Research Article

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

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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, has been believed to have properties of cardiovascular protection, which is likely to be related to its antioxidant 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 platelet-leukocyte aggregation. Gallic acid prevented the elevation of intracellular calcium and attenuated phosphorylation of PKCα/p38 MAPK and Akt/GSK3β on platelets stimulated by the stimulants ADP or U46619. This is the first mechanistic explanation for the inhibitory effects on platelets from gallic acid.

1. Introduction

Platelets are essential for primary hemostasis and the repair of endothelium, but they also play a key role in the development of acute coronary syndromes and contribute to cerebrovascular events. Platelet activation triggered by inflammation is the critical component of atherothrombosis [1]. In addition, platelets participate in the process of forming and extending atherosclerotic plaques [2]. When activated, platelets coaggregate with circulating leukocytes via P-selectin glycoprotein ligand-1 (PSGL-1) and P-selectin interactions. These interactions trigger autocrine and paracrine activation processes leading 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 levels of plasma platelet-leukocyte aggregation [4].

Gallic acid (3,4,5-trihydroxybenzoic acid), a naturally occurring plant phenol, which can be abundantly found in natural plants, tea, and red wines [5], has been demonstrated to have various biological properties, including antioxidant [6], anticancer [7], and anti-inflammatory activities [8]. Epidemiological studies have suggested that red wine consumption is related to a reduction in overall mortality [9]. Although the exact nature of the protective effect of red wine is unclear, it might be partially attributed to its ability to reduce the progression of atherosclerotic lesions [10]. Green tea has also been reported to have protective effects on cardiovascular diseases [11]. Gallic acid itself has 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], but it remains unknown whether or not gallic acid is atheroprotective through nonantioxidant mechanisms, for example, through.
inhibiting platelet activation. Up to date, there have been only scanty and inconsistent data concerning 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.

2. Materials and Methods

2.1. Antibodies and 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), an mAb conjugated with PE that is directed against P-selectin expressed on the platelet surface, and anti-CD14-allophycocyanin (APC) antibody (Becton Dickinson), an mAb which recognizes a myelomonocytic differentiation antigen expressed by monocytes. Polyclonal antibodies against p38 mitogen-activated protein kinase (MAPK), protein kinase C-alpha (PKCa), and Akt were obtained from Cell Signaling (Boston, MA, USA). Polyclonal antibodies against glycogen synthase kinase-3β (GSK3β) were purchased from R&D Systems (Minneapolis, MN, USA). 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) and fluo-3 acetoxyethyl ester (fluor-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 A2 (TXA2) mimetic, was obtained from Cayman Chemical (Ann Arbor, Michigan, USA). Gallic acid was dissolved in dimethylsulfoxide (DMSO).

2.2. Preparation of Platelet Suspension. Human platelets were purified as previously described [14]. Whole blood for the 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 600 × g for 20 minutes at 37°C to prepare platelet-rich plasma (PRP). PRP was first washed with modified Tyrode’s solution (NaH2PO4: 0.42 mM, NaCl: 136.9 mM, KCl: 2.68 mM; NaHCO3: 11.9 mM; CaCl2: 1.85 mM; MgCl2: 1.0 mM; 0.35% BSA and 0.1% glucose) containing heparin (7 U/mL) and PGE2 (0.6 μM) and centrifuged at 600 × g for 15 minutes at 25°C. After descanting the supernatant, pellet was then washed twice with modified Tyrode’s solution containing heparin and PGE2. Finally, washed platelets were resuspended to a final concentration of 3 × 10⁸ platelets/ml in Tyrode’s solution containing 0.35% BSA and incubated at 37°C.

2.3. Platelet Aggregation. Platelet aggregation was measured with an aggregometer (Payton Scientific, Buffalo, NY, USA) as previously described [15]. Briefly, PRP was applied to the aggregometer and stirring was initiated at 900 rpm for 1 minute at 37°C with a small magnetic bar. Then, various concentrations of indicated gallic acid were added and incubated for 3 minutes followed by adding proaggregatory substance ADP (2.5 μM) and TXA2 analog U46619 (1.5 μM). We used PowerLab 8/SP (ADInstruments, Sydney, Australia) to analyze the extent of platelet aggregation that was continuously monitored for 8 minutes by turbidimetry and expressed as increase of light transmission.

2.4. Assess Platelet-Leukocyte Aggregates and P-Selectin Expression by Flow Cytometry. The amount of platelet-leukocyte aggregates (PLAs) and P-selectin expression 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°C. The blood samples were treated for 15 minutes of 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°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 was 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.

