J Ginseng Res xxx (2016) 1–7

Contents lists available at ScienceDirect

Journal of Ginseng Research

journal homepage: http://www.ginsengres.org

Research article

Inhibition of hypoxia-induced cyclooxygenase-2 by Korean Red Ginseng is dependent on peroxisome proliferator-activated receptor gamma

Heewon Song, Young Joo Lee*

Department of Bioscience and Biotechnology, College of Life Science, Sejong University, Kwangjingu, Kunjadong, Seoul, Korea

ARTICLE INFO

Article history:
Received 22 January 2016
Received in Revised form
19 March 2016
Accepted 2 April 2016
Available online xxx

Keywords:
cyclooxygenase-2
hypoxia
Korean Red Ginseng
PPARγ

ABSTRACT

Background: Korean Red Ginseng (KRG) is a traditional herbal medicine made by steaming and drying fresh ginseng. It strengthens the endocrine and immune systems to ameliorate various inflammatory responses. The cyclooxygenase-2 (COX-2)/prostaglandin E2 pathway has important implications for inflammation responses and tumorigenesis. Peroxisome proliferator-activated receptor gamma (PPARγ) is a transcription factor that regulates not only adipogenesis and lipid homeostasis, but also angiogenesis and inflammatory responses.

Methods: The effects of the KRG on inhibition of hypoxia-induced COX-2 via PPARγ in A549 cells were determined by luciferase assay, Western blot, and/or qRT-PCR. The antimigration and invasive effects of KRG were evaluated on A549 cells using migration and matrigel invasion assays.

Results and conclusion: We previously reported that hypoxia-induced COX-2 protein and mRNA levels were suppressed by KRG. This study examines the possibility of PPARγ as a cellular target of KRG for the suppression of hypoxia-induced COX-2. PPARγ protein levels and PPRE-driven reporter activities were increased by KRG. Reduction of hypoxia-induced COX-2 by KRG was abolished by the PPARγ inhibitor GW9662. In addition, the inhibition of PPARγ abolished the effect of KRG on hypoxia-induced cell migration and invasion. Our results show that KRG inhibition of hypoxia-induced COX-2 expression and cell invasion is dependent on PPARγ activation, supporting the therapeutic potential for suppression of inflammation under hypoxia. Further studies are required to demonstrate whether KRG activates directly PPARγ and to identify the constituents responsible for this activity.

1. Introduction

Ginseng is a popular herbal medicine that has been used for over 2,000 y in Oriental countries. Its use is not confined to Asia but has expanded to Western countries as one of the top 10 best-selling herbs [1]. This popularity and its worldwide consumption indirectly demonstrate its efficacy, and accumulating scientific evidence shows that ginseng has a wide range of pharmacological activities in the cardiovascular, endocrine, immune, and central nervous systems [2]. It is especially well established that ginseng ameliorates inflammatory responses [3–5]. Data have shown that ginsenosides are pharmacological compounds with antiinflammatory and anticarcinogenic effects both *in vivo* and *in vitro* [6,7].

Red ginseng is made by steaming and drying fresh ginseng. The pharmacological efficacy of Korean ginseng is known to be enhanced by these special processes, mostly due to changes in the characteristics of the constituent ginsenosides [8,9]. During the steaming process, seven ginsenosides (Rg1, Re, Rb1, Rc, Rb2, Rb3, and Rd) decreased, while five ginsenosides (Rh1, Rg2, 20R-Rg2, Rg3, and Rh2) increased [10].

Hypoxia is a state of reduced overall tissue oxygen availability and a hallmark of solid tumors that leads to cell invasion and metastasis [11]. Cyclooxygenase-2 (COX-2) is induced by various stimuli such as LPS, cytokines, hypertonicity, and hypoxia [12–15]. COX-2 increases the metastatic potential of cancer cells, and silencing COX-2 inhibits metastasis and delays tumor onset in poorly differentiated metastatic cancers [16,17]. Mammary epithelial cells express peroxisome proliferator-activated receptor gamma (PPARγ), and its signaling is critical during breast tumorigenesis and correlated with COX-2 expression [18]. These

