

Aged Garlic Extract Inhibits Human Platelet Aggregation by Altering Intracellular Signaling and Platelet Shape Change^{1–3}

Khalid Rahman,* Gordon M Lowe, and Sarah Smith

School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Liverpool, United Kingdom

Abstract

Background: Increased platelet aggregation plays a pivotal role in the etiology of cardiovascular disease. Upon platelet aggregation, an increase in free cytoplasmic Ca^{2+} results in the inhibition of soluble guanylyl cyclase (sGC) and adenylyl cyclase (AC), leading to a decrease in cyclic guanosine-5'-monophosphate (cGMP) and cAMP, respectively. This leads to the activation of the glycoprotein IIb/IIIa (GPIIb/IIIa) fibrinogen receptor, resulting in platelet shape change. Aged garlic extract (AGE) decreases platelet aggregation; however, the mechanisms involved are not clearly defined.

Objective: Our objective was to investigate the effects of AGE on intraplatelet cell signaling and platelet shape change.

Methods: Platelets from 14 participants were studied. Platelet aggregation was induced by ADP in the presence of AGE up to a concentration of 6.25% (vol:vol) alone or in combination with 3-morpholinolinosydnonimine (Sin-1), a nitric oxide donor. The experiments with AGE were repeated in the presence of 3-isobutyl-1-methylxanthine (IBMX), a phosphodiesterase inhibitor. In a series of separate experiments, platelet aggregation was induced in the presence of either 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), an sGC inhibitor, or 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ22536), an AC inhibitor, or a combination of both in the presence of IBMX and AGE. Intraplatelet cGMP and cAMP were measured. The platelets were also subjected to scanning electron microscopic analysis, and their binding to fibrinogen was determined.

Results: AGE decreased platelet aggregation at all concentrations tested; this decrease was more marked in the presence of Sin-1 and ranged between 15% and 67%. The presence of IBMX also led to a decrease (17–35%) in platelet aggregation at all AGE concentrations and a significant decrease in the amounts of cGMP (24–41%) and cAMP (19–70%), respectively, in the presence of ODQ and SQ22536. The presence of AGE significantly inhibited the binding of activated platelets to fibrinogen, preventing changes in platelet shape.

Conclusion: These results indicate that AGE inhibits platelet aggregation by increasing cyclic nucleotides and inhibiting fibrinogen binding and platelet shape change. *J Nutr* 2016;146(Suppl):410S–5S.

Keywords: garlic, platelet aggregation, GPIIb/IIIa receptor, cAMP, fibrinogen, thrombosis

Introduction

Cardiovascular disease, which can lead to heart attack and stroke, is the leading cause of mortality and morbidity. Many risk factors are associated with this disease (1–4); however, a significant factor in the etiology of cardiovascular disease is an increase in the ability of platelets to aggregate, causing the narrowing of the blood vessels, which can eventually lead to acute coronary syndrome and ischemic stroke (4, 5). An increase in oxidative stress, such as that associated with cigarette smoking, is also strongly correlated with cardiovascular disease; furthermore, smokers display increased platelet aggregation, adhesiveness, and increased fibrinogen concentrations (6, 7).

The cytoplasmic side of the plasma membrane contains phospholipids that serve as substrates for phospholipases, and the intrinsic membrane glycoproteins IIb/IIIa (GPIIb/IIIa)⁴ extrude through the plasma membrane and act as platelet receptors responding to activating and inhibiting agents (4). The resting platelets are of discoid shape and, on activation, become spherical, and long, spiky pseudopods appear, which adhere to each other, forming what is often referred to as the hemostatic plug.

ADP binds to its receptors on the platelet plasma membrane, resulting in major biochemical changes that take place inside the cell, such as an inhibition of the enzyme adenylyl cyclase (AC). This leads to a reduction in the intraplatelet concentrations of

cAMP, which eventually results in platelet shape change and aggregation.

During platelet activation, the intrinsic glycoprotein fibrinogen receptor GPIIb/IIIa also becomes exposed on the plasma membrane, further accelerating platelet aggregation. Other modulators play a part in the activation and aggregation of platelets such as prostaglandins, lipoxygenase metabolites, protein kinase (PK) C, and cyclic guanosine-5'-monophosphate (cGMP) (8–11). NO also has an important role in platelet function in that it stimulates soluble guanylyl cyclase to form cGMP, which prevents the adhesion and aggregation of platelets (11, 12).

