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Induction of cyclooxygenase-2 by ginsenoside Rd via activation of CCAAT-enhancer binding proteins and cyclic AMP response binding protein

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Abstract

Panax ginseng is a widely used herbal medicine in East Asia and is reported to have a variety of pharmacological effects against cardiovascular diseases and cancers. Here we show a unique effect of ginsenoside Rd (Rd) on cyclooxygenase-2 (COX-2) expression in RAW264.7 macrophages. Rd (100 μg/ml), but not other ginsenosides induced COX-2 and increased prostaglandin E_2 production. Gel shift and Western blot analyses using nuclear fractions revealed that Rd increased both the DNA binding of and the nuclear levels of CCAAT/enhancer binding protein (C/EBP)α/β and cyclic AMP response element binding protein (CREB), but not of p65, in RAW264.7 cells. Moreover, Rd increased the luciferase reporter gene activity in cells transfected with a 574-bp mouse COX-2 promoter construct. Site-specific mutation analyses confirmed that Rd-mediated transcriptional activation of *COX-2* gene was regulated by C/EBP and CREB. These results provide evidence that Rd activated C/EBP and CREB, and that the activation of C/EBP and CREB appears to be essential for induction of COX-2 in RAW264.7 cells.

Keywords: Ginsenoside Rd; COX-2; C/EBP; CREB

Panax ginseng is a well-known and popular herbal medicine used worldwide. This herbal root has been used for more than 2000 years throughout Eastern countries. A large number of studies have described the pharmacological effects of *P. ginseng* on certain chronic disease states and on aging [1]. Moreover, these effects are suggested to be due to enhanced immune function and antioxidant capacity [1,2]. Ginsenosides are mixtures of triterpene glycosides, obtained as extracts from various species of ginseng. Among more than 30 ginsenosides, ginsenoside Rd (Rd) is one of the active ingredients in ginseng, and is regarded as the main compound responsible for the many pharmacological actions of ginseng. Rd inhibits proliferation and induces apoptosis in a human cervical cancer cell line [3]. Rd also has an immune-stimulating activity; it induces Th1 and Th2 immune responses by regulating the gene expression of both Th1 and Th2 cytokines [4]. Moreover, the glycoside reduces vascular contractile responses, presumably by blocking receptor-operated calcium channels [5].

Cyclooxygenase (COX) is a rate-limiting enzyme in the conversion of arachidonic acid into prostaglandins and thromboxanes. The enzyme plays several important roles in maintaining physiological homeostasis, such as mucosa secretion and smooth muscle contraction, and in regulating pathological conditions, such as allergic diseases and rheumatoid arthritis [6]. There are two isoforms of cyclooxygenase, i.e., COX-1 and COX-2 [7]. COX-1 functions as a housekeeping gene and is constitutively expressed in most human tissues, whereas COX-2 is an inducible form that is induced by oncogenes, growth factors, cytokines, endotoxin or phorbol esters [8]. Overexpression of COX-2 has been related to chronic inflammation, angiogenesis and carcinogenesis [9]. The

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cis-acting elements in the 5′-flanking promoter region of the *COX-2* gene contain a TATA box and multiple transcription factor binding sites for nuclear factor-κB (NF-κB), specific protein-1, Myb, CCAAT/enhancer-binding protein (C/EBP), and cAMP response element binding protein (CREB) [10]. Among these transcription factors, C/EBP, CREB, and NF-κB play important roles in the induction of COX-2 [11–13].

In the present study, we found that COX-2 expression was selectively up-regulated by ginsenoside Rd, but not by other ginsenosides, in RAW264.7 macrophages. Furthermore, we demonstrated that ginsenoside Rd-inducible COX-2 expression was transcriptionally regulated by two distinct transcription factors: C/EBP and CREB.

