DOI:10.1021/jf101518p

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Antiplatelet Activity of α-Lipoic Acid

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α-Lipoic acid (ALA) is often used as a dietary supplement to prevent and treat chronic diseases associated with excessive oxidative stress. The aim of this study was to investigate the mechanisms of the antiplatelet activity of ALA. ALA significantly inhibited collagen-induced platelet aggregation, thromboxane B_2 (TXB_2) formation, Ca^{2+} mobilization, and protein kinase Cα (PKCα) activation, but ALA itself increased cyclic AMP formation in rabbit washed platelets. However, the effects of ALA on the above platelet responses were markedly reversed by the addition of 2′5′-ddAdo, an adenylate cyclase inhibitor. Additionally, increased reactive oxygen species (ROS) formation and cyclooxygenase-1 activity stimulated by arachidonic acid were inhibited by ALA. In conclusion, we demonstrated that ALA possesses an antiplatelet activity, which may be associated with an elevation of cyclic AMP formation, involving subsequent inhibition of TXA_2, Ca^{2+} mobilization, and PKCα-mediated pathways. Moreover, inhibition of ROS formation and increase of platelet membrane fluidity may also involve its actions.

KEYWORDS: α-Lipoic acid; platelet aggregation; thromboxane A_2; cyclic AMP; Ca^{2+} mobilization

INTRODUCTION

Increasing evidence indicates that platelet adherence and aggregation are involved in intraluminal thrombosis. Hyperactivity of platelet aggregation often observed in patients with cardiovascular diseases plays a critical role in the initiation and pathogenesis of the atherothrombotic process (*1*). Clinical trials have reported that antiplatelet therapy exhibits a beneficial effect in preventing and reducing the incidence of stroke in these patients (*2*, *3*). Platelet activation is a result of a complex signal transduction cascade reaction mediated by various constituents (*4*). When platelets are activated, arachidonic acid (AA) is released and is further converted to thromboxane A_2 (TXA_2) through the actions of cyclooxygenase (COX) and thromboxane synthase. TXA_2, a major metabolite of AA in platelets, is a potent inducer that activates platelet function (*5*). In addition, the rise of intracellular calcium level ($[Ca^{2+}]_i$) and reactive oxygen species (ROS) formation stimulated by agonists also act as essential factors for platelet activation (*6*). However, cyclic AMP, an endogenous negative regulator of platelet responses, inhibits adhesion, aggregation, release of granule contents, production of TXA_2, and intracellular Ca^{2+} mobilization of platelets upon stimulation (*7*). Several studies have shown that several cyclic AMP-elevating agents exhibit antiplatelet activity (*8*, *9*).

α-Lipoic acid (ALA), a thiol compound (**Figure 1**), occurs naturally in the human diet, animal tissues, and plants. ALA and its reduced thiol form, dihydrolipoic acid (DHLA), are powerful antioxidants by scavenging free radicals, chelating metal ions, and regenerating endogenous and exogenous antioxidants, such as ubiquinone, glutathione, and ascorbic acid (*10*). Currently, ALA is used as a dietary supplement to prevent and treat chronic and vascular diseases including atherosclerosis, thrombosis, and diabetes (*11*−*13*). Previous studies have reported that ALA increased cyclic AMP production in T lymphocytes and NK cells and that dietary ALA supplementation decreased platelet $[Ca^{2+}]_i$ in spontaneously hypertensive rats (*14*, *15*), suggesting that ALA may have the potential to inhibit platelet aggregation. However, whether ALA exerts antiplatelet activity is still unknown. Thus, the objective of this study was to investigate the antiplatelet activity of ALA and further study the underlying mechanisms involved.

MATERIALS AND METHODS

Materials. Racemic RS(±)-ALA, collagen (type 1, equine tendon), AA, prostaglandin H_2 (PGH_2), 2′,7′-dichlorfluorescein-diacetate (DCFH-DA), Phorbol 12,13- dibutyrate (PDBu), and nitroblue tetrazolium (NBT) were purchased from Sigma Chemical Company (St. Louis, MO, USA). 2′5′-Dideoxy-adeonsine (2′5′ddAdo) was purchased from Biomol Company (Plymouth Meeting, PA, USA). Thromboxane B_2 (TXB_2), prostaglandin E_2 (PGE_2), cyclic AMP, cyclic GMP EIA kits, and COX inhibitor screening assay kits were purchased from Cayman Chemical Company, (Ann Arbor, MI, USA). Protein kinase Cα (PKCα) and phospho-PKCα antibodies were purchased from Upstate Biotechnology (Lake Placid, NY, USA). ALA was first dissolved in an aqueous alkaline solution (1 N NaOH) followed by the addition of Tyrode solution, and the pH was neutralized with 1 N HCl.

Platelet Aggregation. The present study was approved by the Ethical Committee of Animal Experiments, National Defense Medical Center. Animals were housed in a standard environment and maintained on tap water and rabbit food ad libitum throughout the investigation. Blood was withdrawn from rabbit marginal veins, mixed with anticoagulant and EDTA (100 mM, 14:1 v/v), and centrifuged at 160*g*, 25 °C for 10 min to obtain platelet rich plasma (PRP). Then, platelet suspension was prepared

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 Published on Web 07/20/2010

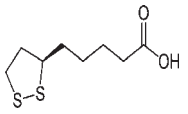

Figure 1. Structure of α-lipoic acid (ALA).

from the PRP according to the washing procedures described previously (*16*). The platelet pellets were finally suspended in Tyrode's solution containing $CaCl_2$ (1 mM), NaCl (136.8 mM), KCl (2.7 mM), $NaHCO_3$ (11.9 mM), $MgCl_2$ (2.1 mM), NaH_2PO_4 (0.4 mM), glucose (10 mM), and bovine serum albumin (0.35%). Platelet concentration was counted by a Coulter counter (Model ZM) and adjusted to 3.0×10^8 platelets/mL. Platelet aggregation was measured turbidimetrically at 37 °C with constant stirring at 1000 rpm by using an aggregometer (Model 560, Chrono-Log Corporation, Havertown, PA, USA). The absorbance of Tyrode's solution was assigned as 100% aggregation and the absorbance of platelet suspension as 0% aggregation. Platelet suspensions (0.4 mL) were preincubated with drugs or an isovolumetric solvent control (Tyrode solution) for 3 min before the addition of collagen (10 μg/mL) or AA (100 μM), and the reaction was allowed to proceed for 6 min. The extent of platelet aggregation was evaluated by measuring the peak height of the aggregation curves. Data were expressed as the percentage of maximal aggregation.