In recent years, the use of natural products such as garlic (*Allium sativum*) for the treatment of chronic diseases has increased substantially (13), and its ability to reduce or prevent cardiovascular disease has been widely reported (6, 14–17). We previously reported that a commercially available aged garlic extract (AGE) inhibited platelet aggregation both in vitro and in vivo (13, 18). We proposed that this inhibition of platelet aggregation was being mediated via a reduction in the expression of the GPIIb/IIIa receptor and an increase in cAMP; however, in our previous studies the effect of AGE on cGMP was not investigated. The present study was designed to investigate the effect of AGE on cell signaling within the platelets. The effect of AGE on intraplatelet cGMP was investigated in the presence of the soluble guanylyl cyclase (sGC) stimulator 3-morpholinosydnonimine (Sin-1), which generates NO, causing stimulation of sGC to induce synthesis of the secondary signaling molecule cGMP (19, 20). The effect of inhibiting sGC by the inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) in the presence of AGE was also investigated. Further experiments were undertaken in which AC was inhibited by 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ22536), and the platelets were subjected to activation by ADP in the presence of AGE to determine its effect on intraplatelet cAMP (21). The platelets were also subjected to

3-isobutyl-1-methylxanthine (IBMX) in order to inhibit the phosphodiesterases (PDEs) (22), and intraplatelet cAMP and cGMP were measured in the presence and absence of AGE. Finally, the binding of platelets to fibrinogen and their shape change in the presence of the above stimulators/inhibitors with and without AGE were determined by scanning electron microscope.

Methods

Chemicals. ADP, fibrinogen fraction 1 type IV from bovine plasma and Rose Bengal, was obtained from Sigma-Aldrich. cGMP and cAMP enzyme immunoassay kits were obtained from Amersham (GE Healthcare). NO donors Sin-1, ODQ, IBMX, SQ22536, and GPIIb/IIIa inhibitor 4-(4-[4-(aminoiminomethyl)phenyl]-1-piperazinyl)-1-piperidineacetic acid trihydrochloride (GR144053) were all obtained from Sigma Aldrich.

AGE (Kyolic) was kindly provided by Wakunaga of America and is formulated by soaking sliced raw garlic (*A. sativum*) in 15–20% aqueous ethanol for up to 20 mo at room temperature. The extract is then filtered and concentrated under reduced pressure at low temperature. The content of water-soluble compounds is relatively high, whereas that of oil-soluble compounds is low. The AGE used in this study contained 305 g/L extracted solids; and S-allylcysteine, the most abundant water-soluble organosulfur compound in AGE, was present at 1.47 g/L.

Preparation of plasma-rich protein. Ethical approval was obtained from Liverpool John Moores University Research and Ethics Committee. Blood was taken via venipuncture from 14 healthy volunteers, and platelet-rich plasma (PRP) was prepared as described by Rahman and Billington (18). Briefly, blood was placed into 3.8% (wt:vol) trisodium citrate at a ratio of 9:1 vol:vol (blood:anticoagulant). PRP was generated via centrifugation of citrated blood at $100 \times g$ for 10 min at room temperature and removed; platelet-poor plasma was isolated via further centrifugation at $1500 \times g$ for 20 min. and platelet count was adjusted to $2.5 \pm 0.5 \times 10^5$ cells/mL with platelet-poor plasma for aggregation studies.

Washed platelets. PRP was centrifuged for 15 min at $1000 \times g$. Pelleted platelets were resuspended in HEPES buffered Tyrode's solution and recentrifuged for a further 10 min at $1000 \times g$ at room temperature followed by a final resuspension in this buffer.

