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Title Page

AMP-activated Protein Kinase Mediates the Antiplatelet Effects of the Thiazolidinediones Rosiglitazone and Pioglitazone

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Running Title Page

Running title: Antiplatelet effects of thiazolidinediones

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Abbreviations

ACD, acid-citrate-dextrose; AMPK, AMP-activated protein kinase; CC, compound C; cGMP, cyclic GMP; C_{max}, maximum plasma concentration; HRP, horseradish peroxidase; PPAR, peroxisome proliferator-activated receptor; PRP, platelet-rich plasma; RXR, retinoid X receptor; WP, washed platelets; 15d-PGJ_2, 15-deoxy-Δ12,14-prostaglandin J_2.

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ABSTRACT

The thiazolidinedione antidiabetic drugs rosiglitazone and pioglitazone exert antiplatelet effects. Such effects are known to be mediated by the peroxisome proliferator-activated receptor γ (PPARγ), an acknowledged target of the thiazolidinediones, although the molecular mechanism is elusive. Recently, AMP-activated protein kinase (AMPK) signaling was reported to inhibit platelet aggregation. Since AMPK is another target of the thiazolidinediones, the impact of rosiglitazone and pioglitazone on platelet AMPK and its involvement in aggregation were investigated to assess the contribution of AMPK to the antiplatelet activity of these agents. Treatment with rosiglitazone stimulated both AMPK and PPARγ in isolated rat platelets. However, the concentration and the treatment time required for activation were distinct from each other. Indeed, stimulation of AMPK and PPARγ were discrete events without any cross-activation in platelets. Activation of AMPK or PPARγ by rosiglitazone rendered platelets less responsive to aggregatory stimuli such as collagen, ADP, and thrombin. However, the resultant efficacy caused by activating AMPK was higher than that attributable to PPARγ stimulation. Similar results were obtained with pioglitazone. Taken together, rosiglitazone and pioglitazone inhibit platelet aggregation by activating AMPK. AMPK functions as a potential target of rosiglitazone and pioglitazone for their antiplatelet activity, although the *in vivo* or clinical relevance remains to be assessed.

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Introduction

The thiazolidinediones, such as rosiglitazone and pioglitazone, are a class of antidiabetic drugs used for the treatment of type 2 diabetes mellitus. Thiazolidinediones function as insulin sensitizers to make cells more responsive to insulin. The therapeutic effect of thiazolidinediones is mainly ascribed to their activity as ligands for peroxisome proliferator-activated receptors (PPARs), a group of nuclear receptors, with the greatest specificity for PPARγ. Upon activation, PPARγ forms heterodimers with retinoid X receptors (RXRs) that regulate the transcription of various genes involved in the metabolism of fatty acids and glucose, improving insulin resistance. In addition to PPARγ, thiazolidinediones are able to stimulate AMP-activated protein kinase (AMPK), an enzyme that plays a major role in cellular energy homeostasis (Boyle et al., 2008; Fryer et al., 2002; LeBrasseur et al., 2006; Saha et al., 2004). AMPK is regarded to be another therapeutic target of thiazolidinediones because AMPK activation ameliorates insulin resistance. However, it has yet to be assessed how much AMPK activation contributes to their clinical efficacy (Coletta et al., 2009).

As with other antidiabetic drugs, thiazolidinediones have been reported to exert vascular protective effects, especially on vascular complications related to diabetes (Patel et al., 2008; Turnbull et al., 2009), although intense debate exists regarding the effect of rosiglitazone on ischemic heart disease in large clinical trials (Bach et al., 2013; Home et al., 2007). In agreement with these reports, rosiglitazone and pioglitazone attenuated platelet activity in *in vitro* and animal experiments, as well as in clinical studies (Bodary et al., 2005; Khanolkar et al., 2008; Li et al., 2005; Sidhu et al., 2004). Platelets are the primary cells that mediate hemostasis under physiological conditions, but their dysfunction induces atherothrombotic disorders by promoting unnecessary or excessive aggregation. Chronic diabetic conditions cause platelet hyperreactivity, which contributes to the pathogenesis and progression of diabetic vascular complications. Hence, the vascular protective effects of thiazolidindiones may be attributed, at least to some extent, to their antiplatelet activity (Randriamboavonjy et al., 2008; Ray et al.,

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2006; Spinelli et al., 2008).

Platelets are cytoplasmic fragments of bone marrow megakaryocytes and thus do not contain nuclei. Despite a lack of nuclei, platelets contain a number of nuclear receptors, which include PPARs, RXRs, and receptors for sex steroids and glucocorticoids (Akbiyik et al., 2004; Bishop-Bailey, 2010). Although the function of PPARs in platelets has not been fully elucidated, they are suggested to be related to aggregation, the inherent function of platelets (Ali et al., 2009b; Ray et al., 2008). Indeed, PPARγ agonists, such as 15-deoxy-Δ12,14-prostaglandin J_2 (15d-PGJ_2) and rosiglitazone, were capable of inhibiting platelet aggregation and suppressing the release of thromboxane A_2 and sCD40L, proinflammatory and proaggregatory mediators from platelets (Akbiyik et al., 2004; Ali et al., 2006; Khanolkar et al., 2008; Moraes et al., 2010). The PPARγ-mediated antiplatelet effects were also reported in a study of the statin class of hypolipidemic drugs, such as simvastatin (Ali et al., 2009a). These experimental evidences indicate that activating PPARγ blunts the aggregatory response of platelets.

Fleming et al. (2003) and our group previously showed that pharmacological AMPK activators negatively regulated platelet aggregation (Fleming et al., 2003; Liu et al., 2013; Liu et al., 2015). AMPK stimulates endothelial nitric oxide synthase and nitric oxide production, thereby activating soluble guanylate cyclase, resulting in cyclic GMP (cGMP) formation and subsequent cGMP-dependent protein kinase signaling. Hence, AMPK activators inhibit aggregation by enhancing the aggregation inhibitory potential inherent in platelets. Indeed, AMPK-mediated antiaggregatory effects were observed with all acknowledged AMPK activators that were evaluated, including AICAR, PT1, and A-769662, as well as the recently developed agent YLF-466D. Therefore, AMPK and its activators were suggested as a novel target and potential antiplatelet agents, respectively (Liu et al., 2013; Liu et al., 2015). This raises the possibility that AMPK, in addition to PPARγ, is related to the aggregation inhibitory effect of rosiglitazone and pioglitazone if it is activated by these drugs in platelets.

In the present study, we investigated the antiplatelet activity of rosiglitazone and pioglitazone, focusing on AMPK activation as a potential mechanism. Rosiglitazone and pioglitazone were capable of

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stimulating both AMPK and PPARγ in platelets. However, activations of AMPK and PPARγ were discrete events without any cross-talk. Stimulation of AMPK or PPARγ by rosiglitazone and pioglitazone rendered platelets less responsive to aggregatory stimuli, but the efficacy attributable to AMPK activation was much higher than that caused by PPARγ stimulation. Thus, AMPK may represent a major target responsible for modulating the antiplatelet effects of the thiazolidinediones, although clinical relevance has yet to be verified.

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Materials and Methods

Reagents. Rosiglitazone, pioglitazone, and 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR) were purchased from Toronto Research Chemicals (North York, Ontario, Canada). cGMP and PPARγ transcription factor assay kits were acquired from R&D Systems (Minneapolis, MN, USA) and Cayman Chemical (Ann Arbor, MI, USA), respectively. All antibodies, including anti-AMPKα, anti-phospho-AMPKα (Thr172, clone 40H9), anti-β-actin, horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and anti-mouse IgG were obtained from Cell Signaling Technology (Beverly, MA, USA). The following chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA): GW9662, GW1929, thrombin, and adenine 9-β-D-arabinofuranoside (ara-A). Other chemicals used included the following: 6-[4-(2-piperidin-1-yl-ethoxy)-phenyl)]-3-pyridin-4-yl-pyrrazolo[1,5-a]-pyrimidine (compound C, CC) (Merck KGaA, Darmstadt, Germany), collagen and ADP (Chrono-log, Havertown, PA, USA), protease inhibitor cocktail and phosphatase inhibitor cocktail tablets (Roche Diagnostics, Indianapolis, IN, USA), and Immobilon Western detection reagents (Millipore, Billerica, MA, USA). All other chemicals used were of the highest purity available and purchased from standard suppliers.

