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Inhibitory Effect of α-Lipoic Acid on Platelet Aggregation Is Mediated by PPARs

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ABSTRACT: Peroxisome proliferator-activated receptors (PPARs) isoforms (α, β/δ, and γ are present in human platelets, and activation of PPARs inhibits platelet aggregation. α-Lipoic acid (ALA), occurring naturally in human food, has been reported to exhibit an antiplatelet activity. However, the mechanisms underlying ALA-mediated inhibition of platelet aggregation remain unknown. The aim of this study was to investigate whether the antiplatelet activity of ALA is mediated by PPARs. ALA itself significantly induced PPARα/γ activation in platelets and increased intracellular amounts of PPARα/γ by blocking PPARα/γ secretion from arachidonic acid (AA)-activated platelets. Moreover, ALA significantly inhibited AA-induced platelet aggregation, Ca^{2+} mobilization, and cyclooxygenase-1 (COX-1) activity, but increased cyclic AMP production in rabbit washed platelets. Importantly, ALA also enhanced interaction of PPARα/γ with protein kinase Cα (PKCα) and COX-1 accompanied by an inhibition of PKCα activity in resting and AA-activated platelets. However, the above effects of ALA on platelets were markedly reversed by simultaneous addition of selective PPARα antagonist (GW6471) or PPARγ antagonist (GW9662). Taken together, the present study provides a novel mechanism by which ALA inhibition of platelet aggregation is mediated by PPARα/γ-dependent processes, which involve interaction with PKCα and COX-1, increase of cyclic AMP formation, and inhibition of intracellular Ca^{2+} mobilization.

KEYWORDS: α-lipoic acid, peroxisome proliferator-activated receptors (PPARs), platelet aggregation, protein kinase Cα

INTRODUCTION

Originally, platelets were viewed as the main cells responsible for hemostasis. Currently, several studies have demonstrated that platelets also play an important role in regulation of immune cell activation and inflammation via production of proinflammatory and bioactive mediators.[1] It is well-known that platelet hyperactivity is implicated in the pathogenesis of thrombus formation and vascular diseases,[2,3] suggesting that treatment with antiplatelet drugs may be a beneficial strategy to prevent and treat vascular thrombosis and inflammatory diseases.[4,5] Although platelets are anuclear cells released from megakaryocytes, they also contain transcription factors, notably the peroxisome proliferator-activated receptors (PPARs). PPARs, members of ligand-activated transcription factors, are known to modulate several important biological effects, including lipid, energy metabolism, inflammation, and thrombosis associated with atherosclerosis.[1,6,7] Exposure to PPAR agonists leads to PPARs heterodimerized with retinoid X receptor followed by binding to peroxisome proliferator response element in the promoter of target genes, which subsequently modulates gene expression.[8] There are three PPAR isoforms (α, β/δ, and γ) found in human platelets, and activation of PPARs results in an inhibition of platelet activation through a nongenomic mechanism.[9] Recent research has further demonstrated that antiplatelet actions of simvasatin and fenofibrate are mediated by activation of PPARγ and PPARα, respectively,[10] suggesting that drugs with a PPAR-activating effect may exhibit an antiplatelet activity.

α-Lipoic acid (ALA), a thiol compound (Figure 1) occurring naturally in human food, animal tissues, and plants, has been regarded as a powerful antioxidant in scavenging free radicals, chelating metal ions, and regenerating endogenous and exogenous antioxidants.[11] Currently, ALA is often used as a dietary supplement to prevent and treat several vascular diseases including atherosclerosis, thrombosis, and diabetes.[12,13] Our previous study has demonstrated that ALA exerts an antiplatelet activity, at least in part, through elevation of cyclic AMP formation and inhibition of the cyclooxygenase-1 (COX-1)/thromboxane A_2 (TXA_2) pathway.[14] Because it has been reported that ALA is a dual PPARα/γ agonist in CV-1 monkey epithelial cells,[15] we propose that PPAR-mediated processes may account for the actions of ALA. However, whether activation of PPARs-dependent pathway contributes to ALA-mediated antiplatelet activity is still unknown. Here, we clearly demonstrate for the first time that the antiplatelet activity of ALA is mediated by PPARα/γ.

MATERIALS AND METHODS

Materials. Racemic *RS*(±)-ALA, AA, rosiglitazone, and anti-rabbit IgG conjugated to horseradish peroxidase were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The enzyme-linked immunosorbent assay (ELISA) kits of cyclic AMP, and protein antibodies of PPARα, PPARγ, and COX-1 as well as COX-1 inhibitor screening assay kits were purchased from Santa Cruz, Biotechnology, Inc. (Santa Cruz, CA). GW9662, GW6471, and GSK0660 were purchased from Tocris (Avonmouth, U.K.). GW9662, a selective PPARγ antagonist, exhibits an IC_{50} in the nanomolar range for PPARγ binding, and is 10- and

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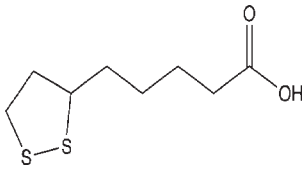

Figure 1. Structure of α-lipoic acid (1,2-dithiolane-3-pentanoic acid).

600-fold less potent in binding PPARα and PPARβ/δ, respectively. GW6471 was designed as a PPARα antagonist by substituting an ethyl amide group for the acid group of GW409544, a potent PPARα agonist, with an IC_{50} of 0.24 μM for GW409544-induced activation of PPARα.[16] GSK0660, a selective PPARβ/δ antagonist, exhibits IC_{50} values of 0.155, >10, and ≥10 μM at PPARβ/δ, PPARα, and PPARγ, respectively.[17] Fluo-4-acetoxymethyl ester (Fluo-4AM) was purchased from Invitrogen Molecular Orobes (Eugene, OR). The PPAR transfactor kit was purchased form Panomics (Redwood City, CA). The pure proteome protein A magnetic beads, PKCα antibody, phospho-PKCα antibody, and ECL reagent were purchased from Upstate Biotechnology (Lake Placid, NY). ALA was first dissolved in an aqueous alkaline solution (1 N NaOH) followed by 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 standard environment and maintained on tap water and rabbit food ad libitum throughout the study. Blood was withdrawn from rabbit marginal vein, mixed with the anticoagulant, EDTA (100 mM, 14:1 v/v), and centrifuged at 160*g* and 25 °C for 10 min to obtain platelet-rich plasma (PRP). Then, platelet suspension was prepared from the PRP according to the washing procedures as previously described.[14] 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 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 Corp., Havertown, PA). The absorbance of Tyrode's solution was assigned as 100% aggregation and the absorbance of platelet suspension as 0% aggregation. Platelet suspensions (0.3 mL) were preincubated with drugs or an isovolumetric solvent control (Tyrode solution) for 3 min before the addition of AA (100 μM) and the reaction was allowed to proceed for 6 min. The platelet aggregation was evaluated by measuring the peak of the aggregation curves. To evaluate the effects of PPAR antagonists on the antiplatelet effect of ALA, the percent inhibitions of platelet aggregation of various agents were presented based on the AA-induced platelet aggregation alone set at 100%.