Lactate Dehydrogenase Assay. Level of lactate dehydrogenase (LDH), acting as an index of platelet damage, was measured. Briefly, platelets were preincubated with ALA (200−800 μM) for 10 min followed by centrifugation at 10,000*g* for 5 min. Then, the supernatant was incubated with phosphate buffer, containing 0.2 mg of β-NADPH for 20 min at room temperature, followed by the addition of 100 μL of pyruvate solution, and the absorbance wavelength was read at 340 nm using an ultraviolet visible recording sepctrophotometer (SUV-2120, Scinco, Seoul, Korea). LDH released was compared with the total LDH activity of platelets dissolved in 0.1% Triton X-100.

Measurement of Levels of Cyclic AMP, Cyclic GMP, and TXB_2. Platelet suspensions (3×10^8/mL) were incubated with drugs or solvent control at 37 °C for 3 min, and the reaction was stopped by adding 10 mM EDTA and immediately boiling for 5 min. After centrifugation at 10,000*g* for 5 min, the levels of cyclic AMP and cyclic GMP in the supernatants were determined by enzyme-linked immunosorbent assay (ELISA) kits. For the determination of TXB_2 formation, platelets were preincubated with drugs or solvent control at 37 °C for 3 min before the addition of agonists for 6 min. Then, 2 mM EDTA and 50 μM indomethacin were added to the suspensions, and the levels of TXB_2 in the supernatants were measured by using an ELISA kit.

Assay of COX-1 Activity. The COX activity was determined according to the instructions in the COX inhibitor screening assay kit. Briefly, ALA or aspirin (200 μM, as positive control) was mixed with COX-1 enzyme for 10 min as provided by this kit. Then, AA (100 μM) was added and incubated for 2 min, followed by the addition of 0.1 N HCl and saturated stannous fluoride solution. The level of PGE_2 was measured with the ELISA kit.

Assay of Thromboxane Synthase Activity. Platelets were pretreated with ALA or solvent control for 3 min before PGH_2 (5 μM) was added and incubated for another 6 min, followed by immediate boiling for 5 min, and centrifugation at 10,000*g* for 5 min. The levels of TXB_2, reflecting the thromboxane synthase activity, in the supernatant were measured with the ELISA kit.

Measurement of Platelet Intracellular Ca^{2+} Mobilization. One milliter of PRP (3.0×10^8 platelets/mL) was incubated with Fluo-4 AM (5 μM) for 30 min at 37 °C in the dark followed by centrifugation at 500*g* for 10 min. Then, the pellets were suspended in 2 mL of Tyrode solution. The fluorescence intensity of 20,000 platelets per sample was analyzed using a flow cytometer (FACScan, Becton Dickinson, Heidelberg, Germany) as described previously (*17*).

Measurement of ROS Production. Washed platelets were preincubated with 10 μM DCFH-DA for 30 min at 37 °C in dark. After washing, 240 μL of platelets (2×10^8/mL) was added to 96-well plates containing 5 μL of various concentrations of ALA or solvent control and incubated for 3 min, followed by the addition of AA for another 6 min. The emitted 2′,7′-dichlorfluorescein DCF fluorescence was measured at excitation and emission wavelengths of 485 and 535 nm, respectively, with a Fluoroskan Ascent FL (Thermo Electron Co. Vantaa, Finland). For the measurement of O_2^- production, platelets incubated with ALA or solvent control at 37 °C for 3 min were added by NBT (2 mg/mL) and AA (100 μM) for 1.5 h. Then, the platelet suspension was centrifuged at 10,000*g* for 5 min to collect the pellets and resolved in 5% Triton-X 100. The absorbance at 560 nm was detected with a spectrometer.

