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RESEARCH PAPER

Antiplatelet activity of nifedipine is mediated by inhibition of NF-κB activation caused by enhancement of PPAR-β/-γ activity

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BACKGROUND AND PURPOSE

The transcription factor NF-κB, stimulates platelet aggregation through a non-genomic mechanism. Nifedipine, a voltage-gated L-type calcium channel blocker, is widely used to treat hypertension. Nifedipine also displays antiplatelet activity, but the underlying mechanisms involved remain unclear. This study was designed to investigate whether the antiplatelet effects of nifedipine are mediated by regulating NF-κB-dependent responses.

EXPERIMENTAL APPROACH

Platelet aggregation was measured turbidimetrically using an aggregometer. NF-κB and PPAR activation, intracellular Ca^{2+} mobilization, PKCα activity, surface glycoprotein IIb/IIIa (GPIIb/IIIa) expression and platelet activation-related signalling pathways were determined in control and nifedipine-treated platelets in the presence or absence of PPAR antagonists or betulinic acid, a NF-κB activator.

KEY RESULTS

Exposure of platelets to nifedipine significantly increased the PPAR-β/-γ activity in activated human platelets. Treatment with nifedipine reduced collagen-induced NF-κB events, including the phosphorylation of IκB kinase-β, IκBα and p65NF-κB, which were markedly attenuated by GSK0660, a PPAR-β antagonist, or GW9662, a PPAR-γ antagonist. Furthermore, the interaction of PPAR-β/-γ with NF-κB and the PPAR-β/-γ-up-regulated NO/cGMP/PKG1 cascade may contribute to inhibition of NF-κB activation by nifedipine. Suppressing PPAR-β/-γ activity or increasing NF-κB activation greatly reversed the inhibitory effect of nifedipine on collagen-induced platelet aggregation, intracellular Ca^{2+} mobilization, PKCα activity and surface GPIIb/IIIa expression.

CONCLUSIONS AND IMPLICATIONS

PPAR-β/-γ-dependent inhibition of NF-κB activation contributes to the antiplatelet activity of nifedipine. These findings provide a novel mechanism underlying the beneficial effects of nifedipine on platelet hyperactivity-related vascular and inflammatory diseases.

Abbreviations

BetA, betulinic acid; GPIIb/IIIa, glycoprotein IIb/IIIa; IKKs, IκB kinases; ODQ, 1H-[1, 2, 4] oxadiazolo[4,3-a] quinoxalin-1-one

Introduction

Traditionally, platelet hyperactivity is recognized as a crucial factor in the pathogenesis of thrombotic vascular events. Currently, it has been believed that the activated platelets also play a key role in modulating inflammatory responses (Rajagopalan *et al.*, 2007). Therefore, inhibiting platelet hyperactivity may be a potential strategy for preventing and alleviating platelet-related thrombotic and inflammatory diseases. Nifedipine, a dihydropyridine derivative, blocks the voltage-gated L-type α1c calcium channel ($Ca_v1.2$) and is widely used in the treatment of hypertension and coronary heart diseases (Yamagishi *et al.*, 2006). Interestingly, nifedipine also exhibits an antiplatelet activity (Ośmiałowska *et al.*, 1990), but the mechanisms involved remain unclear. Accumulating evidence has indicated that the antiplatelet activity of nifedipine may be independent of the inhibition of calcium influx (Doyle and Ruegg, 1985), suggesting that other mechanisms may contribute to this property.

Although platelets are non-nucleated cells, they do contain transcription factors, notably PPARs and NF-κB, which exert negative and positive regulating effects on platelet aggregation respectively. PPARs are ligand-activated transcription factors and modulate several crucial physiological functions such as lipid metabolism and glucose homeostasis (Rosen and Spiegelman, 2001; Yessoufou and Wahli, 2010). Three PPAR isoforms (PPAR-α, PPAR-β/-δ and PPAR-γ) have been found in human platelets (nomenclature follows Alexander *et al.*, 2013). Recent studies have demonstrated that PPARs inhibit platelet aggregation through a non-genomic mechanism (Ali *et al.*, 2009; Chou *et al.*, 2011), suggesting that compounds with PPAR-activating activity may be a new type of antiplatelet drug. Although nifedipine reportedly enhances PPAR-γ activity in macrophages and smooth muscle cells (Hashimoto *et al.*, 2010; Ishii *et al.*, 2010), no information regarding whether PPARs are involved in nifedipine-mediated antiplatelet activity is available.

In unstimulated cells, NF-κB, which exists as an inactive cytoplasmic heterodimer complex composed of p50 and p65 subunits, is tightly bound to an inhibitory protein, IκB-α. Once activated by several pro-inflammatory stimuli, IκB-α is phosphorylated by IκB kinases (IKKs), leading to rapid poly-ubiquitination and degradation by proteasomes, followed by the release of NF-κB from its inhibitors. The NF-κB dimers subsequently translocate to the nucleus, where they bind to and activate the transcription of target genes (Tak and Firestein, 2001). In addition to the critical role of NF-κB in inflammatory responses (Ghosh and Hayden, 2008), NF-κB also exerts a non-genomic function to regulate platelet activation. It is known that IKKβ phosphorylation, IκBα degradation and p65NF-κB phosphorylation in human platelets are greatly amplified in response to thrombin or collagen. Because treatment with NF-κB inhibitors impairs agonist-induced platelet aggregation and granule release (Malaver *et al.*, 2009; Chang *et al.*, 2011), suppressing NF-κB activation may be a way of inhibiting platelet aggregation. Several lines of evidence have confirmed that PPAR-γ and PPAR-β/-δ agonists exhibit anti-inflammatory activity by inhibiting NF-κB activation through the attenuation of IKKs and the DNA-binding activity of NF-κB in activated macrophages, smooth muscle cells and experimental periodontitis (Castrillo *et al.*, 2000; Ikeda *et al.*, 2000; Di Paola *et al.*, 2011). However, the effect of PPAR-γ on NF-κB activation is controversial, and it is proposed that the described mechanisms of PPARs may be cell type and PPAR isoform specific (Ricote and Glass, 2007). To date, whether PPARs or PPAR-regulated NF-κB events contribute to the antiplatelet activity of nifedipine remain unclear. In the present study, we demonstrated that nifedipine initially increases PPAR-β/-γ activity and subsequently attenuates NF-κB activation in platelets, which ultimately inhibits platelet aggregation.