Lactate Dehydrogenase (LDH) Assay. The level of LDH was measured to act as an index of platelet damage. Briefly, platelets were preincubated with ALA (200−800 μM) for 10 min followed by centrifugation at 10000*g* for 5 min. Then, the supernatants were 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 PPAR Activity. Platelets were incubated with drugs for 5 min at 37 °C and lysed in buffer containing 50 μM Tris-HCl, pH 7.4, 0.5 M NaCl, 1 μM EDTA, 0.05% SDS, 0.5% Triton X-100, and 1 μM phenylmethanesulfonyl fluoride (PMSF). Then, the cell lysates were centrifuged at 15000*g* for 10 min at 4 °C to collect the supernatant, which was used to determine PPAR activation by measuring the absorbance at 450 nm using a Panomics PPAR transcription factor ELISA kit (Redwood City, CA) as previously described.[10]

Table 1. Effect of ALA on PPARα/γ Activation in Platelets[a]

	PPAR activation (OD_{450})	
	PPARα	PPARγ
resting	0.061 ± 0.005	0.27 ± 0.03
ALA (200 μM)	0.068 ± 0.006	0.29 ± 0.02
ALA (400 μM)	0.23 ± 0.04*	0.46 ± 0.04*
ALA (800 μM)	0.58 ± 0.02**	0.81 ± 0.16**

[a] Platelets were incubated with ALA for 5 min at 37°C and lysed. The cell lysates were collected to determine PPARα/γ activation by using a specific kit. The solely vehicle-treated platelets acted as resting group. Data were expressed as mean ± SEM ($n = 5$). *, $P < 0.05$, nad **, $P < 0.01$, as compared to resting platelets.

Measurement of Cyclic AMP. 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 the addition of 10 mM EDTA and immediately boiled for 5 min. After centrifugation at 10000*g* for 5 min, the levels of cyclic AMP in the supernatants were determined by ELISA kit.

Measurement of Platelet Intracellular Ca^{2+} Mobilization. PRP (3.0×10^8 platelets/mL) was incubated with Fluo-4AM (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, and the fluorescence intensity of 20000 platelets was measured by using a flow cytometer (FACScan, Becton Dickinson, Heidelberg, Germany) as previously described.[18]

Measurement of COX-1 Activity. The COX-1 activity was determined according to the instruction of COX inhibitor screening assay kit. Briefly, drugs were mixed with COX-1 enzyme provided by this kit for 10 min. 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 formation of PGE_2 was measured by ELISA kit to reflect COX-1 activity.

Western Blotting. Platelets (3×10^8/mL) were preincubated with drugs for 3 min at 37 °C followed by the addition of AA (100 μM) or solvent for 1−10 min. The cell lysates (20 μg) were separated in 8% sodium dodecyl sulfate (SDS)−polyacrylamide gels and electrotransferred by semidry transfer (Bio-Rad, Richmond, CA). The expression of target genes was determined by Western blotting analysis with reference to a cytoplasmic protein (β-actin, Sigma) as previously described.[14]

Immunoprecipitations. The total extracted cellular protein from platelets was added with pure proteome protein A magnetic beads in the presence of primary antibody of PPARα or PPARγ and incubated overnight at 4 °C. Then, the sample tubes were placed into the magnetic rack and the beads were allowed to adhere to the side. The magnetic beads were put into lysis buffer and boiled to separate the beads and bound protein.[10] After centrifugation, expression of PPARα, PPARγ, PKCα, phospho-PKCα, and COX-1 in immunoprecipitation samples (labeled IP:PPAR) was determined by Western blotting.

Statistical Analysis. The experimental results were expressed as means and their standard errors (SEM). One-way ANOVA with post hoc Bonferroni test was used for statistical analysis. Results were considered to show significant difference at a value of $P < 0.05$.

RESULTS

ALA Increases PPARα/γ Activity in Platelets. To test our original hypothesis that activation of PPARs accounts for the antiplatelet activity of ALA, the effect of ALA on PPARs activation was determined. As shown in Table 1, treatment with

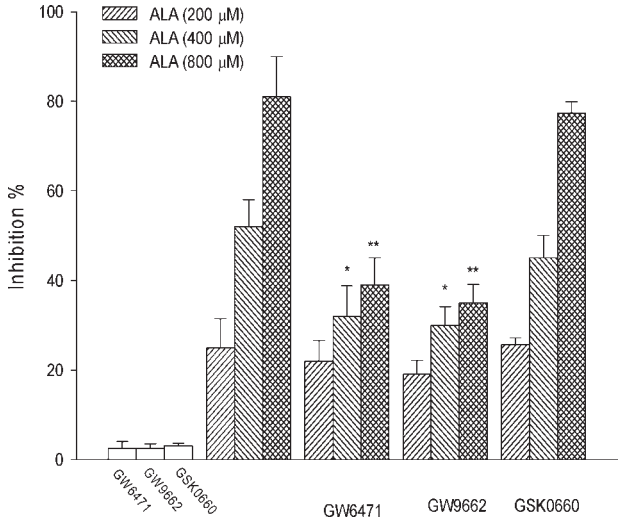

Figure 2. Effect of PPARs on ALA-mediated inhibition of platelet aggregation. Washed platelets were preincubated with selective PPARs antagonists, ALA (200−800 μM) or ALA combination with GW6471 (5 μM), GW6992 (5 μM), or GSK0660 (5 μM), for 3 min followed by addition of AA (100 μM) to trigger platelet aggregation. Percent inhibitions of platelet aggregation were based on AA-induced platelet aggregation alone set at 100%. Data were expressed as mean ± SEM (n = 5). *, $P < 0.05$, and **, $P < 0.01$, as compared to solely AA + ALA-treated platelets.

