



## 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, Seoul, Republic of Korea

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## 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 (PPAR $\gamma$ ) 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 PPAR $\gamma$  in A549 cells were determined by luciferase assay, Western blot, and/or quantitative reverse transcription-polymerase chain reaction (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 PPAR $\gamma$  as a cellular target of KRG for the suppression of hypoxia-induced COX-2. PPAR $\gamma$  protein levels and PPAR $\gamma$ -responsive element (PPRE)-driven reporter activities were increased by KRG. Reduction of hypoxia-induced COX-2 by KRG was abolished by the PPAR $\gamma$  inhibitor GW9662. In addition, the inhibition of PPAR $\gamma$  abolished the effect of KRG on hypoxia-induced cell migration and invasion.

**Discussion:** Our results show that KRG inhibition of hypoxia-induced COX-2 expression and cell invasion is dependent on PPAR $\gamma$  activation, supporting the therapeutic potential for suppression of inflammation under hypoxia. Further studies are required to demonstrate whether KRG activates directly PPAR $\gamma$  and to identify the constituents responsible for this activity.

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## 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 lipopolysaccharide (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 (PPAR $\gamma$ ), and its signaling is critical during breast tumorigenesis and correlated with COX-2

\* Corresponding author. Department of Bioscience and Biotechnology, Sejong University, Kwang-Jin-Gu, Seoul 05006, Republic of Korea.  
E-mail address: [yjlee@sejong.ac.kr](mailto:yjlee@sejong.ac.kr) (Y.J. Lee).

expression [18]. These observations indicate the importance of COX-2 inhibition in preventing hypoxia-induced cell invasion.

PPAR $\gamma$ , 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 PPAR $\gamma$  include fatty acids and prostanoids. PPAR $\gamma$  regulates adipogenesis by differentiating adipocytes, lipid metabolism, inflammation, and angiogenesis [20]. PPAR $\gamma$  regulates COX-2 gene expression through PPAR response elements within the promoter of COX-2 [21,22]. However, depending on the cell type, PPAR $\gamma$  can both activate and inhibit COX-2 through PPAR $\gamma$ -dependent and -independent mechanisms [23–25]. Continuous research is required to understand these complex phenomena. PPAR $\gamma$ -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 PPAR $\gamma$  signaling pathway and that PPAR $\gamma$  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- $\beta$ -actin was purchased from Sigma. Anti-sirtuin-1 (SIRT-1), anti-PPAR $\gamma$  and anti-peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1 $\alpha$ ) 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 Roswell Park Memorial Institute medium (RPMI) containing 10% FBS and penicillin/streptomycin. Cells were grown at 37°C in a humidified atmosphere of 95% air/5% CO<sub>2</sub> 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<sub>2</sub> level of 5% with 1% O<sub>2</sub> balanced with N<sub>2</sub> 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  $\mu$ m 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 AutoLumat LB9507 luminometer (EG & G Berthold, Bad Wildbad, Germany) using the luciferase assay system (Promega Corp., Madison, WI, USA) and expressed as relative light units. PPAR $\gamma$ -responsive element-Luciferase (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  $\mu$ g total RNA was incubated at 70°C for 5 min with 0.5  $\mu$ g of random hexamer and deionized water (up to 11  $\mu$ L). The reverse transcription reaction was performed using 40 units of Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega Corp.) in 5 $\times$  reaction buffer (250 mmol/L Tris-HCl; pH 8.3, 375mM KCl, 15mM MgCl<sub>2</sub>, 50mM dithiothreitol (DTT)), RNase inhibitor at 1 unit/ $\mu$ L, 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:  $\beta$ -actin sense primer, 5'-CAAATGCTTCTAGGCGGACTATG-3';  $\beta$ -actin anti-sense primer, 5'-TGCGCAAGTTAGGTTTTGTCA-3'; COX-2 sense primer, 5'-TGAA-GAAGTACAGGAGAAAA-3'; COX-2 anti-sense primer, 5'-TACCA-GAAGGGCAGGATACA-3'. Using the comparative threshold cycle (Ct), relative expression was calculated and normalized by the expressions of  $\beta$ -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 13,000g. 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  $\beta$ -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  $\mu$ m pores

(Corning Inc., NY, USA). Matrigel invasion assay was performed using membranes coated with matrigel matrix (BD Science, Sparks, MD, USA). A549 cells were seeded into the upper chamber in serum-free media. The lower chambers consisted of RPMI media containing 10% FBS. After incubation under normoxia or hypoxia for 24–48 h, noninvasive cells present on the upper surface of the membrane were scraped with cotton swabs and the invasive cells present on the lower side of the membrane were fixed with ice cold methanol, stained with 0.1% crystal violet. The cells that migrated and invaded to the lower side of the filter were observed using a light microscope and counted.

