. Previous studies also reported the anti-aggregatory effects on platelets of red wine come from interference with the synthesis of TxA_2, which serves in an autocrine loop that accelerates aggregation[25]. Herein, we used in vitro models to show that gallic acid at the concentration greater than 100 μM reduced ADP- or U46619-stimulated aggregation of platelets in a concentration-dependent manner. Gallic acid can inhibit platelet aggregation stimulated by different pro-aggregatorystimulants, which have different action mechanisms responsible for the platelet aggregation. This implied that gallic acid might block a common step shared by these agonists.

PLA was found to increase in patients with acute coronary syndrome[26]. In various inflammatory clinical entities, such as cardiopulmonary bypass, hemodialysis, sepsis and trauma, PLA level was higher than the general population. The role of platelet-leukocyte crosstalk in atherosclerosis and inflammation had been extensively investigated. PLA may trigger a serial activation of platelets, which further leads to leukocyte recruitment into the vascular wall[27]. The formation of platelet-leukocyte conjugates is largely mediated by the binding of P-selectin expressed on activated platelets to PSGL-1 on leukocytes. Platelet P-selectin was also shown to have an important role in arterial thrombogenesis by forming large stable platelet-leukocyte aggregates[27]. Recent reports demonstrate that gallic acid, as a structure-like molecule to P-selectin, can inhibit P-selectin mediated adhesion both in vitro and in vivo[28]. Our study similarly showed that gallic acid could concentration-dependently

reduce ADP- or U46619- induced PLA in whole blood. In our work, we further revealed that gallic acid can concentration-dependently attenuates ADP- or U46619- induced P-selectin expression, which may further play a partial role in the inhibition of PLA by gallic acid. Collectively, gallic acid in our in vitro experiments is shown to regulate platelet aggregation, PLA formation and platelet P-selectin expression induced by its stimulants, which may partially explain the cardiovascular protective effects of gallic acid.

Intracellular free Ca^{2+} concentration controls a number of platelet functions, including aggregation and P-selectinexpression[19]. Platelet stimulants increase Ca^{2+} concentration, which consists of two components: release of Ca^{2+} from intracellular stores and Ca^{2+} entry through plasma membrane channels[19, 29]. Kim et al., found that pre-incubation of gallic acid with mast cells decreased the intracellular calcium level after stimulation[30]. Hence, we further explored the effect of platelet incubation with the gallic acid on Ca^{2+} concentration in these cells. Treatment of washed platelets for with gallic acid of desired concentration (100 μM and 500μM) significantly reduced ADP- or U46619-evoked Ca^{2+} release. As Ca^{2+} is a potent stimulus of platelet granule secretion, the inhibition of Ca^{2+} release may lead to inhibition of platelet granule secretion with P-selectinexpression[31]. This result did provide a mechanistic involvement by which gallic acid inhibits platelet aggregation and P-selectin expression.

Stimulation of platelets by pro-aggregatory agents results in phospholipase C (PLC)-catalyzed hydrolysis of the plasma membrane phospholipid, phosphatidylinositol 4,5-bisphosphate, with concomitant formation of IP3 and diacylglycerol (DAG)[32, 33]. DAG phosphorylates PKC, inducing protein phosphorylation, ATP release and intracellular Ca^{2+} rise, and finally activate platelets. In our work, ADP-induced PKCα phosphorylation was inhibited by gallic acid, suggesting that gallic acid-mediatedantiplatelet activity involves inhibition of PKCα activation. The role of p38 MAPK provides a crucial signal that is necessary for aggregation of platelets caused by collagen or thrombin[20]. Among the numerous downstream

targets of p38 MAPK, the most physiologically relevant one in platelets is cytosolic phospholipase A2 (cPLA2) which catalyzes arachidonic acid release to produce TxA_2[34]; thus, p38 MAPK appears to provide a TxA_2-dependent platelet aggregation pathway. Pre-treatment of gallic acid with the stimulation of ADP reduced the phosphorylation of P38 MAPK in our study, which may at least partially explain the inhibitory effects of gallic acid on platelet aggregation.