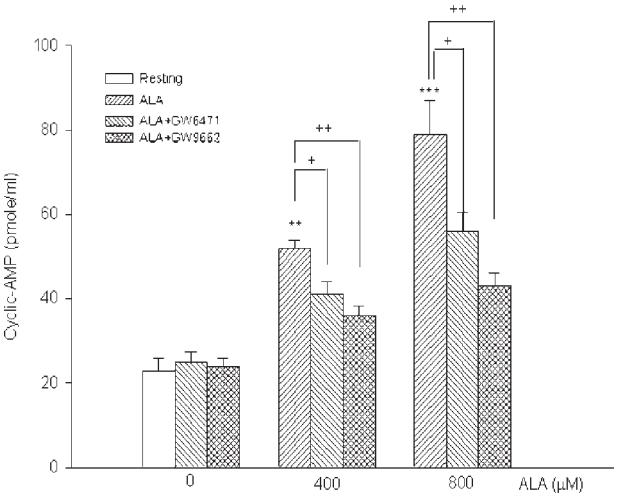

Figure 3. Effect of PPARα/γ on ALA-induced cyclic AMP formation. Platelets were preincubated with GW6471 (5 μM), GW6992 (5 μM), ALA (400, 800 μM), or ALA combination with GW6471(5 μM) or GW6992 (5 μM) for 3 min; then the levels of cyclic AMP were measured. The solely vehicle-treated platelets acted as resting group. Data were expressed as mean ± SEM (n = 5). **, $P < 0.01$, and ***, $P < 0.001$, as compared to the resting group; $^{+}$, $P < 0.05$, and $^{++}$, $P < 0.01$, as compared to solely ALA-treated platelets.

ALA (400−800 μM) alone dose-dependently increased PPARα and PPARγ activity but caused no alteration in PPARβ/δ activity (data not shown), suggesting that ALA is a novel agonist of PPARα/γ in platelets.

PPARα/γ Mediate Antiplatelet Activity of ALA. To evaluate the role of PPARα/γ on the antiplatelet activity of ALA, selective PPARα or PPARγ antagonist was added. Our data showed that in the presence of selective PPARα antagonist (GW6471, 5 μM) or PPARγ antagonist (GW9662, 5 μM), however, the inhibitory effect of ALA on AA-induced platelet aggregation was markedly reduced, especially at doses of ALA of 400 or 800 μM (Figure 2). Similarly, the antiplatelet activity of ALA was not affected by the addition of PPARβ/δ antagonist (GSK0660, 5 μM). These results strongly indicate that the antiplatelet activity of ALA is in part at least mediated by PPARα/γ Moreover, PPARs antagonists alone did not affect AA-induced platelet aggregation (data not shown).

PPARα/γ Mediate Cyclic AMP Formation. To investigate if PPARα or PPARγ contributes to ALA-induced cyclic AMP production in platelets, we determined whether blockage of PPARα/γ impaired ALA-induced cyclic AMP formation. As illustrated in Figure 3, ALA itself dose-dependently increased cyclic AMP formation compared with that in untreated platelets. In the presence of GW6471 or GW6992, however, the increased cyclic AMP formation by ALA was markedly abolished, supporting the possibility that PPARα or PPARγ exerts a positive effect in cyclic AMP formation. Treatment with PPARα or PPARγ antagonist alone did not affect the basal level of cyclic AMP in platelets.

PPARα/γ Mediate AA-Evoked Calcium Mobilization. Once platelets are stimulated by inducers, the rise of $[Ca^{2+}]_i$ is a critical mediator for subsequent platelet activation. Thus, the effect of PPARα/γ on the reduction of calcium mobilization by ALA was determined. As shown in Figure 4, in the presence of GW6471 or GW9662, ALA-mediated inhibition of AA-induced calcium mobilization was markedly reversed, indicating that ALA-mediated reduction of calcium mobilization is also regulated by the actions of PPARα/γ.

ALA Enhances PPARα/γ-PKCα Interaction and Inhibits PKCα activity. Previous studies have indicated that PPARs bind PKCα accompanied by an inhibition of PKCα activity.[10] To elucidate the effect of ALA on PPARα/γ-PKCα interaction and PKCα activation, immunoprecipitation of PPARα/γ-PKCα and PKCα phosphorylation in the immunoprecipitation complexes was determined. Our results showed that in resting and AA-activated platelets, ALA (400, 800 μM) enhanced association of PPARα/γ with PKCα accompanied by a decrease of PKCα activity as evidenced by a lower PKCα phosphorylation in the immunoprecipitation complexes compared with that in respective ALA-untreated control groups (Figure 5). Similarly, blocking PPARα/γ activity markedly abolished ALA-mediated inhibition of PKCα activity (Figure 6). This finding that PPARα or PPARγ associates with PKCα may provide a mechanism by which ALA inhibits PKCα activation in platelets.

ALA Enhances PPARα/γ−COX-1 Interaction. It has been demonstrated that ALA inhibited AA-induced COX-1 activity in platelets.[14] To determine whether PPARα or PPARγ also interacts with COX-1 and affects COX-1 activity, immunoprecipitation of PPARα/γ with COX-1 and the effect of PPARα/γ on COX-1 activity were investigated. As shown in Figure 7, ALA (400, 800 μM) enhanced the interaction of PPARα/γ with COX-1 in both resting and AA-activated platelets. In addition, ALA-induced elevation of PPARα/γ amounts was parallel with the increased amount of COX-1 detected in the immunoprecipitation complexes with no significant difference in the ratio of COX-1/PPARs among these groups, suggesting that ALA-induced PPARα/γ−COX-1 interaction may be mainly due to an increase of PPARα/γ available for binding to COX-1. To further assess the effect of PPARα/γ on ALA-mediated inhibition of COX-1 activity, PPARα/γ was blocked by GW6471 or GW9662. We found that PPARα/γ