### 2.7. Statistical analysis

All data were analyzed and expressed as means and standard deviations. The two-tailed, unpaired Student *t* test was applied using SPSS software (version 23.0; IBM, Armonk, NY, USA). The *t* test was used to compare data between the hypoxia and KRG-treated groups. The criterion for statistical significance was  $p < 0.05$ .

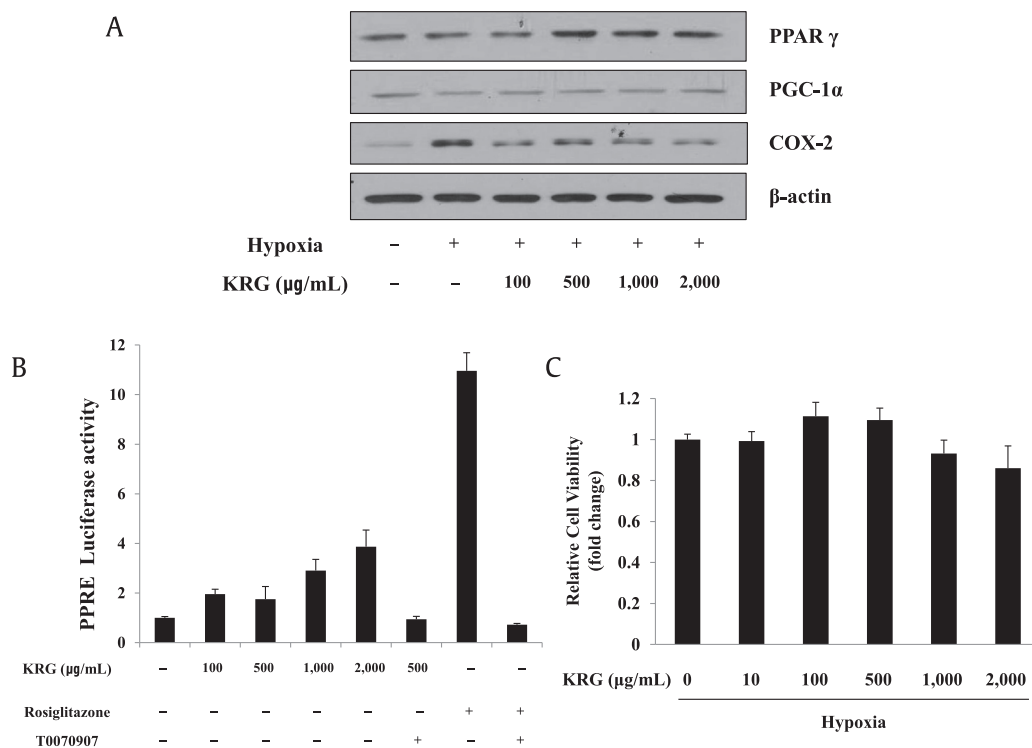
## 3. Results

### 3.1. KRG induces PPAR $\gamma$ and inhibits hypoxic induction of COX-2 expression in A549 cells

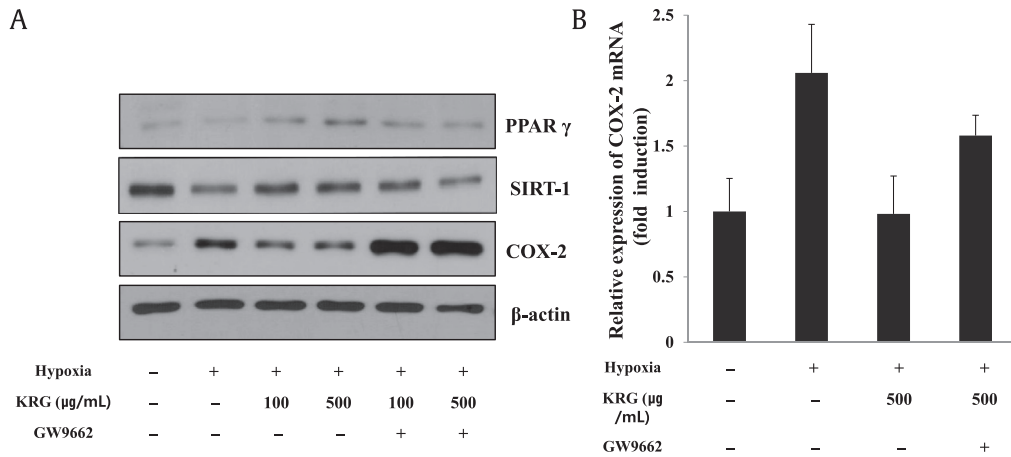
COX-2 is transcriptionally induced by hypoxia and has been implicated in tumor progression and angiogenesis in tumor cells. We have previously shown that KRG inhibits COX-2 expression under hypoxia in A549 lung cancer cells, where COX-2 is also