Platelets stimulated by agonists, thrombin and ADP, could activate G protein-coupled receptors on the platelet surface, which have been shown to activate multiple isoforms of PI3K and Akt[35, 36]. Plateletsfrom Akt-1-deficient micecannot form thrombus upon stimulation with thrombin and collagen[37]. Glycogen synthase kinase (GSK) 3β (GSK3β) had been found in platelets and it can regulate platelet activation as an Akteffector[22, 38]. GSK3β is a ser-thr kinase that is regulated by its phosphorylation on ser9[39]. Phosphorylation of this residue by the ser-thr kinase, Akt, is associated with decreased GSK3 activity, which releases a tonic inhibition of the GSK3 substrate. Therefore, the phosphorylation of GSK3β by Akt suppresses its inhibitory effect on platelet function. It was reported that decreased activity of GSK3β in haploinsufficiency mice or by treatment of platelets with the inhibitor (LiCl or SB216763) enhances agonist-induced dense granule secretion[38]. Our results disclosed that gallic acid reduced the phosphorylation of Akt and GSK3β in platelets stimulated by ADP. Taken together, from our data, gallic acid may exert its anti-platelet effects via regulating the signals of PKCα/p38MAPK and Akt/GSK3β.

Though red wineand teaboth had been known to have anti-oxidant properties, there were scarce studies about the anti-oxidant effects on platelet of gallic acid. Some studies even reported that gallic acid only had a weak inhibitory effect on oxidative stress[6]. Gallic acid was proved to have anti-oxidant effects on human lymphocytes and cardiac myocytes[12, 40]. In rats models, intake of GA was shown to be beneficial for the suppression of high fat diet-

induced hepatosteatosis and oxidative stress[13]. Earlier investigations noticed that oxidative stress could activate platelets and lead to thrombosis through consumption of nitric oxide[41]. Reactive oxygen species also act as a secondary messengers that increase the cytosolic Ca^{2+} during the initial phase of platelet activation processes[42]. In our experiment, after pre-treatment of gallic acid, there is no influence on platelet ROS production with induction of U46619 (2 μM) or ADP (2 μM) (not shown). Therefore, the inhibitory results of platelet function from gallic acid may not come from the anti-oxidative actions.

Conclusion

Our study demonstrated that gallic acid inhibited platelet aggregation, P-selectin expression and PLA formation stimulated by ADP or U46619 possibly through decreasing intracellular Ca^{2+} mobilization. The inhibition of phosphorylation of PKCα/p38 MAPK and Akt/GSK3β in stimulated platelets after gallic acid pre-treatment is the suggestive mechanisms of action. This is the first report about the properties of gallic acid on platelet inhibition and its mechanisms. These findings of gallic acid suggest a possible therapeutic application of this agent in the diseases associated with atherosclerosis.

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Disclosures

All authors have reported no conflict of interest.

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Figure Legends

Fig. 1. Inhibitory effects of gallic acid on ADP- or U46619-induced platelet aggregation.Platelet-rich plasma (PRP) was incubated with 100-1000 μMgallic acid or buffer for 3 min, and aggregation was induced by addition with (A) ADP (2.5 μM) or (B) U46619 (1.5 μM) (B). Gallic acid inhibited platelet aggregation induced by ADP or U46619 in a dose-dependent manner. These experiments are representative results of at least three similar experiments.

Fig. 2. Flow cytometric analysis of the effects of gallic acid on platelet-leukocyte aggregates (PLA) after stimulation with ADP or U46619. Quantitative results of the influence of gallic acid (100 μM, 500 μM) on PLA induced by (A) ADP (2μM) or (B) U46619 (2 μM)by means of the mean fluorescence intensity of CD 42a.Platelet−monocyte (open bars) and platelet−granulocyte (filled bars) aggregates were both inhibited by gallic acid in a concentration-dependent manner. Results are presented as mean±SEM, n=5.*, **$p<0.05$ vs. control samples stimulated with ADP or U46619.