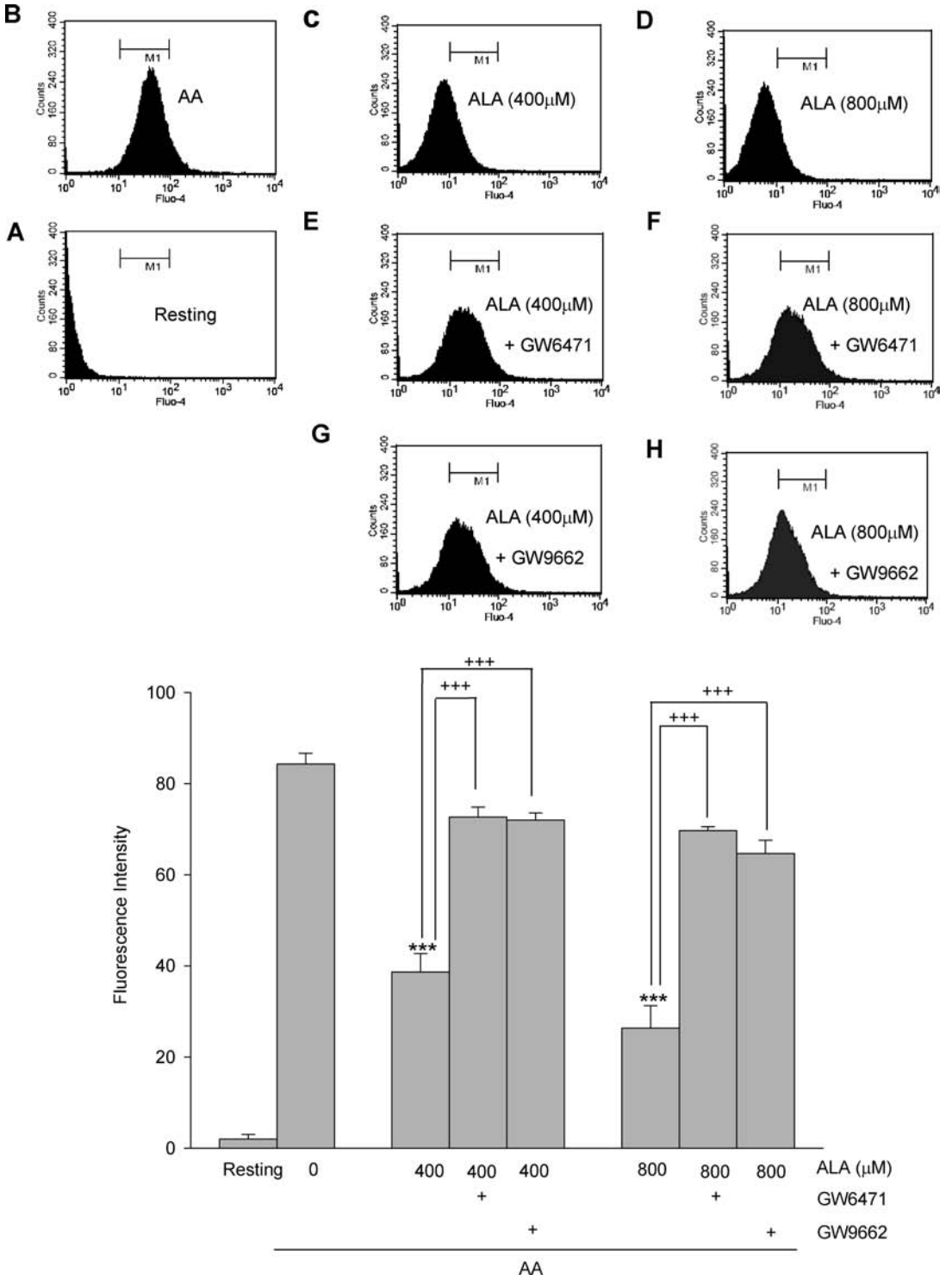

Figure 4. Effect of PPARα/γ on ALA-mediated intracellular Ca^{2+} mobilization. Fluo-4-loaded platelets were preincubated with vehicle, ALA (400, 800 μM), or ALA combination with GW6471 (5 μM) or GW6992 (5 μM) for 3 min followed by addition of AA to evoke Ca^{2+} mobilization. The solely vehicle-treated platelets acted as resting group. Fluorescence intensity was presented as mean ± SEM (n = 5). ***, $P < 0.001$, as compared to solely AA-treated alone platelets. $^{+++}$, $P < 0.001$, as compared to respective solely AA + ALA-treated platelets.

antagonists dramatically reversed ALA-mediated inhibition of COX-1 activity, which was consistent with the result that rosglitazone, a PPARγ agonist, -induced inhibition of COX-1 activity was also reduced by GW9662 (Figure 7). Therefore, binding and repression of COX-1 by PPARα/γ may be a novel explanation of how ALA inhibits COX-1 activity.

ALA Inhibits PPARα/γ Release from Activated Platelets. To gain insight into the mechanism causing elevation of intracellular amounts of PPARα/γ caused by ALA, the effect of ALA on PPARs' movement in activated platelets was determined. In resting platelets, PPARα and PPARγ were mainly detected in platelets, whereas the corresponding