strongly implicated in tumorigenesis [28]. In the course of studying the mechanism of KRG inhibition of COX-2 under hypoxia, the protein levels of PPAR $\gamma$  were examined. A549 cells were preincubated with KRG for 1 h and cotreated with hypoxia. KRG at doses of 500–2,000  $\mu\text{g}/\text{mL}$  increased PPAR $\gamma$  protein levels. At the same time, as observed previously, KRG efficiently blocked the expression of hypoxia-induced COX-2 protein (Fig. 1A). KRG at doses of 100–2,000  $\mu\text{g}/\text{mL}$  activated PPAR $\gamma$  luciferase reporter activity. To confirm that the activities of KRG are PPAR $\gamma$ -mediated, we cotreated the cells with the PPAR $\gamma$  antagonist T0070907 at a concentration of 5  $\mu\text{M}$ , which is enough to block almost all the PPAR $\gamma$  on the cells. A known PPAR $\gamma$  agonist, rosiglitazone, was used as a positive control. The transcriptional activation of the reporter plasmid by KRG was blocked by T0070907, indicating that luciferase gene activation is PPAR $\gamma$ -specific (Fig. 1B). The concentration of 100–500  $\mu\text{g}/\text{mL}$  KRG was chosen as the treatment condition in further experiments because KRG exerted its efficacy with no effect on cell viability at these concentrations (Fig. 1C).

To further confirm the involvement of PPAR $\gamma$  in inhibiting the hypoxic induction of COX-2, COX-2 was examined after treatment with another PPAR $\gamma$  antagonist, GW9662. In accordance with our previous report, SIRT-1 levels were increased with KRG treatments. The enhanced protein levels of SIRT-1, PPAR $\gamma$ , and COX2 were blocked by GW9662, suggesting that the response involves PPAR $\gamma$  (Fig. 2A). Induction of COX-2 occurs mostly at the transcription level. Inhibition of COX-2 at the mRNA level by 500  $\mu\text{g}/\text{mL}$  KRG was blocked by GW9662 (Fig. 2B). These results indicate that the inhibition of hypoxia-induced COX-2 regulation by KRG at the transcription and translation levels in A549 cells is dependent on PPAR $\gamma$ .



**Fig. 1.** Korean Red Ginseng (KRG) induces peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) activity and its expression. (A) A549 cells were treated with KRG at 100–2,000  $\mu\text{g}/\text{mL}$  for 24 h under hypoxia and analyzed by Western using indicated antibodies. (B) A549 cells were transiently transfected with the PPAR $\gamma$ -responsive element (PPRE)-luciferase reporter gene. The following day, A549 cells were cultured in medium containing vehicle or KRG (100–2,000  $\mu\text{g}/\text{mL}$ ) or PPAR $\gamma$  agonist rosiglitazone (1  $\mu\text{M}$ ) or PPAR $\gamma$  antagonist T0070907 (5  $\mu\text{M}$ ) for 48 h and luciferase activities were determined. (C) A549 cells were incubated with KRG at 10–2,000  $\mu\text{g}/\text{mL}$  for 24 h under hypoxia and 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay was performed. All experiments were repeated at least three times.