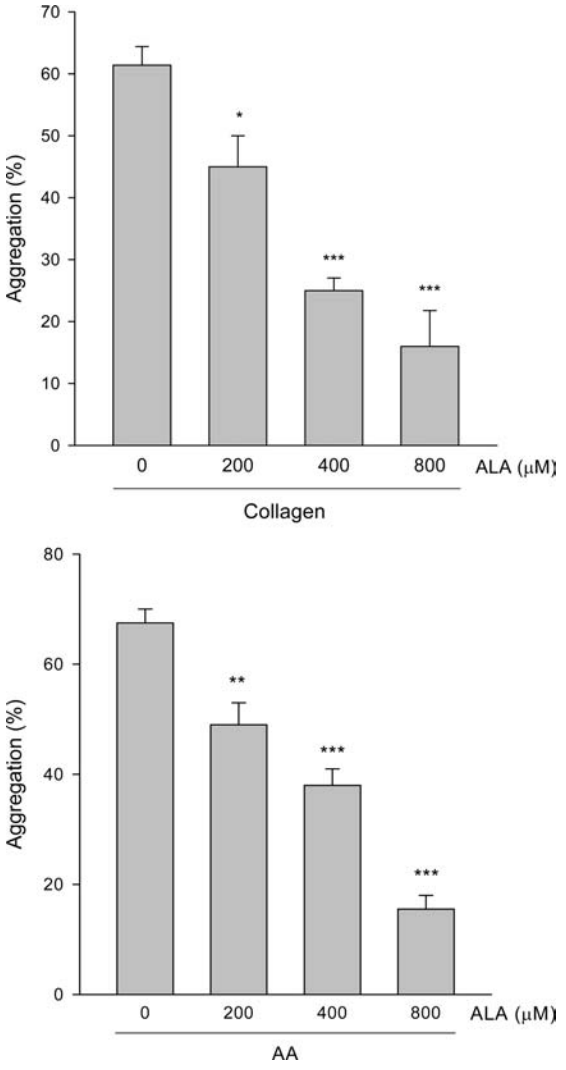

Figure 2. Effect of ALA on collagen or AA-induced platelet aggregation. Washed rabbit platelets were preincubated with Tyrode solution or ALA (200−800 μM) for 3 min, then collagen (10 μg/mL) or AA (100 μM) was added to trigger platelet aggregation. Platelet aggregation is expressed as the percentage of maximal aggregation. Data are expressed as the mean ± SEM ($n = 5$). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ as compared to the respective agonist-stimulated alone platelets.

Assay of PKCα Activation. Platelets (3×10^8/mL) were incubated with drugs for 3 min at 37 °C followed by the addition of collagen (10 μg/mL) for 1 to 10 min. Then, samples were lysed, and the subcellular extracts (20 μg) were collected at specific times and separated in 8% sodium dodecyl sulfate (SDS)−polyacrylamide gels; the proteins were electrotransferred by semidry transfer (Bio-Rad, CA, USA). Blots were blocked with TBST (10 mM Tris-base and 0.01% Tween 20) containing 1% (w/v) skim milk for 30 min and probed with various primary antibodies. Then, the membranes were incubated with horseradish peroxidase-conjugated anti-IgG or anti-IgG (diluted 1;3000 in TBST) for 1 h, and the immunoreative bands were detected by chemiluminescence (ECL) reagent (Amersham International Plc., Buckinghamshire, UK).

Analysis of Membrane Fluidity. The platelet membrane fluidity was evaluated according to our previous study (*18*). Briefly, platelets were preincubated with ALA for 3 min, followed by the addition of 1 μM DPH

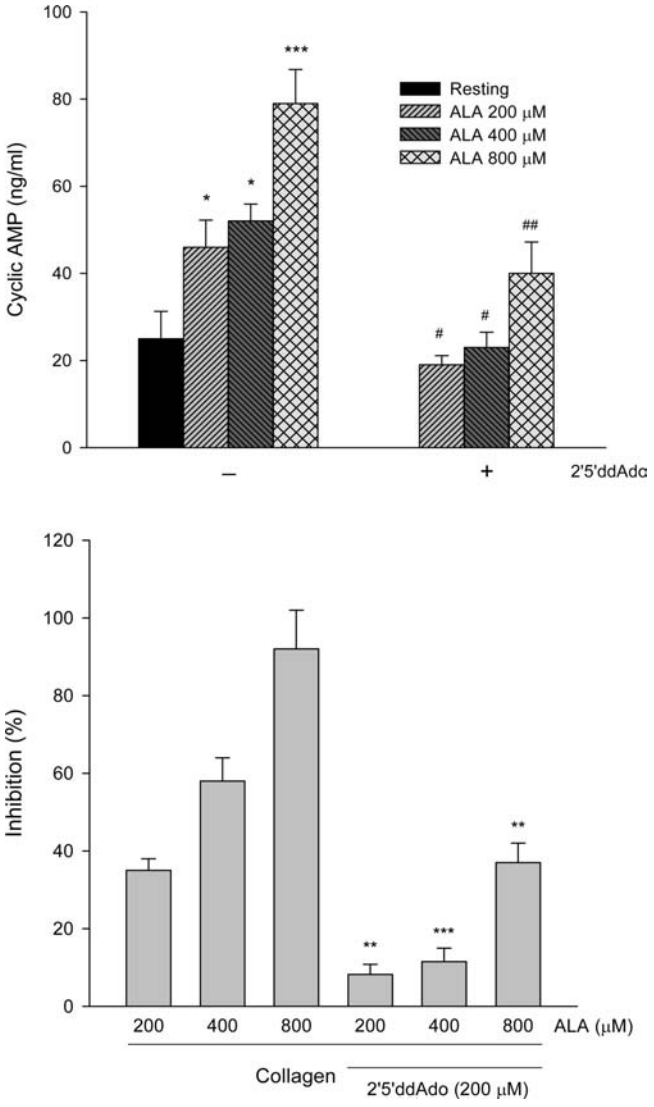

Figure 3. Effect of ALA or ALA in combination with 2′5′ddAdo on cyclic AMP formation and platelet aggregation. Washed platelets were preincubated with Tyrode solution, ALA, or ALA in combination with 2′5′ddAdo (200 μM) at 37 °C for 3 min, then the level of cyclic AMP (upper) or the platelet aggregation induced by collagen (bottom) was measured. Data are expressed as the mean ± SEM (n = 5). In cyclic AMP tests, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ as compared to the resting platelets; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ as compared to the respective ALA-treated alone platelets. In platelet aggregation tests, $^{**}P < 0.01$ and $^{***}P < 0.001$ as compared to the respective ALA + collagen-treated alone platelets.

for 6 min. The fluorescence intensity was measured by a fluorescence spectrophotometer (RF-5301PC, Shimadzu, Kyoto, Japan). The lower fluorescence intensity indicates the higher membrane fluidity.

Statistical Analysis. The experimental data were expressed as the mean ± SEM. One-way ANOVA with post hoc Bonferroni test was used for statistical analysis. Results were considered significantly difference at a value of $P < 0.05$.