Materials and methods

Materials. 5-Bromo-4-chloro-3-indoylphosphate and nitroblue tetrazolium were supplied by Life Technologies (Gaithersburg, MD). Anti-COX-2, C/EBPα, C/EBPβ, CREB and p65 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated donkey anti-rabbit and alkaline phosphatase-conjugated donkey anti-mouse IgGs were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). The reagents used for molecular studies were primarily obtained from Sigma (St. Louis, MO).

Cell culture. RAW264.7 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured at 37 °C in 5% CO_2/95% air in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. For all experiments, cells were grown to 80–90% confluency and subjected to no more than 20 cell-passages.

Preparation of nuclear extract and Western blot analysis. Cells were removed using a cell scraper and centrifuged at 2500*g* for 5 min at 4 °C. The cells were then swollen with 100 μl of lysis buffer [10 mM Hepes (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.5% Nonidet-P40, 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonylfluoride]. Tubes were vortexed to disrupt cell membranes, and samples were incubated for 10 min on ice and then centrifuged for 5 min at 4 °C. Pellets containing crude nuclei were resuspended in 100 μl of extraction buffer [20 mM Hepes (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonylfluoride], incubated for 30 min on ice, and centrifuged at 15,800*g* for 10 min; the supernatants containing the nuclear extracts were collected and stored at −80 °C until required. Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and immunoblot analyses were performed as described previously [14]. Cell lysates were fractionated by 10% gel electrophoresis, and electrophoretically transferred to nitrocellulose membranes. The membranes were subsequently incubated with primary antibody, and then with alkaline phosphatase- or horseradish peroxidase-conjugated secondary antibodies. Finally, the membranes were developed using either 5-bromo-4-chloro-3-indoylphosphate and nitroblue tetrazolium or using an ECL chemiluminescence detection kit.

Gel shift assay. Double-stranded DNA probes (1.75 pmole/μl, each) for the consensus sequences of NF-κB (5′-AGTTGAGGGGACTTTCCC AGGC-3′), C/EBP (5′-TGCAGATTGCGCAATCTGCA-3′), and CRE (5′-CAGTCATTTCGTCACATGGG-3′) were obtained from Promega (Madison, WI) and used for gel shift analyses after end-labeling the probe with [γ-^{32}P]ATP and T_4 polynucleotide kinase. The reaction mixture contained 2 μl of 5× binding buffer with 20% glycerol, 5 mM $MgCl_2$, 250 mM NaCl, 2.5 mM EDTA, 2.5 mM dithiothreitol, 0.25 mg/ml poly(dI–dC), 50 mM Tris–Cl (pH 7.5), 10 μg of nuclear extracts, and sterile water to a total volume of 10 μl. Incubations were carried out at room temperature for 20 min by adding 1 μl probe (10^6 cpm) after a 10 min pre-incubation. The specificity of DNA/protein binding was determined through competition reactions using a 10-fold molar excess of unlabeled NF-κB, C/EBP or CRE oligonucleotide. Samples were loaded onto 5% polyacrylamide gels at 100 V. After electrophoresis, the gels were removed, dried, and autoradiographed.

Construction of a COX-2 promoter-luciferase construct and reporter gene assays. To determine the transcriptional activity of the *COX-2* gene, we used the pGL-COX-2-574 luciferase reporter gene. To construct the luciferase (LUC) reporter gene plasmid, COX-2-LUC(-574), a DNA fragment containing −574 bp of 5′-flanking sequences and 113 bp of 5′-untranslated region (UTR) from the human COX-2 gene was first amplified by PCR (forward: 5′-ACGCGTAAGCGTCGTCACTAAAAC AT-3′; reverse: 5′-AGGGGTAGGCTTTGCTGTCTG-3′) using a human genomic clone as the template. The PCR fragment was then cloned into pGL3-Basic (Promega, Madison, WI). Site-directed mutagenesis of NF-κB, CRE, and CEBP binding sites was performed using a LAPCR *in vitro* Mutagenesis Kit (TAKARA SHUZO Ltd., Japan). The construct pGL-COX-2-574 was used as the template for the other constructs. The sense primers were as follows (the NF-κB, CRE or CEBP core is underlined and bold letters represent mutated nucleotides compared with the wild-type sequence): NF-κB wild-type, 5′-GGAGAGTGGGGACTACC CCC-3′; NF-κB mutant, 5′-GGAGAGTG**TAACG**TACCCCC-3′; CEBP wild-type, 5′-CCGGGCTTACGCAATTTTTTTA-3′; CEBP mutant, 5′-C CGGGCTTAC**TACG**TTTTTTTA-3′; CRE wild-type, 5′-AGTCATTTC GTCACATGGGCTT-3′; CRE mutant, 5′-AGTCATTTC**TAAT**CATG GGCTT-3′.

