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Research Article

Korean Red Ginseng suppresses bisphenol A-induced expression of cyclooxygenase-2 and cellular migration of A549 human lung cancer cell through inhibition of reactive oxygen species

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ABSTRACT

Background: Korean Red Ginseng (KRG) is a natural product with antiinflammatory and anticarcinogenic effects. We have previously reported that the endocrine-disrupting compound bisphenol A (BPA)-induced cyclooxygenase-2 (COX-2) via nuclear translocation of nuclear factor-kappa B (NF-κB) and activation of mitogen-activated protein kinase and promoted the migration of A549. Here, in this study, we assessed the protective effect of KRG on the BPA-induced reactive oxygen species (ROS) and expression of COX-2 and matrix metalloproteinase-9 (MMP-9) in A549 cells.

Methods: The effects of KRG on the upregulation of ROS production and COX-2 and MMP-9 expression by BPA were evaluated by fluorescence-activated cell sorting (FACs) analysis, quantitative reverse transcription polymerase chain reaction, and western blotting. Antimigration ability by KRG was evaluated by migration assay in A549 cells.

Results: KRG significantly suppressed the BPA-induced COX-2, the activity of NF-κB, the production of ROS, and the migration of A549 cells. These effects led to the downregulation of the expression of MMP-9.

Conclusions: Overall, our results suggest that KRG exerts an antiinflammatory effect on BPA-treated A549 cells via the suppression of ROS and downregulation of NF-κB activation and COX-2 expression which leads to a decrease in cellular migration and MMP-9 expression. These results provide a new possible therapeutic application of KRG to protect BPA-induced possible inflammatory disorders.

1. Introduction

Bisphenol A (BPA) is an endocrine-disrupting chemical (EDC) that is used to manufacture polycarbonate plastics and epoxy resins for many consumer products, including plastic cups, food storage containers, and beverage cans [1,2]. BPA exposure causes reproductive disorders, such as precocious puberty, prostate developmental abnormalities, decreased sperm production, and reproductive organ malignancies [3,4]. The cellular effects of BPA are mediated by binding to estrogen receptors (ERs) alpha and beta [5]. Disrupted ER signaling can cause reproductive disorders and inflammatory dysregulation [6,7]. In an endometriosis animal model, ER hyperstimulation caused enhanced cell proliferation and vascularization and increased neuron innervation and the inflammatory responses [8]. Hyperestrogenic stimulation and inflammation are linked by a feed-forward loop [9]. There is accumulating evidence of cross-talk between ER signaling and the inflammasome; therefore, an ER agonistic ligand-like BPA very likely affects inflammation [10–12]. BPA exposure stimulates interferon signaling and activates inflammasome activity, leading to the deterioration of autoimmune diseases such as systemic lupus erythematosus [13]. Studies have examined the effects of BPA on hormone-dependent and other tumors [14]. BPA produces inflammation and reactive oxygen species (ROS) in human lung cancer cells and promotes the development of pulmonary inflammatory diseases [15,16]. Previously, we showed that BPA induces cyclooxygenase-2 (COX-2) and the migration in A549 human lung cancer cells [15]. Although precautions to minimize exposure to

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environmental chemicals are useful, remedies that will alleviate possible adverse effects of these environmental chemicals are important.

Ginseng has been used in traditional medicine for thousands of years. Ginseng is divided into two species: *Panax ginseng* (Korean ginseng) and *Panax quinquefolius* (American ginseng) [17]. The word *Panax* means "all healing" and it comes from the traditional belief that ginseng can cure all illnesses [18]. However, the two ginseng species have different ginsenoside contents and some compounds are unique to Korean ginseng, such as ginsenoside Rf [19,20]. When Korean ginseng is steamed and dried, its ginsenoside and polysaccharide components change [21]. The resulting product, called Korean Red Ginseng (KRG), has obtained approval of the Korean Food and Drug Administration and is reported to improve immunity, relieve fatigue, improve the blood circulation, have antioxidative effects, and improve the symptoms of menopause in women [17]. With these activities, KRG is therapeutically effective against various diseases [18,22]. Many studies have shown that KRG targets oxidative stress by inhibiting ROS formation and blocking inflammation, making it effective in various inflammatory diseases, such as atherosclerosis, coronary artery dysfunction, cancer, and neurodegenerative diseases [23–26]. We hypothesized that KRG could protect against the inflammation-related phenomena caused by BPA that we observed previously. In this study, we investigated whether KRG prevents BPA-induced COX-2 expression and cellular migration in A549 cells.