Aggregation studies. Platelet aggregation was determined in a PAP-4D platelet aggregation profiler (Bio/Data), as reported previously (18). AGE at a concentration of up to 6.25% (vol:vol) was added to the PRP; after a minimum time of 1 min, aggregation was initiated by the addition of 8 μ M ADP, and each plot was analyzed for total percentage aggregation. In some experiments, aggregation was induced in the presence of Sin-1, SQ22536, ODQ, and IBMX (21–24).

cGMP and cAMP assay. The cGMP and cAMP assay was essentially performed as outlined in the manufacturer's protocol. Briefly, washed platelets were incubated with 100 μ M IBMX for 30 min followed by further incubation with either 100 μ M SQ22536, 100 μ M ODQ, or PBS for 10 min and subjected to 1% AGE (vol:vol) for 1 min. Platelets were then activated with 8 μ M ADP for a maximum of 15 s before being placed directly onto ice for 5 min to stop the reaction. Samples were then centrifuged at $1000 \times g$ and resuspended in lysis buffer; the nucleotides were quantified with reference to a standard curve.

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⁴ Abbreviations used: AC, adenylyl cyclase; AGE, aged garlic extract; cGMP, cyclic guanosine-5'-monophosphate; GPIIb/IIIa, glycoprotein IIb/IIIa; GR144053, 4-(4-[4-(aminoiminomethyl)phenyl]-1-piperazinyl)-1-piperidineacetic acid trihydrochloride; IBMX, 3-isobutyl-1-methylxanthine; ODO, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PDE, phosphodiesterase; PK, protein kinase; PRP, platelet-rich plasma; sGC, soluble guanylyl cyclase; Sin-1, 3-morpholinosydnonimine; SQ22536, 9-(tetrahydro-2-furanyl)-9H-purin-6-amine.

*To whom correspondence should be addressed. E-mail: k.rahman@ljmu.ac.uk.

Fibrinogen assay. The fibrinogen assay was performed in a 96-well plate coated with fibrinogen (human plasma; Merck Millipore), as reported previously with slight modifications (13). Essentially, PRP was incubated with PBS, 1 μ M of the GPIIb/IIIa inhibitor GR 144053, or AGE to a concentration of 6.25% (vol:vol). Platelets were then activated with 8 μ M ADP and nonadherent cells were removed via aspiration. This was followed by adherent platelets being stained with 0.2% (wt:vol) Rose Bengal in PBS, and after lysis the released Rose Bengal was measured at a wavelength of 540 nm.

Scanning electron microscope imaging of platelets. Washed platelets (1×10^8 /mL) were treated either with 5% (vol:vol) AGE or PBS, washed and resuspended in HEPES buffered Tyrode's solution, followed by activation with 8 μ M ADP. Aliquots of platelets were taken at time 0 and at 60 s, and placed directly into a solution of 2.5% (vol:vol) glutaraldehyde. After a further wash, the platelets were placed onto a standard 13-mm alloy scanning electron microscope specimen stub and left to air-dry before being coated by using EMITECH (Quorum Technology, Lewes, East Sussex, United Kingdom) k550X gold sputter coater for 2.5 min at 25 mA; images were obtained by using an Inspect S (FEI, Oregon) scanning electron microscope at 12.5 kV.

Fluorescent imaging of platelet actin was also performed, and in another series of experiments aliquots of activated platelets were placed directly into a 2% (vol:vol) paraformaldehyde solution, briefly washed, and then permeabilized with 0.1% Triton X-100. This was followed by the addition of monoclonal mouse anti-actin antibody conjugated to fluorescein isothiocyanate (Sigma Aldrich) at a dilution of 1:50 in PBS, with the addition of 1% BSA, and left to incubate in the dark at room temperature for 1 h. The platelets were then washed and air-dried; after the addition of 1 drop of anti-fade mounting medium (Invitrogen), the platelets were imaged in the dark by using either an Olympus fluorescence microscope with Openlab software (4.0.4) or on a Zeiss 510 laser scanning confocal microscope.

Statistical analysis. Experimental results were assessed by using SPSS version 22 software. Data are expressed as means \pm SEMs and were assessed by using a 2-factor ANOVA with post hoc analysis with the use of Dunnett's test. *P* values < 0.05 were considered significant.

