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The classical NFκB pathway is required for phloroglucinol-induced activation of murine lymphocytes

Ginnae Ahn [a], Eunjin Park [b], Hyun Jeong Park [b], You-Jin Jeon [a], Jehee Lee [a], Jae Woo Park [c], Youngheun Jee [b,*]

[a] Faculty of Marine Life Science, Jeju National University, Jeju 690-756, Korea
[b] College of Veterinary Medicine and Applied Radiological Science Institute, Jeju National University, Jeju 690-756, Korea
[c] Department of Nuclear and Energy Engineering, Jeju National University, Jeju 690-756, Korea

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ABSTRACT

Background: Phloroglucinol (PG), a polyphenolic compound, has been proposed to show free radical scavenging, anti-tumor, and immunomodulation effects on immune cells. In this study, we investigated PG for its immunological activity and their molecular mechanisms, specifically focusing on the functional activation of nuclear factor-κB (NFκB), an important transcription factor involved in the activation and differentiation of lymphocytes, and subsequent recruitment of murine lymphocytes.
Methods: We tested whether PG may contribute to the activation of immune response through the NFκB pathway by ^{3}H-thymidine incorporation assay, Western blot, intracellular cytokine assays and Electrophoretic mobility shift assay (EMSA) in murine lymphocytes.
Results: PG markedly enhanced the proliferation of lymphocytes by inducing the degradation and phosphorylation of IκB, which leads to the activation of NFκB p65. Also, PG induced the activation of mitogen-activated protein kinases (MAPKs) such as ERK1/2, JNK, and p38, upstream molecules of NFκB pathway. In addition, PG augmented the secretion of interleukin (IL)-2 in mature T lymphocytes and their production of IL-2. Furthermore, the application of TPCK significantly reduced the activation of lymphocytes by PG via inhibiting the NFκB activation.
Conclusion: These results suggest that PG induces the activation of lymphocytes by inducing the proliferative activity and secretion of IL-2 through the classical NFκB.
General significance: The effect of PG on lymphocyte activation was assayed for the first time. These results would also elucidate its underlying mechanism.

1. Introduction

Nuclear factor-κB (NFκB), a well known transcription factor, is a key regulator of many inflammatory processes such as activation, differentiation, and proliferation of immune cells [1]. The classical NFκB signal cascade is initiated from the formation of p50-RelA (p65) complex which has a c-terminal transcriptional activator and localizes in the cytoplasm in an inactive form bound to inhibitory proteins IκBα or IκB [2–4]. The ubiquitination and degradation of IκB in T lymphocytes, then, releases active NFκB complex which subsequently translocates into the nucleus, promoting the transcription of essential target genes required for T cells' survival, cytokine and chemokine production, up-regulation of adhesion molecules, organogenesis and apoptosis [5]. The classical NFκB signaling cascade is particularly important for encoding and synthesizing cytokines such as interleukin (IL)-2, which in turn stimulates the activation, proliferation, and differentiation of T lymphocytes as well as the expression of IL-2 receptors [5–11].

Polyphenolic compounds including flavonoids, phloroglucinols (PGs), and PG derivatives such as eckol and triphloroethol-A are abundant in certain vegetables, fruits, seeds, and seaweeds and regarded as a class of semi-essential nutrients for humans [12]. These polyphenolic compounds were reported to have such beneficial effects as scavenging oxygen radicals, killing bacteria, and combating cancer [13,14]. In particular, previous reports documented that PGs derived from *Ecklonia cava* have anticancer and antioxidant activity [15,16]. Elsewhere, PG and its derivatives such as triphloroethol-A and eckol from *Ecklonia cava* were also shown to have antioxidant and matrix metalloproteinase (MMP)-1 inhibitory activities *in vitro* and radioprotective effects that induced the repair of damaged DNA in immune cells and enhanced their proliferation when administered to