RESULTS

Effect of ALA on Platelet Aggregation and Cyclic Nucleotide Formation. ALA dose-dependently inhibited platelet aggregation induced by collagen or AA (**Figure 2**). The antiplatelet activity of ALA was not due to its cytotoxicity because no evident LDH release was observed (data not shown). In addition, ALA itself significantly increased cyclic AMP formation, but the increase was markedly reversed by the addition of 2′5′ddAdo, an inhibitor

Table 1. Effect of ALA or ALA in Combination with 2′5′ddAdo on TXB_2 Formation Stimulated by Collagen or AA[a]

	TXB_2 (ng/mL) formation stimulated by	
treatment	collagen (10 μg/mL)	AA (100 μM)
resting	0.25 ± 0.02	0.26 ± 0.03
control	26.63 ± 5.24	55.62 ± 4.97
ALA (200 μM)	11.31 ± 1.80*	33.91 ± 3.85*
ALA (400 μM)	4.12 ± 0.12**	17.36 ± 1.92**
ALA (800 μM)	1.67 ± 0.10***	8.34 ± 0.26***
ALA (200 μM) + 2′5′ddAdo (200 μM)	15.4 ± 1.25	34.53 ± 2.14
ALA (400 μM) + 2′5′ddAdo (200 μM)	6.33 ± 0.51#	19.77 ± 2.75
ALA (800 μM) + 2′5′ddAdo (200 μM)	2.56 ± 0.04#	7.11 ± 0.63

[a] Washed platelets were preincubated with Tyrode solution, ALA, or ALA + 2′5′ddAdo for 3 min at 37 °C, then collagen or AA was added for another 6 min to trigger TXB_2 formation. Data were expressed as the mean ± SEM (n = 5). * <0.05, $^{**}P < 0.01$, and $^{***}P < 0.001$ as compared to that of respective the control group. $^{\#}P <$ 0.05 as compared to that of the respective collagen-treated alone platelets.

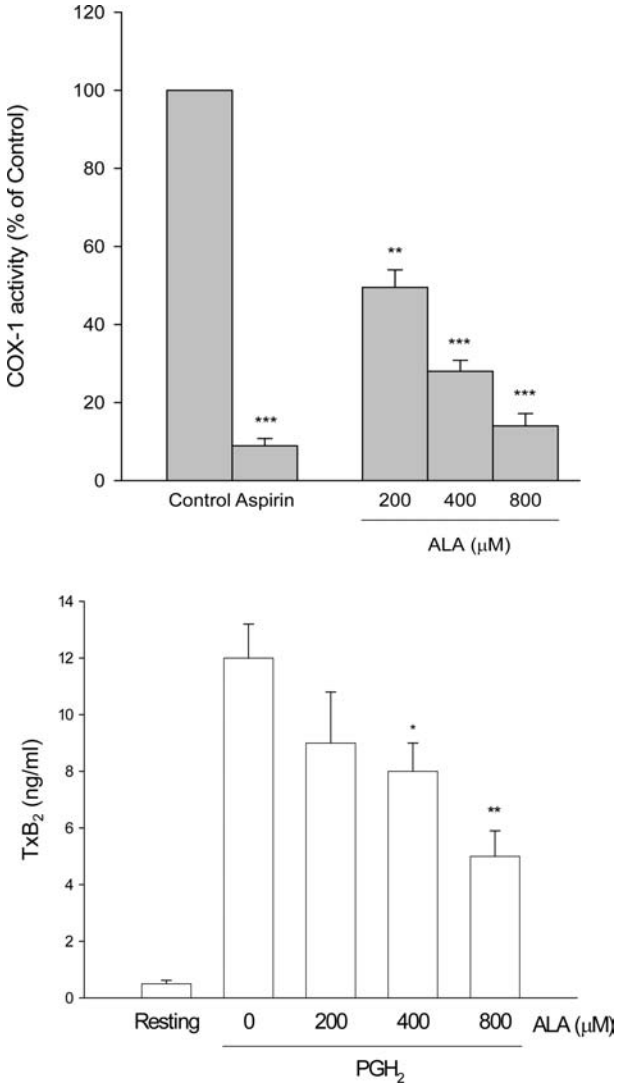

Figure 4. Effect of ALA on COX-1 and thromboxane synthase activity. In the upper panel, ALA (200−800 μM) or aspirin (200 μM, as positive control) was mixed with COX-1 enzyme for 10 min followed by the addition of AA (100 μM) for 2 min, and the levels of PGE_2 were measured to reflect the COX-1 activity. The AA-treated alone platelets acted as a control group. $^{**}P < 0.01$ and $^{***}P <$ 0.001 as compared to the control group. In bottom panel, washed platelets were preincubated with ALA for 3 min at 37 °C, then PGH_2 (5 μM) was added to trigger TXB_2 formation. Data are expressed as the mean ± SEM (n = 5). $^{*}P < 0.05$ and $^{**}P < 0.01$ as compared to PGH_2-treated alone platelets.