* Corresponding author. Department of Bioscience and Biotechnology, Sejong University, Kwang-Jin-Gu, Seoul 143-747, Korea.
E-mail address: yjlee@sejong.ac.kr (Y.J. Lee).

http://dx.doi.org/10.1016/j.jgr.2016.04.001
p1226-8453 e2093-4947/$ – see front matter

Please cite this article in press as: Song H, Lee YJ, Inhibition of hypoxia-induced cyclooxygenase-2 by Korean Red Ginseng is dependent on peroxisome proliferator-activated receptor gamma, Journal of Ginseng Research (2016), http://dx.doi.org/10.1016/j.jgr.2016.04.001

observations indicate the importance of COX-2 inhibition in preventing hypoxia-induced cell invasion.

PPARγ, a member of a nuclear receptor superfamily, heterodimerizes with the retinoid X receptor and activates transcription by binding to the PPAR response elements of its target genes [19]. Endogenous ligands for PPARγ include fatty acids and prostanoids. PPARγ regulates adipogenesis by differentiating adipocytes, lipid metabolism, inflammation, and angiogenesis [20]. PPARγ regulates COX-2 gene expression through PPAR response elements within the promoter of COX-2 [21,22]. However, depending on the cell type, PPARγ can both activate and inhibit COX-2 through PPARγ-dependent and -independent mechanisms [23–25]. Continuous research is required to understand these complex phenomena. PPARγ-activating natural products and plant extracts have been extensively sought after and studied because of their great potential for use in the treatment of a variety of metabolic syndromes [26,27].

We previously showed that Korean Red Ginseng (KRG) efficiently blocks hypoxia-induced COX-2 mediated by sirtuin-1 (SIRT-1), the pathway of which differs from that of dexamethasone [28]. This provides scientific evidence of KRG being effective for the suppression of the inflammatory response and tumorigenesis under hypoxia through mechanisms other than those of steroids. We present herein further evidence that KRG suppresses hypoxia-induced COX-2 and is dependent on the PPARγ signaling pathway and that PPARγ activation by KRG may reduce the tumorigenesis of pulmonary epithelial cells.

2. Materials and methods

2.1. Materials

KRG was kindly supplied by the Korea Ginseng Cooperation (Daejeon, Korea). KRG is prepared from roots of 6-yr-old KRG. Voucher specimen (KGC No. 201-3-1081) of KRG was deposited at the herbarium located at KGC Central Research Institute (Daejeon, Korea). Yield of KRG extract was 75%. The water content of the pooled extract was 36% of total weight. Phytochemical characteristics of KRG with standard ginsenosides were identified by HPLC analysis as reported previously [29,30]. HPLC analysis result of standard ginsenosides is provided by Korea Ginseng Cooperation [28]. The ginsenoside content in KRG is 7%, and it is composed of major ginsenosides (G-Rg1, 1.79 mg/g; G-Re, 1.86 mg/g; G-Rf, 1.24 mg/g; G-Rh1, 1.01 mg/g; G-Rg2s, 1.24 mg/g; G-Rb1, 7.44 mg/g; G-Rc, 3.04 mg/g; G-Rb2, 2.59 mg/g; and G-Rd, 0.91 mg/g), and other minor ginsenoside components [29,30]. GW9662 and celecoxib were purchased from Sigma (St. Louis, MO, USA). T0070907 was purchased from Selleckchem (Houston, TX, USA). Fetal bovine serum (FBS), Trizol Reagent, and penicillin/streptomycin were purchased from GIBCO Invitrogen (Grand Island, NY, USA). Anti-COX-2 was obtained from Cayman Chemical (Ann Arbor, MI, USA). Anti-β-actin was purchased from Sigma. Anti-sirtuin-1 (SIRT-1), anti-PPARγ and anti-peroxisome proliferator-activated receptor gamma coactivatior-1 alpha (PGC-1α) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Cell culture and hypoxic conditions

Human pulmonary epithelial A549 cells were maintained in RPMI containing 10% FBS and penicillin/streptomycin. Cells were grown at 37°C in a humidified atmosphere of 95% air/5% CO_2 and fed every 2–3 d. Before treatment, the cells were washed with phosphate-buffered saline and cultured in RPMI/5% charcoal–dextran stripped FBS (CD-FBS) for 2 d. For the hypoxic condition, cells were incubated at a CO_2 level of 5% with 1% O_2 balanced with N_2 using a hypoxic chamber (Thermo Fisher Scientific, Waltham, MA, USA). KRG stock was prepared at 10 mg/mL in phosphate buffer saline and diluted with media to 1 mg/mL just prior to use and sterilized by filtration with a 0.22 μm bottle top filter (Thermo Fisher Scientific).

