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Suppression of NF-κB signaling by andrographolide with a novel mechanism in human platelets: Regulatory roles of the p38 MAPK-hydroxyl radical-ERK2 cascade

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ABSTRACT

Andrographolide, a novel nuclear factor-κB (NF-κB) inhibitor, is isolated from leaves of *Andrographis paniculata*. Platelet activation is relevant to a variety of coronary heart diseases. Our recent studies revealed that andrographolide possesses potent antiplatelet activity by activating the endothelial nitric oxide synthase (eNOS)-NO-cyclic GMP pathway. Although platelets are anucleated cells, they also express the transcription factor, NF-κB, that may exert non-genomic functions in platelet activation. Therefore, we further investigated the inhibitory roles of andrographolide in NF-κB-mediated events in platelets. In this study, NF-κB signaling events, including IKKβ phosphorylation, IκBα degradation, and p65 phosphorylation, were time-dependently activated by collagen in human platelets, and these signaling events were attenuated by andrographolide (35 and 75 μM). ODQ and KT5823, respective inhibitors of guanylate cyclase and cyclic GMP-dependent kinase (PKG), strongly reversed andrographolide-mediated inhibition of platelet aggregation, relative $[Ca^{2+}]i$ mobilization, and IKKβ, and p65 phosphorylation. In addition, SB203580 (an inhibitor of p38 MAPK), but not PD98059 (an inhibitor of ERKs), markedly abolished IKKβ and p65 phosphorylation. SB203580, NAC (a free-radical scavenger), and BAY11-7082 (an inhibitor of NF-κB) all diminished ERK2 phosphorylation, whereas PD98059, BAY11-7082, and NAC had no effects on p38 MAPK phosphorylation. Furthermore, SB203580, but not BAY11-7082 or PD98059, reduced collagen-induced hydroxyl radical (•HO) formation. KT5823 also markedly reversed andrographolide-mediated inhibition of p38 MAPK and ERK2 phosphorylation, and hydroxyl radical formation in platelets. In conclusion, this study demonstrated that andrographolide may involve an increase in cyclic GMP/PKG, followed by inhibition of the p38 MAPK/•HO-NF-κB-ERK2 cascade in activated platelets. Therefore, andrographolide may have a high therapeutic potential to treat thromboembolic disorders and may also be considered for treating various inflammatory diseases.

1. Introduction

Andrographolide, a labdane diterpenoid, is the most active and important constituent of the medicinal plant *Andrographis paniculata* [1], which has long been used as an herbal medicine to prevent and treat upper respiratory tract infections, diarrhea, rheumatoid arthritis, and laryngitis in Asia and Scandinavia [1,2]. Recently, this plant was used in cooking as a valuable health food, and the extract is used as a nutritional supplement for preventing inflammatory diseases in Taiwan. Several studies demonstrated that andrographolide possesses anticancer and hepatoprotective activities [3,4]. It was also reported to suppress v-Src transformation and sensitize cancer cells to tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-induced apoptosis [5]. It was shown to interfere with T-cell activation and dendritic cell maturation [6]. In addition, andrographolide inhibits inducible nitric oxide synthase (iNOS) expression and subsequent NO production in lipopolysaccharide (LPS)-stimulated macrophages [7]. Several lines of evidence demonstrated that inhibition of nuclear factor-kappa B (NF-κB) transcriptional activity contributes to andrographolide's protective anti-inflammatory actions

Abbreviations: BSA, bovine serum albumin; $cPLA_2$, cytosolic phospholipase A_2; eNOS, endothelial nitric oxide synthase; ESR, electron spin resonance; •HO, hydroxyl radical; IKK, IκB kinase; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NF-κB, nuclear factor-kappa B; PKA, cyclic AMP-dependent kinase; PKG, cyclic GMP-dependent kinase; PP2A, protein phosphatase 2A; PRP, platelet-rich plasma; PGE_1, prostaglandin E_1; PPAR, peroxisome proliferator-activated receptor; ROS, reactive oxygen species; TxA_2, thromboxane A_2; TNF, tumor necrosis factor.

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[3,8–10]. Recently, we also reported that andrographolide enhances NF-κB subunit p65 Ser^{536} dephosphorylation through activation of protein phosphatase (PP)2A in vascular smooth muscle cells [11].

NF-κB, a transcription factor, regulates diverse cell functions ranging from inflammation to cell death. As the term, "nuclear factor" implies, the actions of NF-κB require its translocation from the cytosol to nuclei to bind cognate nuclear DNA sequences. Recently, we reported that andrographolide exhibits potent activity of inhibiting platelet activation [12], and its mechanism may involve activation of the endothelial nitric oxide synthase (eNOS)/NO-cyclic GMP pathway resulting in inhibition of platelet aggregation [12]. Arterial thromboses are quite distinct from venous thromboses in that arterial thromboses are mostly composed of platelets that adhere to ruptured endothelial surfaces [13]. Venous thromboses, which are enriched in fibrin and erythrocytes, can occur in the absence of vessel wall damage. Therefore, platelet aggregation may play a crucial role in the atherothrombotic process [14].

Platelets are anucleated, do not differentiate or proliferate, and thus are a good model for studying non-genomic functions of NF-κB in cells. In our preliminary study, andrographolide obviously suppressed p65 phosphorylation in washed human platelets. We therefore systematically examined the regulatory signaling events of andrographolide-mediated inhibition of NF-κB activation in washed human platelets.