Fig. 3. Inhibitory effects of gallic acid (GA) on CD62P expression of platelets stimulated with ADP or U46619. Platelets were pretreated with gallic acid (50 μM, 100 μM, 500 μM) and then stimulated with (A) ADP (5 μM) or (B) U46619 (5 μM). Surface expression of CD62P on platelets was quantified by flow cytometry. The percentage of platelets positive for CD62P expression (open bars) and the corresponding mean fluorescence intensity (MFI) (filled bars) of the positive platelets were determined. The results were presented as means±SEM, n = 5. *, ** $p<0.05$ vs. control samples stimulated by ADP or U46619.

Fig. 4. Effects of gallic acid (GA) on intracellular Ca^{2+} concentration of platelets measured by flow cytometry. Gallic acid at a concentration of 100 μM and 500 μM inhibited the intracellular Ca^{2+} rise, which were stimulated by (A) ADP (10μM) and (B) U46619 (2 μM). These results were confirmed in 3 separate experiments.

Fig. 5. Effects of gallic acid (GA) on activation of protein kinase C alpha (PKCα), P38 mitogen-activated protein kinases (MAPK), Akt and glycogen synthase kinase 3β (GSK3β) in platelets. Platelets were pretreated with gallic acid (50 - 1000 μM) for 15 minutes prior to stimulation with ADP 2.5 μM and the phosphorylation of PKCα and p38 (A) and Akt and GSK3β (B) was assayed by Western blot (n=3).

Fig. 6. Effects of gallic acid (GA) on reactive oxygen species (ROS) production in platelets assessed by flow cytometry. Gallic acid at a concentration of 500 μM had no inhibitory effects on platelet ROS stimulated with U46619 (2 μM). This cytofluorimetric histogram of fluorescence of 2′7′-dichlorodihydrofluorescein (DCF) was representative of three similar experiments.

Fig 1.

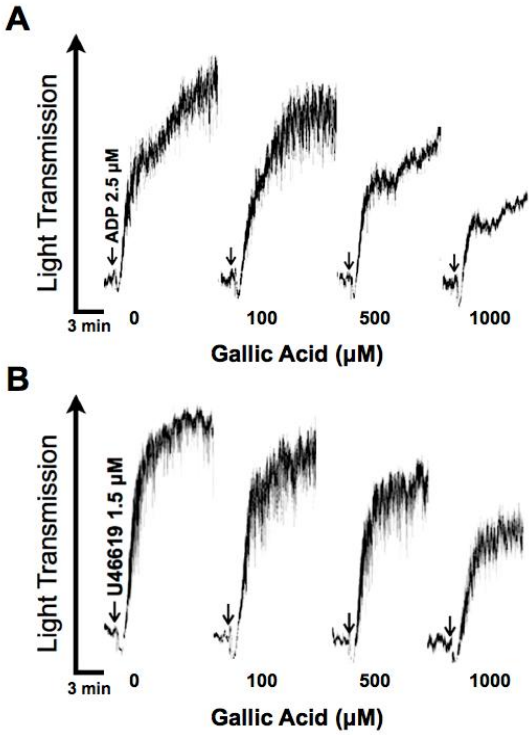

A
Light Transmission
← ADP 2.5 µM
3 min
0
100
500
1000
Gallic Acid (µM)
B
Light Transmission
← U46619 1.5 µM
3 min
0
100
500
1000
Gallic Acid (µM)

Fig. 2.