Methods

Determination of PPAR activity

Platelets were incubated with various drugs or solvent control for 5 min at 37°C; lysed in a buffer containing 50 μM of Tris-HCl (pH 7.4), 0.5 M of NaCl, 1 μM of EDTA, 0.05% SDS, 0.5% Triton X-100 (Sigma Chemical Company, St. Louis, MO, USA) and 1 μM of PMSF; and subsequently centrifuged at 15 000× *g* for 10 min at 4°C. The PPAR activity in supernatants was determined using a PPAR transcription factor ELISA kit, and the absorbance at 450 nm was measured (Chou *et al.*, 2011).

Platelet aggregation

Blood samples were taken from healthy human volunteers who had not taken any medicine during the preceding 2 weeks, were mixed with ACD solution (75 mM of trisodium citrate, 42 mM of citric acid and 136 mM of glucose, pH 5.2) and centrifuged at 160× *g* and 25°C for 10 min to produce PRP. Centrifugation was subsequently performed to produce platelet pellets and suspended in Tyrode solution (pH 7.4). To prevent the contamination of platelet samples with leukocytes, platelet suspension was filtered through a 5 μm syringe-adaptable filter to remove white blood cell contaminants as previously described (Freedman *et al.*, 2010), and platelet concentration was adjusted to 3.0×10^8 platelets·mL^{-1}. Platelet aggregation was measured turbidimetrically using an aggregometer at 37°C with constant stirring at 1000 r.p.m. (Model 560; Chrono-Log Corporation, Havertown, PA, USA). Tyrode solution was assigned as 100% aggregation, and platelet suspension was assigned as 0% aggregation. Platelet suspensions (0.3 mL) were preincubated with various drugs or solvent control (0.1% DMSO) for 3 min, and collagen (10 μg·mL^{-1}) was subsequently added for 6 min. The extent of platelet aggregation was expressed as a percent-

age of the Tyrode solution in light transmission units as described previously (Chou *et al.*, 2011).

Determination of cGMP formation

Washed platelets were preincubated with various drugs or solvent control for 3 min at 37°C, and collagen (10 μg·mL^{-1}) was subsequently added for 6 min. The reaction was stopped by adding EDTA (10 mM) followed by immediate boiling for 5 min. After centrifugation at 10 000× *g* for 5 min at 4°C, the cGMP content of the supernatant was measured using an ELISA kit.

Determination of nitrate + nitrite formation

Washed platelets were preincubated with various drugs or solvent control for 3 min at 37°C, and collagen (10 μg·mL^{-1}) was subsequently added for 6 min. Centrifugation was performed at 10 000× *g* for 5 min at 4°C. The amount of nitrate + nitrite (NOx), a stable end product of NO, in the supernatants was measured using a Sievers NO analyser (Sievers 280 NOA; Sievers, Boulder, CO, USA) as described previously (Chou *et al.*, 2008). Nitrate concentrations were calculated with reference to a standard solution of sodium nitrate.

Measurement of platelet intracellular Ca^{2+} mobilization

One mL of PRP (3.0×10^8 platelets·mL^{-1}) was incubated with Fluo-4 AM (5 μM; Sigma Chemical Company) for 30 min at 37°C in the dark and subsequently centrifuged at 500× *g* for 10 min. The pellets were then suspended in 2 mL of Tyrode solution. The fluorescence intensity of 20 000 platelets per sample was analysed using a flow cytometer equipped with CellQuest software (FACScan; Becton Dickinson, Heidelberg, Germany) (Chou *et al.*, 2011).

Measurement of platelet surface glycoprotein IIb/IIIa (GPIIb/IIIa) expression

A platelet suspension (10 μL) was placed into polystyrene tubes containing 35 μL of HEPES buffer and 5 μL of CD41/CD61-FITC that was raised against a platelet GPIIb/IIIa complex. Various drugs were then added and incubated at 37°C for 5 min, and collagen (10 μg·mL^{-1}) was subsequently added for 10 min. The reaction was stopped by adding 500 μL of 1% paraformaldehyde, and the fluorescence intensity of 20 000 platelets per sample was analysed using a flow cytometer.

Immunoprecipitation

The total cellular protein of platelets (1×10^9 cells) was extracted and incubated with pure proteome protein A magnetic beads for 1 h at 4°C. The sample tubes were subsequently placed onto the magnetic rack, and the beads were allowed to adhere to the side to remove non-specific binding. After centrifugation and collecting the supernatant, the primary antibody for PPAR-β or PPAR-γ was added in the presence of protein A magnetic beads and incubated overnight at 4°C. The sample tubes were subsequently placed onto the magnetic rack, and the beads were allowed to adhere to the side, which enabled the collection of the magnetic beads. Subsequently, a lysis buffer was added and boiled to separate the beads and bound protein (Chou *et al.*, 2011). The expression of the target protein in the samples was determined using Western blotting.

Western blotting

Platelets (3×10^8 mL^{-1}) were incubated with various drugs for 3 min at 37°C and were subsequently lysed in RIPA buffer (150 mM of NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM of Tris, pH 8.0) containing a mixture of protease and phosphatase inhibitors. The cell lysates were heated at 95°C for 10 min, and proteins (20 μg) were separated in 8% SDS-PAGE and electrotransferred using semi-dry transfer (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Various primary antibodies were then incubated with transferred membranes for 1.5 h, and a peroxidase-conjugated secondary antibody was added in PBS Tween 20 (Sigma Chemical Company) for 1 h. The immunoreactive bands of target genes were detected using an ECL kit (Amersham International Plc., Buckinghamshire, UK) with reference to a cytoplasmic protein (β-actin).

Data analysis

Experimental results are expressed as means ± SEM. A one-way ANOVA with a *post hoc* Bonferroni test was used for statistical analysis. Results were considered significant difference at a value of $P < 0.05$.

Materials

N^G-nitro L-arginine methyl ester (L-NAME), 1H-[1, 2, 4] oxadiazolo[4,3-a] quinoxalin-1-one (ODQ) and other chemical agents were obtained from Sigma Chemical Company (St. Louis, MO, USA). Collagen (type I, equine tendon) was obtained from Chrono-Log Corporation (Broomall, PA, USA). RIPA buffer was obtained from Pierce Biotechnology Inc. (Rockford, IL, USA). A PPAR transfactor kit and PPAR-γ (NR1C3) antibody were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). GW6471, GSK0660, GW9662 and betulinic acid (BetA) were purchased from Tocris (Avonmouth, Bristol, UK). An enhanced chemiluminescence (ECL) reagent was purchased from Upstate Biotechnology (Lake Placid, NY, USA). PPAR-β (NR1C2) and β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phospho-p65NF-κB, total-p65NF-κB, phospho-IKK and total-IKK antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Nifedipine and rosiglitazone were purchased from Sigma Chemical Company, dissolved in DMSO and diluted with Tyrode solution; the final concentration of DMSO was fixed at 0.1%. Other chemical agents were obtained from Sigma Chemical Company.