One microgram of the plasmid was transfected into the cells using LipofectAMINE2000 according to the manufacturer's instructions. After 18 h, the transfection medium was replaced with basal medium. The cells were then treated with Rd for 12 h, and lysed. The luciferase activities in the cell lysates were then measured using a luminometer. The relative luciferase activity was calculated by normalizing the promoter-driven luciferase activity versus β-galactosidase.

Prostaglandin E_2 (PGE2) determination. A commercial enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI) was used to determine PGE2 concentrations in medium, according to the manufacturer's protocol. Briefly, cells were plated in 24-well culture plates in serum-free medium. Ginsenosides were then added, and the cells were incubated for 24 h. PGE2 concentrations were determined by measuring absorbance at 415 nm.

Statistics. One-way analysis of variance (ANOVA) was used to determine the significance of differences between treatment groups. The Newman–Keuls test was used for multi-group comparisons. Statistical significance was accepted for p values of <0.05 or <0.01.

Results

Selective induction of COX-2 by Rd

The effect of various ginsenosides on the expression of COX-2 protein was determined in RAW264.7 macrophages. Western blot analyses revealed that COX-2 expression was up-regulated by Rd in quiescent macrophages. Up to 30 μg/ml, Rd did not induce COX-2, but 100 μg/ml Rd potently increased the expression of COX-2 (Fig. 1A). 1 μg/ml of lipopolysaccharide (LPS) was used as a positive control. At concentrations of up to 100 μg/ml, ginsenoside Rb1, Re, Rg1 or Rg3 did not actively induce COX-2 (data not shown). Because prostaglandin E_2 (PGE2) is one of the stable autacoids produced by COX-2, we then determined PGE2 levels in the culture medium. When cells were treated with 100 μg/ml Rd, PGE2 levels increased significantly, but lower concentration ranges of Rd and other ginsenosides did not (Fig. 1B). These results clearly demonstrated that COX-2 was selectively induced by Rd, but not by other ginsenosides.