Animals. All animal experiments were conducted in accordance with protocols approved by the Ethics Committee of Animal Service Center at Dongguk University. Male Sprague–Dawley rats (5–6 weeks of age) were purchased from Daehan Biolink (Eumseong, Korea) and acclimated for 1 week before experiments. The laboratory animal facility was maintained at a constant temperature and humidity with a 12 hr light/dark cycle. Food and water were provided *ad libitum*.

Preparation of Washed Platelets. Washed platelets (WP) were prepared, as previously described (Liu et al., 2013). Briefly, rat blood was collected from the abdominal aorta of rats anesthetized with ether using acid-citrate-dextrose (ACD; 85 mM trisodium citrate, 66.6 mM citric acid, 111 mM glucose) as an anticoagulant (ACD:blood = 1:6). After centrifugation at 250 *g* for 15 min, platelet-rich plasma (PRP)

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was obtained from the supernatant. Platelets were spun down by further centrifugation of PRP at 500 *g* for 10 min and washed once with washing buffer (138 mM NaCl, 2.8 mM KCl, 0.8 mM $MgCl_2$, 0.8 mM Na_2HPO_4, 10 mM HEPES, 0.55 mM glucose, 22 mM trisodium citrate, and 0.35% bovine serum albumin; pH 6.5). After resuspending the platelet pellets in suspension buffer (138 mM NaCl, 2.8 mM KCl, 0.8 mM $MgCl_2$, 0.8 mM Na_2HPO_4, 10 mM HEPES, 5.6 mM glucose, 1 mM $CaCl_2$, and 0.3% bovine serum albumin; pH 7.4), the platelet concentration was adjusted to 2×10^8 platelets/ml.

Platelet Aggregation Studies. Platelet aggregation experiments were performed using a four-channel aggregometer (Chrono-log Corp.), as previously described (Liu et al., 2013). Platelets were treated with testing materials for the indicated times and aggregation was induced by the addition of either 2.5 μg/ml collagen, 0.12–0.14 U/ml thrombin, or 16 μM ADP, which was the minimal concentration eliciting submaximal aggregation.

Assessment of AMPK Activation in Platelets. Activation of AMPK was examined by conventional Western blotting employing a specific antibody to detect activation-dependent phosphorylation at Thr172 in AMPKα and a suitable horseradish peroxidase-conjugated secondary antibody. To avoid protein loss during membrane stripping and reprobing, phospho-protein and total protein were detected separately with the same samples in different gels. After treating platelets with the testing materials, the platelet pellet was obtained by centrifugation at 12,000 *g* for 2 min and lysed by lysis buffer (50 μM HEPES, 50 μM NaCl, 50 μM sucrose, 1% Triton X-100, protease inhibitor cocktail, and phosphatase inhibitor cocktail). Protein content was quantified with a bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA) and cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After transfer to a polyvinylidene difluoride membrane, immunoreactive proteins were detected with primary antibodies for AMPK, phospho-AMPK, and β-actin, HRP-conjugated secondary antibodies, and Immobilon Western detection reagents (Millipore), as previously described (Liu et al., 2013). Chemiluminescence images were obtained and analyzed with a ChemiDoc XRS+ system equipped with Image Lab software (Bio-Rad Laboratories, Hercules, CA).

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PPARγ Activation Assay. PPARγ activation was assessed by measuring DNA binding activity. Platelets were treated with testing materials for the indicated times and then lysed with lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 1 mM dithiothreitol, protease inhibitor cocktail, and phosphatase inhibitor cocktail) and brief sonication. After centrifugation at 12,000 *g* for 10 min, the supernatant was obtained and used for assay. PPARγ DNA binding was measured with a PPARγ transcription factor assay kit (Cayman Chemical, Item Number 10006855) according to the procedure provided by manufacturer. Optical density at a 450 nm wavelength was measured with a SpectraMax M3 fluorescence microplate reader (Molecular Devices, Sunnyvale, CA).

Analysis of cGMP in Platelets. Platelets treated with testing materials were disrupted by sonication on ice. Lysed platelets were treated with 10% ice-cold trichloroacetic acid and kept on ice for 30 min. After centrifugation at 12,000 *g* for 10 min, the supernatant was extracted three times with water-saturated ether. The water layer containing cGMP was lyophilized in a centrifugal vacuum concentrator (Hanil Science Industrial, Incheon, Korea). Dried samples were stored at -70°C until assayed. The amount of cGMP was analyzed using a cGMP assay kit (R&D Systems) according to the manufacturer's instructions.

Statistical Analyses. The means and standard errors (SE) of the means were calculated for all experimental groups. The data were analyzed using one-way analysis of variance (ANOVA) followed by Dunn's test to determine significant differences from the control. Statistical analyses were performed using SigmaStat software, version 3.5 (Systat Software, San Jose, CA, USA). A P value < 0.05 was considered statistically significant.

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Results

Rosiglitazone Inhibits Platelet Aggregation by Activating AMPK. The impact of rosiglitazone on AMPK and aggregation was examined to investigate whether rosiglitazone stimulated platelet AMPK, thereby attenuating aggregation. Treatment of isolated platelets with rosiglitazone for 10 min resulted in AMPK activation, which was proportional to the concentration of rosiglitazone in the range of 50–200 μM (Fig. 1A, left panel). AICAR, an acknowledged AMPK activator, was tested as a positive control (PC). In evaluating time course, AMPK activation by 200 μM rosiglitazone gradually increased and peaked at 10 min after treatment, thereafter declining quickly (Fig. 1A, right panel). Consistent with these results, rosiglitazone blunted the aggregatory response to 2.5 μg/ml collagen under the same experimental conditions, as described in Fig. 1A (Fig. 1B). This antiaggregatory effect was concentration- and time-dependent at 50–200 μM and 3–10 min, respectively. The maximal inhibition observed was 59 ± 6%. No significant effect was observed at a concentration lower than 50 μM, and the response plateaued at concentrations greater than 200 μM (data not shown). Similar aggregation inhibitory effects of rosiglitazone were also observed upon treatment with ADP and thrombin (Fig. 1C), indicating that its antiaggregatory effect is not platelet activator-specific but common regardless of activator type.

The effect of AMPK inhibitors on AMPK activation and aggregation inhibitory effects was tested to confirm the involvement of AMPK in the antiaggregatory activity of rosiglitazone. Rosiglitazone-induced AMPK activation was nearly completely abolished by pretreatment with the AMPK inhibitors CC and ara-A at 10 μM and 500 μM, respectively (Fig. 2A), which are the concentrations commonly used to inhibit AMPK *in vitro* (Henin et al., 1996; Liu et al., 2013; Musi et al., 2001; Zhou et al., 2001). CC and ara-A also attenuated the antiaggregatory effect of rosiglitazone (Fig. 2B). However, restoration of aggregation inhibition was not complete (Fig. 2B) and higher concentrations of CC or ara-A did not exhibit stronger effects (data not shown), implying that other effects than AMPK activation may be related to antiaggregatory activity. Neither CC nor ara-A alone affected the basal level of phosphorylated

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AMPK or the aggregation response to collagen (data not shown) (Liu et al., 2013). According to our previous study, cGMP elevation was a major consequence of AMPK activation related to aggregation inhibition (Liu et al., 2013). Hence, cGMP was quantified in rosiglitazone-treated platelets as supportive evidence for a link between AMPK stimulation and aggregation inhibition. As expected, rosiglitazone elevated cGMP and such elevation was prevented by treatment with CC and ara-A (Fig. 2C). Taken together, these results indicate that AMPK mediates the antiplatelet effects of rosiglitazone.