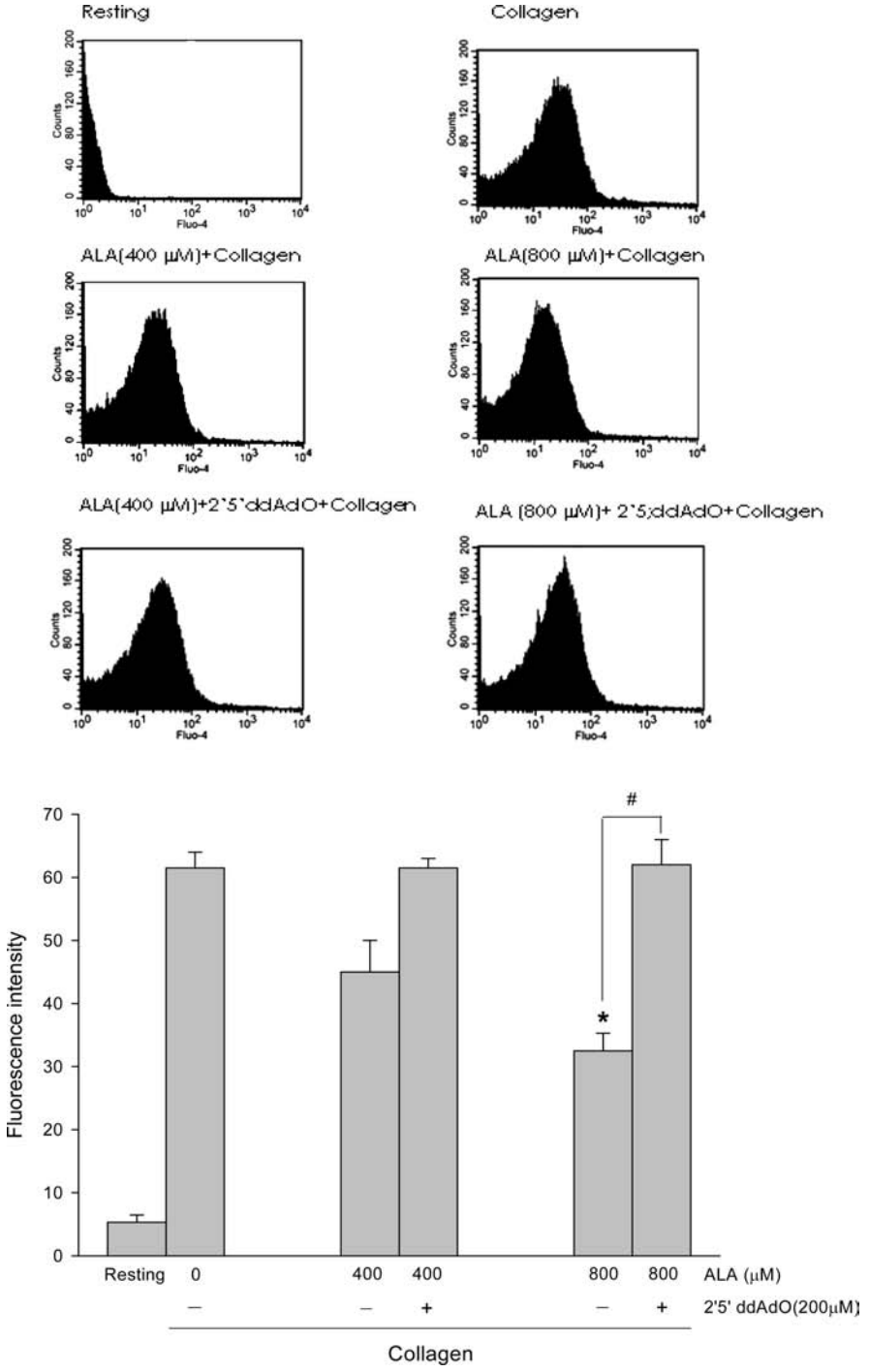

Figure 5. Effect of ALA or ALA in combination with 2′5′ddAdo on intracellular Ca^{2+} mobilization stimulated by collagen. Fluo-4-loaded platelets were preincubated with Tyrode solution, ALA, or ALA in combination with 2′5′ddAdo at 37 °C for 3 min, then collagen was added to evoke Ca^{2+} mobilization. Fluorescence intensity is presented as the mean ± SEM (n = 5). $^{*}P < 0.05$ as compared to collagen-treated alone platelets. $^{\#}P < 0.05$ as compared to respective ALA + collagen-treated alone platelets.

of adenylate cyclase (**Figure 3**). In the presence of 2′5′ddAdo, only the inhibitory effect of ALA on collagen-induced platelet aggregation was markedly reversed (**Figure 3**). However, ALA did not affect the cyclic GMP level (data not shown).

Effect of ALA on TXB_2 Production and COX Activity. AA or collagen induced-TXB_2 formation, a stable metabolite of TXA_2, was markedly inhibited by ALA. Similarly, in the presence of 2′5′-ddAdo, only the inhibitory effect of ALA on collagen-induced TXB_2 formation was reversed (**Table 1**). Additionally, ALA suppressed COX-1 activity compared to that of AA-stimulated alone platelets (**Figure 4**), suggesting that ALA is a COX inhibitor. Moreover, a lower inhibition of PGH_2-induced TXB_2 formation by ALA was found, indicating that ALA may also inhibit thromboxane synthase activity especially at higher concentrations of ALA (400, 800 μM). In resting platelets, ALA itself did not affect the basal TXB_2 level (data not shown).

Effect of ALA on Ca^{2+} Mobilization. In Fluo-4-loaded platelets, ALA suppressed the rise of platelet Ca^{2+} mobilization evoked by collagen, which was reversed by the addition of 2′5′-ddAdo (**Figure 5**). In addition, the inhibitory potency of ALA on Ca^{2+} mobilization was similar in the presence of external Ca^{2+} (1 mM) or in the Ca^{2+}-free solution (data not shown).