Results

Effect of AGE on platelet aggregation in the presence of Sin-1 and IBMX. The effect of AGE on platelet aggregation in the presence of Sin-1 is shown in Figure 1A. AGE at all concentrations tested inhibited platelet aggregation when compared with the ADP-activated control (Figure 1A). A significant interaction between AGE with and without Sin-1 on platelet aggregation was observed ($P < 0.001$). The interaction between Sin-1 and AGE on the inhibition of platelet aggregation was also significant ($P < 0.001$) (Figure 1A). The reduction in platelet aggregation in the presence of 8 μ M Sin-1 was $\sim 32\%$; however, when AGE and Sin-1 were used in combination, a synergistic inhibition of platelet aggregation was apparent, mainly at the lower concentration of AGE when compared with the ADP-activated control (Figure 1A).

Figure 1B shows the effect of AGE on platelet aggregation in the presence of IBMX, which at a concentration of 100 μ M, reduced platelet aggregation by 31% compared with the ADP-activated control. A significant interaction between AGE with and without IBMX on the inhibition of platelet aggregation was observed ($P < 0.001$). The interaction between IBMX and AGE on platelet aggregation was also significant ($P < 0.001$) (Figure 1B).

Effect of AGE on intraplatelet cyclic nucleotides. AGE in the presence of ODQ and/or SQ22536 decreased cGMP concentrations in ADP-stimulated platelets ($P < 0.01$); this decrease was

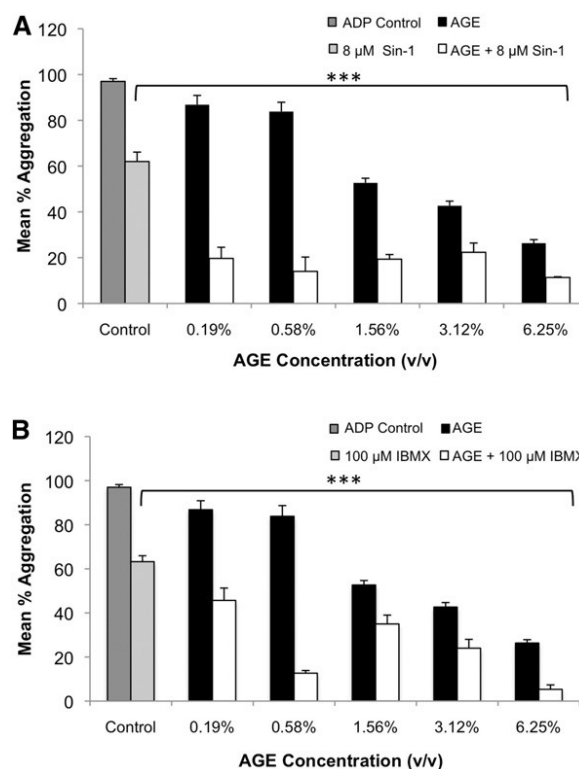


FIGURE 1 Effects of AGE and Sin-1 and their combination (A) and AGE and IBMX and their combination (B) on aggregation of ADP-activated human platelets. Platelets were treated with Sin-1 or IBMX and were then challenged with 0.19–6.25% (vol:vol) AGE followed by ADP activation. Platelet aggregation was then determined. Values are means \pm SEMs, $n = 9$ independent samples. ***Different from ADP-induced platelet aggregation, $P \leq 0.001$. AGE, aged garlic extract; IBMX, 3-isobutyl-1-methylxanthine; Sin-1, 3-morpholinopyridone; v/v, vol:vol.

$\sim 20\%$ in the presence of ODQ ($P < 0.01$) and $\sim 39\%$ in the presence of SQ22536 ($P < 0.001$) compared with that observed in ADP-activated AGE platelets (Figure 2A). The presence of AGE, ODQ, and SQ22536 further decreased the concentration of cGMP in ADP-activated platelets ($P < 0.001$) (Figure 2A). However, there was no significant interaction between ODQ and SQ22536 when compared with their combined effect on the lowering of cGMP in ADP-activated platelets ($P > 0.05$) (Figure 2A).