2.3. Transfection and luciferase assays

A549 cells were transiently transfected with plasmids by using the polyethylenimine (Polysciences, Warrington, PA, USA). Luciferase activity was determined 48 h after treatment with an Auto-Lumat LB9507 luminometer (EG & G Berthold, Bad Widbad, Germany) using the luciferase assay system (Promega Corp., Madison, WI, USA) and expressed as relative light units. PPRE-Luc, a firefly luciferase reporter construct containing PPRE-elements, was kindly provided Dr. Ron Evans (The Salk Institute, San Diego CA, USA).

2.4. Reverse transcription-polymerase chain reaction

Total RNA was extracted using Trizol Reagent according to the manufacturer's instruction. To synthesize first strand cDNA, 3 μg total RNA was incubated at 70°C for 5 min with 0.5 μg of random hexamer and deionized water (up to 11 μL). The reverse transcription reaction was performed using 40 units of M-MLV reverse transcriptase (Promega Corp.) in 5× reaction buffer (250 mmol/L Tris-HCl; pH 8.3, 375mM KCl, 15mM $MgCl_2$, 50mM DTT), RNase inhibitor at 1 unit/μL, and 1mM dNTP mixtures at 37°C for 60 min. Real-time polymerase chain reaction (PCR) was performed with STEP ONE (Applied Biosystems, Foster City, CA, USA) using a SYBR green premix according to the manufacturer's instructions, as reported previously [31–33]. The primers used were: β-actin sense primer, 5′-CAAATGCTTCTAGGCGGACTATG-3′; β-actin anti-sense primer, 5′-TGCGCAAGTTAGGTTTTGTCA-3′; COX-2 sense primer, 5′-TGAAGAACTTACAGGAGAAAA-3′; COX-2 anti-sense primer, 5′-TACCAGAAGGGCAGGATACA-3′. Using the comparative threshold cycle (Ct), relative expression was calculated using $2^{-\Delta Ct}$ method and normalized by the expressions of β-actin from the same samples.

2.5. Western blot analysis

Protein was isolated in lysis buffer (150mM NaCl, 50mM Tris-HCl, 5mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate, 1% SDS) with protease inhibitor cocktail (Sigma) on ice for 1 h and then centrifuged for 20 min at 13,000*g*. Supernatant was collected and protein concentrations were measured using the Bradford method (Bio-Rad, Hercules, CA, USA). Proteins were dissolved in sample buffer and boiled for 5 min prior to loading onto an acrylamide gel. After SDS-PAGE, proteins were transferred to a polyvinylidene difluoride membrane, blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 60 min at room temperature. The membranes were incubated for 2 h at room temperature with antibody. Equal lane loading was assessed using β-actin monoclonal antibody (Sigma). After washing with TBST, blots were incubated with 1:5,000 dilution of the horseradish peroxidase conjugated-secondary antibody (Invitrogen, Grand Island, NY, USA), and washed again three times with TBST. The transferred proteins were visualized with an enhanced chemiluminescence detection kit (GE Healthcare Life Sciences, Pittsburgh, PA, USA).

2.6. Cell migration and invasion assays

The migration assay was performed with transwell inserts that have 6.5 mm polycarbonate membranes with 8.0 μm pores

Please cite this article in press as: Song H, Lee YJ, Inhibition of hypoxia-induced cyclooxygenase-2 by Korean Red Ginseng is dependent on peroxisome proliferator-activated receptor gamma, Journal of Ginseng Research (2016), http://dx.doi.org/10.1016/j.jgr.2016.04.001