2.5. Measurements of Intracellular Ca²⁺ Concentration. Intracellular Ca²⁺ levels were determined with the Ca²⁺-sensitive fluorescent chromo-fluor-3 AM using flow cytometry as previously described [16]. Briefly, washed human platelets (3 × 10⁶ platelets/mL) were loaded with 8 μM fluo-3 AM for 30 minutes at 37°C in the dark. After being washed once, platelets were resuspended and the external Ca²⁺ was adjusted to 1 mM and 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 minutes in the dark and analyzed by flow cytometry.

2.6. Determination of Reactive Oxygen Species Formation. The influence of gallic acid on reactive oxygen species (ROS) production of stimulated platelets was 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-DA for 30 minutes at 37°C followed by stimulation of U46619 (2 μM). Oxidation was quantified by measuring the increase in fluorescence of 2′,7′-dichlorodihydrofluorescein
3. Results

3.1. Gallic Acid Inhibits Platelet Aggregation. To test the influence of gallic acid on platelets, we performed in vitro platelet aggregation studies. PRP was incubated with different concentrations of gallic acid for 3 minutes before the addition of ADP or TxA2 analog U46619. Gallic acid significantly inhibited platelet aggregation induced by ADP (2.5 μM) and U46619 (1.5 μM) in a concentration-dependent manner (Figures 1(a) and 1(b)). The aggregation of platelets could not be fully inhibited by gallic acid. Gallic acid exerted no effects on the initial phase of platelet aggregation induced by ADP and 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).

3.2. Gallic Acid Inhibits Platelet-Leukocyte Aggregates (PLAs). The influence of gallic acid on PLA was determined by flow cytometry in whole blood stimulated with ADP (Figure 2(a)) and U46619 (Figure 2(b)). 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 whole blood.

3.3. Gallic Acid Inhibits P-Selectin Expression of Platelets. The influence of gallic acid on CD62P surface expression after stimulation of ADP (Figure 3(a)) and U46619 (Figure 3(b)) in PRP was measured by flow cytometry. The percentage of CD62P-positive platelets and MFI of CD62P on platelets was quantitatively assessed. Preincubation with increasing gallic acid concentrations (50, 100, and 500 μM) had inhibitory effects of the P-selectin expression on platelets in response to ADP or U46619, and the inhibitory influence of gallic acid on platelets was concentration dependent.

3.4. Gallic Acid Inhibits Intracellular Ca2+ of Platelets. The effects of gallic acid on intracellular Ca2+ level of the platelets were studied by stimulation with ADP (Figure 4(a)) and U46619 (Figure 4(b)). As shown in Figure 4, ADP (10 μM) and U46619 (2 μM) could evoke a marked increase in Ca2+ concentration of platelets. Intracellular Ca2+ level of platelets was concentration-dependently inhibited by pre-incubation of gallic acid (100 μM and 500 μM).
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3.5. Effects of Gallic Acid on Activities of PKCa, P38MAPK, Akt, and GSK3β. To evaluate the effects on the phosphorylation of P38 MAPK and PKCa from gallic acid, ADP (2.5 μM) was added to PRP and the amount of the phosphorylated P38 MAPK and PKCa was assessed. The level of phosphorylated PKCa and p38MAPK of stimulated platelets increased apparently as compared with the other ones. Gallic acid (100 μM, 500 μM) could inhibit the phosphorylation of PKCa and p38MAPK in platelets receiving ADP stimulation (Figure 5(a)). In addition, the attenuation of PKCa phosphorylation from gallic acid manifested concentration-dependent manner. To assess the effects of gallic acid on...
Figure 4: Effects of gallic acid 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 (10 µM) and (b) U46619 (2 µM). These results were confirmed in 3 separate experiments. GA: gallic acid.

Figure 5: Effects of gallic acid on activation of protein kinase C alpha (PKC\(\alpha\)), P38 mitogen-activated protein kinases (MAPK), Akt, and glycogen synthase kinase 3\(\beta\) (GSK3\(\beta\)) 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 PKC\(\alpha\) and p38 (a) and Akt and GSK3\(\beta\) (b) was assayed by western blot (n = 3). GA: gallic acid.

Akt and GSK3\(\beta\), ADP (2.5 µM) was added to PRP and the level of phosphorylated Akt and GSK3\(\beta\) was determined (Figure 5(b)). The phosphorylation of Akt and GSK3\(\beta\) increased after stimulation of ADP as compared with other platelets, which was further reduced by gallic acid (1000 µM, 500 µM, and 100 µM) concentration-dependently.

3.6. Effects of Gallic Acid on Reactive Oxygen Species of Platelets. To test whether the inhibitory effects of gallic acid on stimulated platelets came from antioxidant ability, the influence of ROS production after stimulation with U46619 and ADP was determined by the fluorescence of DCF. The representative histogram (Figure 6) showed that pretreatment 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 with no influence on ROS production of platelets stimulated with U46619 or ADP.