2. Materials and methods

2.1. Experimental design

We have previously found that COX-2 and cell migration are increased by BPA in human lung adenocarcinoma A549 cells [15]. Therefore, we evaluated the protective effect of KRG against BPA-induced COX-2 in A549 cells. First, to ensure that the increased COX-2 by BPA is suppressed by KRG, two concentrations of KRG were pretreated for 1 h and BPA was processed and incubated for 24 h to check COX-2 protein and mRNA expression. And the amount of ROS generation was measured by FACs analysis to determine whether KRG inhibits BPA-induced ROS. To determine whether COX-2 and matrix metalloproteinase-9 (MMP-9) increased by BPA increased dependent on ROS, we examined the protein and mRNA expression of COX-2 and MMP-9 by treating with N-acetyl-L-cysteine (NAC), a ROS inhibitor. In addition, the effect of KRG on BPA-induced cellular migration was analyzed by using transwell migration assay in A549 cells.

2.2. Materials (reagents and antibodies)

BPA, 2′,7′-dichlorofluorescin diacetate (DCF-DA), NAC, dimethyl sulfoxide, thiazolyl blue tetrazolium bromide (MTT), celecoxib and Anti-β-actin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Roswell Park Memorial Institute 1640 Medium and TOM™ Transfection Optimized Medium were purchased from WelGENE Inc. (Daegu, Korea). Trizol reagent, fetal calf serum, and antibiotic-antimycotic were purchased from GIBCO Invitrogen (Grand Island, NY, USA). Polyethylenimine was purchased from Polyscience (Warrington, PA, USA). Enhanced chemiluminescence was obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). Anti-COX-2 was used from Cayman Chemical Co. (Ann Arbor, MI, USA). Anti–nuclear factor-kappa B (NF-κB) p65 and lamin B were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-MMP-9 (Matrix Metalloproteinase-9) was used from Cell Signaling Technology (Beverly, MA, USA).

2.3. Preparation of KRG

KRG was manufactured and kindly provided by the Korea Ginseng Corporation (KGC, Daejeon, Korea) [27]. The analysis of KRG was based on the reported method [28]. The phytochemical characteristics of KRG with standard ginsenosides were confirmed by high-performance liquid chromatography (HPLC) analysis (Rb1, 7.34 mg/g; Rb2, 2.54 mg/g; Rc, 3.41mg/g; Rd, 0.97 mg/g; Re, 1.64 mg/g; Rf, 1.26 mg/g; Rg1, 1.21 mg/g; Rg2 (s), 1.36 mg/g; Rg3(s), 2.21 mg/g; Rg3(r), 1.17mg/g and Rh1, 1.56 mg/g).

2.4. Cell culture conditions

Human adenocarcinoma A549 cells were maintained in Roswell Park Memorial Institute 1640 Medium containing 10% FCS, 100 units/mL of penicillin, 100 μg/mL of streptomycin and 250 ng/mL of Fungizone® (amphotericin B) mixed antibiotic-antimycotic. The cells were cultured at 37°C in the humidified 5% CO_2 atmosphere.

2.5. Luciferase reporter assay

The NF-κB luciferase was constructed using the enhanced luciferase reporter gene pELAM promotor. A549 cells were transiently transfected with plasmid using polyethylenimine reagent and Tom medium. Luciferase assay was performed according to a previously reported method [29].

2.6. Reverse transcription polymerase chain reaction

Total RNA was extracted using the trizol reagent, and the first-strand cDNA was synthesized according to a previously reported method [30]. Quantitative real-time polymerase chain reaction was performed with StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using a AccuPower® GreenStar™ qPCR Pre Mix (Bioneer Corporation, Daejeon, Korea) according to the manufacturer's instructions [31]. The primers used were MMP-9: 5′-ACTTTGACAGCGACAAGAAGTG-3′, 5′-GGCACTGAGGAATGATCTAAGC-3′. The primers of COX-2 and β-actin were described previously [15]. The relative expression was calculated and standardized by the expression of β-actin taken from the same sample using the comparative cycle threshold (Ct).