Abbreviations: NFκB, nuclear factor-κB; MAPKs, mitogen-activated protein kinases; PG, phloroglucinol; MMP, matrix metalloproteinases; MTT, 3-[4,5-dimethylthiazole-2-yl]-2,5 diphenyltetrazoliumbromide; DMSO, dimethylsulfoxide; TPCK, N-p-Tosyl-L-phenylalanine chloromethyl ketone; PMA, phorbol myristate acetate; HRP, horseradish peroxidase; FBS, fetal bovine serum; EMSA, Electrophoretic mobility shift assay; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gels; ELISA, Enzyme linked immunosorbent assay; SPSS, statistical package for the social science

* Corresponding author. College of Veterinary Medicine and Applied Radiological Science Research Institute, Cheju National University, Jeju 690-756, South Korea. Tel.: +82 64 754 3374; fax: +82 64 756 3354.
E-mail address: yhjee@jejunu.ac.kr (Y. Jee).

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animals exposed to ionizing irradiations [16–18]. Although, PG derivatives have well-established defensive and protective functions such as the antioxidant effect on free radicals and oxidative stress-induced cell damages [16,19], no previous reports have identified its role in the activation of immune response or its underlying mechanism. In the present study, we demonstrate that PG elicited the proliferation of lymphocytes without cytotoxicity and enhanced IL-2 production by activating the NFκB signaling pathway.

2. Materials and Methods

2.1. Chemicals

ICR mice (male) were obtained from SLC, Inc. (Shizuoka, Japan). PG (phloroglucinol), MTT (3-[4,5-dimethylthiazole-2-yl]-2,5 diphenyltetrazoliumbromide), DMSO (dimethylsulfoxide), TPCK (N-*p*-Tosyl-L-phenylalanine chloromethyl ketone), brefeldin-A, ionomycin and PMA (phorbol myristate acetate) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). ^{3}H-thymidine and ECL reagents came from Amersham (Arlington Heights, IL, USA). Phospho-IκBα, IκBα, NFκB p65, ERK, phospho-ERK, JNK, phospho-JNK, p38, and phospho-p38 were from Santa Cruz Biotechnology, Inc. (CA, USA) and Upstate Biotechnology (Lake Placid, NY, USA). Horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit IgG, NFκB oligonucleotides and nitrocellulose transfer membranes were from Santa Cruz Biotechnology, Inc. (CA, USA) and Schleicher and Schuell (Keene, NH, USA)Anti-CD3 (145-2C11 an American Hamster IgG1*,κ)-FITC and IL-2 (JES6-5H4)-PE were purchased from BD Biosciences (San Jose, CA, USA). An ELISA Kit from eBioscience (San Diego, CA, USA) was used for measuring IL-2. The NE-PER^R Nuclear and Cytoplasmic Extraction Reagents, nylon membrane, biotin 3′-end DNA labeling and LightShift™ chemiluminescent EMSA kit were purchased from Pierce (Rockford, IL, USA).

2.2. Preparation of lymphocytes

Spleens were removed aseptically from ICR mice, aged 8 to 9 weeks; lymphocytes were isolated and suspended in RPMI medium containing 10% fetal bovine serum (FBS) and 1% antibiotic (100 U/ml penicillin and 100 mg/ml streptomycin) after removing the attached cells including monocytes and macrophages. Red blood cells were lysed by immersion in ACK lysis buffer including 0.84% NH_4Cl, 0.1 mM Na_2EDTA, 1 mM $KHCO_3$. After washing with DPBS, the purified cells were obtained and used directly for experiments in which they were treated with PG dissolved in RPMI medium or with RPMI-only medium, hereafter called “untreated control cells.”

2.3. Assessment of cytotoxicity by MTT assay

To ascertain the viability of lymphocytes exposed to various compounds, we performed MTT assay, a test of metabolic competence predicated upon the assessment of mitochondrial performance. MTT is also used for a colorimetric assay that is dependent on the conversion of yellow tetrazolium bromide to its purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells [20]. For these assays, 2×10^4 cells were incubated with PG at various concentrations (from 1 μg/ml to 30 μg/ml) for 24 and 72 h and MTT stock solution (50 μl; 2 mg/ml) was applied to each of the wells for 4 h. The control cells were treated with only RPMI medium. The absorbance of formazan crystals dissolved in 150 μl of DMSO was measured at 540 nm. The optical density of the formazan generated in control cells that were treated with RPMI-only medium was considered to represent 100% viability. The data are expressed as mean percentages of the viable cells versus the respective control.