Antiaggregatory Effects Attributable to PPARγ Stimulation Are Weak and Thus Observable Only against Mild Aggregatory Stimuli. The ability of rosiglitazone to induce PPARγ/RXR heterodimer formation and its DNA binding was monitored as a proxy for PPARγ activation. Treatment with 10–200 μM rosiglitazone for 30 min led to PPARγ stimulation, which was biphasic, reaching a maximum at 25 μM, but declining at higher concentrations (Fig. 3A). The time course of PPARγ stimulation was evaluated over a 60 min period with two different rosiglitazone concentrations, 25 and 200 μM which elicited maximal activations of PPARγ and AMPK, respectively (Figs. 1A and 3A). PPARγ stimulation by 25 μM rosiglitazone was time-dependent up to 30 min and decreased afterwards (Fig. 3B). However, 200 μM rosiglitazone failed to activate PPARγ over the entire 60 min treatment period. Pretreatment with a specific PPARγ antagonist GW9662 prevented rosiglitazone-induced PPARγ activation, which was nearly complete at 5 μM (Fig. 3C).

Treatment with 25 μM rosiglitazone for 30 min showed minimal effects on platelet aggregation induced by 2.5 μg/ml collagen (Fig. 3D). As with other general antiplatelet agents, the antiaggregatory effect of the PPARγ agonist 15d-PGJ_2 was inversely proportional to the strength of aggregatory stimuli and was prominent in weak aggregation (Moraes et al., 2010). Accordingly, aggregation was tested with a mild stimulus. Rosiglitazone marginally but significantly attenuated aggregation by 1 μg/ml collagen which induced approximately 16% aggregation in the control (Fig. 3D). GW9662 abolished this aggregation inhibitory effect. In short, the concentration and treatment time required for AMPK and PPARγ activation are quite different from each other. Rosiglitazone, under the condition that activated

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PPARγ, was effective only against weak aggregation and failed to inhibit fully-induced platelet aggregation. PPARγ mediates the antiaggregatory activity of rosiglitazone, but the efficacy resulting from PPARγ stimulation is not as high as that attributable to AMPK activation.

Activations of AMPK and PPARγ by Rosiglitazone Are Discrete Events. A potential interaction between AMPK and PPARγ were examined in platelets based on reports of cross-talk in other cell types, such as hepatoma and cardiac muscle cells (Sozio et al., 2011; Xiao et al., 2010). The specific AMPK activators A-769662 and PT1 significantly stimulated AMPK at concentrations of 250 and 100 μM, respectively (Fig. 4B), as reported previously (Liu et al., 2013). However, they failed to stimulate PPARγ under the same experimental conditions (Fig. 4A). In addition, the specific PPARγ agonist, GW1929, did not stimulate AMPK at concentrations ranging from 5-20 μM (Fig. 4B), which is the concentration capable of stimulating PPARγ (Supplemental Fig. 1). Consistent with these results, 5 μM GW9662 affected neither AMPK activation nor aggregation inhibition by 200 μM rosiglitazone (Fig. 4C and D). Taken together, AMPK does not have an influence on PPARγ in platelets and *vice versa*. This finding is in agreement with the results described above showing that treatment conditions required for stimulating AMPK are distinct from those for activating PPARγ. AMPK and PPARγ are not interrelated but generate discrete signals at least in platelets, although both can be stimulated by rosiglitazone.

Pioglitazone Inhibits Platelet Aggregation in a Similar Manner to Rosiglitazone. Antiaggregatory activity and its relationship to AMPK or PPARγ were examined with pioglitazone. Treatment with pioglitazone for 3 min caused AMPK activation in the range of 50–200 μM, which was comparable to the effective concentration of rosiglitazone (Fig. 5A). Under the same experimental conditions, pioglitazone attenuated the aggregatory response to collagen, which reached statistical significance at 200 μM (Fig. 5B). Higher concentrations than 200 μM or longer treatments than 3 min was confirmed to not exhibit stronger effects (data not shown). AMPK activation by 200 μM pioglitazone was prevented by the pretreatment with 10 μM CC or 500 μM ara-A, but not by 5 μM GW9662 (Fig. 5C). In accordance with this result, CC and ara-A abolished the aggregation inhibitory effect of pioglitazone,

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whereas GW9662 showed minimal effects (Fig. 5D). Pioglitazone increased cGMP in platelets and this cGMP elevation was suppressed by CC or ara-A (Fig. 5E). Pioglitazone was capable of stimulating PPARγ, which was also showed a biphasic response; PPARγ activation was maximal at 10 and 25 μM, and decreased at higher concentrations (Fig. 5F). Pioglitazone-induced PPARγ stimulation was prevented by GW9662 (Fig. 5F, gray bar). As with rosiglitazone, 10 μM pioglitazone exhibited little effect on platelet aggregation induced by 2.5 μg/ml collagen (Fig. 5G). However, it could attenuate mild aggregation induced by 1.0 μg/ml collagen, which disappeared upon pretreatment with GW9662.

Low Efficacy of PPARγ-mediated Antiaggregation Is Not Ascribed to Weak Activation of PPARγ. The antiplatelet effect attributable to PPARγ stimulation was weak and not sufficient enough to inhibit full aggregation (Figs. 3 and 5). This observation implies that rosiglitazone and pioglitazone cannot fully stimulate PPARγ to inhibit aggregation or that the antiaggregatory function of PPARγ is fundamentally too weak to inhibit full aggregation. These interpretations were examined by evaluating the antiaggregatory activity of GW1929. GW1929 stimulated platelet PPARγ, with a maximal effectiveness at a 20 μM concentration and 10 min of treatment time (Supplemental Fig. 1A). Higher concentrations than 20 μM or longer incubation times than 10 min did not further activate PPARγ (data not shown). Under these experimental conditions, GW1929 successfully inhibited platelet aggregation induced by 2.5 μM collagen (Supplemental Fig. 1B and Fig. 6B). Pretreatment with GW9662 prevented GW1929-induced PPARγ activation and abolished the aggregatory inhibitory effect of GW1929 (Figs. 6A and B), confirming that the effect of GW1929 is entirely attributable to PPARγ (Fig. 6B). PPARγ activation by rosiglitazone or pioglitazone was compared to that by GW1929 in the same sets of experiments. Interestingly, all the tested compounds, including rosiglitazone, pioglitazone and GW1929, were able to stimulate PPARγ to a similar extent under their optimal conditions (Fig. 6C). Therefore, the low efficacy of PPARγ-mediated antiaggregation is not ascribed to weak activation of PPARγ.

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Discussion

The contribution of AMPK to the antiplatelet effects of thiazolidinediones was hypothesized based on the aggregation inhibitory function of platelet AMPK and the ability of thiazolidinediones to stimulate AMPK. As expected, rosiglitazone and pioglitazone were capable of inhibiting platelet aggregation by activating AMPK. Besides AMPK, PPARγ is also involved in the antiaggregatory effect of these drugs, as previously reported (Ali et al., 2009a; Moraes et al., 2010). However, the efficacy associated with PPARγ stimulation appears to be much lower than that attributable to AMPK activation. The concentration and the treatment time required for activating AMPK or PPARγ were different from each other, with no evidence of any cross-activation between the two. Therefore, AMPK and PPARγ are reciprocally independent and generate distinct signals in platelets, although both can be activated by rosiglitazone and pioglitazone. Thus, the results of this study suggest that platelet AMPK functions as a potential target of thiazolidinediones in the mediation of their antiplatelet activity, although *in vivo* or clinical significance has yet to be assessed.