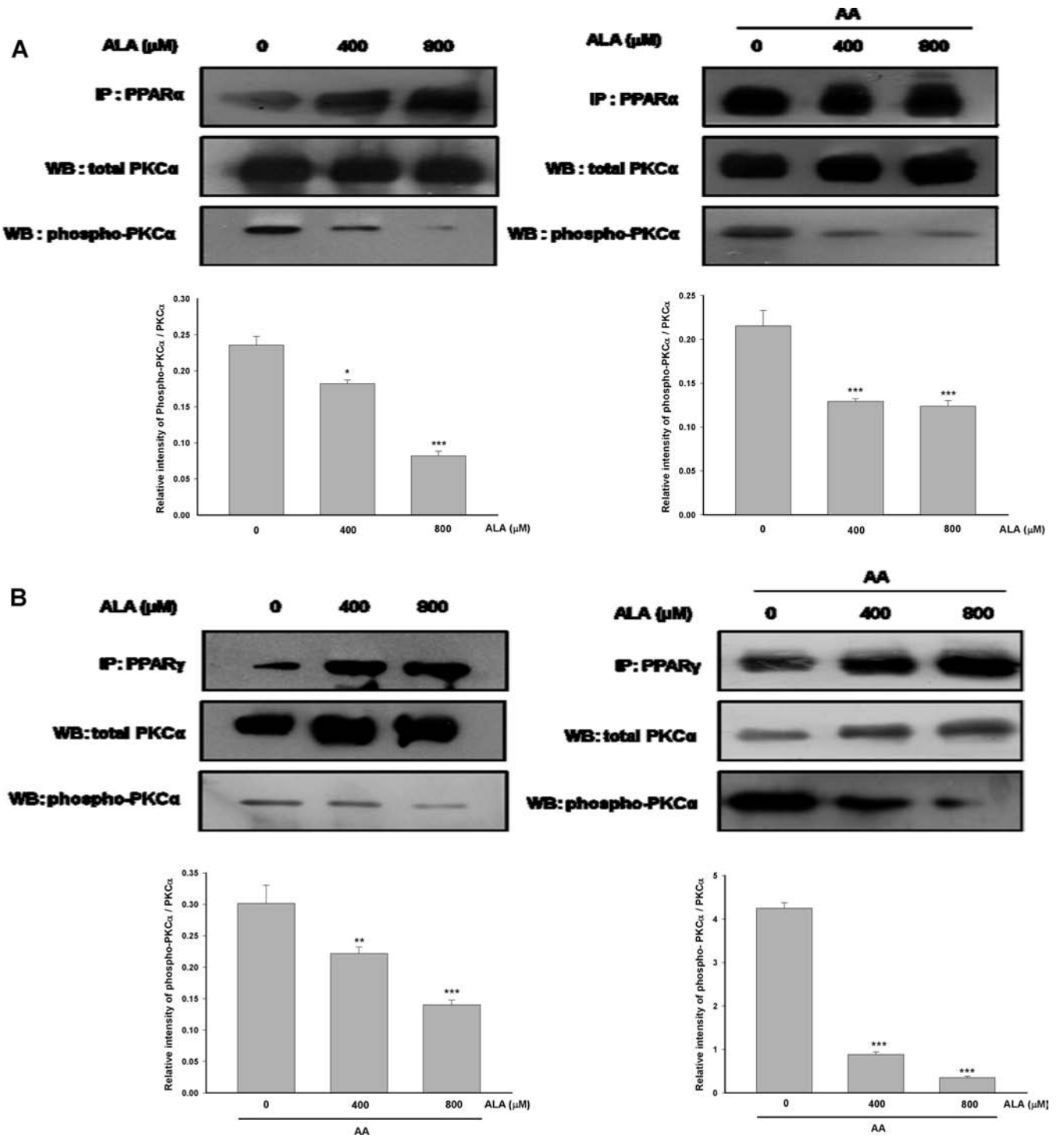

Figure 5. Effect of ALA on the interaction of PPARα/γ with PKCα and PKCα activation in resting and AA-activated platelets. Platelets were incubated with vehicle or ALA (400, 800 μM) for 3 min followed by addition of solvent or AA (100 μM) for 10 min. Then, the extracted protein was immunoprecipitated (IP) with PPARα (A) or PPARγ (B). Expression of PPARα, PPARγ, total PKCα, and phospho-PKCα in the immunoprecipitated complexes was determined by Western blotting (WB) method. Blots were representative of four experiments. *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$, as compared to resting or solely AA-treated platelets.

extracellular amount of PPARα/γ (supernatants) was small. Once platelet activation by AA had been perfomred for 1−10 min, the intracellular amounts of PPARα/γ were time-dependently decreased, accompanied by a marked increase of PPARα/γ amounts in supernatants. Interestingly, treatment with ALA dramatically blocked AA-induced PPARα/γ release, resulting in an accumulation of PPARα/γ amounts in platelet cytosol (Figure 8). These data are the first to demonstrate that ALA can increase intraplatelet amounts of PPARα/γ by blocking PPARα/γ release, which may increase the amounts of PPARα/γ available for binding to PKCα and COX-1.

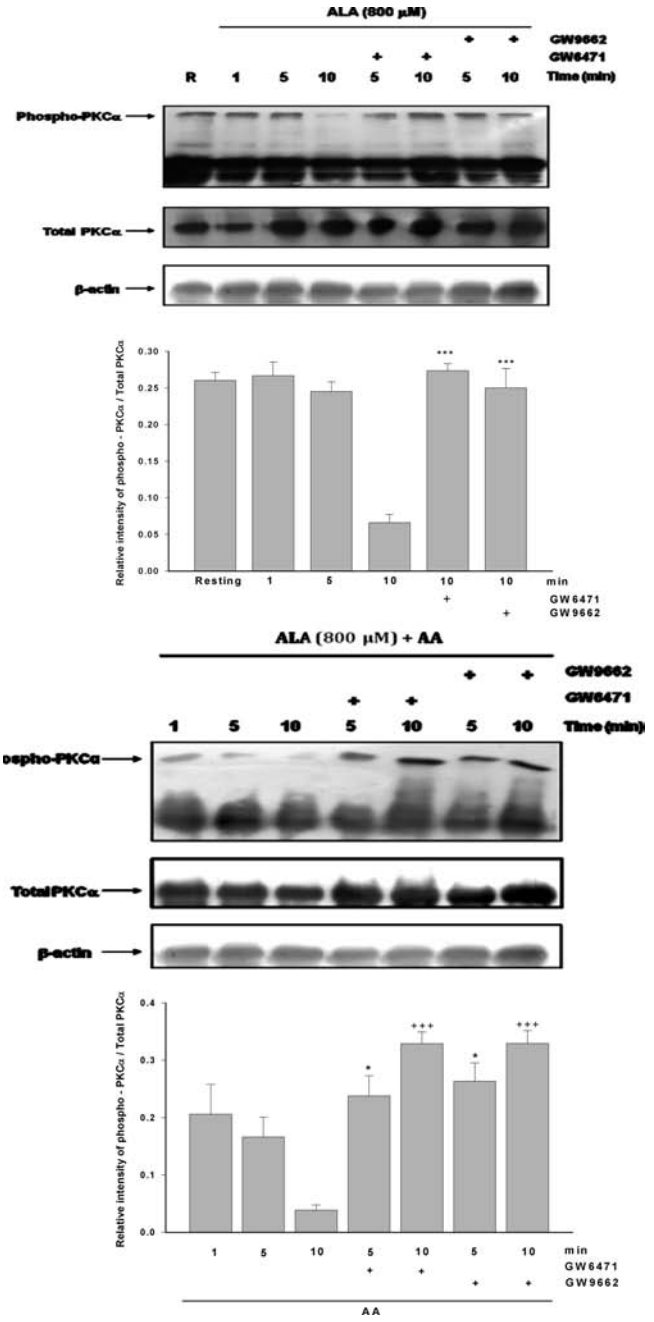

Figure 6. Effect of PPARα/γ on ALA-mediated inhibition of PKCα activation in resting and AA-activated platelets. Platelets were preincubated with ALA (800 μM), ALA (800 μM) + GW6471 (5 μM), or ALA (800 μM) + GW9662 (5 μM) for 3 min followed by addition of vehicle or AA for 1−10 min. Expression of phospho-PKCα, total PKCα, and β-actin was determined by Western blotting method. The solely vehicle-treated platelets acted as resting group (R). Blots were representative of four experiments. In the upper panel, ***, $P < 0.001$, for ALA + PPARs antagonists versus solely ALA-treated (10 min). In the bottom panel, *, $P < 0.05$, for AA + ALA + PPARα/γ antagonists versus solely AA + ALA-treated (5 min), $^{+++}$, $P < 0.001$, for AA + ALA + PPARα/γ antagonists versus solely AA + ALA-treated (10 min).

