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The antiplatelet activity of magnolol is mediated by PPAR-β/γ

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ABSTRACT

Activation of peroxisome proliferator-activated receptor (PPAR) isoforms (α, β/δ, and γ) is known to inhibit platelet aggregation. In the present study, we examined whether PPARs-mediated pathways contribute to the antiplatelet activity of magnolol, a compound purified from *Magnolia officinalis*. Magnolol (20–60 μM) dose-dependently enhanced the activity and intracellular level of PPAR-β/γ in platelets. In the presence of selective PPAR-β antagonist (GSK0660) or PPAR-γ antagonist (GW9662), the inhibition of magnolol on collagen-induced platelet aggregation and intracellular Ca^{2+} mobilization was significantly reversed. Moreover, magnolol-mediated up-regulation of NO/cyclic GMP/PKG pathway and Akt phosphorylation leading to increase of eNOS activity were markedly abolished by blocking PPAR-β/γ activity. Additionally, magnolol significantly inhibited collagen-induced PKCα activation through a PPAR-β/γ and PKCα interaction manner. The arachidonic acid (AA) or collagen-induced thromboxane B_2 formation and elevation of COX-1 activity caused by AA were also markedly attenuated by magnolol. However, these above effects of magnolol on platelet responses were strongly reduced by simultaneous addition of GSK0660 or GW9662, suggesting that PPAR-β/γ-mediated processes may account for magnolol-regulated antiplatelet mechanisms. Similarly, administration of PPAR-β/γ antagonists remarkably abolished the actions of magnolol in preventing platelet plug formation and prolonging bleeding time in mice. Taken together, we demonstrate for the first time that the antiplatelet and antithrombotic activities of magnolol are modulated by up-regulation of PPAR-β/γ-dependent pathways.

1. Introduction

Initiation of intraluminal thrombosis is believed to be responsible for platelet adherence and aggregation, and platelet hyperactivity is a crucial factor causing the pathogenesis of thrombsis and related vascular diseases [1,2]. Therefore, treatment with antiplatelet agents may be a beneficial strategy to prevent and improve thrombosis-related cardiovascular diseases [3,4]. Platelet activation is a result of a complex signal transduction cascade reaction mediated by several bioactive mediators [4]. Although, platelets are anucleated cells released from megakaryocytes, they also contain transcription factors, notably the peroxisome proliferator-activated receptors (PPARs) that belong to the ligand-activated transcription factors [5]. Exposure to PPAR agonists results in PPARs heterodimerized with retinoid X receptor (RXR), followed by binding to peroxisome proliferator response element in the promoter of target genes leading to induction or repression of several genes. PPARs have been demonstrated to exhibit a variety of homeostatic functions including energy metabolism, cell proliferation, differentiation, and inflammation [6–8]. So far, three PPAR isoforms (PPAR-α, PPAR-β/δ, and PPAR-γ) have been found in human platelets, and up-regulation of PPARs inhibits platelet activation through a nongenomic mechanism [9]. Previous study has shown that the antiplatelet activity of lipid lowing drugs such as simvasatin and fenofibrate is mediated by activation of PPAR-γ and PPAR-α, respectively [10]. Furthermore, our recent study has demonstrated that the anti-aggregatory effect of alpha-lipolic acid may attribute to the activation of PPAR-α/γ [11]. Taken together, these findings suggest that agents with PPARs-activating effect may exert an antiplatelet activity.

Magnolol extracted from a Chinese traditional medicinal herb (*Magnolia officinalis*) has been demonstrated to exhibit multiple pharmacological effects, including anti-atherosclerosis, anti-oxidative, anti-inflammatory and anti-bacterial properties [12]. Although, the antiplatelet activity of magnolol has been reported [13], the actual molecular mechanisms or signaling pathways

Abbreviations: PPAR, peroxisome proliferator-activated receptor; AA, arachidonic acid; cyclic GMP, 3′,5′-cyclic monophosphate; NOS, nitric oxide synthase; NO, nitric oxide; PRP, platelet rich plasma; LDH, lactate dehydrogenase; TXA_2, thromboxane A_2; TXB_2, thromboxane B_2; PKCα, protein kinase Cα; PI3K, phosphatidylinositol-3 kinase.

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involved have not yet been clarified. Based on the finding that magnolol is a PPAR-γ agonist through direct binding to the PPAR-γ ligand-binding domain [14], we propose that the actions of magnolol may be regulated by PPARs-mediated pathways. However, little is known about the role of PPARs on magnolol-mediated antiplatelet activity. In the present study, we demonstrate for the first time that activation of PPAR-β/γ-dependent pathways contributes to the antiplatelet activity of magnolol.