Results

Nifedipine increases PPAR-β/-γ activity in human platelets

Nifedipine (1 and 5 μM) concentration-dependently increased PPAR-β and PPAR-γ activity but did not affect PPAR-α (NRIC1) activity in collagen-stimulated platelets. Adding GW7647 (20 μM), a PPAR-α agonist; GW0742 (20 μM), a PPAR-β agonist; or rosiglitazone (20 μM), a PPAR-γ agonist, as positive controls, markedly enhanced the activity of PPAR-α, PPAR-β and PPAR-γ respectively (Figure 1A).

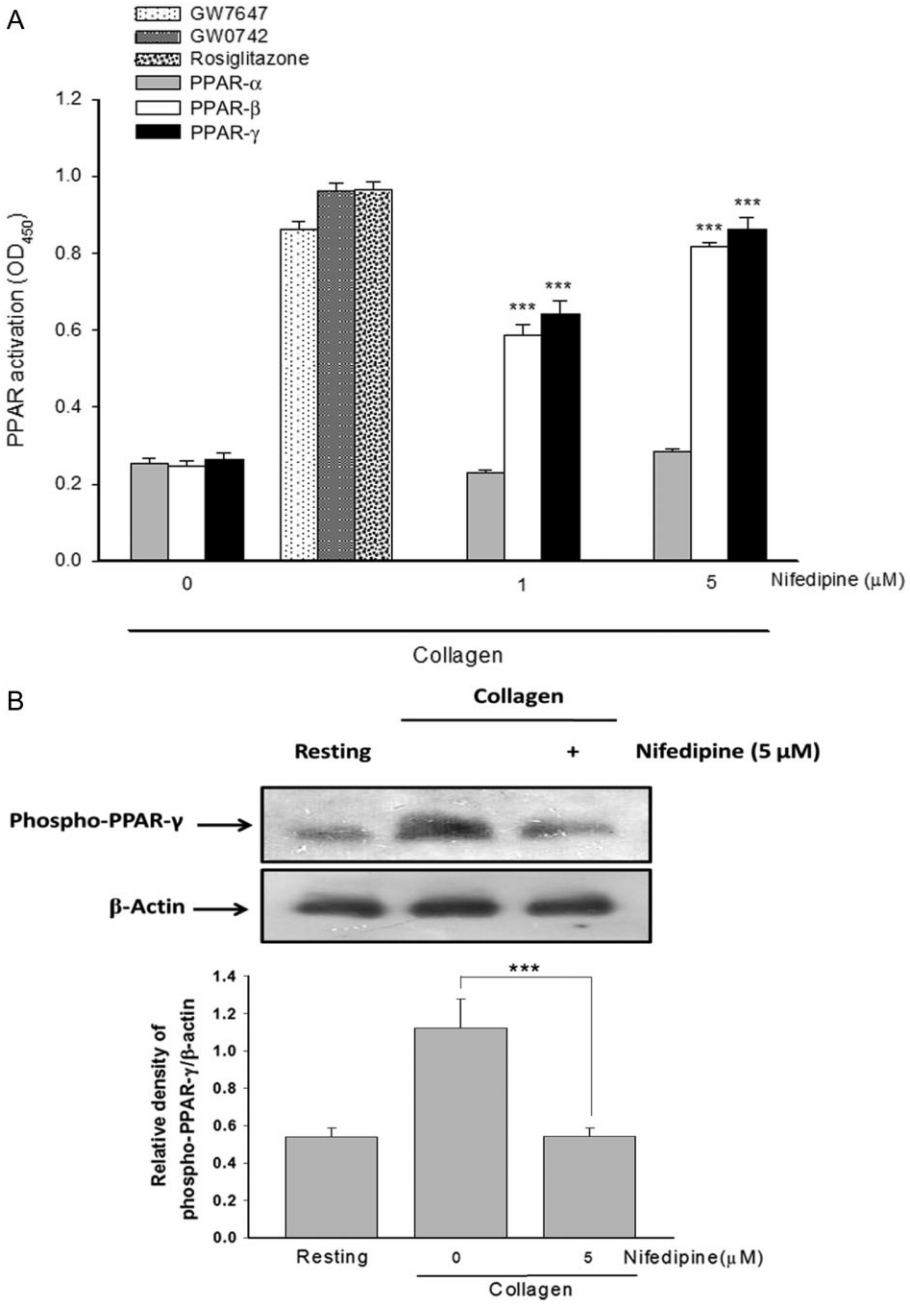

Figure 1

Effect of nifedipine on PPAR activity in activated platelets. (A) Platelets were incubated with GW7647 (20 μM), GW0742 (20 μM), rosiglitazone (20 μM) or nifedipine (1 or 5 μM) for 5 min followed by addition of collagen (10 μg·mL^{-1}) for 6 min and lysed. PPAR activity was measured as described. (B) Platelets were preincubated with nifedipine (5 μM) at 37°C for 3 min followed by addition of collagen (10 μg·mL^{-1}) for 6 min; the PPAR-γ phosphorylation was detected by Western blotting. Platelets treated with vehicle alone served as controls (resting). Data were expressed as mean ± SEM (n = 4). ***P < 0.001, significantly different from collagen-stimulated platelets.

Moreover, nifedipine significantly attenuated collagen-induced PPAR-γ phosphorylation (Figure 1B).

PPAR-β/-γ involve nifedipine-mediated inhibition of NF-κB activation

Collagen-induced NF-κB events, including the phosphorylation of IKK-β, IκBα and p65NF-κB in human platelets, were significantly inhibited by nifedipine, GW0742 and rosiglitazone. However, the decreased NF-κB events caused by nifedipine were reversed by GSK0660 (5 μM), a PPAR-β antagonist, or GW9662 (5 μM), a PPAR-γ antagonist (Figure 2). In addition, nifedipine concentration-dependently attenuated p65NF-κB phosphorylation in the PPAR-β/-γ-NF-κB complexes of collagen-stimulated platelets (Figure 3A).