2.4. ^{3}H-thymidine incorporation assay

The proliferation of lymphocytes was assessed by ^{3}H-thymidine incorporation assay that measures the level of radioactive ^{3}H-thymidines incorporated into newly replicated cell DNAs. 4×10^5 cells were incubated with PG (1 μg/ml, 3 μg/ml and 10 μg/ml) in the presence or absence of TPCK (45 μM) for 3 days and 1 μCi of ^{3}H-thymidine added to the cells. After 18 h, the cells were harvested onto glass fiber filters by an automatic cell harvester. The radioactivity was determined with a liquid scintillation spectrometer.

2.5. Preparation of cytoplasmic and nuclear proteins

Cytoplasmic and nuclear proteins were lysed from lymphocytes incubated in the presence or absence of PG (1, 3, 10, 30, and 50 μg/ml) and/or TPCK (45 μM and 90 μM) by NE-PER^R Nuclear and Cytoplasmic Extraction Reagents according to manufacturer's instructions. Protein extracts were used for Western blot analysis and EMSA (Electrophoretic mobility shift assay).

2.6. Western blot analysis

We performed Western blot analysis to assess whether PG is related to the NFκB pathway in lymphocytes. Cytoplasmic (60 μg/well) and nuclear protein (40 μg/well) preparations were loaded into each lane of SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gels) and electrophoresed under denaturing conditions. Subsequently, proteins were electro-transferred onto nitrocellulose transfer membrane. After blocking with 5% nonfat milk for 1 hr, blots were incubated with phospho-IκBα (1:500 dilution), IκBα (1:500 dilution), NFκBp65 (1:1000 dilution), ERK (1:1000 dilution), phospho-ERK (1:1000 dilution), JNK (1:1000 dilution), phospho-JNK (1:1000 dilution), p38 (1:1000 dilution), phospho-p38 (1:1000 dilution), or β-action (1:3000 dilution) antibodies for 60 min followed by incubation with HRP-conjugated anti-mouse or anti-rabbit IgG (1:2,000) for 45 min. Visualization was achieved by using ECL reagents.

2.7. EMSA

The EMSA with an oligonucleotide harboring a consensus NFκB binding element was used to identify whether PG has NFκB p65 DNA binding activity. NFκB binding oligonucleotides (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) were 3′-biotinylated and annealed using the biotin 3′-end DNA labeling kit. The 20 fmol of biotin-end-labeled target DNA and 10 μg of nuclear proteins were used for binding reactions using LightShift™ chemiluminescent EMSA kit. The binding mixture was loaded onto 4% polyacrylamide gels and electrophoresed at 100 V before being transferred onto a positively charged nylon membrane (Hybond™-N$^+$) in 0.5×Tris borate/EDTA at 380 mA for 1 h. Transferred DNA samples were cross-linked and detected using HRP-conjugated streptavidin (LightShift™ chemiluminescent EMSA kit) according to manufacturer's instructions.

2.8. Intracellular cytokine staining assay

To assess the production of IL-2, which is a key event in Th1 and Th2 cell activation, by the NFκB pathway, we performed an intracellular cytokine staining assay. Briefly, lymphocytes incubated for 3 days with PG were re-incubated with a cocktail containing brefeldin-A, ionomycin and PMA from 5 h. Then, cells were blocked with anti-mouse IgG solution and stained with fluorescently labeled CD3, a specific marker for mature T lymphocytes and the mAb IL-2-PE. After the reaction, cells were washed and fixed with 1% formalin. Twenty thousand viable cells per treatment (as determined by light scatter profiles) were analyzed by using a BD FACSCalibur™ flow cytometer and CellQuest software (BD Biosciences).