In comparison, AGE in the presence of ODQ and/or SQ22536 also decreased the concentration of cAMP in ADP-activated platelets ($P < 0.01$). The decrease in cAMP concentrations in the presence of AGE and ODQ was $\sim 18\%$ in ADP-activated platelets ($P < 0.05$), whereas the presence of SQ22536 led to a significant reduction in intraplatelet cAMP concentrations of $\sim 75\%$ ($P < 0.001$) when compared with the concentrations observed in ADP-activated AGE platelets (Figure 2B). This reduction was $\sim 53\%$ when platelets were activated with ADP in the presence of AGE, ODQ, and SQ22536 ($P < 0.001$) (Figure 2B). However, as with cGMP, there was no significant interaction between ODQ and SQ22536 when compared with their combined effect on the lowering of cAMP in ADP-activated platelets ($P > 0.05$) (Figure 2B).

Effect of AGE on platelet shape change and actin structure. When platelets were stimulated with ADP, they aggregated and formed a clot by linking to each other, and an increase in filopodia was observed (Figure 3A). In the presence of AGE, a decrease in platelet aggregation was observed, as

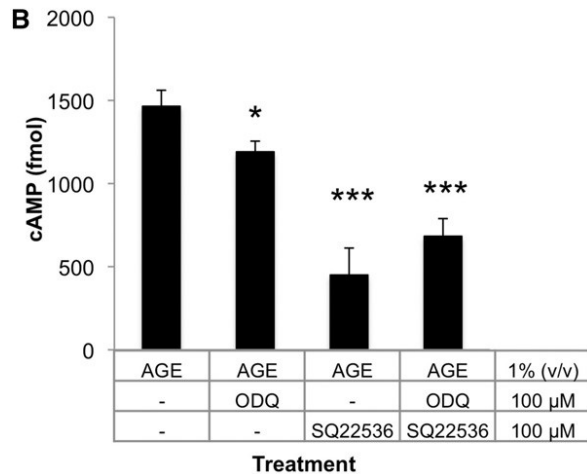
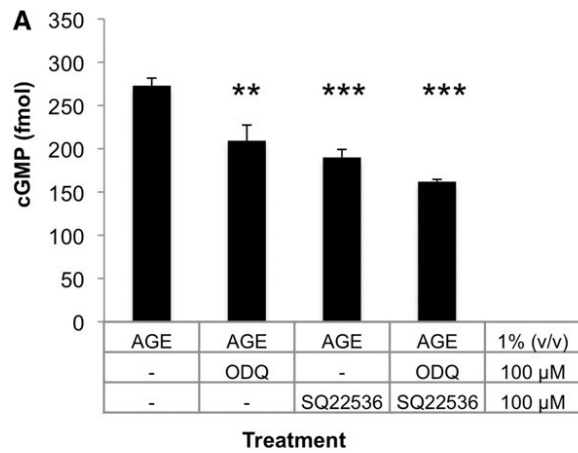


FIGURE 2 Effects of ODQ and SQ22536 and their combination on cGMP (A) and cAMP (B) in AGE-challenged, ADP-activated human platelets. Platelets were treated with IBMX and incubated separately with ODQ and SQ22536 or their combination and then challenged with 1% AGE (vol:vol) followed by ADP activation. cGMP and cAMP concentrations were then determined. Values are means \pm SEMs, $n = 6$ independent samples. ****Different from AGE alone: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. AGE, aged garlic extract; cGMP, cyclic guanosine-5'-monophosphate; IBMX, 3-isobutyl-1-methylxanthine; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; SQ22536, 9-(tetrahydro-2-furanyl)-9H-purin-6-amine; v/v, vol:vol.

evidenced by a decrease in filopodia and hence a decrease in clot formation (Figure 3B). On ADP-induced platelet aggregation, the platelet cytoskeleton reorganized and a change in actin was clearly observed (Figure 3C); the presence of AGE prevented this shape change and actin stayed mainly unaffected (Figure 3D).

Effect of AGE on platelet adhesion to fibrinogen. AGE significantly reduced platelet adhesion to fibrinogen ($P < 0.001$), and a significant interaction between AGE and adhesion to fibrinogen was also apparent. When platelets were activated with ADP in the presence of AGE at a concentration of 1.56% and 3.12% (vol:vol), a significant decrease in binding to fibrinogen occurred as judged by a decrease in OD at 540 nm ($P < 0.05$). This decrease was more marked at a concentration of 6.25% (vol:vol) AGE ($P < 0.001$) (Figure 4). In the presence of GR144053, a significant reduction in the binding of platelets to fibrinogen was also observed ($P < 0.001$) (Figure 4). The presence of AGE resulted in the inhibition of platelets binding to fibrinogen, supporting the data presented in Figure 3; hence, it is