4. 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]. Antiplatelet therapies, including aspirin, cilostazol, and clopidogrel have 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 reduce Ca\(^{2+}\) mobilization, and this involved a decrease in the phosphorylation of PKC\(\alpha\)/p38 MAPK and Akt/GSK3\(\beta\).

Platelet aggregation is known to be the result of the complex signal transduction cascades caused by the certain stimulants. Vessel wall injury triggers rolling and adhering of platelets to subendothelial matrix with their surface receptors. 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 semisynthetic antioxidant (hydroxy-tyrosyl gallate) related to gallic acid had been demonstrated to exert an inhibitory effect on platelet aggregation stimulated by thrombin [19]. Previous studies also reported that the antiaggregatory effects on platelets of red wine came from interference with the synthesis of TXA2, which served as an autocrine loop that accelerates aggregation [20]. Herein, we used in vitro models to show that gallic acid could reduce ADP- or U46619-stimulated aggregation of platelets in a concentration-dependent manner. Gallic acid could inhibit platelet aggregation stimulated by different proaggregatory stimulants, which had different action mechanisms responsible for 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 [21]. In various inflammatory clinical entities, such as cardiopulmonary bypass, hemodialysis, sepsis, and trauma, PLA level was higher than in the general population. PLA may trigger serial activation of platelets, ADP-induced PKC, induce protein phosphorylation, ATP release, and intracellular Ca2+ rise, and finally activate platelets. In our work, ADP-induced PKCα phosphorylation was inhibited by gallic acid, suggesting that gallic-acid-mediated antiplatelet activity involved inhibition of PKCα activation. P38 MAPK provides a crucial signal that is necessary for aggregation of platelets caused by collagen or thrombin [30]. 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 TXA2 [31]. Therefore, p38 MAPK appears to be necessary in TXA2-dependent pathways of platelet aggregation. Pre-treatment of gallic acid with the stimulated platelets reduced the phosphorylation of P38 MAPK in our study, which might also partially explain the inhibitory effects of gallic acid on platelet aggregation.

Platelet stimulated by agonists, for example, thrombin and ADP, could activate G protein-coupled receptors on platelet surface, which have been shown to activate multiple isoforms of PI3K and Akt [32, 33]. Platelets from Akt-1-deficient mice cannot form thrombus upon stimulation with thrombin and collagen [34]. In addition, glycogen synthase kinase (GSK) 3β (GSK3β) has been found in platelets and it can regulate platelet activation as an Akt effector [35, 36]. GSK3β is a ser-thr kinase that is regulated by its phosphorylation on ser9 [37]. Phosphorylation of this residue by Akt is related to 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 GSK3β inhibitors (LiCl or SB216763) enhanced agonist-induced dense granule secretion [36]. 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 antiplatelet effects via regulating the signals of PKCa/p38 MAPK and Akt/GSK3β.

Both red wine and tea have been known to have antioxidant properties, but 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, 38]. In rat models, the intake of gallic acid 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 [39]. Reactive oxygen species also act as a secondary messengers that increase the cytosolic Ca\(^{2+}\) during the initial phase of platelet activation processes [40]. Contrarily, previous reports showed that gallic acid of similar concentrations could exhibit prooxidant effects [7, 41]. In our experiment, after pre-treatment of gallic acid, there was no influence on platelet ROS production with induction of U46619 (2\(\mu\)M) or ADP (2\(\mu\)M) (not shown). Therefore, the inhibitory results of platelet function from gallic acid might not come from the anti-oxidative actions.

Though our present studies demonstrated that gallic acid had the possible cardiovascular protective roles from inhibiting platelet activation and its interaction with leukocytes, notably, gallic acid has been shown to induce apoptosis in tumor cells with higher sensitivity than that of normal cells in the comparative concentrations [7, 42, 43]. In fact, some of the concentrations of gallic acid used in our in vitro experiments are higher than those detected in plasma after acute intake of 50 mg gallic acid (as pure compound) or one cup of Assam black tea, which were within a low micromolar range (∼4 \(\mu\)mol/L) [5]. Nonetheless, gallic acid is largely consumed through tea, nuts, and even fruits in daily life [44, 45]. Studies are lacking that report the bioavailability after repeated intake of gallic acid.

In conclusion, our study demonstrated that gallic acid inhibited platelet aggregation, P-selectin expression, and PLA formation stimulated by ADP or U46619, which is likely to be through decreasing intracellular Ca\(^{2+}\) mobilization. The inhibition of phosphorylation of PKCa/p38 MAPK and Akt/GSK3β in stimulated platelets after gallic acid pre-treatment was 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 related to atherothrombosis.

**Conflict of Interests**

All authors have reported no conflict of interests.

Authors’ Contribution

S.-S. Chang and V. S. Y. Lee contributed equally to this work.

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References


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