DISCUSSION

Activation of PPARs inhibits platelet function,[19] suggesting PPAR-activating agents may be a new class of antiplatelet drugs. Although our recent study has demonstrated that ALA exerts an antiplatelet activity,[14] the mechanisms underlying ALA-mediated inhibition of platelet aggregation remain unknown. On the basis of the finding that ALA is a PPARα/γ agonist in CV-1 monkey epithelial cells,[15] we propose that activation of the PPARα/γ-dependent pathway may play a role in ALA-mediated antiplatelet activity. There is evidence supporting our initial hypothesis that the antiplatelet activity of ALA is mediated by PPARα/γ. First, we found that ALA itself increases PPARα/γ activity accompanied by an accumulation of PPARα/γ amounts in platelets. Second, the inhibitory effect of ALA on AA-induced platelet aggregation was markedly impaired in the presence of PPARα/γ antagonists. These data indicated that PPARα/γ-mediated processes may, at least in part, contribute to the antiplatelet activity of ALA.

Several in vitro studies have reported that a wide range of doses of ALA (0.1−1 mM) was used to investigate its various pharmacological effects in different cells,[20−23] suggesting that the dose of ALA required to achieve its effects may be dependent on cell and pathological states. Moreover, the half-life of ALA in plasma is short (30 min), suggesting that ALA is rapidly taken up into tissues, metabolized, and excreted. Cells contain some special transport systems including monocarboxylate transport and Na^+-dependent multivitamin transport,[24,25] accounting for ALA bioavailability. It is possible that the expression and activity of these transports for ALA uptake in platelets may be different from those of other types of cells, which may explain why a higher dose of ALA (200−800 μM) is required to exert its antiplatelet activity in vitro. However, whether ALA exhibits an antiplatelet action in vivo by diet or supplementation is worthy of further investigation.

A novel finding of this study was that ALA (400−800 μM) dose-dependently activates PPARα/γ in platelets, suggesting that ALA is a novel PPARα/γ agonist in platelets. To confirm our hypothesis that PPARα and PPARγ contribute to the antiplatelet activity of ALA, PPARα/γ antagonists were added. Our results clearly demonstrated that the inhibitory effect of ALA on AA-induced platelet aggregation was markedly impaired by PPARα/γ antagonists, suggesting that antiplatelet activity of ALA is in part at least mediated by a PPARα/γ-dependent pathway. Furthermore, recent study has reported that PPARγ agonists inhibit collagen-induced platelet aggregation via suppression of glycoprotein VI (GP VI).[26] Thus, ALA-mediated inhibition of collagen-induced platelet aggregation observed in our previous study[14] may be also associated with actions of PPARs.

It is well-known that cyclic AMP is regarded as an endogenous negative regulator of platelet activation through inhibition of platelet adhesion, aggregation, and release of granule contents via the cyclic AMP-dependent protein kinase (PKA)-mediated signal pathway.[27] Additionally, cyclic AMP-induced activation of PKA is required for transcriptional activation of PPAR-γ,[28] suggesting that cyclic AMP is a stimulator for PPARα/γ activation. Blockage of PKCα completely reversed the phorbol ester, an activator of PKC-induced attenuation of isoproterenol-induced cyclic AMP formation in murine Swiss 3T3 fibroblasts,[29] suggesting that PKCα is a negative mediator for cyclic AMP production. Exposure to PPARα and PPARγ ligands causes PPARs to interact and inhibit PKCα activity,[10] suggesting that PPARα/γ-mediated inhibition of PKCα may be an important mechanism leading to cyclic AMP formation. Therefore, PPARα/γ and cyclic AMP are positively regulated mutually. Our data showed that ALA-induced cyclic AMP formation was markedly