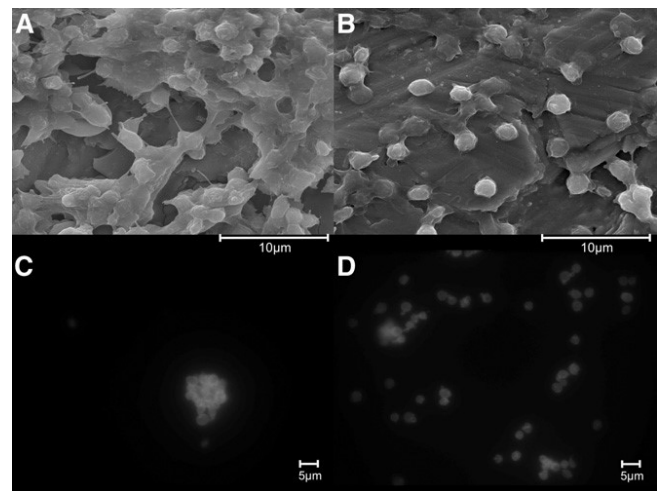


FIGURE 3 Scanning electron microscope images of human platelet aggregation in ADP-activated platelets (A) and AGE-challenged, ADP-activated platelets (B). Fluorescence-labeled platelet actin in ADP-activated platelets (C) and in AGE-challenged, ADP-activated platelets (D). Panels C and D are at $\times 100$ original magnification. Images are representative of 3 separate experiments. AGE, aged garlic extract.

likely that AGE prevented the formation of platelet aggregates by inhibiting their binding to fibrinogen (Figure 4).

Discussion

Cardiovascular disease is characterized by many risk factors, including an increase in platelet aggregation, which involves multiple pathways. The resting platelets are of discoid shape and, on aggregation, become spherical, extending their long, spiky pseudopods, which adhere to each other and lead to the formation of the hemostatic plug (11). The plasma membrane of platelets contains receptors to which agonists such as ADP can bind and induce platelet aggregation. Once this binding has taken place, a transient increase in free cytoplasmic calcium takes place with a concomitant inhibition of AC activity, followed by an increase in thromboxane A_2 formation and activation of the fibrinogen GPIIb/IIIa receptor (25, 26). In addition, platelet function is modulated by lipoxygenase metabolites, PKC, cAMP, cGMP, and NO (11, 13, 27). It has been previously reported that AGE inhibits platelet aggregation both in vitro and in vivo and may do so by altering intracellular messengers such as cAMP and by decreasing the affinity of binding of GPIIb/IIIa to fibrinogen (13, 18). This study was undertaken to further explore the mechanisms by which AGE inhibits platelet aggregation in vitro.

When platelets were stimulated with ADP in the presence of AGE, inhibition of platelet aggregation was observed. The range of AGE used in this study was between 0.19% and 6.25% (vol:vol), and inhibition of platelet aggregation was observed at all concentrations of AGE when compared with the ADP-activated control (Figure 1A). This is in agreement with our previous study in which AGE at a concentration of 10% (vol:vol) caused maximal inhibition of platelet aggregation. Platelets possess NO, which stimulates guanylyl cyclase to form cGMP, resulting in the inhibition of platelet aggregation (28). Because the effect of AGE on intraplatelet cGMP is unknown, platelet aggregation was induced by ADP in the presence of Sin-1, an NO donor that induces cGMP via sGC. When platelet aggregation was initiated in the presence of 8 μ M Sin-1, inhibition of platelet aggregation of $\sim 32\%$ was observed when compared with the ADP-activated control. AGE with and without Sin-1 displayed a