The antiplatelet effect of thiazolidinediones has gained attention regarding their cardiovascular protective properties and ameliorative effects on diabetic vascular complications. The impact of thiazolidinediones on platelet activity has long been studied in a variety of experiments. Rosiglitazone reduced platelet activity in patients with coronary artery disease (Sidhu et al., 2004) or diabetes (Khanolkar et al., 2008). However, these clinical studies did not describe the direct effect of the drug on platelet activity. For instance, by acting on megakaryocytes, rosiglitazone restored altered sarco/endoplasmic reticulum Ca^{2+}-ATPase expression, thus correcting platelet hyperreactivity in diabetes (Randriamboavonjy et al., 2008). Although few studies investigated the direct effect on platelets, the *in vitro* exposure of rosiglitazone obviously reduced the aggregatory response of platelets (Ali et al., 2006; Khanolkar et al., 2008). In addition, rosiglitazone reduced thromboxane A_2 production and prevented the surface expression and release of sCD40L induced by thrombin (Akbiyik et al., 2004). Notably, most of

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these studies regarded rosiglitazone as only a PPARγ agonist and tended to ascribe these platelet effects to PPARγ stimulation without proper verification (Akbiyik et al., 2004; Ali et al., 2006; Bishop-Bailey, 2010; Moraes et al., 2010; Spinelli et al., 2008). Such interpretation was also attributed to accompanying experimental evidence; the antiaggregatory activity of the PPARγ agonist 15d-PGJ_2 (Akbiyik et al., 2004; Moraes et al., 2010). There is currently sufficient evidence that stimulating PPARγ causes the attenuation of aggregation. A PPARγ-dependent inhibition of platelet aggregation was demonstrated with another PPARγ agonist, GW1929 (Fig. 6), and simvastatin (Ali et al., 2009a; Ali et al., 2009b). In addition, Moraes et al. (2010) reported the inhibitory effect of rosiglitazone against collagen-induced platelet aggregation and its abolition by GW9662 (Moraes et al., 2010), confirming the PPARγ-mediated antiaggregatory effect of rosiglitazone. The effective concentration used in their study was 1–20 μM, which was comparable to that used in this study. However, the quantitative aspect of the antiaggregatory effect was not presented clearly and higher concentrations of rosiglitazone were not tested. Rosiglitazone and pioglitazone have never been tested for antiaggregatory effects under conditions that stimulate AMPK, therefore making our study the first to evaluate AMPK-related antiplatelet activity.

Higher than 50 μM concentrations are required for rosiglitazone and pioglitazone to activate platelet AMPK (Figs. 1 and 5), which is hardly achieved with a clinical dosage. According to clinical pharmacokinetic studies, the maximal plasma concentrations (C_{max}) reached only 598 ng/ml (≈ 1.7 μM) and 1599 ng/ml (≈ 4.5 μM) in oral, single administration of 8 mg rosiglitazone (GlaxoSmithKline, 2014) and 30 mg pioglitazone (Sripalakit et al., 2006), respectively. However, AMPK activation could be detected in long-term, *in vivo* studies using clinically relevant doses, although the concentration for stimulating AMPK was generally high in short-term, *in vitro* studies. Rosiglitazone was effective at several tens to hundreds of μM in cultured cells, such as endothelial and muscle cells (Boyle et al., 2008; Fryer et al., 2002), which is quite comparable to the results of this study. However, rosiglitazone could elevate AMPK activity in various tissues, including liver, muscle, heart of diabetic animals and humans and animals fed high-fat diets if administered for several weeks to months at doses of several mg/kg/day

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(Bandyopadhyay et al., 2006; Dallaire et al., 2008; Guo et al., 2012; Song et al., 2010; Ye et al., 2006). AMPK activation could also be observed in liver, adipose tissue and vein grafts from animals and humans with impaired glucose tolerance when a clinical dose of pioglitazone was administered for weeks to months (Coletta et al., 2009; Morisaki et al., 2011; Rasouli et al., 2012; Saha et al., 2004). Accordingly, the plasma concentrations for stimulating platelet AMPK may be much lower after prolonged exposure than the concentrations effective in *in vitro* systems; thus, the activation of platelet AMPK may be a clinically relevant event. Long-term studies will be required to test this speculation. However, chronic treatment is not feasible for platelets since platelets cannot be cultured and should be used immediately after preparation because of aging problem, a gradual change of platelet activity (Brown et al., 2000). Furthermore, *in vivo* studies may have limitations because of the ambiguity of result interpretation. Although rosiglitazone or pioglitazone exert antiplatelet or antithrombotic effects, it is hard to distinguish the individual contributions of platelet AMPK and PPARγ. It is because nonplatelet-mediated effects may also be involved in *in vivo* situation. For instance, AMPK can phosphorylate and activate eNOS in endothelial cells, which stimulates nitric oxide production, thereby resulting in the suppression of platelet activity (Morrow et al., 2003; Zhang et al., 2006). An indirect effect on AMPK cannot be excluded either. Thiazolidinediones, for example, may activate platelet AMPK indirectly by increasing expression and secretion of adiponectin, which is positively regulated by PPARγ and activates AMPK in various tissues (Higuchi et al., 2010; Li et al., 2010). Because of these redundancies, it is complicated to demonstrate the present findings in animal experiments. AMPK in tissues other than platelets may be related with the platelet effect of thiazolidinediones in *in vivo* or clinical conditions.

There are a few results that need further investigation. Antiaggregatory effects exerted by activated AMPK were not sufficiently potent to fully inhibit submaximal aggregation and the maximal inhibition was about 50% (Figs. 1C and 5B). The efficacies of rosiglitazone and pioglitazone appear to be lower than those of the specific AMPK activators, AICAR and YLF-466D, which inhibited 60–80% of submaximal aggregation under optimal experimental conditions (Liu et al., 2013; Liu et al., 2015).

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Rosiglitazone and pioglitazone may not be able to activate AMPK as strongly as AICAR and YLF-466D (Fig. 1A) or may have additional effects interfering with AMPK downstream signaling. The restoration of aggregation inhibition by CC or ara-A was not complete, while AMPK activation and subsequent cGMP production were nearly completely prevented (Fig. 2). These results also imply that other factors than AMPK activation are involved in the antiaggregatory activity under these experimental conditions. Furthermore, a similar interpretation is applicable to low efficacy under the conditions that activate PPARγ. Although rosiglitazone and pioglitazone can activate PPARγ to the same degree as GW1929, their antiplatelet activity was weaker than GW1929, suggesting that they may additionally interfere with the antiaggregatory signaling generated by PPARγ. Rosiglitazone and pioglitazone do not appear to be completely specific for AMPK or PPARγ and other off-target activities have been reported (Wright et al., 2014). Further studies will be needed to clarify these issues.

The question still remains how PPARγ activation reduces platelet aggregation. Although platelets lack nuclei, they express nuclear receptors. More than a decade has passed since the presence of these receptors in platelets has been reported, but little is known about their functions and working mechanisms. Platelets express all subtypes of PPARs, including PPARα, PPARβ/δ and PPARγ, and the ligands for PPARs commonly exhibit antiplatelet activity (Spinelli et al., 2008). Indeed, simvastatin and 15d-PGJ_2 attenuate the aggregatory response to platelet activators in a PPARγ-dependent manner, and PPARα mediates the antiaggregatory activity of fibrates, such as fenofibrate (Ali et al., 2009a; Phipps and Blumberg, 2009). PPARγ maintains DNA binding ability in platelets, but it is unlikely that PPARγ exerts its antiplatelet function by working as a traditional transcription factor (Moraes et al., 2010; Phipps and Blumberg, 2009). Although DNA binding itself may not be the mechanism for aggregation inhibition, it must be the final consequence of ligand-PPARγ binding in platelets and thus can be a measure encompassing a series of PPARγ activation process including ligand-PPARγ binding, PPAR/RXR heterodimerization and subsequent events. Hence, DNA binding was examined as a proxy of PPARγ activation in this study. Indeed, DNA binding was assessed as a measure of PPARγ activation in all the

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reports studying the functions of PPARs in platelets without exception (Akbiyik et al., 2004; Ali et al., 2009a; Ali et al., 2006; Ali et al., 2009b; Khanolkar et al., 2008; Moraes et al., 2010; Ray et al., 2008). Fortunately, non-genomic, non-transcriptional activities of PPARγ are being uncovered (Bishop-Bailey, 2010; Luconi et al., 2010) and such progress may allow us to elucidate the working mechanism of platelet PPARγ.