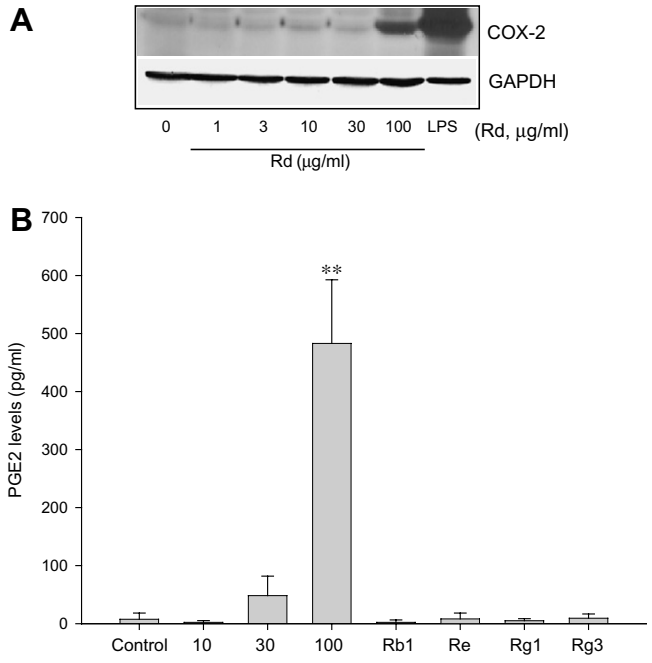

Fig. 1. Selective induction of COX-2 by Rd. (A) Immunoblot analysis of COX-2 protein. RAW264.7 cells were incubated in a medium containing Rd (1–100 μg/ml) for 18 h. (B) Effect of ginsenosides on PGE2 production. RAW264.7 cells were incubated with 10–100 μg/ml Rd, 30 μg/ml ginsenoside Rg3 or 100 μg/ml ginsenoside Rb1, Re, and Rg1 for 24 h and PGE2 amounts in medium was determined using PGE2-specific ELISA assays. The results shown represent the means ± SD ($n = 3$) (significant as compared to control, $^{**}p < 0.01$).

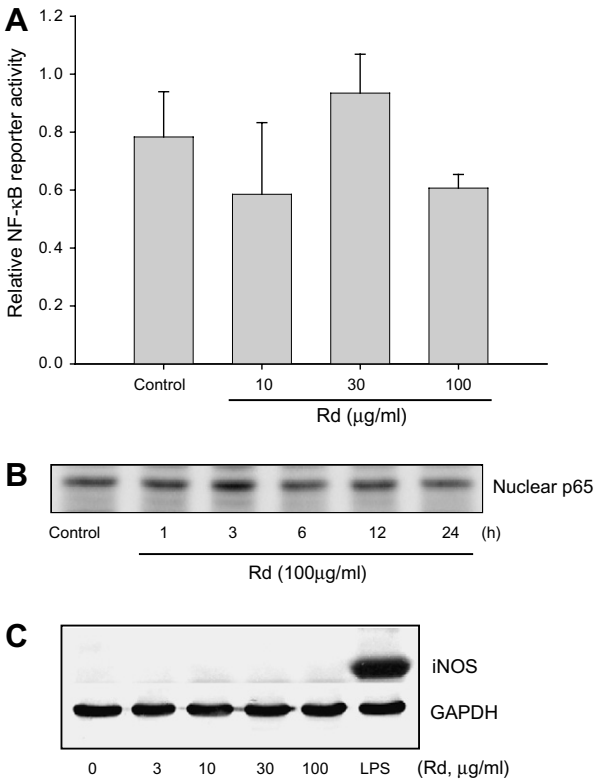

Fig. 2. Effect of Rd on NF-κB activity. (A) NF-κB reporter gene assay. RAW264.7 cells were transiently transfected with pNF-κB Luc reporter and treated with 10–100 μg/ml Rd. The cells were incubated in serum-free medium for 12 h after Rd exposure. Data represent the mean ± SD ($n = 3$). (B) Effect of Rd on p65 nuclear translocation. RAW264.7 cells were treated with 100 μg/ml Rd for 1–24 h, and the nuclear levels of p65 was determined immunochemically using specific antibody. (C) Immunoblot analysis of iNOS protein. RAW264.7 cells were incubated in a medium containing Rd (3–100 μg/ml) for 18 h.

No activation of NF-κB by Rd

There are two NF-κB consensus sequences in the promoter region of the *COX-2* gene, and *COX-2* gene expression is dependent on NF-κB activation [15]. Hence, we first assessed whether Rd activates NF-κB in quiescent macrophages. The NF-κB minimal reporter activity was not significantly altered by 10–100 μg/ml Rd (Fig. 2A). Since p65 is the major component of NF-κB complex in activated macrophages, we further determined the nuclear levels of p65 in cells treated with Rd for 1–24 h. The nuclear levels of p65 were marginally affected by Rd (Fig. 2B). These results demonstrate that Rd-mediated COX-2 induction is not associated with NF-κB activation.