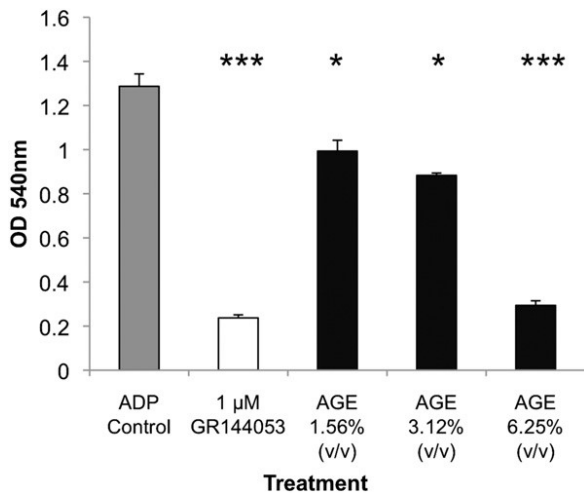


FIGURE 4 Effect of AGE on binding of human platelets to fibrinogen. The control represents ADP-activated PBS platelets (positive control), whereas platelets stimulated with ADP in the presence of GR144053 represent the negative control (GR144053). Decreased release of Rose Bengal absorbance at 540 nm indicates a decrease in platelet binding to fibrinogen. Values are means \pm SEMs, $n = 6$ independent samples. ****Different from ADP control: * $P \leq 0.05$, *** $P \leq 0.001$. AGE, aged garlic extract; GR144053, 4-(4-(aminoiminomethyl) phenyl)-1-piperazinyl)-1-piperidineacetic acid trihydrochloride; v/v, vol:vol.

significant interaction on the inhibition of platelet aggregation ($P < 0.001$). The interaction between Sin-1 and AGE on the inhibition of platelet aggregation was also significant ($P < 0.001$) (Figure 1A).

PDE regulates the concentration of platelet cAMP and cGMP; hence, the effect of AGE on platelet aggregation was investigated in the presence of a PDE inhibitor, IBMX (100 μ M) (24). When platelet aggregation was initiated in the presence of IBMX, a reduction of 31% in platelet aggregation was observed when compared with the ADP-activated control (Figure 1B). A significant interaction between AGE with and without IBMX on the inhibition of platelet aggregation was also observed ($P < 0.001$) (Figure 1B). This indicates that AGE may be inhibiting platelet aggregation by increasing either cGMP or cAMP; hence, these nucleotides were measured in platelets stimulated in the presence of ODQ and SQ22536, which are inhibitors of AC and sGC, respectively (21, 23). PDE was also inhibited by IBMX in these experiments in order to measure the maximum amount of induced intraplatelet cyclic nucleotides.

The cGMP concentration in the control platelets subjected to aggregation in the presence of AGE was ~ 263 fmol; in the presence of the sGC inhibitor ODQ, the concentration of cGMP decreased by 20% ($P < 0.01$); and when AC was inhibited by SQ22536, the concentration decreased further, by $\sim 28\%$ ($P < 0.001$) (Figure 2A). When both inhibitors were combined, the decrease in cGMP was $\sim 39\%$ ($P < 0.001$). However, there was no significant interaction between SQ22536 and ODQ on the lowering of cGMP concentrations in ADP-stimulated platelets ($P > 0.05$) (Figure 2A). This indicates that one of the mechanisms by which AGE inhibits platelet aggregation may be by increasing cGMP concentrations, because AGE inhibits platelet aggregation (Figure 1A) and cGMP concentrations are higher in platelet aggregation induced in the presence of AGE when compared with the concentrations in the presence of the 2 inhibitors (Figure 2A).

It was reported that an increase in 1 nucleotide can influence the concentration of other nucleotides (29, 30). Because AGE is

reported to increase cAMP concentrations (13), it is likely that cGMP may influence the concentration of cAMP. Hence, to investigate this further, cAMP was quantified in platelets subjected to aggregation by ADP in the presence of AGE, ODQ, and SQ22536 and their combination (Figure 2B). When platelet aggregation was initiated in the presence of AGE, the concentration of cAMP was measured at ~ 1500 fmol; this was reduced in the presence of ODQ, and the decrease was more prominent in the presence of SQ22536. AGE in the presence of ODQ and/or SQ22536 decreased the cAMP concentrations in ADP-stimulated platelets ($P < 0.01$) (Figure 2B). There was no significant interaction between SQ22536 and ODQ on the lowering of cAMP concentrations in ADP-stimulated platelets ($P > 0.05$). This result confirms the data from cGMP studies that one of the mechanisms by which AGE inhibits platelet aggregation may be via an increase in these 2 nucleotides (Figure 2A) (13).

cGMP can influence cAMP concentrations (22, 29), and it has been suggested that cGMP may control the concentration of cAMP via PDE3 (20, 31) or that cGMP increases cAMP via phosphorylation of PKA and PKG (32, 33). Because the decrease in cAMP is greater than with cGMP, the results indicate that AGE inhibits platelet aggregation by influencing both platelet cyclic nucleotides, with the majority of the mechanism of inhibition attributed to an increase in intraplatelet cAMP.