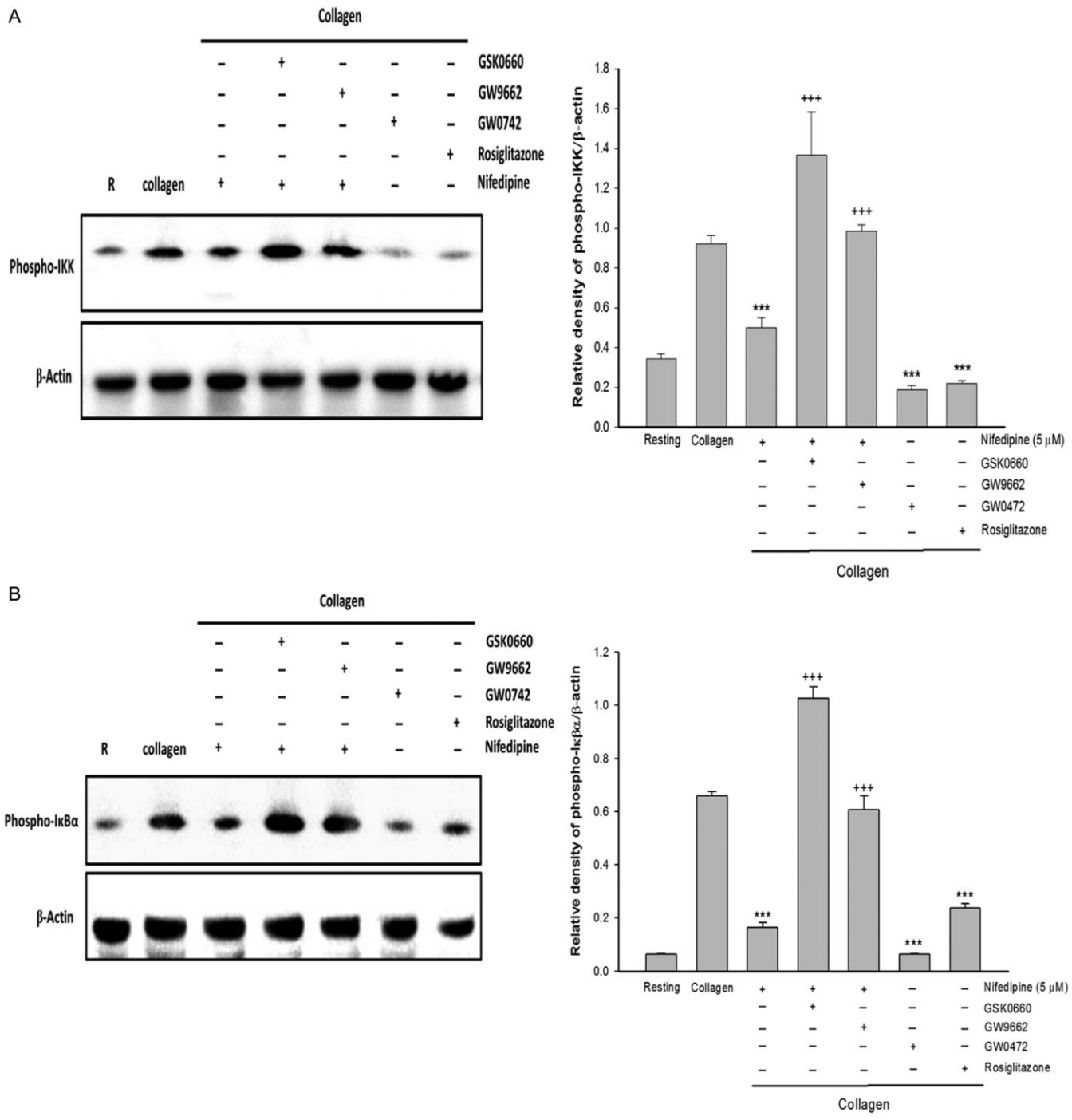

Figure 2

Effects of PPAR-β/-γ on nifedipine-mediated suppression of NF-κB activation in activated platelets. Platelets were pretreated with GW0742, rosiglitazone, nifedipine (5 μM) or nifedipine combination with GSK0660 (5 μM) or GW9662 (5 μM) at 37°C for 3 min followed by addition of collagen (10 μg·mL^{-1}) for 6 min. Then, the expression of (A) phospho-IKK, (B) phospho-IκBα and (C) phospho-p65NF-κB were determined by Western blot. Data were expressed as means ± SEM (n = 4). ***P < 0.001, significantly different from collagen-stimulated platelets; ^{+++}P < 0.001, significantly different from collagen + nifedipine-treated platelets.

Role of PPAR-β/-γ and NF-κB in nifedipine-mediated antiplatelet activity

Nifedipine (1, 5 μM) concentration dependently inhibited collagen-induced platelet aggregation, which was markedly attenuated by GSK0660, GW9662 and BetA (10 μM), a NF-κB activator (Figure 3B). These findings were also observed in thrombin-stimulated platelets (Supporting Information; Figure S1). Moreover, the PPAR-β/-γ antagonists and BetA did