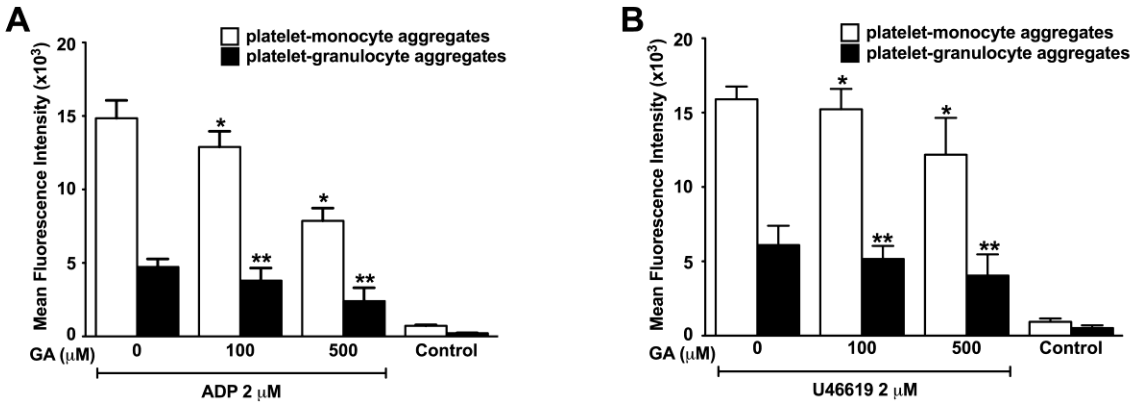

A
platelet-monocyte aggregates
platelet-granulocyte aggregates
Mean Fluorescence Intensity (x10³)
20
15
10
5
0
*
**
GA (µM)
0
100
500
Control
ADP 2 µM
B
platelet-monocyte aggregates
platelet-granulocyte aggregates
Mean Fluorescence Intensity (x10³)
GA (µM)
0
100
500
Control
U46619 2 µM

Fig 3.

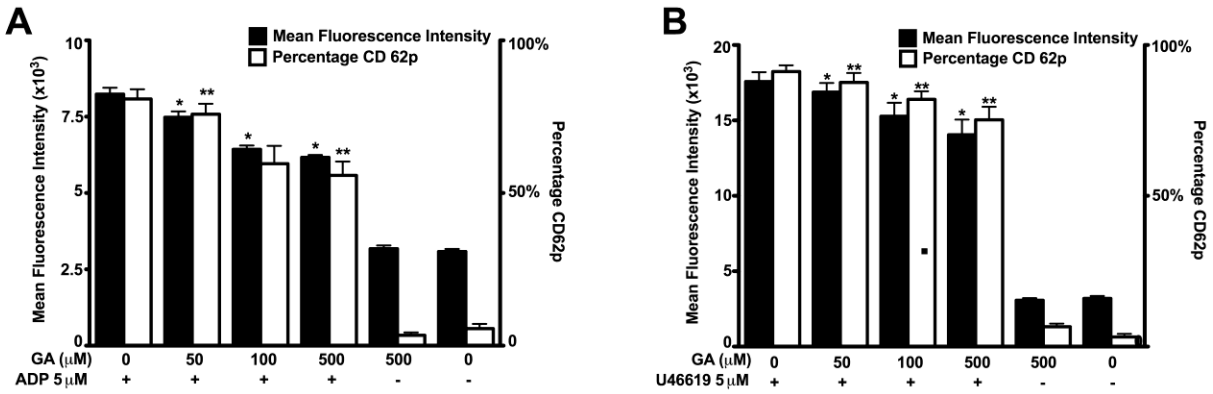

A
Mean Fluorescence Intensity
Percentage CD 62p
Mean Fluorescence Intensity (x10³)
Percentage CD62p
10
7.5
5
2.5
0
100%
50%
0
GA (μM) 0 50 100 500 500 0
ADP 5 μM + + + + - -
B
Mean Fluorescence Intensity
Percentage CD 62p
Mean Fluorescence Intensity (x10³)
Percentage CD62p
20
15
10
5
0
100%
50%
GA (μM) 0 50 100 500 500 0
U46619 5 μM + + + + - -

Fig 4.