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Authorship Contributions

Participated in research design: Y. Liu and M.Y. Lee.

Conducted experiments: Y. Liu, J.M. Park, K.W. Chang, H.J. Huh, and K. Lee.

Performed data analysis: Y. Liu, J.M. Park, K.W. Chang, H.J. Huh, K. Lee, and M.Y. Lee.

Wrote or contributed to the writing of the manuscript: Y. Liu and M.Y. Lee.

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Footnotes

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Figure Legends

Fig. 1. Rosiglitazone activates platelet AMPK and inhibits aggregation. (A) Activation of platelet AMPK by rosiglitazone. Platelets were incubated with the indicated concentrations of rosiglitazone for 10 min (left panel) or with 200 μM rosiglitazone for the indicated times (right panel). AMPK activation was assessed by detecting activation-dependent phosphorylation at Thr172. As a positive control (PC), platelets were treated with 500 μM AICAR for 30 min. β-Actin was used as a loading control. Representative images and the relative band intensities are presented in the upper and lower panels, respectively. (B) Concentration- and treatment time-dependent antiaggregatory effect of rosiglitazone. After treating platelets with rosiglitazone, as described in A, aggregation was induced by the addition of 2.5 μg/ml collagen (indicated by arrowheads). Representative tracings are presented from at least three repeated experiments. (C) Aggregation inhibitory effect of rosiglitazone against the platelet activators, collagen, ADP and thrombin. Platelets were treated with rosiglitazone for 10 min and aggregation was induced with 2.5 μg/ml collagen, 16 μM ADP, or 0.12–0.14 U/ml thrombin. Values are means ± standard errors (n=6 for the left panel of A, 5 for the right panel of A, and 4–6 for C). $^{*}P < 0.05$ vs. control.

Fig. 2. AMPK mediates the antiplatelet effects of rosiglitazone. (A) Prevention of rosiglitazone-induced AMPK activation by the AMPK inhibitors, compound C (CC) and ara-A. Platelets were pretreated with 10 μM CC or 500 μM ara-A for 15 min and incubated with 200 μM rosiglitazone for an additional 10 min. (B) Abolition of the aggregation inhibitory effect of rosiglitazone by CC and ara-A. After treating platelets with CC, ara-A and rosiglitazone, as described in (A), aggregation was elicited with the addition of collagen (indicated with the arrowhead). Representative tracings and the aggregation percentages are presented in the left and right panels, respectively. (C) Increase in cGMP by rosiglitazone and its suppression by CC and ara-A. Platelets were treated with CC, ara-A and rosiglitazone, as described in A,

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and cGMP levels in platelets were quantified. Values are means ± standard errors (n = 8 for A, 5 for B, and 4 for C). $^{*}P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. rosiglitazone only. Rosi: rosiglitazone.

Fig. 3. Antiaggregatory effect attributable to PPARγ stimulation is weak and thus observable only against mild aggregatory stimuli. (A) Activation of platelet PPARγ by rosiglitazone. Platelets were incubated with the indicated concentrations of rosiglitazone for 30 min and PPARγ activation was assessed. (B) Time course of PPARγ stimulation by rosiglitazone. PPARγ activation was measured in platelets treated with 25 or 200 μM rosiglitazone for the indicated times. (C) Prevention of rosiglitazone-induced PPARγ activation by the PPARγ antagonist, GW9662. Following pretreatment with 1–5 μM GW9662 for 5 min, platelets were treated with 25 μM rosiglitazone for an additional 30 min. (D) Antiaggregatory effect of 25 μM rosiglitazone and its abolition by pretreatment with GW9662. Platelets untreated or pretreated with 5 μM GW9662 for 5 min were further incubated with 25 μM rosiglitazone for 30 min. Aggregation was induced with 1 or 2.5 μg/ml collagen. Representative tracings and the aggregation percentages are presented in the left and right panels, respectively. Values are means ± standard errors (n = 3 for A and C, 4 for B, and 5 for D). $^{*}P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. rosiglitazone only. Rosi: rosiglitazone.

Fig. 4. Activation of AMPK and PPARγ by rosiglitazone are discrete events. (A) Effect of the specific AMPK activators, A-769662 and PT1, on PPARγ. Platelets were incubated with 250 μM A-769662 or 100 μM PT1 for 30 or 20 min, respectively, and PPARγ activation was measured. For comparison, platelets were treated with 25 μM rosiglitazone for 30 min. (B) Effect of the PPARγ agonist, GW1929, on AMPK. Platelets were treated with 5–20 μM of GW1929 for 10 min. As positive controls, 250 μM A-769662 or 100 μM PT1 was added for 30 or 20 min, respectively. (C and D) Effect of GW9662 on rosiglitazone-mediated AMPK activation and aggregation inhibition. After pretreating with 5 μM GW6992 for 5 min, platelets were treated with 200 μM rosiglitazone for an additional 10 min.

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Aggregation was elicited with 2.5 μg/ml collagen. Representative images and the relative band intensities are presented in the left and right panels (B and C), respectively. Values are means ± standard errors (n = 3 for A and D, and 6 for B and C). $*P < 0.05$ vs. control. Rosi: rosiglitazone.

Fig. 5. Pioglitazone inhibits platelet aggregation in a similar manner as rosiglitazone. (A and B) Activation of AMPK and inhibition of platelet aggregation by pioglitazone. Platelets were incubated with pioglitazone for 3 min, and aggregation was induced with 2.5 μg/ml collagen. (C and D) Prevention of 200 μM pioglitazone-mediated AMPK activation and aggregation inhibition by CC and ara-A. After CC, ara-A or GW9662 were added to platelets, as described in Figs. 2 and 3, platelets were further treated with pioglitazone for 3 min. Aggregation was induced with 2.5 μg/ml collagen. (E) AMPK-dependent cGMP elevation by pioglitazone. (F) PPARγ stimulation by pioglitazone. Platelets untreated or pretreated with 5 μM GW9662 for 5 min were incubated with pioglitazone for an additional 30 min. (G) Antiaggregatory effects of 10 μM pioglitazone and its abolition by pretreatment with GW9662. Platelets were treated with 5 μM GW9662 and 10 μM pioglitazone, as described in F and aggregation was induced with 1 or 2.5 μg/ml collagen. Values are means ± standard errors (n = 6 for A, 8 for B and D, 7 for C and G, and 3 for E and F). $*P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. pioglitazone only. Pio: pioglitazone.

Fig. 6. Low efficacy of PPARγ-mediated antiaggregation is not ascribed to weak activation of PPARγ. (A) PPARγ activation by GW1929 and its inhibition by GW9662. Platelets were untreated or pretreated with 5 μM GW9662 for 5 min and then incubated with 20 μM GW1929 for an additional 10 min. (B) PPARγ-mediated antiaggregatory activity of GW1929. Platelets were untreated or treated with 5 μM GW9662 for 5 min and then further treated with 20 μM GW1929 for 10 min. Aggregation was induced by the addition of 2.5 μg/ml collagen (indicated by arrowhead). Representative tracings and the aggregation percentages are presented in the left and right panels, respectively. (C) Comparison of PPARγ activation by

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rosiglitazone, pioglitazone and GW1929. Platelets were treated with 25 μM rosiglitazone for 30 min, 10 μM pioglitazone for 30 min or 20 μM GW1929 for 10 min. Values are means ± standard errors (n = 5 for A, 4 for B, and 3 for C). $^{*}P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. GW1929 only.