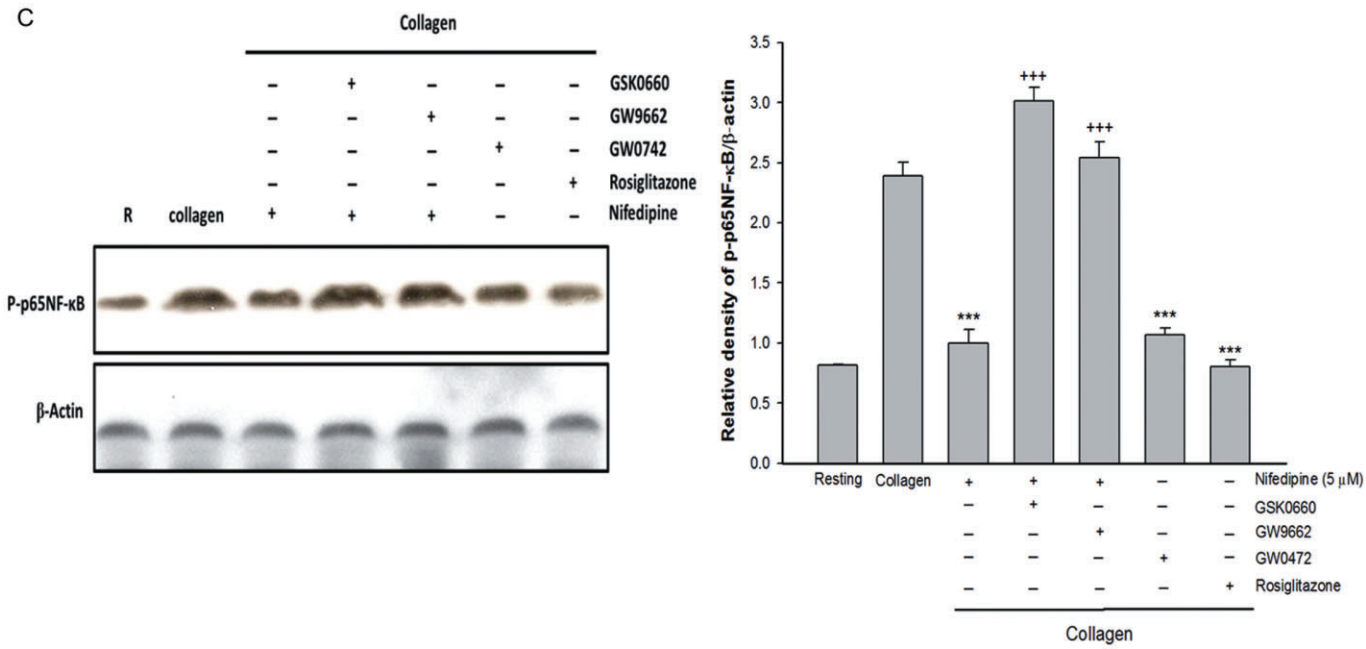

Figure 2
Continued

not affect the platelet aggregation in both resting and collagen-stimulated platelets (Supporting Information; Figure S2).

The PPAR-β/-γ-regulated NO/cGMP/PKG1 cascade involves NF-κB inactivation by nifedipine

Treatment with nifedipine further enhanced NOx and cGMP formation in collagen-stimulated platelets, compared with platelets stimulated with collagen alone (Figure 4A,B). Adding GSK0660 or GW9662 significantly diminished the enhancement of NOx and cGMP formation by nifedipine. In the presence of L-NAME (100 μM), an inhibitor of NOS; ODQ (10 μM), an inhibitor of soluble GC; or KT-5823 (5 μM), a PKG1 inhibitor, the inhibitory effect of nifedipine on collagen-induced p65NF-κB phosphorylation was markedly decreased (Figure 4C).

The PPAR-β/-γ/NF-κB cascade involves the nifedipine-mediated reduction of intracellular calcium mobilization and surface GPIIb/IIIa expression

Nifepidine concentration-dependently reduced collagen-induced intracellular calcium mobilization (Figure 5A) and surface GPIIb/IIIa expression (Figure 5B). These effects of nifedipine were decreased after adding GSK0660, GW9662 or BetA. Similarly, the PPARs antagonists and BetA did not affect the parameters measured in this study.

Discussion and conclusions

Several studies have reported that activation of NF-κB induces platelet activation, and blocking NF-κB activation by BAY 11-7082 or Ro106-9920 inhibits platelet aggregation via suppressing the PLA_2-TxA_2 cascade and surface GPIIb/IIIa expression (Malaver *et al.*, 2009; Chang *et al.*, 2011). These results suggest that agents able to inhibit NF-κB activation may exhibit antiplatelet activity. The present study is the first to demonstrate that nifedipine attenuates NF-κB activation in activated human platelets, possibly providing a novel mechanism underlying the antiplatelet activity of nifedipine.

The molecular mechanisms underlying the inhibitory effect of nifedipine on NF-κB activation were then investigated. A critical finding of this study is that nifedipine increases both PPAR-β and PPAR-γ activity in human platelets. Our supplemental data show that other calcium channel blockers (CCBs) such as amlodipine, but not lacidipine, are PPAR-β activators in platelets (Supporting Information; Figure S3). The effects of different CCBs on PPAR activity in platelets is diverse and chemical structure specific. Ishii *et al.* (2010) reported that activation of ERK1/2 suppressed PPAR-γ activity through the phosphorylation of PPAR-γ. We observed that nifedipine inhibited collagen-induced PPAR-γ phosphorylation accompanied by an attenuation of ERK1/2 activation (data not shown), which may be a possible mechanism of increasing the PPAR-γ activity. In the work described here, to determine the role of PPAR-β/-γ in regulating NF-κB activation, PPAR-β/-γ antagonists were added. In the presence of GSK0660 or GW9662, nifedipine's inhibition of NF-κB events, including the phosphorylation of IKK, IκBα and p65NF-κB in activated platelets, were markedly reduced, effects comparable to those observed in nucleated cells (Castrillo *et al.*, 2000; Ikeda *et al.*, 2000). Accordingly, PPAR-β/-γ are upstream negative regulators of NF-κB activation. We then investigated whether PPAR-β/-γ and NF-κB were involved in the antiplatelet activity of nifedipine. Blocking PPAR-β/-γ activity using GSK0660 or GW9662 or activating