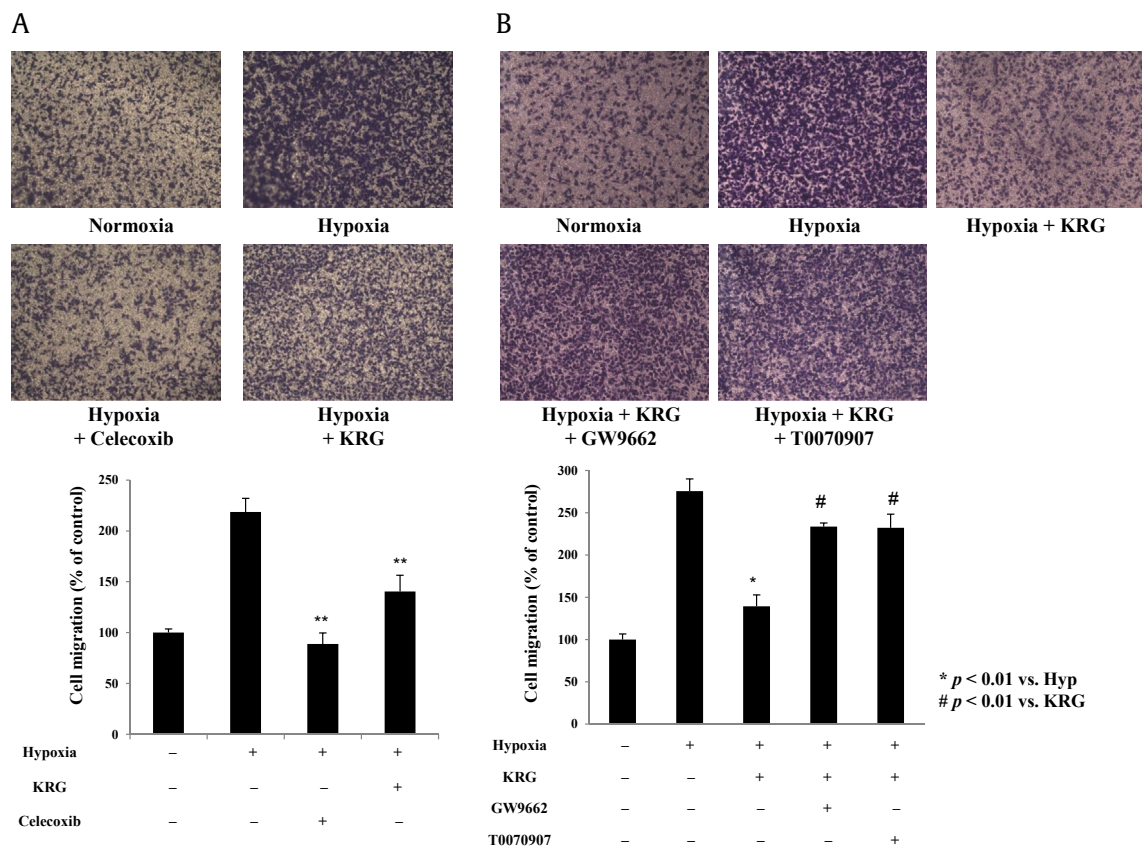


**Fig. 2.** Korean Red Ginseng (KRG) downregulates cyclooxygenase-2 (COX-2) expression through peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) activation. (A) A549 cells were pretreated with KRG (100  $\mu\text{g/mL}$  or 500  $\mu\text{g/mL}$ ) and/or GW9662 (5 $\mu\text{M}$ ) for 1 h before treatment with hypoxia for 24 h. Total proteins were prepared and protein levels of sirtuin-1 (SIRT-1), PPAR $\gamma$ , and COX-2 were determined by western blot. (B) A549 cells were pretreated with KRG (500  $\mu\text{g/mL}$ ) and/or GW9662 (5 $\mu\text{M}$ ) for 1 h before treatment with hypoxia for 24 h. Total RNA from A549 cells were analyzed for COX-2 mRNA expression by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). All experiments were repeated at least three times.

**3.2. KRG inhibits cellular migration and invasion of A549 cells under hypoxia**

To determine whether hypoxia induces cell migration via COX-2-dependent PGE2 production in A549 cells, cell migration was examined with the COX-2 inhibitor, celecoxib. Hypoxia-induced

cell migration was inhibited by celecoxib, suggesting that COX-2 plays an important role in hypoxia-induced cell migration (Fig. 3A). KRG decreased the migration of A549 cells under hypoxic conditions by approximately 50%. KRG inhibition of cell migration was significantly blocked by GW9662 and T0070907, indicating that the effects of KRG on hypoxia-induced migration are mediated



**Fig. 3.** Korean Red Ginseng (KRG) inhibits migration ability of A549 cells under hypoxia. (A) A549 cells were pretreated with KRG (100  $\mu\text{g/mL}$ ) and/or celecoxib (5 $\mu\text{M}$ ) treatment with hypoxia for 24 h. (B) A549 cells were pretreated with KRG (100  $\mu\text{g/mL}$ ) and/or GW9662 (5 $\mu\text{M}$ ) or T0070907 (5 $\mu\text{M}$ ) treatment with hypoxia for 24 h. Cells that migrated through the membranes were fixed and counted using light microscopy. Bar graph shows relative migrated cells. The cells in the lower side were counted and are graphed below. Values represent the mean  $\pm$  standard deviation ( $n = 3$ ). \*  $p < 0.05$  vs. hypoxia, \*\*  $p < 0.01$  vs. hypoxia, #  $p < 0.01$  vs. KRG. All experiments were repeated at least three times.