2.7. Western blot analysis

Protein isolation of whole cells and western blot analysis were performed according to previously reported methods [32]. The blots were incubated for overnight or 1 h with primary antibodies (NF-κB-p65, Lamin B, MMP-9, COX-2, and β-actin) diluted to 1:1000 or 1:5000 in Tris Buffered Saline with Tween 20 (TBST). The blots were washed and incubated with secondary antibody diluted to 1:5000 with skim milk for 1 h. The bands were detected the using enhanced chemiluminescence. Quantity One analysis software (Bio-Rad, Hercules, CA, USA) was used to quantify the strength of the band.

2.8. Extraction of nuclear and cytoplasmic proteins

Cytoplasmic and nuclear proteins of A549 cells were detached using the nuclear and cytoplasmic protein extraction kits (Abcam, Cambridge, UK) according to the manufacturer's instructions. Fractions were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and western blot with specific antibodies.

2.9. Cell migration assays

As previously described, the migration assay was performed using transwell inserts (Corning Inc., NY, USA) that have 6.5 mm polycarbonate membranes with 8.0 μm pores [27]. After 24 h of incubation under BPA or KRG or celecoxib, the migrated cells were fixed and stained with crystal violet.

2.10. MTT assays

A549 cells were seeded in a 96-well plate at a density of 5000 cells/well. Next day, A549 cells were treated with the BPA or KRG into the culture media and incubated for 24 h. MTT assays were performed as reported previously [33].

2.11. Measurement of reactive oxygen species production

The levels of intracellular ROS were measured with a FACSCalibur flow cytometer using the BD CellQuest Pro software (BD Biosciences, San Jose, CA, USA) according to previously reported methods [15]. FlowJo software (TreeStar, Inc., Ashland, OR, USA) was used to analyze data.

2.12. Statistical analysis

Data were described as the means ± standard deviation. We compared each group using one-way analysis of variance and Tukey's multiple-comparison posttest using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA). Differences between groups were considered significant at a *P* value of less than 0.05.

3. Results

3.1. KRG suppresses BPA-induced COX-2 expression

Inflammation is closely associated with cancer progression, and release of inflammatory molecules is known to induce the cancer progression and metastasis [34]. Among various inflammatory mediators, COX-2 is a major factor in inflammation [35]. Previously, we showed that BPA upregulates COX-2 expression in A549 cells [15]. We also investigated whether KRG inhibited BPA-induced COX-2 expression. KRG did not affect cell viability at concentrations of 100–500 μg/mL under BPA treatment (Fig. 1A). Therefore, this concentration was selected as the treatment condition in the following experiment. KRG at a dose of 500 μg/mL efficiently blocked BPA-induced COX-2 mRNA levels (Fig. 1B). KRG also significantly suppressed BPA-induced COX-2 protein (Fig. 1C). These results indicate that KRG exerts an inhibitory effect on COX-2 expression by BPA in A549 cells.

3.2. KRG suppresses BPA-induced NF-κB promoter activity and NF-κB nuclear translocation

NF-κB is a transcriptional regulator of the expression of various genes, including COX-2, which is involved in cell proliferation and