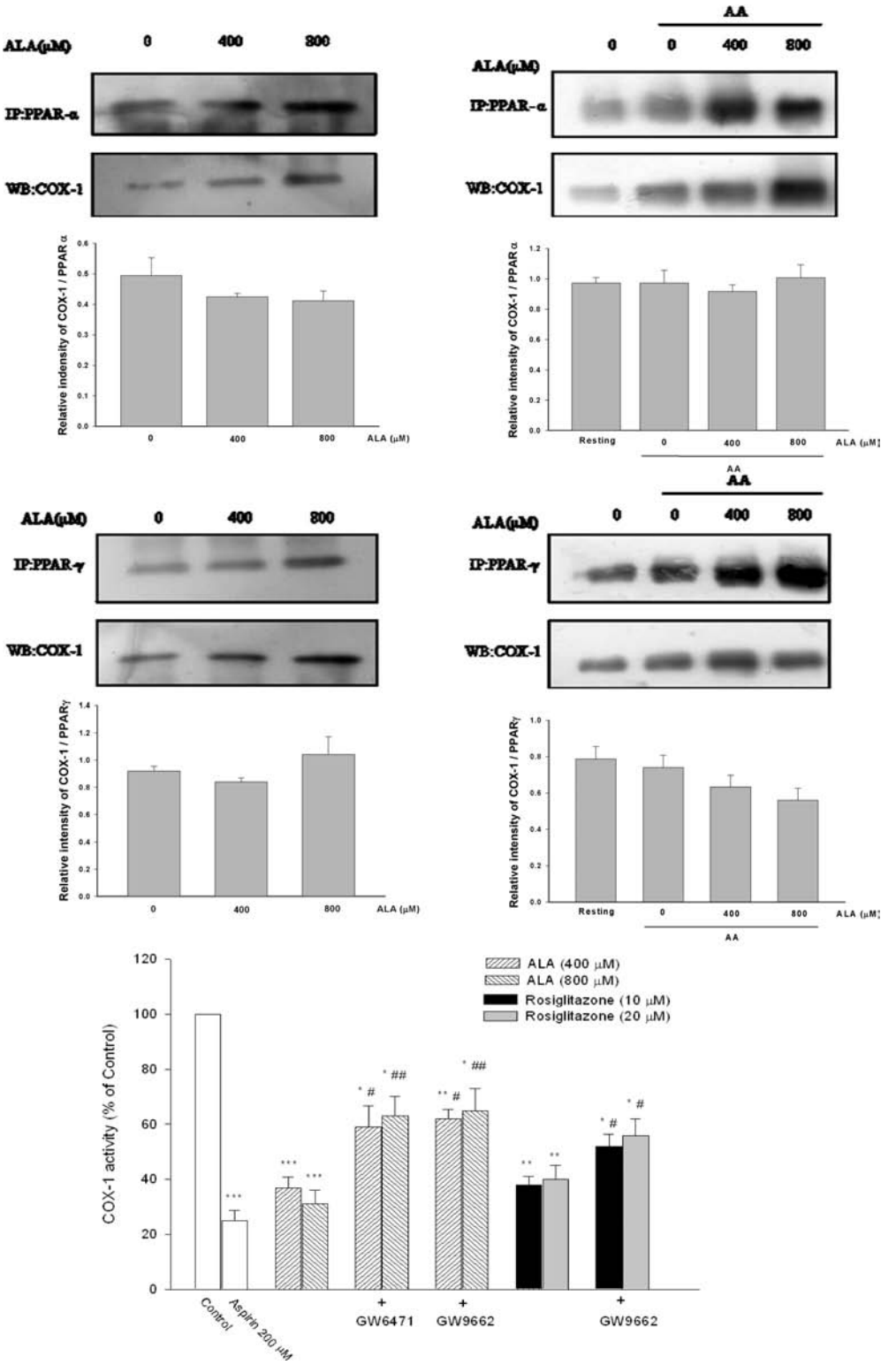

Figure 7. Effect of ALA on the interaction of PPARα/γ with COX-1 and COX-1 activity in resting and AA-activated platelets. Platelets were preincubated with vehicle (left panel) or ALA (400, 800 μM) (right panel) for 3 min followed by addition of solvent or AA (100 μM) for 10 min. Then, the extracted protein was immunoprecipitated (IP) with PPARα or PPARγ. Expression of PPARα, PPARγ, and COX-1 in the immunoprecipitated complexes was determined by Western blotting method (WB). Blots were representative of four experiments. In COX-1 activity assay, vehicle, aspirin (200 μM, as positive control), ALA (400, 800 μM), ALA combination with GW6471 (5 μM) or GW9662 (5 μM), rosiglitazone (10, 20 μM), or rosiglitazone + GW9662 (5 μM) was mixed with COX-1 enzyme for 10 min followed by addition of AA (100 μM) for 2 min. The levels of PGE_2 were measured to reflect the COX-1 activity. The solely AA-treated platelets acted as a control group and set the COX-1 activity at 100%. COX-1 activity (% of control) was presented as mean ± SEM ($n = 5$). *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$, as compared to control group. #, $P < 0.05$, and ##, $P < 0.01$, as compared to solely AA + ALA or AA + rosiglitazone-treated platelets.

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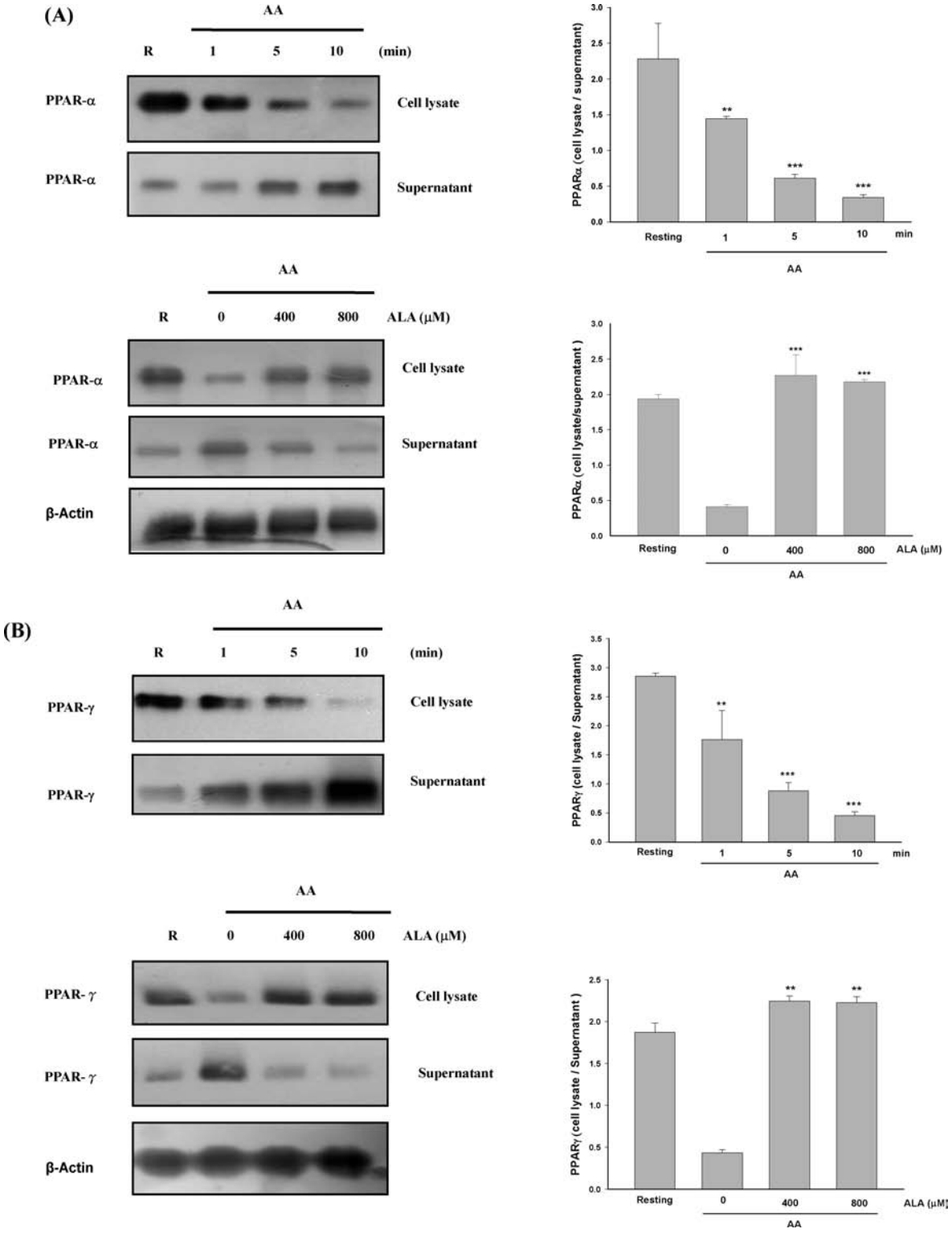