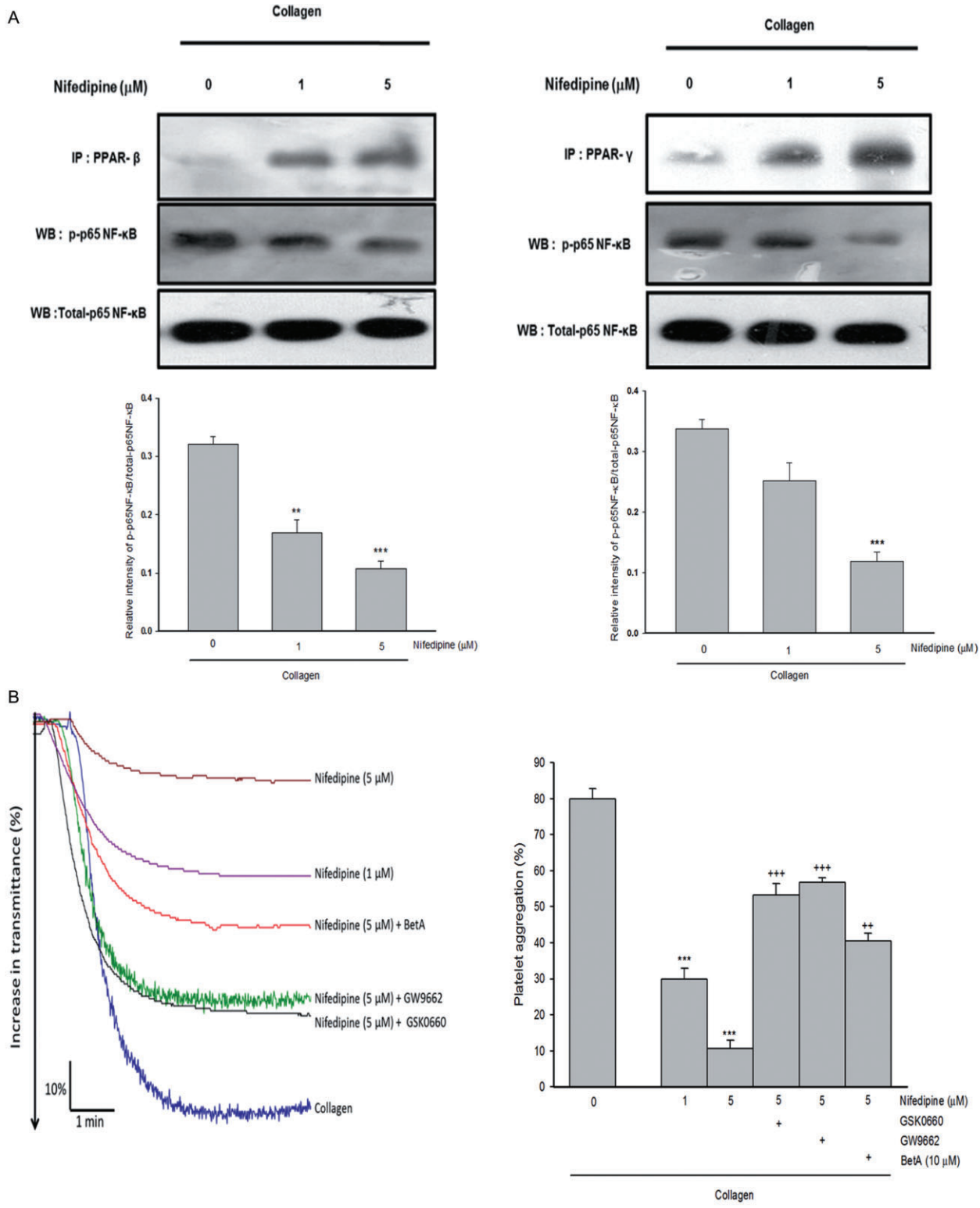

Figure 3

The interaction of PPAR-β/-γ with NF-κB and role of PPAR-β/-γ and NF-κB on nifedipine-mediated antiplatelet activity. Platelets were treated with various drugs for 3 min at 37°C followed by addition of collagen (10 μg·mL^{-1}) for 6 min. (A) The extracted protein was immunoprecipitated (IP) with PPAR-β or PPAR-γ. Then, the expression of target genes in the IP complexes was determined by Western blotting (WB). (B) The changes of platelet aggregation in various groups were measured, and the representative image was shown. Data were expressed as means ± SEM (n = 4). $^{**}P < 0.01$, $^{***}P < 0.001$, significantly different from collagen-stimulated platelets; $^{++}P < 0.01$, $^{+++}P < 0.001$, significantly different from corresponding collagen + nifedipine-treated platelets.