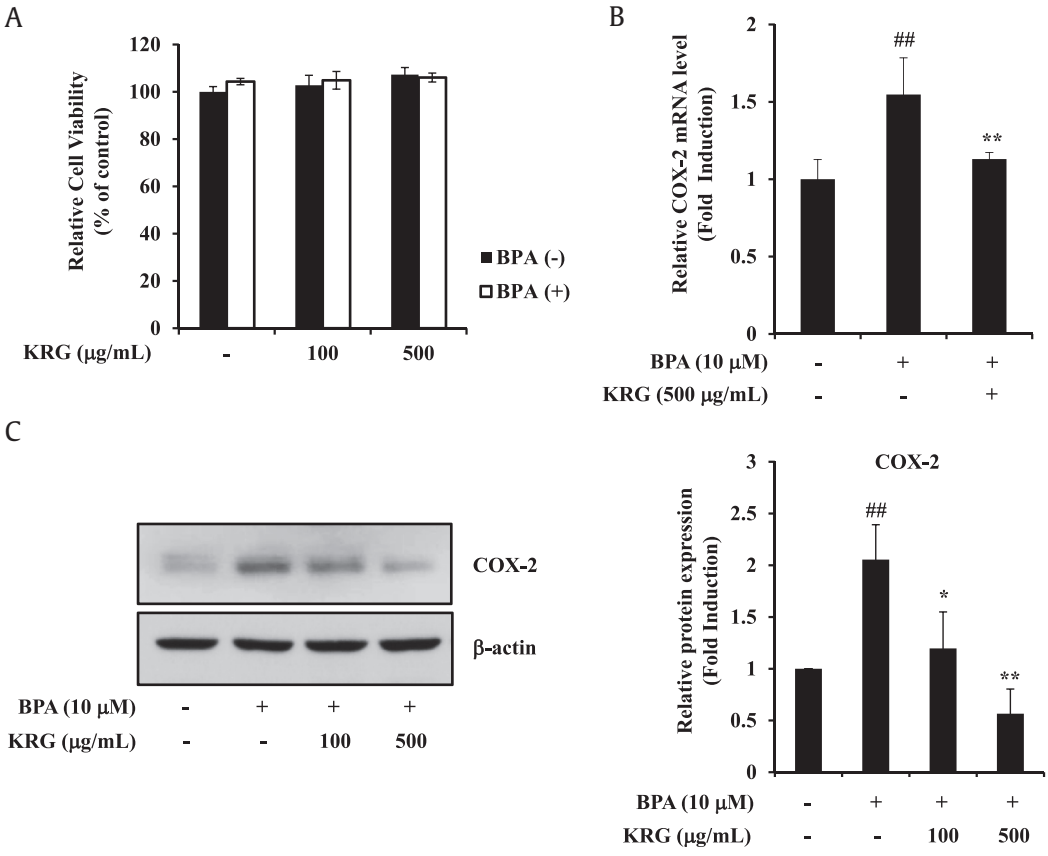

Fig. 1. Effects of KRG on BPA-induced COX-2 expression. (A) A549 cells were preincubated with KRG for 1h and treated with BPA. After 24 h incubation, cell viability was measured by MTT assay. (B) A549 cells were pretreated with KRG (500 μg/mL) for 1 h and treated with BPA for 24 h. The levels of COX-2 mRNA were determined by qRT-PCR. (C) A549 cells were treated as described in (A). COX-2 and β-actin were evaluated by western blot analysis. ##$p < 0.01$, CON vs. BPA; *$p < 0.05$ and **$p < 0.01$, BPA vs. BPA + KRG. KRG, Korean Red Ginseng; BPA, bisphenol A; MTT, thiazolyl blue tetrazolium bromide; qRT-PCR, quantitative reverse transcription polymerase chain reaction; COX-2, cyclooxygenase-2.

inflammatory responses [36]. Previously, we confirmed that BPA upregulates NF-κB activity in A549 cells [15]. Next, we examined whether the increase in NF-κB activity induced by BPA was reduced by KRG treatment. KRG inhibited the BPA-induced NF-κB promoter activity at concentrations of 100 and 500 μg/mL (Fig. 2A). The activation of NF-κB is initiated by translocation of the NF-κB p65 subunit from the cytoplasm into the nucleus [37]. Nuclear p65 acts as a transcription factor that causes the expression of inflammatory genes, such as COX-2 [38]. Treatment with BPA (10 μM) increased the nuclear expression of p65 protein. KRG (500 μg/mL) significantly attenuated BPA-induced translocation of NF-κB p65 into the nucleus (Fig. 2B). These results demonstrate that KRG blocked the BPA-induced NF-κB promoter activity and NF-κB nucleus translocation.

3.3. KRG inhibits BPA-induced cell migration

COX-2 is also strongly implicated in tumor progression by promoting important cellular functions, including cell migration [39]. To determine whether KRG inhibited the BPA-induced migration of A549 cells via COX-2, cell migration was investigated under the presence of the COX-2 inhibitor celecoxib. Celecoxib and KRG significantly suppressed the BPA-induced cell migration by approximately 39% and by 51% compared with BPA treatment (Fig. 3). Because celecoxib blocked cell migration induced by BPA, it suggests that COX-2 plays an important role in the regulation of cell migration by BPA. These results demonstrate that KRG suppresses BPA-induced cell migration.