2. Materials and methods

2.1. Materials

Andrographolide, collagen (type I), dimethyl sulfoxide (DMSO), bovine serum albumin (BSA), prostaglandin E_1 (PGE_1), heparin, 5,5-dimethyl-1 pyrroline N-oxide (DMPO), *N*-acetyl-L-cysteine (NAC), (E)-3-(4-methylphenylsulfonyl)-2-propenenitrile (BAY11-7082), 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059), N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H89), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), KT5823, 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ22536), and 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580) were purchased from Sigma (St Louis, MO). Fura 2-AM was from Molecular Probe (Eugene, OR). The anti-phospho-IKKα (Ser^{180})/IKKβ (Ser^{181}) polyclonal antibody (pAb), anti-IκBα (44D4) monoclonal antibody (mAb), anti-phospho-IκBα($Ser^{32/36}$) (5A5) mAb, anti-phospho-NF-κB p65 (Ser^{536}) pAb, anti-phospho-p38 mitogen-activated protein kinase (MAPK) (Ser^{180}/Tyr^{182}) pAb, anti-p38 MAPK (5F11) mAb, anti-phospho-p44/42 MAPK (ERK1/2) (Thr^{202}/Tyr^{204}) pAb, and anti-p44/42 MAPK (137F5) mAb were all from Cell Signaling (Beverly, MA). The anti-α-tubulin mAb was from NeoMarkers (Fremont, CA); and the Hybond-P polyvinylidene difluoride (PVDF) membrane, enhanced chemiluminescence (ECL) Western blotting detection reagent and analysis system, horseradish peroxidase (HRP)-conjugated donkey anti-rabbit immunoglobulin G (IgG), and sheep anti-mouse IgG were from Amersham (Buckinghamshire, UK). Andrographolide was dissolved in dimethyl sulfoxide (DMSO) and stored at 4 °C until used.

2.2. Platelet aggregation

Human platelet suspensions were prepared as previously described [14]. This study was approved by the Institutional Review Board of Taipei Medical University and conformed to the principles outlined in the Helsinki declaration, and all human volunteers provided informed consent. In brief, blood was collected from healthy human volunteers who had taken no medicine during the preceding 2 weeks, and was mixed with acid-citrate-dextrose solution (9:1, v/v). After centrifugation, the supernatant (platelet-rich plasma; PRP) was supplemented with prostaglandin E_1 (PGE_1) (0.5 μM) and heparin (6.4 IU/ml). Washed platelets were finally suspended in Tyrode's solution containing bovine serum albumin (BSA) (3.5 mg/ml). The final concentration of Ca^{2+} in Tyrode's solution was 1 mM.

A turbidimetric method was applied to measure platelet aggregation [14], using a Lumi-Aggregometer (Payton, Scarborough, Ontario, Canada). Platelet suspensions (3.6×10^8 cells/ml) were preincubated with various concentrations of andrographolide or an isovolumetric solvent control (0.5% DMSO, final concentration) for 3 min before the addition of agonists. The reaction was allowed to proceed for 6 min, and the extent of aggregation was expressed as a percentage of the control (in the absence of andrographolide) in light-transmission units.

2.3. Measurement of platelet $[Ca^{2+}]i$ by Fura 2-AM fluorescence

Citrated whole blood was centrifuged at $120 \times g$ for 10 min. The supernatant was incubated with Fura 2-AM (5 μM) for 1 h. Human platelets were then prepared as described above. Finally, the external Ca^{2+} concentration of the platelet suspensions was adjusted to 1 mM. The $[Ca^{2+}]i$ rise was measured using a fluorescence spectrophotometer (CAF 110, Jasco, Tokyo, Japan) with excitation wavelengths of 340 and 380 nm, and an emission wavelength of 500 nm [14].

2.4. Immunoblotting study

Washed platelets (1.2×10^9 cells/ml) were preincubated with andrographolide (35 and 75 μM) or various inhibitors for 3 min, followed by the addition of collagen (1 μg/ml) to trigger platelet activation. The reaction was stopped, and platelets were immediately re-suspended in 200 μl of lysis buffer for 1 h. Lysates were centrifuged at $5000 \times g$ for 5 min. Samples containing 80 μg of protein were separated by 12% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE); proteins were electrotransferred by semidry transfer (Bio-Rad, Hercules, CA). Blots were blocked with TBST (10 mM Tris-base, 100 mM NaCl, and 0.01% Tween 20) containing 5% BSA for 1 h and then probed with various primary antibodies. Membranes were incubated with HRP-linked anti-mouse IgG or anti-rabbit IgG (diluted 1:3000 in TBST) for 1 h. Immunoreactive bands were detected by an enhanced chemiluminescence (ECL) system. The bar graph depicts the ratios of semiquantitative results obtained by scanning reactive bands and quantifying the optical density using videodensitometry (Bio-profil; Biolight Windows Application V2000.01; Vilber Lourmat, France).

2.5. Measurement of hydroxyl radicals by electron spin resonance (ESR) spectrometry

The ESR method used a Bruker EMX ESR spectrometer as described previously [15]. In brief, platelet suspensions (3.6×10^8 cells/ml) were preincubated with various inhibitors for 3 min before the addition of collagen (1 μg/ml). The reaction was allowed to proceed for 5 min, followed by the addition of DMPO (100 μM) for the ESR study. The rate of free radical-scavenging activity was defined by the following equation: inhibition rate = 1–[signal height (andrographolide)/signal height (control)] [15].

2.6. Data analysis

Experimental results are expressed as the means ± S.E.M. and are accompanied by the number of observations (*n*). Values of *n* refer