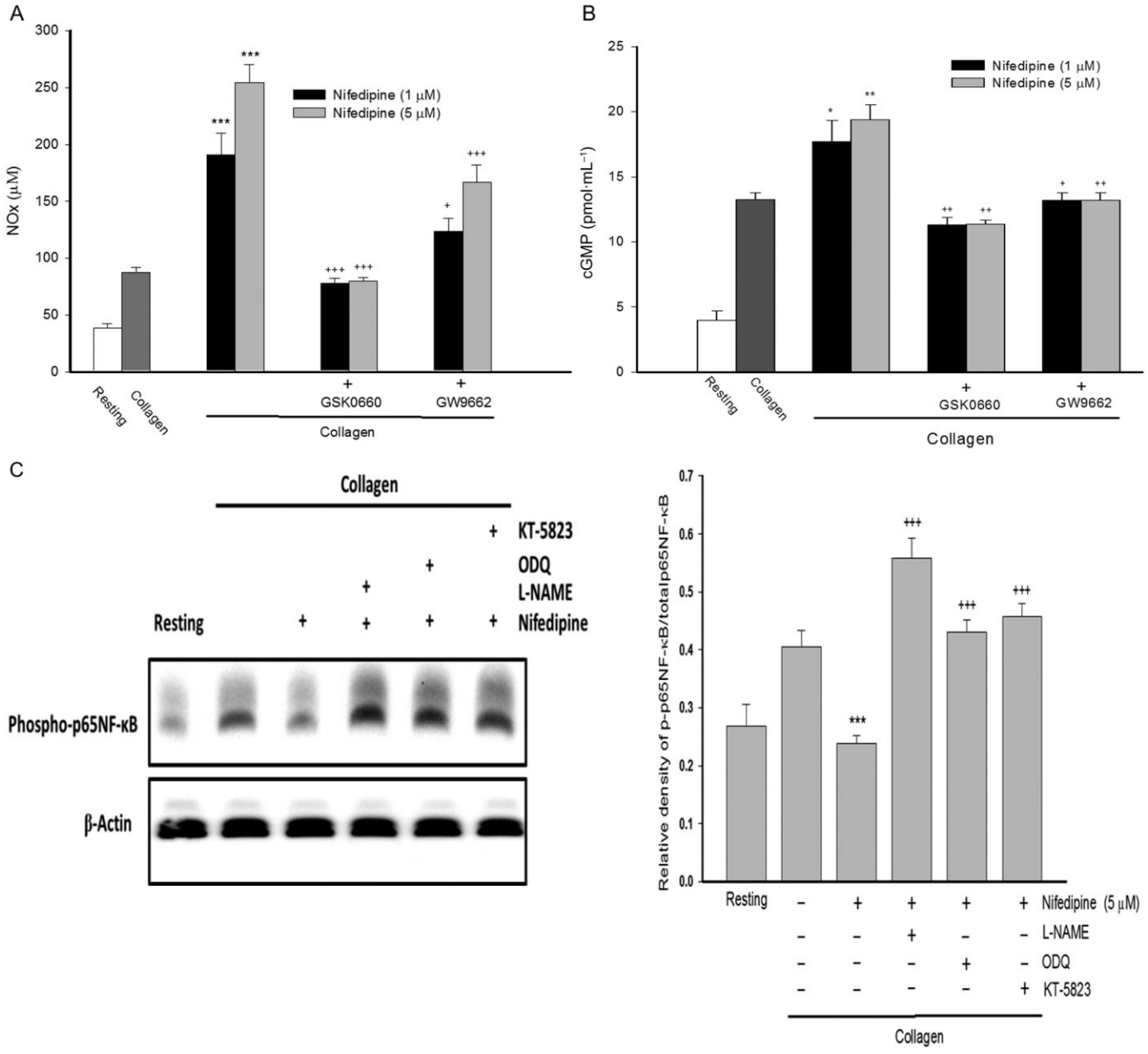

Figure 4

Effects of GSK0660 (5 μM) or GW9662 (5 μM) on nifedipine-induced formation of NOx (A) and cGMP (B) in collagen-stimulated platelets were determined. (C) The effects of L-NAME (100 μM), ODQ (10 μM) or KT-5823 (5 μM) on nifedipine-mediated inhibition of p65NF-κB phosphorylation were examined. Data were expressed as means ± SEM (n = 4). *P < 0.05, **P < 0.01, ***P < 0.001, significantly different from collagen-stimulated platelets; ^{+}P < 0.05, ^{++}P < 0.01, ^{+++}P < 0.001, significantly different from corresponding collagen + nifedipine-treated platelets.

NF-κB using BetA significantly decreased the inhibitory effect of nifedipine on platelet aggregation. Therefore, the PPAR-β/-γ-dependent attenuation of NF-κB activation contributed to the antiplatelet activity of nifedipine in our system.

The activation of PKCα is crucial for stimulating platelet secretion and aggregation (Konopatskaya *et al.*, 2009). Our recent study confirmed that PPAR-α/-γ-mediated inhibition of PKCα activity in activated platelets is associated with an interaction of PPAR-α/-γ with PKCα (Chou *et al.*, 2011), suggesting that protein–protein interaction is a novel mechanism in which they exert their functions. In this study, we demonstrated for the first time that nifedipine increases the interaction of PPAR-β/-γ with NF-κB in collagen-stimulated platelets accompanied by decreased p65NF-κB phosphorylation in the complex. Thus, the association of PPAR-β/-γ with NF-κB may play a role in the attenuation of NF-κB activation caused by nifedipine.

Up-regulation of the NO/cGMP/PKG1 signalling pathway reportedly inhibits platelet activation by modulating the dynamics of actin filaments, integrin activation and intracel-

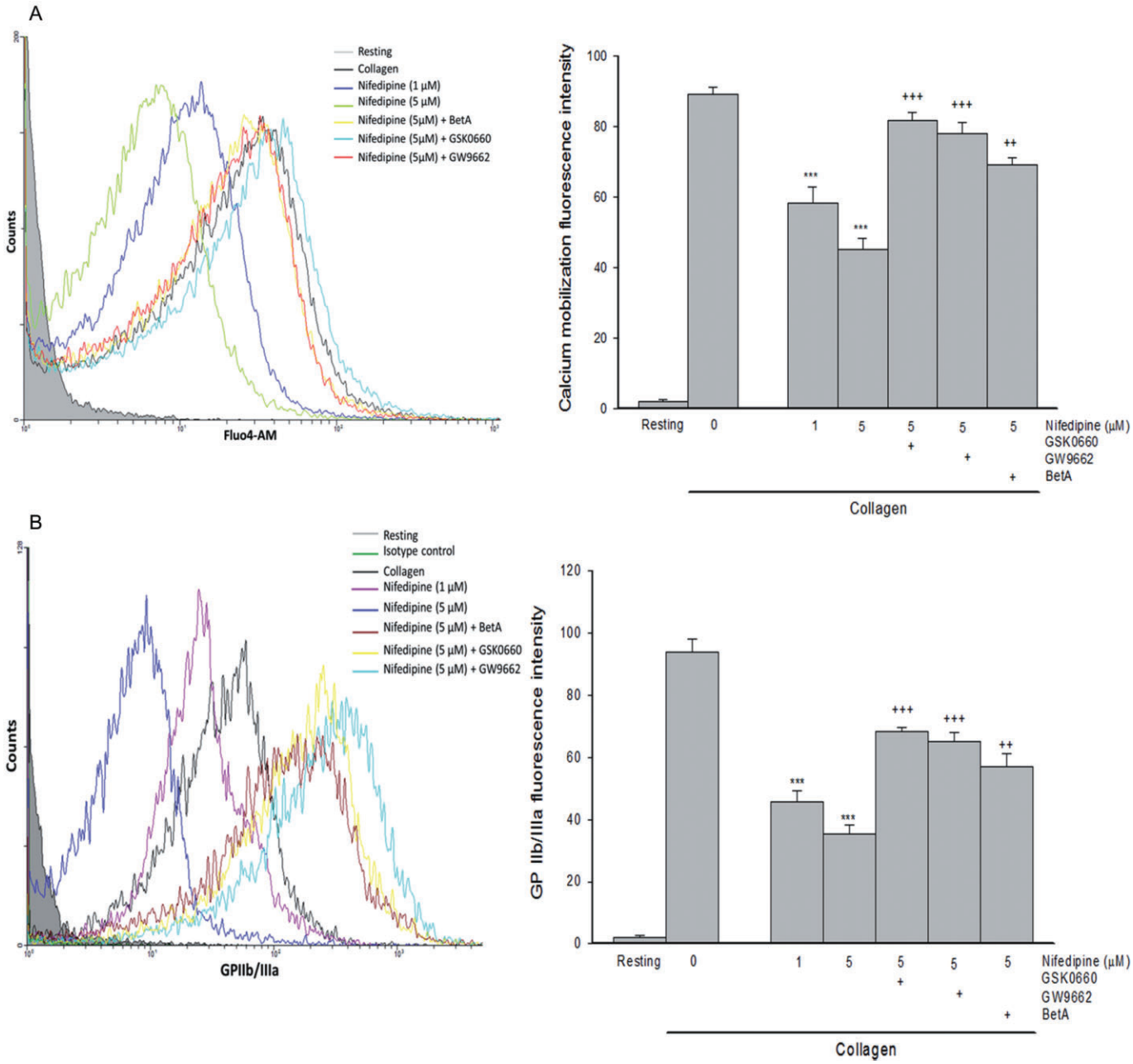

Figure 5

Role of PPAR-β/-γ and NF-κB in nifedipine-mediated attenuation of intracellular Ca^{2+} mobilization and GPIIb/IIIa expression in activated platelets. Platelets were pretreated with nifedipine or nifedipine combination with GSK0660 (5 μM), GW9662 (5 μM) or BetA (10 μM) for 3 min followed by addition of collagen (10 μg·mL^{-1}) for 6 min. The intracellular Ca^{2+} mobilization (A) and surface GPIIb/IIIa expression (B) were determined. Data were expressed as means ± SEM ($n = 4$). ***$P < 0.001$ as compared with collagen-stimulated alone platelets. $^{++}P < 0.01$, $^{+++}P < 0.001$ as compared with respective collagen + nifedipine-treated alone platelets.