3.4. KRG inhibits BPA-induced ROS production

To understand the upstream modulation of NF-κB, we confirmed whether KRG inhibited BPA-induced ROS production in A549 cells by flow cytometry. ROS is involved in NF-κB activation and leads to increased cytokine expression [40]. Moreover, ROS activates signaling pathways, such as in cancer development and progression [41]. As shown Fig. 4, BPA markedly elevated the ROS levels in A549 cells compared with the control, whereas the ROS scavenger NAC almost completely blocked ROS production. Treatment with KRG (500 μg/mL) effectively suppressed the levels of BPA-induced ROS. These results indicate that KRG inhibited BPA-induced ROS production.

3.5. KRG suppresses BPA-induced COX-2 and MMP-9

MMP-9 plays an important role in the migration and invasion of various cancer cells [42]. In addition, BPA increases MMP-9 expression in A549 cells [43]. NF-κB is centrally involved in the induction of MMP-9 expression by BPA [44,45]. To confirm whether the suppression of BPA-induced MMP-9 and COX-2 by KRG is associated with its ROS-scavenging activity, we confirmed the expression of MMP-9 and COX-2 on treatment of KRG and NAC. The BPA-induced COX-2 mRNA and protein levels were blocked by KRG and NAC (Fig. 5A and B). KRG and NAC also inhibited the BPA-induced MMP-9 mRNA and protein expression (Fig. 5C and D). These results demonstrate that KRG blocks BPA-induced COX-2 and MMP-9 via ROS signaling.

4. Discussion

This study demonstrated that KRG inhibits the BPA-induced COX-2 expression and cell migration via the inhibition of ROS and downregulation of NF-κB activation and MMP-9 expression in A549 human lung cancer cells. These results point to a new possible therapeutic application for KRG to protect against inflammatory disorders induced by EDCs. We and others have shown that BPA induces COX-2 in cancer cell lines [15,46]. BPA induced COX-2 expression via the activation of mitogen-activated protein