Overexpression of inducible nitric oxide synthase (iNOS) in activated macrophages is an important inflammatory response. Moreover, NF-κB is a key transcription factor for *iNOS* gene transcription [16]. Thus, we studied whether Rd induced iNOS protein in RAW264.7 cells to confirm whether or not Rd activate NF-κB. Western blot analysis showed that exposure of cells to Rd (1–100 μg/ml) for 18 h did not evoke an increase in iNOS protein levels (Fig. 2C).

Activation of C/EBPs and CREB by Rd

Since C/EBP and CRE elements are involved in cytokine- or ultraviolet B-inducible COX-2 gene transcription [12,17], their DNA-protein binding reactions were examined by gel shift analyses using radiolabeled C/EBP- and CRE-specific probes. After RAW264.7 cells were exposed to Rd (100 μg/ml) for 1, 3, 6, 12, and 24 h, C/EBP or CRE DNA binding activities were analyzed. Treatment of cells with 100 μg/ml Rd for 3–24 h resulted in sustained increases in the band intensity of C/EBP (left panel) and CRE-DNA (right panel) binding compared with control (Fig. 3A). Addition of a 20-fold excess of unlabeled C/EBP- or CRE-binding oligonucleotide to nuclear extract completely abolished binding to the DNA (Fig. 3A). These data indicate that the C/EBP family and CREB are activated by Rd.

We then determined the nuclear levels of C/EBPα and C/EBPβ after exposing RAW264.7 cells to 100 μg/ml Rd. Subcellular fractionation and immunoblot analyses showed that both the nuclear levels of C/EBPα and C/EBPβ were increased by Rd treatment (Fig. 3B). Moreover, Rd persistently enhanced the nuclear CREB levels after 3 h (Fig. 3B). These results showed that CREB and the C/EBP family were activated by Rd and implied that Rd-induced activation of C/EBP and CREB was associated with COX-2 expression in macrophages.