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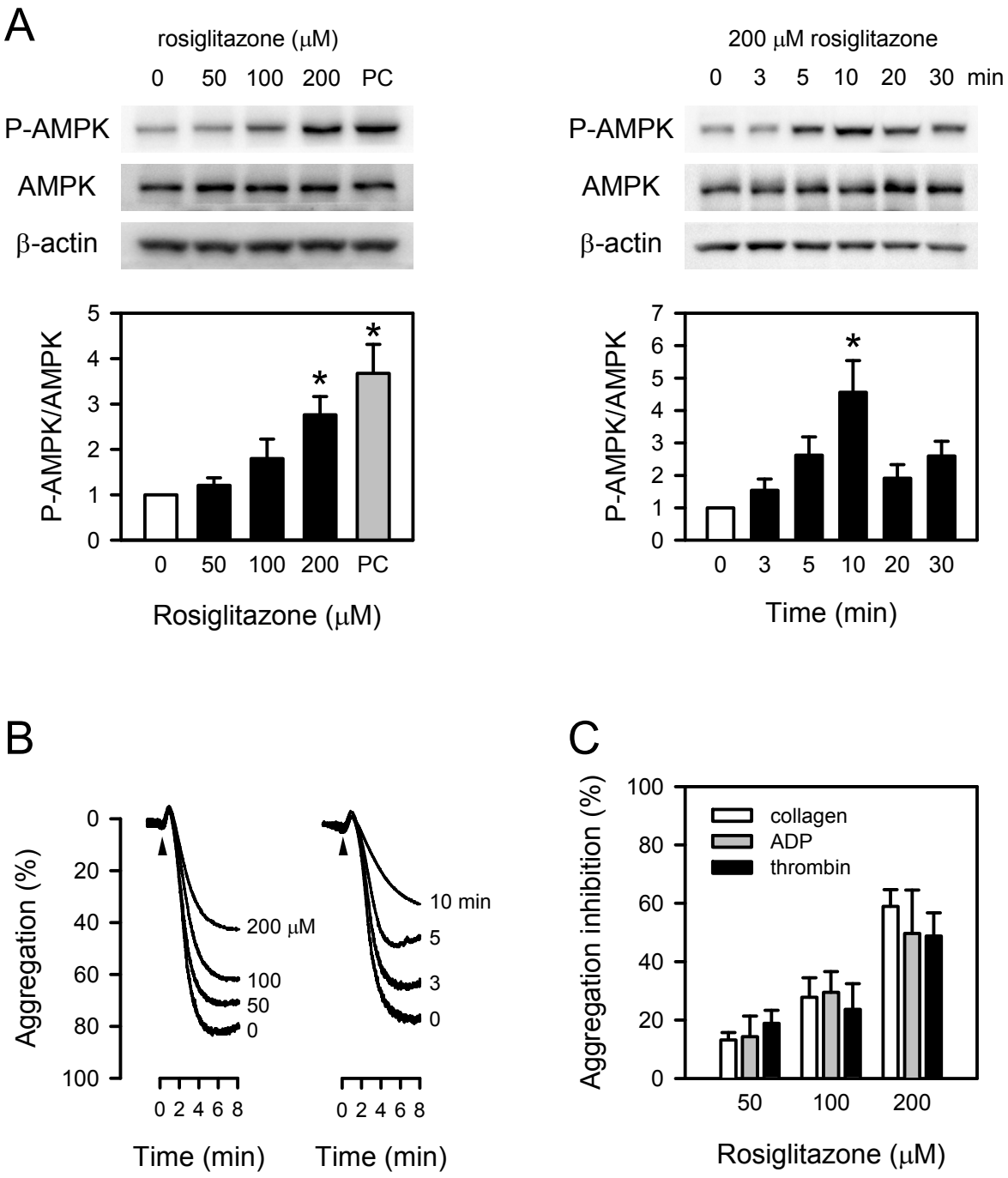

Fig. 1

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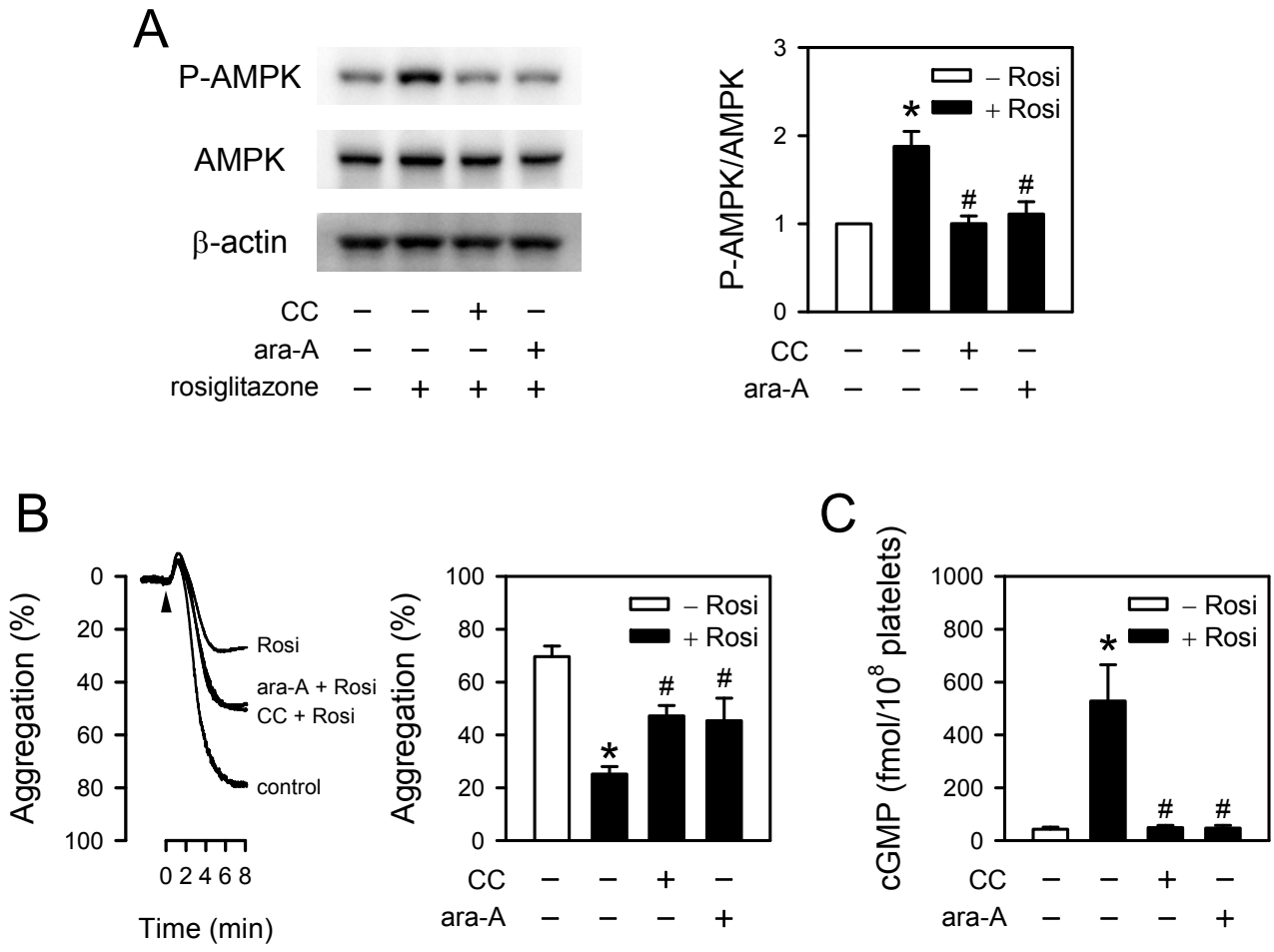