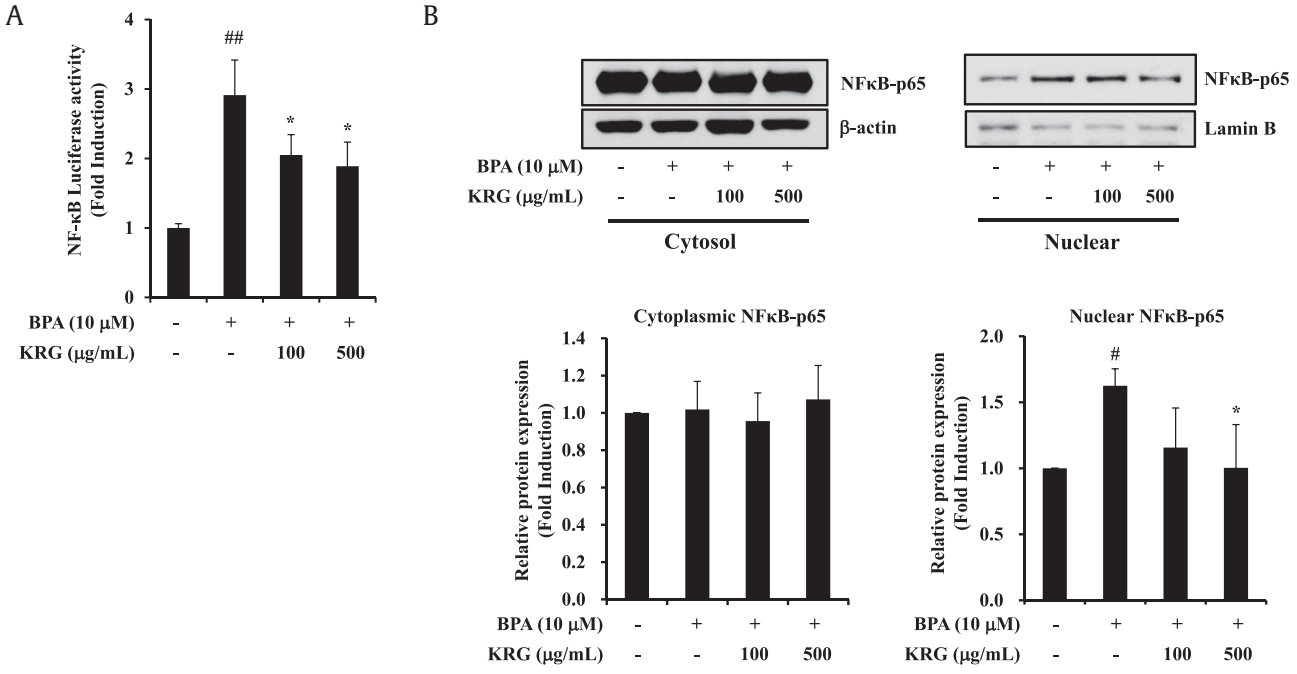

Fig. 2. Effects of KRG on BPA-induced NF-κB activation. (A) A549 cells were transfected with the NF-κB luciferase reporter gene. The next day, A549 cells were pretreated with KRG for 1 h and then treated with BPA for 24 h. Luciferase activities of NF-κB were measured. (B) A549 cells were treated as described in (A). The cytoplasmic and nuclear protein extracts were isolated with different lysis buffers. NF-κB p65, lamin B, and β-actin were evaluated by western blot analysis. #$p < 0.05$ and ##$p < 0.01$, CON vs. BPA; *$p < 0.05$, BPA vs. BPA + KRG.
NF-κB, nuclear factor-kappa B; KRG, Korean Red Ginseng; BPA, bisphenol A.

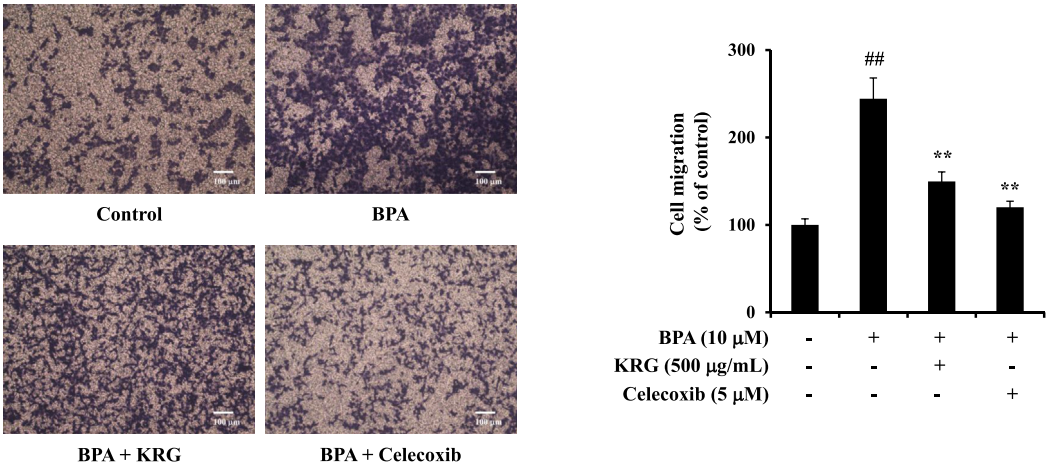

Fig. 3. Effects of KRG on BPA-induced cellular migration. A549 cells were coincubated with BPA or KRG or celecoxib in the upper chamber of transwell for 24 h. The migrated cells were counted using light microscopy. The bar graph shows the cells that have been migrated relatively. Scale bar represents 100 μm. ##$p < 0.01$, CON vs. BPA; **$p < 0.01$, BPA vs. BPA + KRG or BPA + celecoxib.
KRG, Korean Red Ginseng; BPA, bisphenol A.

kinase and promoted the cellular migration of A549 and MDAMB-231 cells [15,47]. BPA induces COX-2 expression in human mesenchymal stem cells derived from uterine myoma tissue, human endometrial carcinoma cells [46,48]. In two epidemiological studies conducted with repeated urine and serum samples from 700 Korean elderly people, the associations of BPA with six inflammation markers (white blood cells, C-reactive protein, interleukin-10, alanine aminotransferase, aspartate transaminase and γ-glutamyl transpeptidase levels) were evaluated, and significant positive correlations between the levels of BPA and six inflammation markers were found [15]. A Taiwanese cohort study suggested that nonylphenol (NP) and BPA increase oxidative stress and decrease antioxidant activity during pregnancy and inflammation [49]. The generation of ROS increased with a decrease in mitochondrial membrane potential in BPA-exposed lymphoblastoid cell lines of children with autism [50]. Accumulating *in vitro* and *in vivo* studies strongly support the prooxidant role of BPA [51]. Induction of ROS and inflammation by BPA leads to activation of the mitogen-activated protein kinase, PI3K/AKT, and NF-κB pathways, inducing mitochondrial dysfunction and promoting changes in several cell signaling pathways, partly via nuclear or membrane ER signaling mechanisms [52–54]