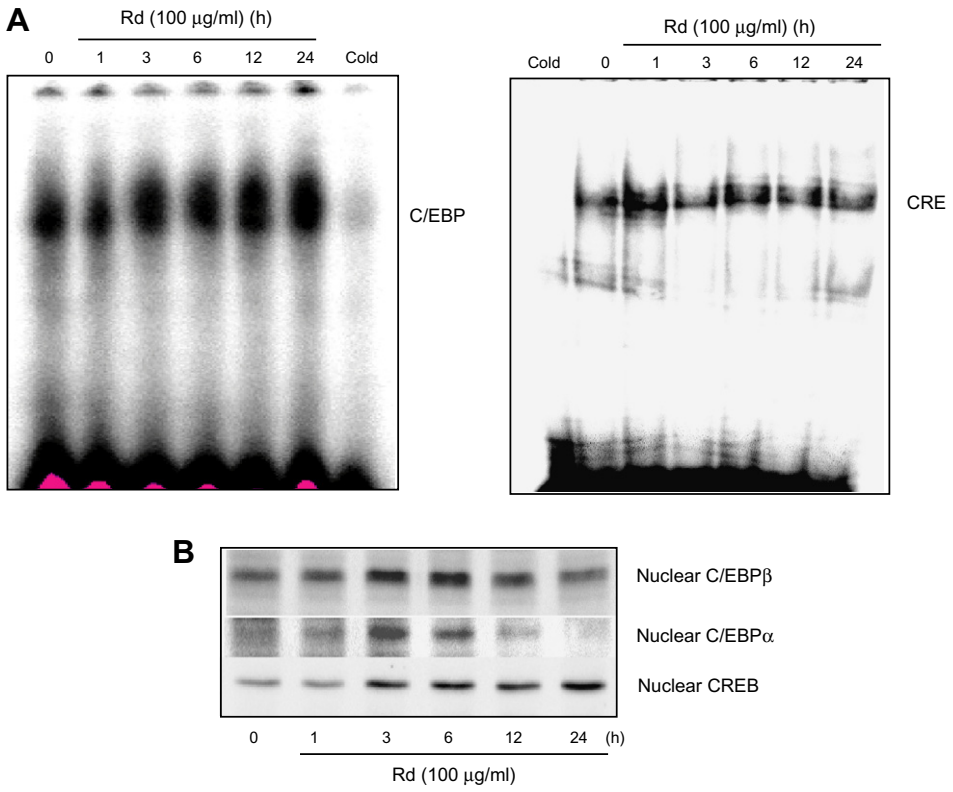

Fig. 3. Effect of Rd on C/EBP and CREB activity. (A) C/EBP (left panel) and CRE (right panel) binding activities. Gel shift assays were performed with nuclear extracts prepared from RAW264.7 cells incubated with Rd (100 μg/ml) for 1–24 h. All lanes were loaded with 10 μg of nuclear extracts and labeled C/EBP and CRE DNA consensus sequences. (B) Effect of Rd on nuclear levels of C/EBPα, C/EBPβ, and CREB. RAW264.7 cells were treated with 100 μg/ml Rd for 1–24 h, and the nuclear levels of each transcription factor was determined immunochemically using specific antibodies.

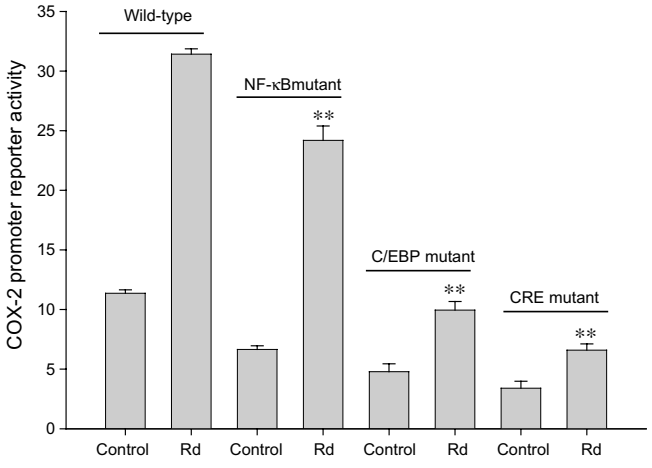

Fig. 4. Essential role of C/EBP/CREB activation in Rd-inducible COX-2 gene expression. Induction of luciferase activity by Rd (100 μg/ml) in RAW264.7 cells transiently transfected with pGL-COX-2-574, NF-κB mutant, C/EBP mutant or CRE mutant construct, was confirmed using a luminometer. Reporter gene activations were expressed as changes relative to β-galactosidase activity. The results shown represent the means ± SD of 3 separate experiments (significant as compared to the Rd-inducible reporter activity values of pGL-COX-2-574-transfected cells, $^{**}p < 0.01$).

Involvement of C/EBP and CREB activation in Rd-mediated transcription of COX-2 gene

To identify the precise roles of each transcription factor in the regulation of Rd-induced COX-2 expression, RAW264.7 cells were transfected with the wild-type COX-2 promoter-luciferase chimeric construct that contained the 574-bp 5′-flanking region of *COX-2* gene, or with the NF-κB mutant with NF-κB site (−223/−214) mutation, C/EBP mutant with C/EBP site (−132/−124) mutation or CRE mutant with CRE site (−59/−53) mutation [18]. Promoter reporter activities were then analyzed by measuring luciferase activity. As shown in Fig. 4A, wild-type COX-2 promoter activity increased up to ~3-fold after cells were exposed to 100 μg/ml Rd. NF-κB mutation slightly reduced Rd-inducible reporter activity (23% inhibition), but the basal reporter activity was also decreased by NF-κB mutation (Fig. 4). However, Rd-inducible COX-2 promoter activity was more distinctly diminished in cells transfected with C/EBP (68% inhibition) or CRE mutant (79% inhibition) (Fig. 4), which demonstrate that C/EBP and CRE elements are essential for Rd-mediated transactivation of the *COX-2* gene.