During platelet activation and aggregation a series of platelet shape changes take place, ultimately leading to the formation of an aggregate. The effect of AGE on this aggregate formation was investigated by imaging the platelets with the use of scanning electron microscopy. Once the platelets are stimulated with ADP, they form a clot within 60 s, and the pseudopods linking to adjacent platelets are clearly visible (Figure 3A). During platelet activation, reorganization of the platelet cytoskeleton and, more specifically, platelet actin takes place. This change in actin reorganization was observed by fixing and permeabilizing the platelets, followed by labeling them with a monoclonal, fluorescently fluorescein isothiocyanate-labeled actin-specific antibody. During platelet aggregation, the actin can be clearly seen in the aggregate form (Figure 3C), confirming the data presented in Figure 3A. In contrast, when AGE is present at a concentration of 5% (vol:vol) during ADP-induced activation, platelet aggregation is inhibited and undergoes only a minimal shape change, and very little pseudopods are visible, which suggests that AGE has prevented the formation of the platelet aggregate (Figure 3B). The presence of AGE also has a minimal effect on the actin shape change, confirming the data in Figure 3B and indicating that AGE may prevent platelet aggregation by inhibiting GPIIb/IIIa receptor and actin reorganization (Figure 3D)

The activation of platelets by ADP is followed by a process termed “inside-out” signaling and leads to a conformational change within the GPIIb/IIIa receptor and increases its affinity to its ligand fibrinogen. This binding of fibrinogen is the last step in the aggregation process in all agonist-induced platelet aggregation (27). Because AGE inhibits ADP-induced platelet aggregation and prevents reorganization of actin, the binding of platelets to fibrinogen in the presence of AGE was investigated by using a simple assay with the use of immobilized fibrinogen. The inhibitor GR144053 prevents fibrinogen binding by inhibiting the fibrinogen receptor GPIIb/IIIa (34) and was used as a negative control, whereas PBS was used as a positive control. When platelets were activated with ADP, binding to fibrinogen was observed as judged by the increase in OD at 540 nm; in contrast, the inhibitor GR144053 reversed this binding of platelets to fibrinogen. The presence of AGE

significantly reduced the adhesion of platelets to fibrinogen ($P < 0.001$) (Figure 4) and there was significant interaction between AGE and fibrinogen adhesion in ADP-activated platelets. The reduction in adhesion of platelets to fibrinogen was similar at 1.56% and 3.12% (vol:vol) of AGE ($P < 0.05$); however, at the higher dose of 6.25% (vol:vol) of AGE, the decrease in adhesion of platelets to fibrinogen was more marked ($P < 0.001$). This is in agreement with the results reported by Allison et al. (13). The NO donor Sin-1 is also reported to inhibit fibrinogen binding, and these results taken together further support the role of AGE in inhibiting platelet aggregation (35). Although this is primarily an in vitro study, we previously showed in vivo that AGE inhibits platelet aggregation when taken daily at a dose of 5 mL (13) and AGE also displays antioxidant properties (6). It is likely that AGE exerts its inhibitory effect on platelet aggregation in vivo by mechanisms similar to those reported here.

In conclusion, AGE inhibits platelet aggregation by increasing cAMP and cGMP, probably via stimulation of sGC and AC and inhibition of PDE activity, with an increase in cAMP being the dominant pathway. Furthermore, AGE inhibits platelet shape change and inhibits the binding of GPIIb/IIIa receptor to fibrinogen and may have a role in reducing the risk of thrombotic episodes in individuals with cardiovascular disease.

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SS conducted the research; KR and GML wrote the manuscript; and KR had primary responsibility for the final content. All authors read and approved the final manuscript.

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