2. Materials and methods

2.1. Materials

Collagen (type I, equine tendon), rosiglitazone, arachidonic acid (AA), and fluorescein sodium were purchased from Sigma Chemical Company (St. Louis, MO, USA). Fluo-4-acetoxymethyl ester (Fluo-4AM) was purchased from Invitrogen Molecular Orobes (Eugene, OR, USA). The GW6471, GSK0660 and GW9662 were purchased from Tocris (Avonmouth, Bristol, U.K.). The pure proteome protein A magnetic beads, PKCα antibody and ECL reagent were purchased from Upstate biotechnology (Lake Placid, NY, USA). The antibodies of PPAR-β, COX-1, and phospho-PKCα were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phospho-Akt (Ser^{473}) antibody was purchased from Cell Signaling Technology (Beverly, MA, USA). Phospho-eNOS (Ser1177), Total-eNOS, and PPAR-γ antibodies were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). The PKG antibody was purchased from GeneTex Inc Company (Alton Parkway Irvine, CA, USA). Magnolol (Fig. 1) was purchased from the Medical and Pharmaceutical Industry Technology and Development Center (Taipei, Taiwan) and dissolved in dimethylsulfoxide (DMSO) followed by dilution with Tyrode solution and the final concentration of DMSO was fixed at 0.1%. Other chemical agents used in the study were at least analytical grade (Sigma).

2.2. Platelet aggregation

The present study was approved by the Ethical Committee of Animal Experiments, National Defense Medical Center, Taipei, Taiwan. Animals were housed in standard environment and maintained on tap water and food *ad libitum* throughout the study. The blood withdrawn from rabbit marginal vein was mixed with anticoagulant, EDTA (100 mM, 14:1 v/v), and centrifuged at 160 g, 25 °C for 10 min to obtain platelet rich plasma (PRP). Then, platelet suspension was prepared from the PRP according to the washing procedures described previously [11], and 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 Corporation, Havertown, PA, USA). The absorbance of Tyrode's solution was assigned as 100% aggregation and the absorbance of platelet suspension as 0% aggregation. Platelet suspensions (0.3 ml) were preincubated with drugs or solvent control (0.1% DMSO) for 3 min before addition of collagen (10 μg/ml) or AA (100 μM), and the reaction was allowed to proceed for 6 min. The platelet aggregation was evaluated by measuring the peck of the aggregation curves. Data were expressed as the percentage of maximal aggregation.

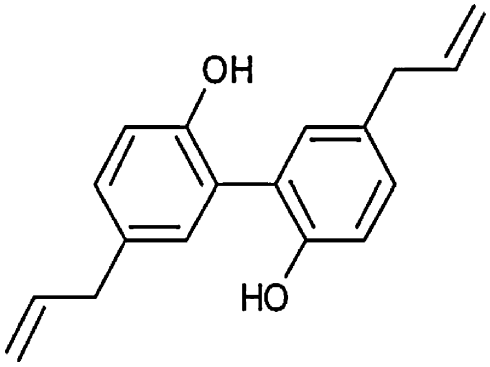

Fig. 1. The structure of magnolol.

2.3. Lactate dehydrogenase (LDH) assay

Level of LDH was measured to act as an index of platelet damage. Briefly, platelets were pre-incubated with magnolol (20–60 μM) for 10 min. After centrifugation at $10{,}000 \times g$ for 5 min, the supernatants were incubated with phosphate buffer containing β-NADPH (120 μM) for 20 min at room temperature followed by addition of pyruvate (600 μM). The absorbance wavelength at 340 nm was read by 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.

2.4. Determination of PPAR activity

Platelets incubated with drugs for 3 min at 37 °C and were lysed in buffer containing 50 μM Tris–HCl, pH 7.4, 0.5 M NaCl, 1 μM EDTA, 0.05% SDS, 0.5% TritonX-100, and 1 μM phenylmethylsulphonyl fluoride. After centrifugation at $15{,}000 \times g$ for 10 min at 4 °C, the supernatant, was used to determine the PPAR activity by using a PPAR transcription factor ELISA kit (Cayman Chemical Co, Ann Arbor, MI, USA), and the absorbance at 450 nm was measured [10].

2.5. Determination of cyclic GMP and TXB_2 formation

Platelet suspensions (3×10^8/ml) were pre-incubated with various drugs or solvent control at 37 °C for 3 min, and stopped the reaction by adding 10 mM EDTA and immediately boiling for 5 min. After centrifugation at $10{,}000 \times g$ for 5 min, the levels of cyclic GMP in the supernatants were determined by ELISA kit. For measurement of TXB_2 formation, platelets were preincubated with drugs for 3 min followed by addition of collagen or AA for another 6 min. Then, 2 mM EDTA and 50 μM indomethacin were added, and the levels of TXB_2 were determined in the supernatants by ELISA kit (Cayman Chemical Co, Ann Arbor, MI, USA).

2.6. Determination of COX-1 activity

The COX-1 activity was measured according to the instruction of COX inhibitor screening assay kit (Cayman Chemical Co, Ann Arbor, MI, USA). Briefly, after platelets treated with drugs for 3 min, AA (100 μM) was added for another 2 min, followed by addition of 0.1 N HCl and saturated stannous fluoride solution to stop the reaction. The COX-1 activity was evaluated by direct measurement of $PGF_{2\alpha}$ production by $SnCl_2$ reduction of COX-derived PGH_2 at 415 nm.

2.7. Determination of nitric oxide (NO) formation

Platelet suspensions were preincubated with drugs or solvent control for 3 min, followed by addition of collagen (10 μg/ml) for 6 min and centrifugation. The amount of nitrat + nitrite (NOx) in the supernatants was measured by a Sievers Nitric Oxide Analyzer (Sievers 280 NOA, Boulder, CO, USA). Nitrate concentrations were calculated by comparison with standard solution of sodium nitrate.