Discussion

Ginsenosides are the biologically active fraction of *Panax ginseng*. Ginsenosides exert various pharmacological effects to control many diseases including cancer [19]. Excess nitric oxide activates the immune system to produce prostanoids and cytokines, and hence exerts pro-inflamma-

tory effects [20]. Numerous groups have previously reported that ginsenosides affect nitric oxide synthesis through the control of inducible nitric oxide synthase (iNOS) expression. Ginseng extracts or ginsenoside Rg3 induce iNOS in murine macrophages via NF-κB activation [21,22]. In contrast, ginsenoside Rg1, Re, and Rb1 suppress NF-κB activity and NO production in N9 microglia or RAW264.7 cells [23,24]. However, the effects of ginsenosides on COX-2 expression are not fully understood. Ginsenoside Rh inhibits COX-2 expression in a microglial cell line, which seems to be associated with its inhibition of AP-1 [25]. Although, Park et al. recently reported that ginsenoside Rb1 blocks the phorbol ester-inducible activation of COX-2 promoter reporter [26], there has been still no report that ginsenosides induce COX-2 and prostanoid production. In the present study, we report for the first time that ginsenoside Rd stimulates PGE2 production and induces COX-2 with an increase in COX-2 promoter reporter activity in macrophages. Thus, the enhanced COX-2 expression caused by Rd seems to be caused by stimulation of transcription. We also found that other ginsenosides (Rg1, Rg3, Rb1, and Re) did not induce COX-2, which suggests that increased COX-2 expression by Rd is a novel action.

Several studies have shown that COX-2 expression is transcriptionally regulated by C/EBP, CREB, and NF-κB and that these transcription factors are synergistically or independently involved in *COX-2* gene expression [17,18,27]. The present studies show that C/EBPs or CREB transcription factors, but not NF-κB, are transcriptionally active to induce COX-2 expression after treatment of macrophages with Rd. Our data using gel shift, reporter gene, and Western blot assays clearly showed that C/EBP members and CREB are activated by Rd, while p65-dependent NF-κB activity was marginally affected by Rd in RAW264.7 cells. We recently found that nuclear levels of p65 were enhanced by Rd in H4IIE cells, a rat hepatoma cell line (Kim et al., unpublished data). This discrepancy may result from differences between cell types or in coupling effectors. We also previously reported that ginsenoside Rg3 failed to induce COX-2, and C/EBP-DNA binding was not noticeably affected by the ginsenoside Rg3 [22]. Thus, the mechanism for COX-2 induction by Rd appears to be unique and differs from the nonspecific activation of diverse inflammatory transcription factors by LPS or inflammatory cytokines.

Chronic inflammation is believed to be associated with carcinogenesis [28,29]. In particular, exaggerated prostanoid production from COX-2 overexpression is involved in the pathogenesis of cancer [30,31]. COX-2 expression increases during tumor progression in the stomach, suggesting that COX-2 participates in gastric tumorigenesis [32]. In addition, several anticancer drugs induce COX-2 expression [33,34]. Microtubule- or actin-interfering agents increase COX-2 levels [33] and the combined use of selective COX-2 inhibitor(s) with conventional cancer chemotherapeutic agents has been suggested [35]. Although ginseng administration was clinically shown to reduce the odds ratios of a number of tumor types such as lung, stomach, and liver [36] and many cell culture studies supported a notion that ginsenosides caused apoptosis in several cancer cell lines [37,38], COX-2 induction by Rd may result in unexpected harmful effects during long-term treatment with ginseng. In summary, the present data demonstrate that Rd, a component in total ginsenosides, selectively produces PGE2 through COX-2 expression and that COX-2 induction by Rd is dependent on C/EBP and CREB activation.

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