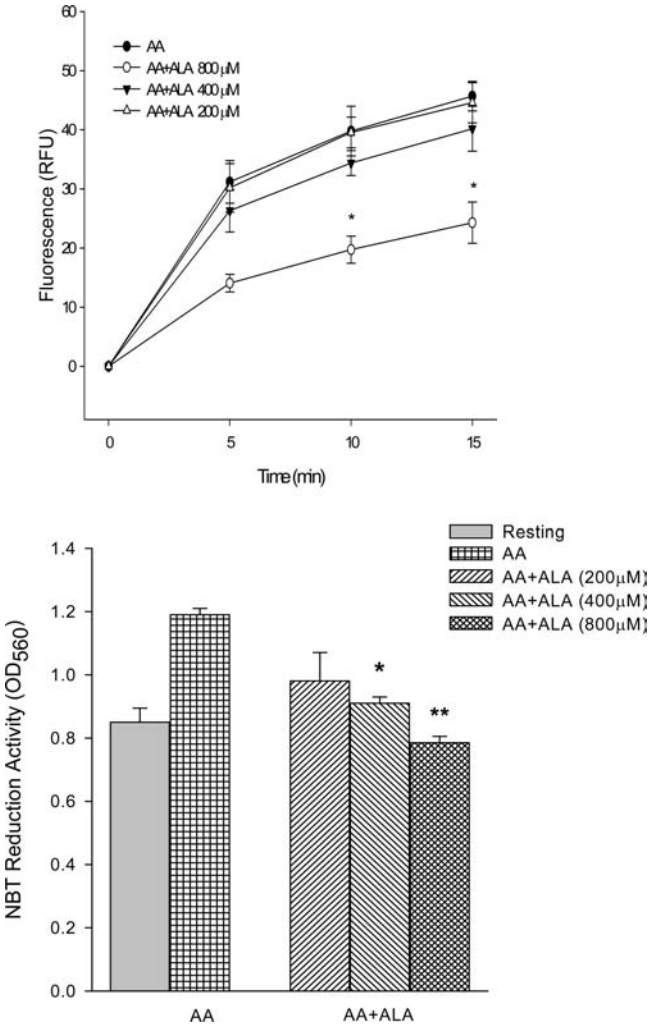

Figure 6. Effect of ALA on AA-induced ROS formation. In the upper panel, DCFH-DA-labeled platelets were pretreated with Tyrode solution or ALA for 3 min, followed by the addition of AA. The DCF fluorescence in platelets was measured. In the bottom panel, platelets were preincubated with ALA or solvent control at 37 °C for 3 min, followed by the addition of NBT (2 mg/mL) and AA (100 μM) for 1.5 h. Then, the absorbance at 560 nm was measured. Data are expressed as the mean ± SEM ($n = 5$). *$P < 0.05$ and **$P < 0.01$ as compared to AA-treated alone platelets.

Effect of ALA on ROS Formation. ALA (800 μM) significantly inhibited AA-induced H_2O_2 formation reflected by a decrease of DCF fluorescence from 46 to 22 relative fluorescent units (RFU) at 15 min compared to that of AA-stimulated alone platelets (**Figure 6**). In addition, the AA-induced O_2^- production reflected by NBT reduction activity was also reduced by ALA (**Figure 6**). However, the addition of 2′5′-ddAdo did not affect the inhibition of ALA on AA-induced ROS formation (data not shown). However, exposure of platelets with collagen showed no significant changes in ROS formation compared to that of resting platelets (data not shown).

Effect of ALA on PKCα Activation. In resting or collagen-activated platelets, the PKCα activation evaluated by the phosphorylation of PKCα was inhibited by ALA in a time-dependent manner, but the total PKCα was unchanged. Similarly, the addition of 2′5′-ddAdo markedly reversed the attenuation of collagen-induced PKCα activation by ALA at 10 min (**Figure 7A**,**B**). However, ALA did not affect the PDBu-induced platelet aggregation and PKCα phosphorylation, suggesting that ALA has no direct inhibition on PKCα activation (**Figure 7C**,**D**).

Effect of ALA on Platelet Membrane Fluidity. Alteration of platelet membrane fluidity was evaluated by the change of the intensity of fluorescence in DPH-labeled platelets. ALA dose-dependently reduced DPH-related fluorescence intensity (**Figure 8**), indicating that ALA increased the platelet membrane fluidity.

DISCUSSION

Under pathological conditions, endothelial cells and platelets may become more proadhesive/procoagulant, which leads platelets to adhere to the injured area, and subsequently release a lot of active constituents and induce thrombotic plug formation (*19*). Therefore, antiplatelet therapy is a reasonable strategy to alleviate vascular thrombosis and disorders. Several researchers have shown that several cyclic AMP-elevating agents exert antiplatelet activity through a cyclic AMP-dependent protein kinase (PKA)-mediated signal pathway (*20*). The steady-state level of cyclic AMP is maintained by a balance between the rate of synthesis by adenylate cyclase and the rate of degradation by cyclic AMP phosphodiesterase (PDE). Interestingly, ALA itself significantly increased the cyclic AMP level in platelets, but the increase was markedly reversed by the addition of 2′5′-ddAdo. In addition, a previous study has shown that ALA did not inhibit PDE activity in T lymphocytes and NK cells (*14*). Accordingly, the enhanced cyclic AMP formation by ALA may be mainly through the activation of adenylate cyclase. Moreover, it has been demonstrated that ALA enhances cyclic AMP formation via the activation of the EP2 and EP4 prostanoid receptors by using pharmacological inhibitors and receptor transfection experiments in NK cells (*21*), suggesting that the activation of EP2 and EP4 prostanoid receptors may also be involved in the action of ALA in cyclic AMP production. In the present study, we demonstrate for the first time that ALA (200−800 μM) dose-dependently inhibited platelet aggregation induced by collagen or AA in vitro. In the presence of 2′5′-ddAdo, only the inhibition of collagen-induced platelet aggregation by ALA was reversed but not AA-induced platelet aggregation, indicating that the cyclic AMP-mediated pathway involves the reduction of collagen-induced platelet aggregation caused by ALA. However, ALA did not affect the intracellular cyclic GMP level, suggesting that its antiplatelet activity might be not likely mediated by the elevation of the cyclic GMP level.

The concentrations of ALA used in this study (0.2−0.8 mM) is similar, lower, or higher compared to those used in other in vitro studies, in which various doses of ALA (0.1 to 1 mM) were used for different cell types to investigate its pharmacological effects (*22*−*25*). Accordingly, the dose of ALA required to achieve its effects may be dependent on the type of cells and condition investigated. Pharmacokinetic studies of ALA have reported that after a single orally administered dose of 10 mg/kg body weight, the plasma concentration reaches up to 70 μM, and higher plasma concentrations can be achieved to a level of 100 to 200 μM if ALA (600 mg) is administered intravenously (*26*). Moreover, the half-life of ALA in plasma is short (30 min), suggesting that it is rapidly taken up into tissues or metabolized, and it is therefore possible that higher concentrations may accumulate in target tissues or cells such as platelets. Furthermore, the uptake rate, metabolism, and absorption of ALA may have big differences between in vivo and in vitro conditions, and the concentrations of ALA used in vitro corresponding to clinical doses are difficult to define. Our findings showed that ALA (200 μM) significantly inhibited platelet activation, suggesting that the dose may be within the clinically relevant range. However, further studies are required in humans to confirm the antiplatelet activity and to determine optimum dosage, form of administration, or preferred type of ALA.