Various studies have searched for protective and preventive natural products that are effective against EDC-induced pathological conditions [55,56]. Oleuropein- and hydroxytyrosol-rich extracts from olive leaves attenuated the liver injury and lipid accumulation induced by BPA in male rats via hypolipidemic and hepatoprotective effects by enhancing the antioxidative defense system and regulating inflammation [57]. Selenium and nanoselenium protected against the reproductive toxicity induced by BPA via improved antioxidant activity [58]. Data from 88,962 adults collected as part of the National Health Interview Survey to assess trends in the use of complementary health approaches found that ginseng was the ninth most commonly used nonvitamin, nonmineral dietary supplement in a question asking about usage in the previous 30 days after fish oil, glucosamine, probiotics, melatonin, coenzyme q-10, Echinacea, garlic supplements, and cranberry [59]. The many studies of the antioxidant activity of KRG induced by various oxidative stresses motivated our study, and our results show that KRG is effective against the increases in intracellular ROS and COX-2 induced by BPA. Only a few antioxidant studies have been conducted in humans, so clinical data on the beneficial effects of KRG on exposure to EDCs will be useful for extending the application of KRG.

MMP-9 plays a critical role in the progression of cancer, and the overexpression of MMP-9 is mostly related to the migration and invasion of various cancer cells [60]. In addition, MMP-9 is involved in the pathophysiology of cancer progression and inflammation-

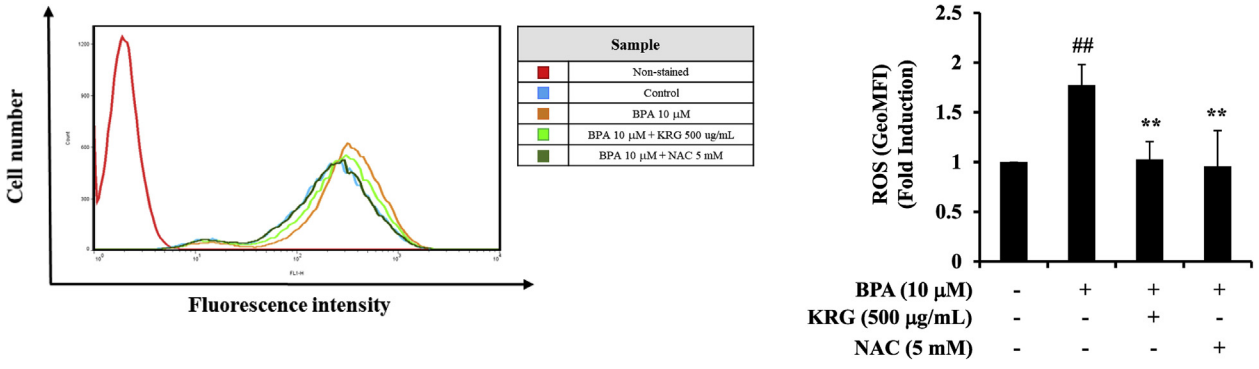

Fig. 4. Effects of KRG on BPA-induced ROS production. A549 cells were pretreated with KRG for 1 h and treated with BPA. After 24h incubation, A549 cells were stained with cell-permeable dye 2′,7′-dichlorofluorescin diacetate (DCF-DA) (1 μM). ROS production was measured by flow cytometry. ##$p < 0.01$, CON vs. BPA; **$p < 0.01$, BPA vs. BPA + KRG or BPA + NAC.
KRG, Korean Red Ginseng; BPA, bisphenol A; ROS, reactive oxygen species; NAC, N-acetyl-L-cysteine.