lular Ca^{2+} mobilization, in turn suppressing PLC and PKC activity (Antl *et al.*, 2007; Chou *et al.*, 2008). Our results showed that nifedipine treatment significantly increased NO and cGMP formation in activated platelets, which was markedly reduced by PPAR-β/-γ antagonists, indicating that nifedipine-induced NO/cGMP formation is regulated by PPAR-β/-γ. Furthermore, Liang *et al.* (2009) and Jimenez *et al.* (2010) have proposed that activation of the PI3K/Akt signaling pathway is involved in PPAR-β/-γ-induced NO/cGMP production. Suppressing the NO/cGMP/PKG1 cascade with appropriate inhibitors reversed the inhibition of p65NF-κB phosphorylation induced by nifedipine. Overall, the molecular mechanisms underlying decreased NF-κB activation by nifedipine may involve a direct association of PPAR-β/-γ with NF-κB and consequent up-regulation of the NO/cGMP/PKG1 pathway.

When platelets are activated by agonists, an increase in intracellular Ca^{2+} concentration as a result of either Ca^{2+} influx and/or Ca^{2+} release from intracellular stores is essential for platelet activation. Nifedipine exhibited a similar inhibitory potency on the collagen-evoked rise of intracellular Ca^{2+} mobilization either in the presence of extracellular calcium (1 mmol·L^{-1}) or in a Ca^{2+}-free solution, implying that the inhibition of intracellular Ca^{2+} mobilization may predominantly be caused by the attenuation of Ca^{2+} release from intracellular Ca^{2+} stores. Previous studies have indicated that blocking NF-κB activation reduces collagen-induced platelet intracellular Ca^{2+} mobilization by inhibiting PLCγ2-derived inositol 1,4,5-trisphosphate receptor formation (Singer *et al.*, 1997; Ragab *et al.*, 2007; Chang *et al.*, 2011). Activation of PPARs decreased intracellular Ca^{2+} mobilization in activated platelets through elevation of the NO/cGMP/PKG1 signalling cascade (Masuda *et al.*, 2010; Shih and Chou, 2012). As expected, adding PPAR-β/-γ inhibitors or BetA significantly reduced inhibition of intracellular Ca^{2+} mobilization by nifedipine, suggesting that PPAR-β/-γ-mediated NF-κB inactivation contributed to the decrease of intracellular Ca^{2+} mobilization.

The binding of fibrinogen to the surface GPIIb/IIIa complex is a critical step for platelet aggregation (Shattil *et al.*, 1998; Salanova *et al.*, 2007). Inhibiting NF-κB activation reduces the outside-in/inside-out signalling of GPIIb/IIIa and fibrinogen binding (Malaver *et al.*, 2009). Interestingly, the GPIIb/IIIa complex is required for NF-κB activation (Joo, 2012), suggesting that there is a mutual regulation between NF-κB and GPIIb/IIIa. Therefore, PPAR-β/-γ-dependent attenuation of NF-κB activation may in part account for the decreased surface GPIIb/IIIa expression by nifedipine. This is supported by the fact that inhibiting PPAR-β/-γ activity or enhancing NF-κB activation displays higher surface GPIIb/IIIa expression than that of collagen + nifedipine-treated platelets. Arachidonic acid and COX-derived TXA_2 formation is a potent stimulator for platelet aggregation. Our supplemental data showed that nifedipine inhibits platelet activity in the presence of arachidonic acid (Supporting Information; Table 1). Moreover, production of arachidonic acid or collagen-induced TXB_2, a stable metabolite of TXA_2, was concentration-dependently decreased by nifedipine (Supporting Information; Figure S4). However, adding GSK0660 or GW9662 did not affect the inhibitory effects of nifedipine on platelet COX-1 activity and arachidonic acid-induced TXB_2 formation, but partly attenuated the inhibition of collagen-induced TXB_2 formation by nifedipine. Accordingly, PPAR-β/-γ may inhibit the release of arachidonic acid rather than directly affect COX-1 activity, subsequently attenuating TXB_2 formation, which may be another mechanism involved in the antiplatelet activity of nifedipine. Although several studies have confirmed that nifedipine inhibits platelet aggregation *in vitro* and *in vivo* (Takahara *et al.*, 1985; Ośmiałowska *et al.*, 1990), there is one contradictory finding conflicting with previous studies (Murphy *et al.*, 1985). Reasons for this discrepancy remain unclear, but the concentration of nifedipine used, treatment duration, the different condition of human subjects chosen and preparation of platelet samples may all contribute to the differences reported. In conclusion, we have demonstrated that the antiplatelet activity of nifedipine was, at least in part, mediated by the inhibition of NF-κB activation in a PPAR-β/-γ-dependent manner. These findings not only provide a novel molecular mechanism accounting for the antiplatelet activity of nifedipine but also suggest that nifedipine may have potential in the treatment of inflammation-related vascular disease therapy, because it modulates NF-κB activation.

Acknowledgements

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Conflict of interest

The authors declare no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Figure S1 Effects of PPAR-β/-γ antagonists on nifedipine-mediated inhibition of thrombin-induced platelet aggregation (A) and phosphorylation of p65NF-κB (B) in platelets.
Figure S2 Effect of PPAR-β/-γ antagonists and BetA on collagen-induced platelet aggregation.
Figure S3 Effects of various CCBs on platelet PPARs activity (A) and PPARs antagonists on their antiplatelet activity (B).
Figure S4 Effects of PPAR-β/-γ antagonists on nifedipine-mediated attenuation of TXB_2 formation in AA or collagen-stimulated platelets.
Table S1 Effects of PPAR-β/-γ antagonists on nifedipine-mediated attenuation of COX-1 activity in platelets.