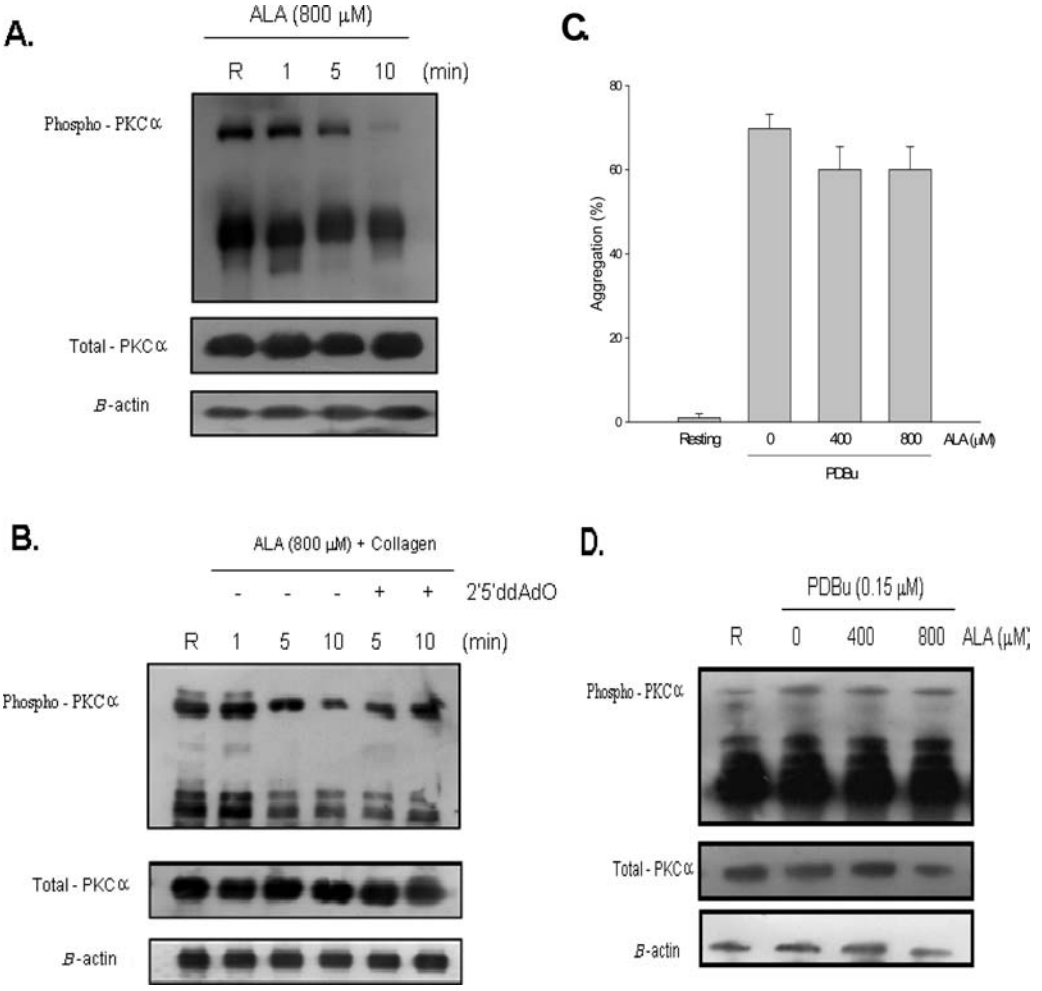

Figure 7. Effect of ALA on PKCα activation and PDBu-induced platelet aggregation. Washed platelets were preincubated with Tyrode solution, ALA, or drugs for 3 min, followed by the addition of solvent (**A**), collagen (**B**), or PDBu (0.15 μM) (**D**). Cells were collected, and subcellular extracts were analyzed for phospho-PKCα and total PKCα as described in Materials and Methods. The profiles are representative examples of four similar experiments. The effect of ALA on PDBu-induced platelet aggregation was also measured as described previously. Data are expressed as the mean ± SEM (n = 5).

Upon platelet activation, AA is released from membrane phospholipids by means of Ca^{2+}-dependent phospholipase A_2, diglyceride lipase, and phosphatidic acid specific phospholipase A_2. Then, AA is converted to TXA_2 by cyclooxygenase-1 (COX-1) and thromboxane synthase and subsequently activates platelet aggregation (*27*). Similarly, in the presence of 2′5′-ddAdo, only the inhibition of ALA on collagen-induced TXB_2 formation was reversed but not in AA-activated platelets, suggesting that the cyclic AMP-mediated pathway involves the attenuation of AA release in collagen-stimulated platelets. This concept was supported by another study demonstrating that cyclic AMP inhibited phospholipase A_2-mediated AA release and subsequent TXB_2 formation (*28*) but did not directly affect COX activity (*29*). Upon AA formation, most AA-derived TXA_2 production in platelets is through the action of COX-1 and thromboxane synthase. Thus, direct inhibition of COX-1 activity and thromboxane synthase activity by ALA may also play a role in the reduction of agonist-induced TXA_2 formation.