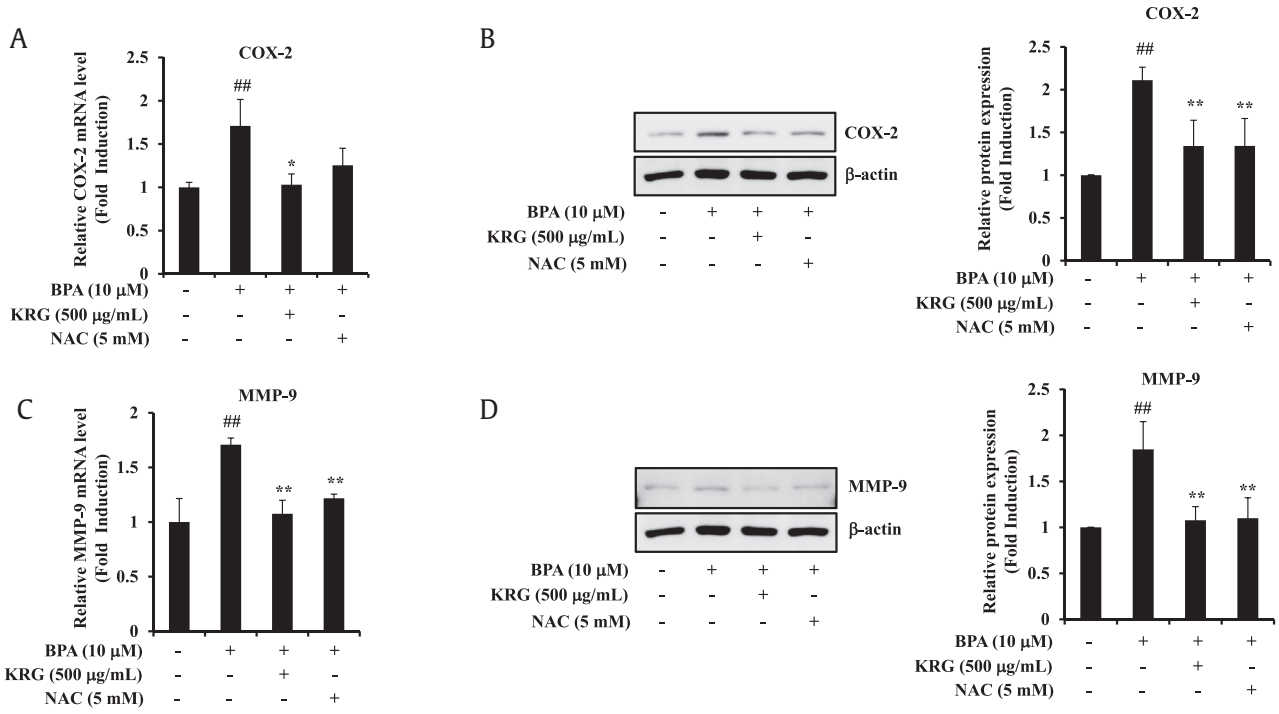

Fig. 5. Effects of KRG on involvement of ROS in COX-2 and MMP-9 expression. (A) A549 cells were preincubated with KRG and NAC for 1 h and treated with BPA for 24 h. The levels of COX-2 mRNA were determined by qRT-PCR. (B) A549 cells were treated as described in (A). COX-2 and β-actin were evaluated by western blot analysis. (C) A549 cells were treated and total RNA was extracted as described in (A). The expression of MMP-9 mRNA was determined by qRT-PCR. (D) A549 cells were treated as described in (A). MMP-9 and β-actin were evaluated by western blot analysis. ##$p < 0.01$, CON vs. BPA; *$p < 0.05$ and **$p < 0.01$, BPA vs. BPA + KRG or BPA + NAC.
KRG, Korean Red Ginseng; BPA, bisphenol A; ROS, reactive oxygen species; COX-2, cyclooxygenase-2; qRT-PCR, quantitative reverse transcription polymerase chain reaction; MMP-9, matrix metalloproteinase-9; NAC, N-acetyl-L-cysteine.

related diseases [61,62]. Recent research studies have supported that MMP-9 inhibitors have significant protective effects for tumor promotion by partially blocking the expression of proinflammatory enzymes, such as COX-2 [63]. Exposure of BPA induces cell migration and MMP-9 expression in several cancer cells [43,64]. Thus, targeting MMP-9 inhibition is another strategy for cancer prevention and treatment [65]. To identify the molecular mechanisms that determine the antimetastatic and antiinflammatory effect of KRG, a key question in this study asked whether KRG downregulates BPA-induced COX-2 and MMP-9 expression in A549 cells.

In summary, these above data demonstrated that KRG inhibits BPA-induced MMP-9 and COX-2 by inhibiting ROS in A549 cells. Therefore, our results suggest that KRG could be used as a potential therapeutic agent for inflammatory disorders by BPA in lung cancer cells.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2020.01.002.

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