Fig. 2

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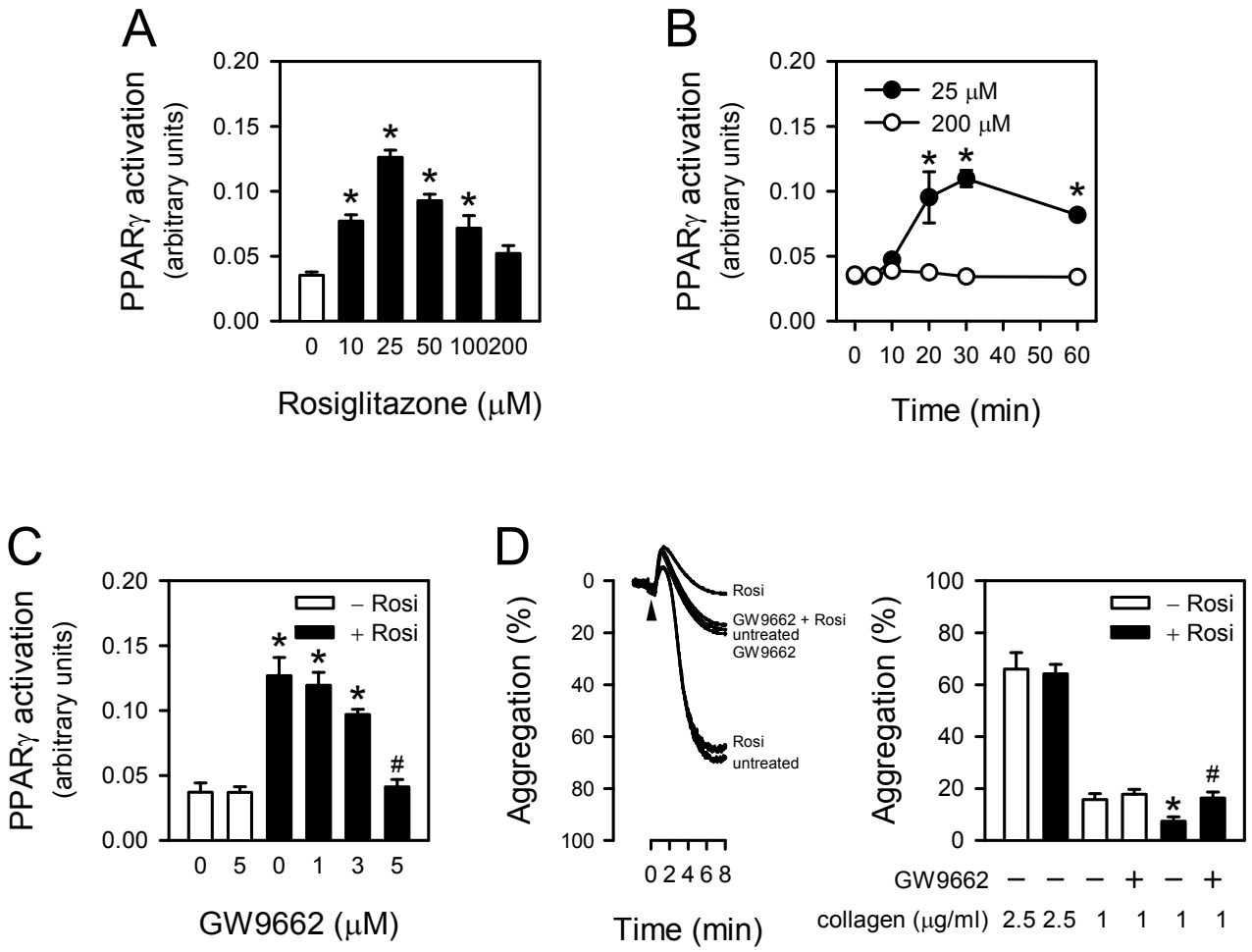

Fig. 3

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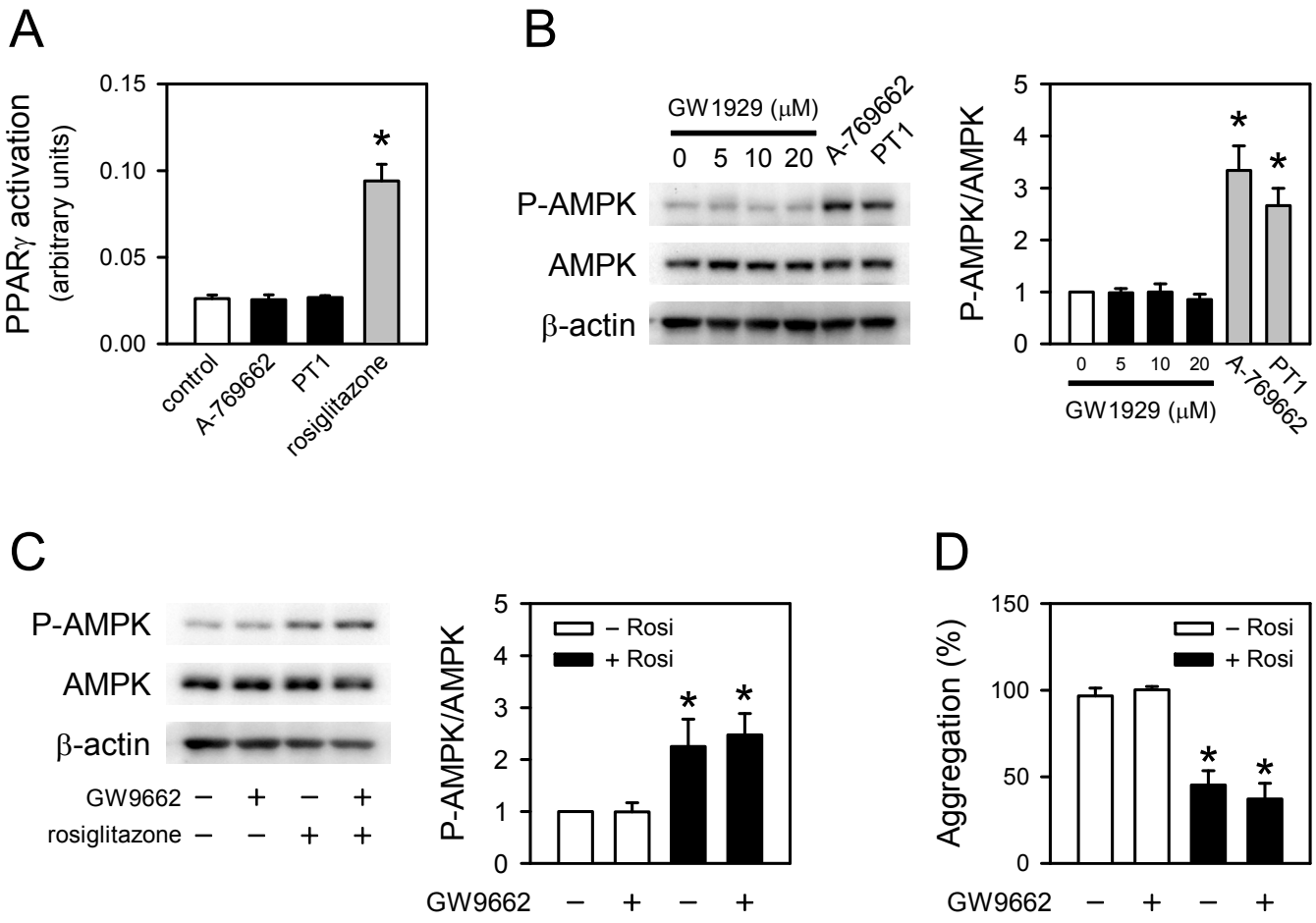