Figure 8. Effect of ALA on PPARα/γ movement in AA-treated platelets. Platelets were preincubated with vehicle or ALA (400, 800 μM) for 3 min followed by addition of AA (100 μM) for 1−10 or 10 min for ALA-treated platelets. Expression of PPARα (A) and PPARγ (B) was measured by Western blotting method in cell lysates and supernatants. The solely vehicle-treated platelets acted as resting group (R). Blots were representative of four experiments. Folds of PPARs (cell lysate/supernatant) were presented as mean ± SEM ($n = 4$). **, $P < 0.01$, and ***, $P < 0.001$, as compared to resting or solely AA-treated platelets.

reduced by PPARα/γ antagonists, further indicating that increase of cyclic AMP formation by ALA is regulated by PPARα/γ. However, whether PPARα or PPARγ also affects adenylate cyclase activity is still unknown and needs further investigation.

When platelets are activated by agonists, the rise of $[Ca^{2+}]_i$ is fundamental for subsequent platelet activation. In the present study, we found that the ALA-induced reduction of AA-evoked Ca^{2+} mobilization in platelets was markedly inhibited by

GW6471 or GW6992, suggesting that PPARα and PPARγ relate to the decrease of Ca^{2+} mobilization. Moreover, it has been reported that cyclic AMP decreases intracellular Ca^{2+} concentration by inhibiting phospholipase C (PLC)-mediated inositol 1,4,5-trisphosphate (IP_3) production and stimulating phosphorylation of ATP-dependent calcium pumps.[30,31] Thus, inhibition of ALA on Ca^{2+} mobilization may be also associated with increase of cyclic AMP formation by ALA.

At least seven PKC isoforms (α, β, δ, θ, ε, η, and ζ) exist in platelets, and PKCα is an abundant isoform. It is well-known that activation of PKCα is a critical mechanism stimulating platelet activation through the action of Syk-dependent phosphorylation mediated by PLC-dependent and PLC-independent manners.[32,33] In nucleated cells and anucleate cells such as platelets, exposure to PPAR ligands causes an association with PKCα and inhibits PKCα activation.[34,35] Similarly, here we demonstrate for the first time that in resting and AA-activated platelets, ALA induces an association of PPARα/γ with PKCα accompanied by a reduction of PKCα activity, as evidenced by an interaction of PKCα phosphorylation in the immunoprecipitation complexes. Furthermore, ALA-induced PKCα inhibition was markedly prevented by selective PPARα/γ antagonists. These findings suggest that interaction of PPARα/γ with PKCα may be associated with ALA-mediated inhibition of PKCα activity and platelet activation.

Upon platelet activation, AA is released from membrane phospholipids and is further converted to TXA_2, a potent inducer of platelet aggregation, through the actions of COX and thromboxane synthase.[36] Although we have demonstrated that ALA inhibited AA-induced thromboxane B_2, a stable metabolite of TXA_2, formation mainly by suppressing COX-1 activity,[14] the underlying mechanisms remain unknown. Here, we found for the first time that both in resting and AA-stimulated platelets, increase of the intraplatelet amount of PPARα/γ by ALA was parallel with enhancement of PPARα/γ−COX-1 association with a similar ratio of COX-1/PPARs among these groups (Figure 7). Accordingly, we propose that the enhanced PPARα/γ−COX-1 interaction caused by ALA may be mainly due to an increase of the amounts of PPARα/γ available for binding COX-1. Similarly, addition of PPARα or PPARγ antagonist significantly reduced ALA-mediated inhibition of COX-1 activity, which is consistent with the result that reduction of COX-1 activity by rosiglitazone was also attenuated by GW9662. Therefore, we hypothesize that ALA-induced PPARα/γ−COX-1 interaction may be a possible mechanism suppressing COX-1 activity.

Recent study has shown that in the presence of inducers such as AA, PPARγ is quickly released from α-granule of platelets into extracellular supernatants,[37] which may exert a systemic effect. Interestingly, ALA markedly blocked the release of PPARα/γ from AA-activated platelets into extracellular supernatants, resulting in an accumulation of PPARα/γ amounts in platelet cytosol. This novel characteristic of ALA may increase the availability of PPARα/γ for interaction with PKCα and COX-1 and subsequently enhance the antiplatelet action of PPARα/γ. However, how ALA inhibits PPARα/γ release from platelets is still unknown and needs further investigation.

We also found that PPARα/γ agonists do not completely reverse the antiplatelet activity of ALA, suggesting that other PPAR-independent mechanisms may be involved. It has been reported that stimulation of G protein (Gs)-coupled PGE_1-receptor (EP2) and histamine-2 receptor (H2R) exerts an antiplatelet action through a cyclic AMP/PKA/vasodilator-stimulated phosphoprotein (VASP) pathway.[38] Recent study has indicated that ALA increases cyclic AMP production via activation of EP2/4 and H2 receptors,[39,40] suggesting that activation of EP2/4 and H2 receptor-mediated processes may be another possible mechanism contributing to the antiplatelet activity of ALA. In conclusion, the present study is the first to demonstrate that the antiplatelet activity of ALA is mediated by a PPARα/γ-dependent pathway that involves interaction with PKCα and COX-1, an increase of cyclic AMP formation, and the inhibition of intracellular Ca^{2+} mobilization.

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ABBREVIATIONS USED

ALA, α-lipoic acid; PPARs, peroxisome proliferator-activated receptors; PKCα, protein kinase Cα; AA, arachidonic acid; COX-1, cyclooxygenase-1; TXA_2, thromboxane A_2; IP_3, inositol 1,4,5-trisphosphate; PLC, phospholipase C; Fluo-4AM, fluo-4-acetoxymethyl ester; PGE_2, prostaglandin E_2; PKA, cyclic AMP-dependent protein kinase.

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