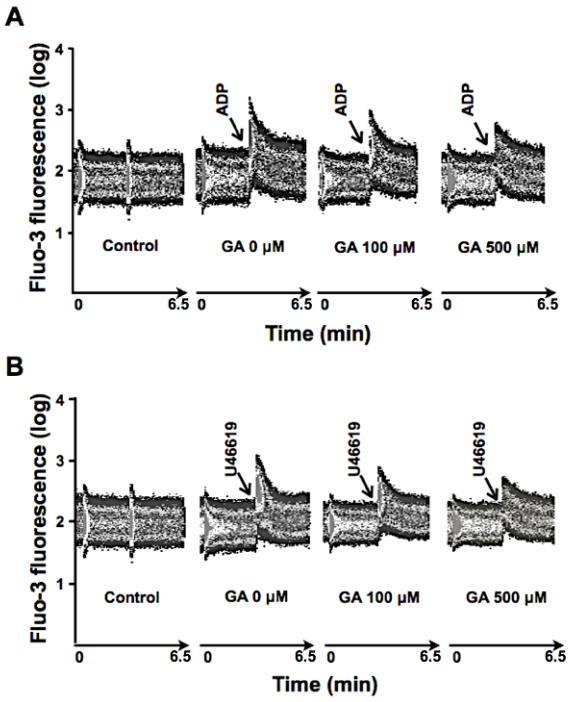

A
Fluo-3 fluorescence (log)
ADP
ADP
ADP
Control
GA 0 μM
GA 100 μM
GA 500 μM
0
6.5
Time (min)
B
Fluo-3 fluorescence (log)
U46619
U46619
U46619
Control
GA 0 μM
GA 100 μM
GA 500 μM
Time (min)

Fig 5.

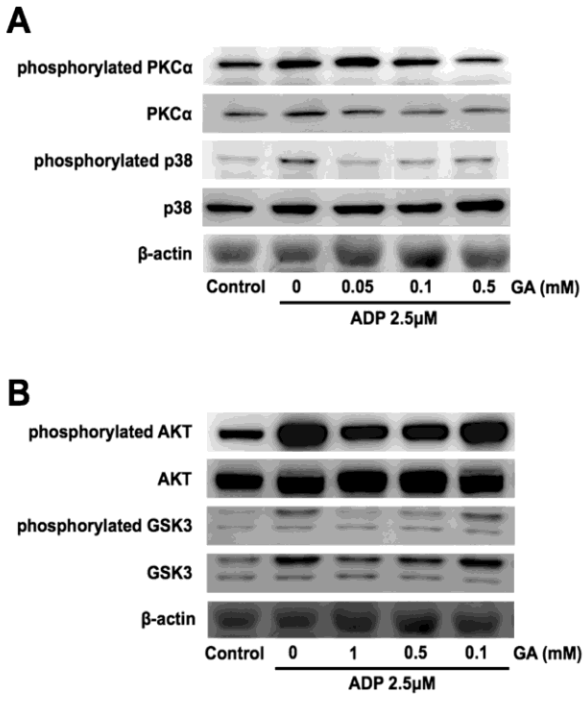
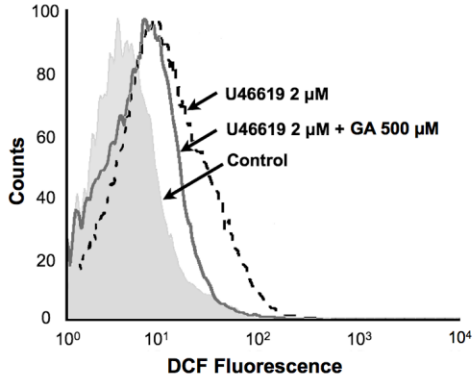

A
phosphorylated PKCα
PKCα
phosphorylated p38
p38
β-actin
Control
0
0.05
0.1
0.5
GA (mM)
ADP 2.5μM
B
phosphorylated AKT
AKT
phosphorylated GSK3
GSK3
β-actin
Control
0
1
0.5
0.1
GA (mM)
ADP 2.5μM

Fig 6.


100
80
60
40
20
0
Counts
U46619 2 µM
U46619 2 µM + GA 500 µM
Control
10⁰
10¹
10²
10³
10⁴
DCF Fluorescence