Fig. 4

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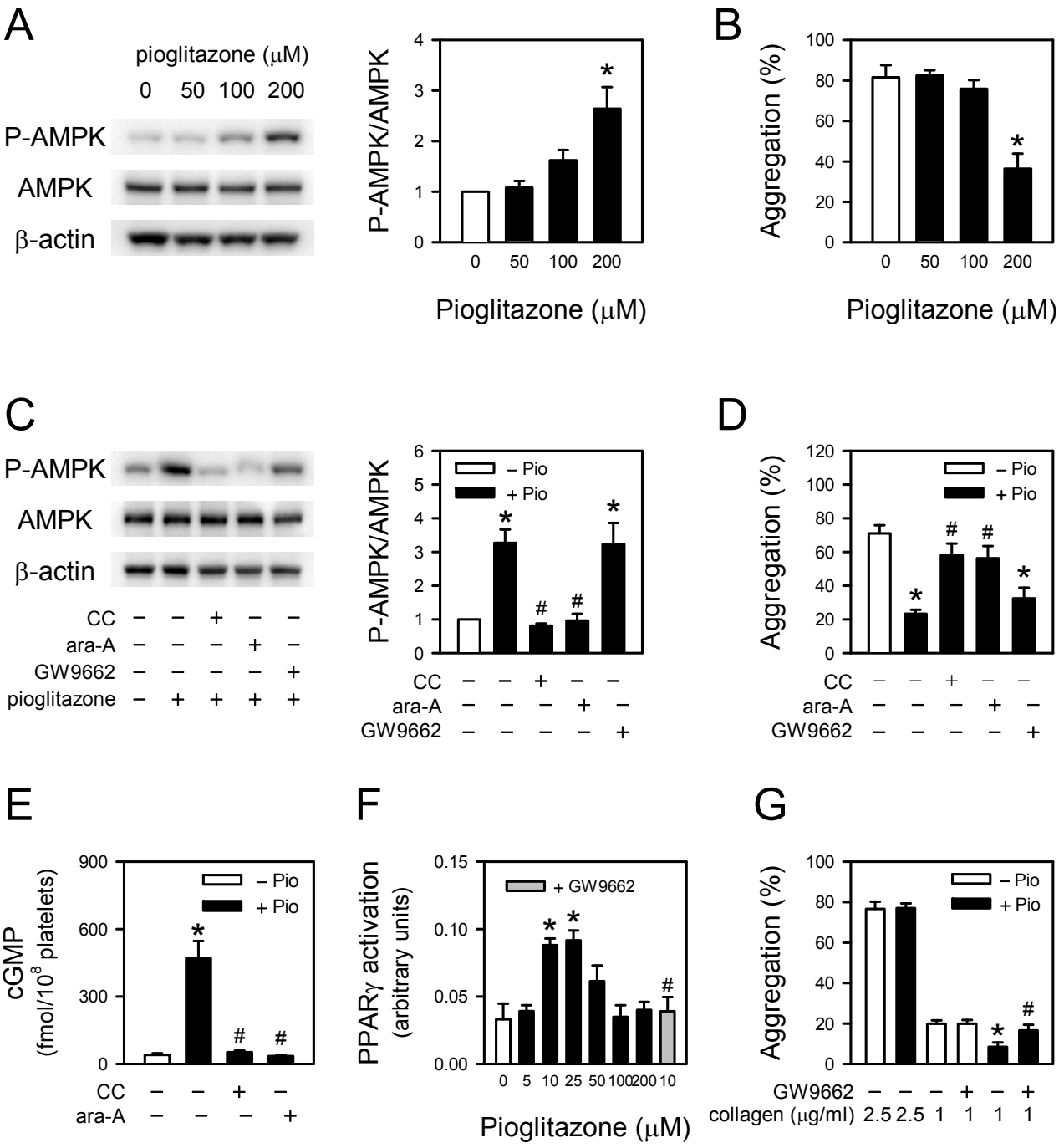

Fig. 5

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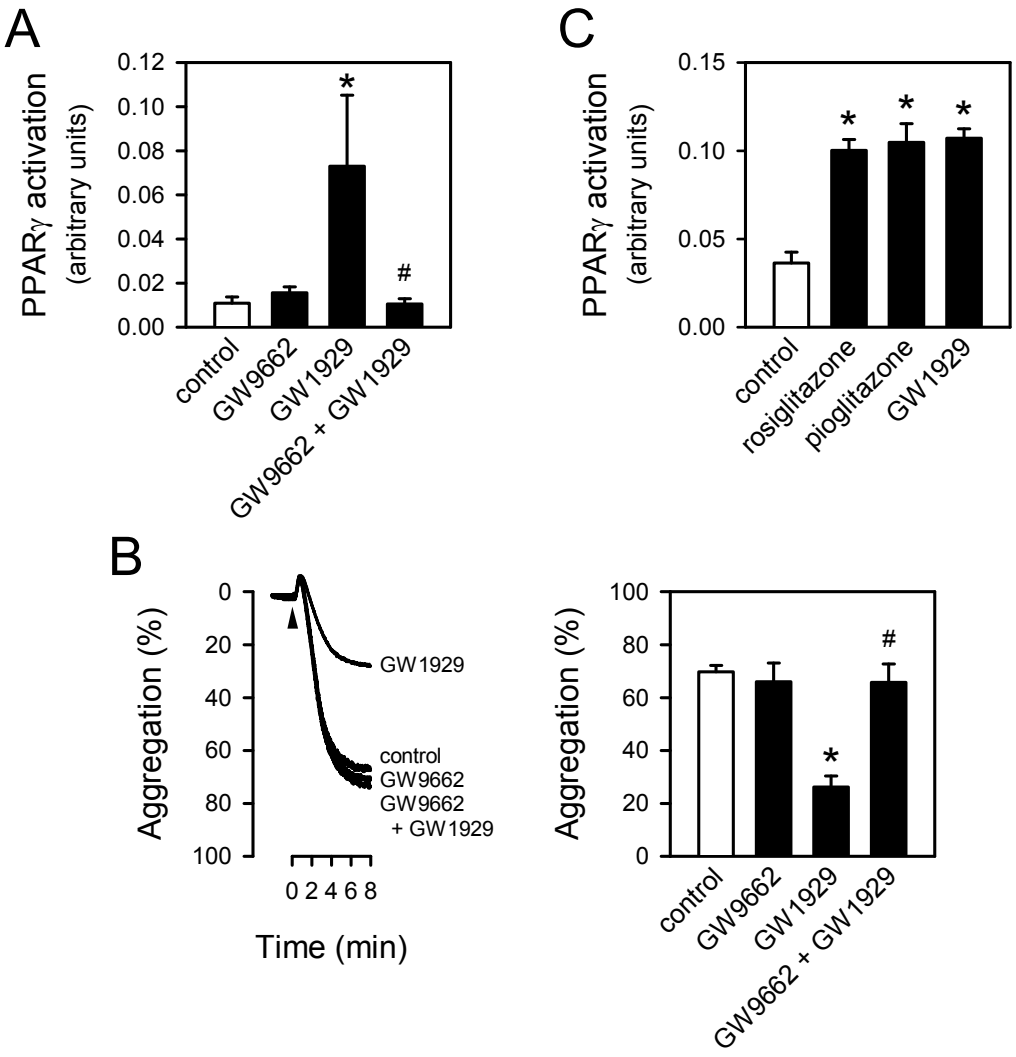

Fig. 6