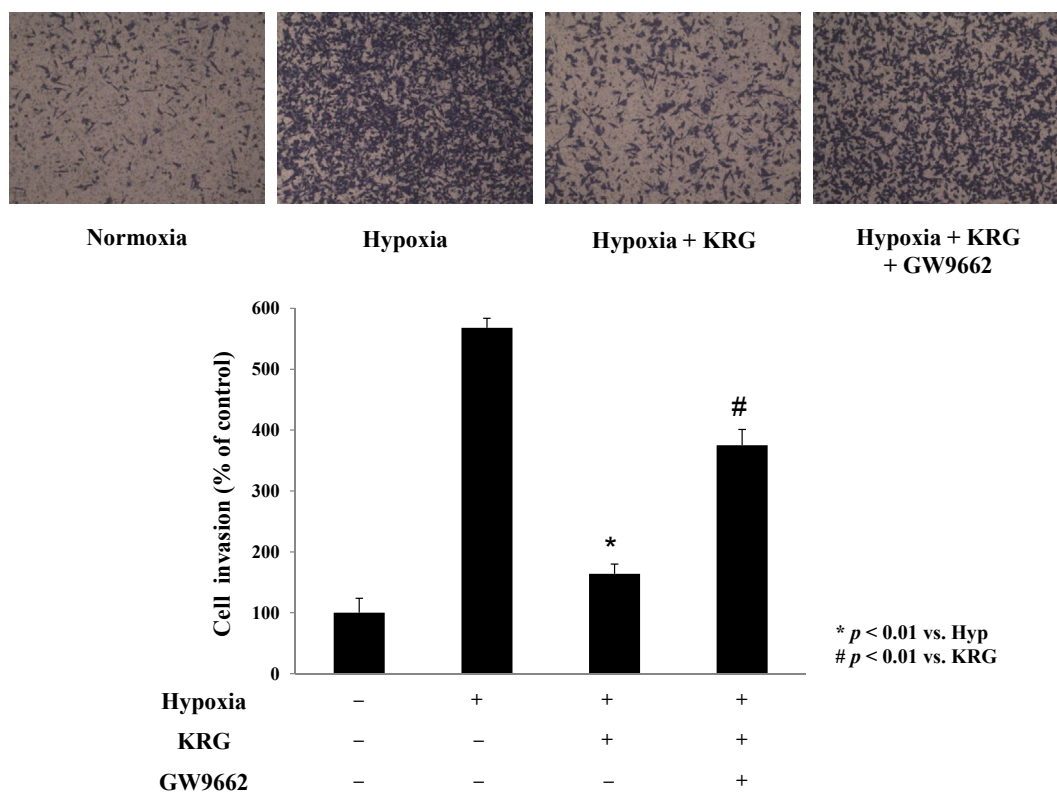
by PPAR $\gamma$  (Fig. 3B). Similarly, hypoxia enhanced A549 cell invasion and KRG treatments significantly decreased this effect (Fig. 4). Inhibition of invasiveness was absent in the presence of GW9662, indicating that the anti-invasive effects of KRG under hypoxic conditions are dependent on PPAR $\gamma$ . The invasiveness was absent in the presence of PPAR $\gamma$  inhibitors, indicating that the anti-invasive properties of KRG under hypoxic microenvironments require PPAR $\gamma$  activation and occur via a COX-2/PGE2-dependent pathway. However, this migration and invasion were also inhibited by SIRT-1 inhibitors [28], suggesting that this cellular movement is a complicated phenomenon relying on multiple factors.

#### 4. Discussion

We have recently reported that SIRT-1 is an important player in the suppression of hypoxia-induced COX-2 by KRG in A549 cells [28]. We suggested that SIRT-1 activation by KRG has potential therapeutic value in the suppression of inflammation and in cancer therapies under hypoxic conditions. COX-2 is rapidly induced by various stimuli such as LPS, high osmolarity, and hypoxia, and plays a pivotal role in the production of proinflammatory eicosanoids. High levels of PGE2 synthesized by COX-2 are an important mediator in airway inflammatory responsiveness and angiogenesis involved in tumor development [34]. Many reports have shown that KRG possesses antiinflammatory and antioxidant properties both *in vitro* and *in vivo* [35,36]. However, better understanding of the molecular mechanisms underlying the inhibitory effect of KRG on hypoxia-induced COX-2 is still needed. In this study, we examined the PPAR $\gamma$ -mediated KRG inhibition of COX-2 under hypoxia and showed that PPAR $\gamma$  activation is responsible for COX-2 suppression under hypoxia.