When platelets are activated, the increase of $[Ca^{2+}]_i$, as a result of either calcium influx and/or calcium release from intracellular stores, is fundamental to platelet activation. In the presence of extracellular Ca^{2+} (1 mM) or in the Ca^{2+}-free solution, ALA exhibited a similar inhibitory potency in collagen-evoked rise of Ca^{2+} mobilization, implying that the reduction of intracellular Ca^{2+} mobilization may be due to the attenuation of calcium release from intracellular calcium stores. Enhanced cyclic AMP stimulates protein kinase-mediated phosphorylation of ATP-dependent calcium pumps and subsequently removes calcium

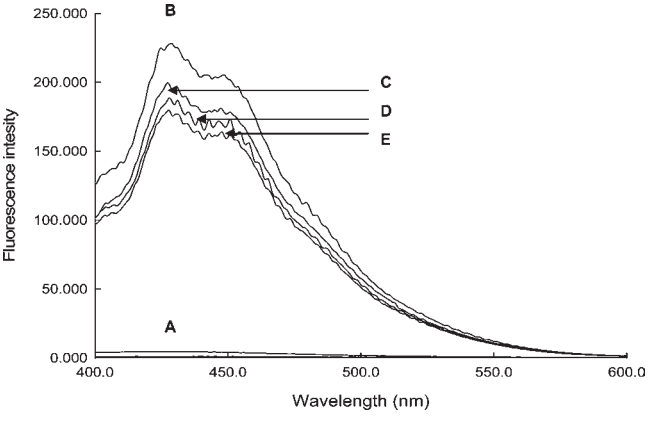

Figure 8. Effect of ALA on platelet membrane fluidity. Fluorescence emission spectra of platelet membranes in the absence (A) or presence of DPH (1 μM) (B). In the presence of ALA (200 μM) (C), ALA (400 μM) (D), or ALA (800 μM) (E), the emission spectra of membranes labeled with DPH are shown. Profiles are representative examples of four similar experiments.

from the cytosol. Also, cyclic AMP inhibits phospholipase C (PLC)-mediated inositol 1,4,5-trisphosphate (IP_3) production (*30*), which leads to the reduction of calcium release from the dense tubular system (*31*). Accordingly, agents that increase cyclic AMP formation may have an ability to inhibit calcium enhancement upon activation. Our data further support the idea that the inhibition of collagen-evoked Ca^{2+} mobilization caused by ALA was significantly reversed by 2′5′-ddAdo, indicating that cyclic AMP plays an important role in the action of ALA in modulating Ca^{2+} mobilization.

Once PLC is activated by agonists, phosphatidylinositol 4,5-bisphosphate in the plasma membrane's inner leaflet is hydrolyzed to IP_3 and diacylglycerol. Importantly, diacylglycerol serves as a stimulator to activate protein kinase C (PKC), an essential mediator for agonist-induced platelet aggregation and granule secretion (*32*). At least seven PKC isoforms (α, β, δ, θ, ε, η, and ζ) are expressed in platelets. Among PKC isoforms, the activation of PKCα through phosphorylation in a Syk-dependent manner is a critical mediator causing platelet activation (*33*−*35*). In the present study, we found that PKCα activation was inhibited by ALA both in resting and collagen-activated platelets. However, the inhibition of PKCα activation was markedly reversed by the addition of 2′5′-ddAdo, suggesting that cyclic AMP accounts for the attenuation of PKCα activation. The concept is confirmed by previous findings that the cyclic nucleotide negatively regulated the activation of PKC (*31*). Moreover, TXA_2 is also an important inducer that activates PKC through the stimulation of a PLC-dependent pathway (*36*, *37*). Thus, the inhibition of TXA_2 formation caused by ALA may also contribute to the reduction of PKCα activation. However, ALA had no direct inhibition on PKC activation, as it did not suppress PDBu (a PKC activator)-induced PKCα phosphorylation and platelet aggregation. Taking these findings together, we propose that ALA initially enhances cyclic AMP formation that subsequently inhibits TXA_2 formation and PLC-mediated Ca^{2+} mobilization and diacylglycerol production, which finally leads to the suppression of PKCα activation.

ROS, including hydrogen peroxide and the superoxide anion released from activated platelets, also induces platelet activation (*38*). In human platelets, it has been demonstrated that AA-induced superoxide anion formation is mainly via PKC-dependent NAD(P)H oxidase activation (*39*). Therefore, it is plausible that the inhibition of AA-induced ROS formation by ALA may be through the reduction of the PKC-mediated process. However, simultaneous addition of 2′5′-ddAdo did not significantly affect the action of ALA in ROS formation, suggesting that cyclic AMP might not directly contribute to its antioxidative activity. Additionally, change in membrane fluidity may affect platelet function, and decreased platelet membrane fluidity (relative rigid membrane) results in the hyperactivity of platelet agonists (*40*). In this study, we found that ALA itself increased platelet membrane fluidity, which may provide another mechanism accounting for its antiplatelet activity. In summary, we demonstrate for the first time that ALA exerts antiplatelet activity through the attenuation of TXA_2 formation, Ca^{2+} mobilization, and PKCα activation, which may be associated with an elevation of cyclic AMP formation. Moreover, suppression of ROS formation and increase of platelet membrane fluidity may be also involved in its effects. Accordingly, ALA may exhibit a therapeutic benefit for platelet hyperaggregation-based diseases.

ABBREVIATIONS USED

ALA, α-lipoic acid; AA, arachidonic acid; PGH_2, prostaglandin H_2; DCFH-DA, 2′,7′-dichlorfluorescein-diacetate; PDBu, Phorbol 12,13- dibutyrate; NBT, nitroblue tetrazolium; 2′5′ddAdo, 2′5′-dideoxy-adeonsine; TXB_2, thromboxane B_2; TXA_2, thromboxane A_2; COX-1, cyclooxygenase-1; PGE_2, prostaglandin E_2; PKCα, protein kinase Cα; ROS, reactive oxygen species; PLC, phospholipase C; PDE, phosphodiesterase; PKA, cyclic AMP-dependent protein kinase; LDH, lactate dehydrogenase; SDS, sodium dodecyl sulfate.

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Received for review April 23, 2010. Revised manuscript received June 18, 2010. Accepted June 21, 2010. This study was partially supported by a research grant from the National Science Council of Taiwan, Republic of China (NSC 90-2315-B016-006).