Peroxisome Proliferator-Activated Receptors (PPARs) is a ligand-activated transcription factor and regulates diverse biological functions including adipocyte differentiation, lipogenesis, inflammation, and insulin sensitivity. PPAR $\gamma$  is one pathway that modulates the thriving of cancer cells by multiple complex pathways, thereby sustaining uncontrolled tumor growth. Endogenous ligands including prostaglandins (15d-PGJ2) and synthetic ligands including the antidiabetic thiazolidinediones are known to bind PPAR. Upon ligand binding, PPAR is modified by phosphorylation, sumoylation, ubiquitination, and acetylation. PPAR $\gamma$  is acetylated by CBP/p300 and deacetylated by SIRT-1 [37].

Some PPAR $\gamma$ -activating ligands that modulate inflammation have been discovered from natural products such as curcumin, alpha-linolenic acid, magnolol, and orange peel extract [38–41]. A recent report showed that fisetin, a flavonol present in vegetables and fruits, upregulates adiponectin with antiobesity, antidiabetic, and antiatherosclerotic functions through the activation of SIRT-1 and PPAR [42]. Ginsenosides from ginseng showed differential response to PPAR activation. Ginsenosides Re and Rg1 showed inhibitory effects on the differentiation of 3T3-L1 adipocytes [43]. However, ginsenosides Rb2, Rb3, and Rc displayed promotional activities [44]. Ginsenoside-Rg3 induces inhibition of adipogenesis through the activation of AMP-activated protein kinase (AMPK) and the inhibition of PPAR $\gamma$  transcriptional activity in 3T3-L1 adipocytes [45]. Protopanaxatriol is a novel PPAR $\gamma$  antagonist with moderate binding activity [44]. By contrast, Ginsenoside-Rb1 binds to PPAR $\gamma$ , attenuates central inflammation and leptin resistance, reduces the release of free fatty acid, and alleviates the ectopic deposit of triglyceride by upregulating the expression of perilipin in adipose tissue [26]. Ginsenoside-Re reduces insulin resistance through activation of PPAR $\gamma$  pathway and inhibited the production



**Fig. 4.** Korean Red Ginseng (KRG) inhibits cellular invasion of A549 cells under hypoxia. A549 cells were pretreated with KRG (100  $\mu$ g/mL) and/or GW9662 (5 $\mu$ M) for 1 h before treatment with hypoxia for 48 h. Matrigel invasion assay was done under normoxia or hypoxia. The cells in the lower side were counted and are graphed below. Values represent the mean  $\pm$  standard deviation ( $n = 3$ ). \*\*  $p < 0.01$  vs. hypoxia, #  $p < 0.01$  vs. KRG. All experiments were repeated at least three times.

of inflammatory cytokine [43]. Ginsenoside-Rg1 can increase the insulin-degrading enzyme expression in the hippocampus by upregulating PPAR $\gamma$ , attenuated hippocampal histopathological abnormalities, and improved learning and memory in a rat model of Alzheimer's disease [46].

Our study showed that total ginseng extract exhibited PPAR $\gamma$  and SIRT-1 activation with simultaneous suppression of COX-2. Our observation was derived from the sum of activity from each component and interactions between the components. These results will aid our understanding of how ginseng is beneficial in the treatment of metabolic disorders. Our data imply that KRG inhibits COX-2 expression by increasing SIRT-1 deacetylase activity, leading to increased interactions with the PPAR complex and ultimately transcriptional activation. The activation of SIRT-1 by KRG may deacetylate PGC-1 $\alpha$ , which in turn may increase its interaction with the PPAR complex to suppress COX-2 transcription. Our ongoing study is designed to characterize the COX-2 suppression and PPAR $\gamma$  activation functions of each ginsenoside in A549 cells, as used in this study, and 3T3L1 preadipocytes. Our findings are important for the clinical usage of ginseng and provide a mechanistic explanation of its effects on metabolic disorders and cancer.

### Conflicts of interest

All authors have no conflicts of interest to declare.

### Acknowledgments

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