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The potent protective effect of wild ginseng (*Panax ginseng* C.A. Meyer) against benzo[α]pyrene-induced toxicity through metabolic regulation of CYP1A1 and GSTs

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Abstract

Wild *Panax ginseng* C.A. Meyer (WG) is a well-known medicinal herb. In this study, the protective effects of a water extract from the root of WG on benzo[α]pyrene (BP)-induced hepatotoxicity and the mechanism of these effects were investigated for the first time. The effects of WG on liver toxicities induced by BP were assessed by blood biochemical and histopathological analyses. BP caused severe liver injury in rats, as indicated by elevated plasma ALT, AST and LPO levels. Pretreatment with WG for 4 weeks completely abrogated increases in the ALT, AST and LPO levels when challenged with BP. Reductions in GSH content and GST activity by BP were reversed by WG. These protective effects of WG against BP-induced toxicity were consistent with the results of histopathological examinations. We next examined the effects of WG on the gene expression of the enzymes that metabolize BP in H4IIE cells. CYP1A1 mRNA and protein expression were increased by BP. WG moderately inhibited BP-induced CYP1A1 gene expression. Moreover, GSTA2, GSTA3 and GSTM2 gene expressions were significantly increased by WG through the Nrf2/antioxidant responsive element pathway for enzyme induction. In summary, WG is efficacious in protecting against BP-induced hepatotoxicity as results of metabolic regulations through both the inhibition of metabolic enzyme activation and the enhancement of electrophilic detoxification, implying that WG should be considered a potential chemopreventive agent.

Keywords: Wild ginseng; Benzo[α]pyrene; CYP1A1; GST; Hepatoprotection

1. Introduction

Ginseng is a well-known medicinal herb in traditional Asian medicine and is considered an adaptogen. *Panax ginseng* C.A. Meyer (Araliaceae), which grows in China and Korea, has a variety of beneficial biological actions that include anti-carcinogenic, anti-diabetic and anti-inflammatory effects, as well as cardiovascular protection and neuroprotection (Zhang et al., 1996; Yun et al., 2001a; Joo et al., 2005; Jung et al., 2005). *Panax ginseng* appears to inhibit some characteristics associated with cancer in animal models and human studies (Shin et al., 2000). As cancer is quickly becoming the leading cause of death in the world, many reports have focused on chemoprevention trials with *Panax ginseng*. Most of the pharmacological actions of ginseng are attributed to a variety of ginsenosides, which are triterpenoid saponins (Attele et al., 1999; Huang et al., 2005). The physiological and medicinal effects of the various ginsenosides differ and can even be oppositional (Sengupta et al., 2004; Joo et al., 2005). Since ginsenosides produce effects that differ from one another, and a single ginsenoside initiates multiple actions, the overall pharmacology of ginseng is complex. Thus, ginseng extracts have been studied to examine the

Abbreviations: ARE, antioxidant response element; BP, benzo[α]pyrene; CYP450 1A1, cytochrome P450 1A1; FCS, fetal calf serum; GST, glutathione *S*-transferase; MEM, minimal essential medium; NF-E2 related factor2, Nrf2; PBS, phosphate-buffered saline; SDS, sodium dodecylsulfate

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final activity of a wide range of biological actions (Tsang et al., 1985; Nishino et al., 2001; McElhaney et al., 2004).

Wild ginseng is not cultivated domestically; rather, it grows naturally and is harvested from wherever it is found growing. It is considered superior to domestic ginseng, and it has been shown to contain higher levels of ginsenoside (Betz et al., 1984; Foster, 2005), although Lui and Staba (1980) reported minimal differences in total ginsenoside content between wild and cultivated ginseng. Ginsenoside levels were consistently lower from the more intensively cultivated garden locations, but growth was consistently higher (Lim et al., 2005). It is widely accepted in both Korea and China that wild ginseng is more active than cultivated ginseng in chemoprevention. However, little has actually been reported on the effectiveness of wild ginseng.

Chemical carcinogenesis is caused by exposure to environmental pollutants known as polycyclic aromatic hydrocarbons (PAH) (Varanasi et al., 1989; Topinka et al., 1998). Exposure to PAH increases the risk of cancer in multiple organ sites. The carcinogenicity of PAH requires metabolic activation to generate electrophilic intermediates that covalently bind to critical DNA targets to initiate carcinogenesis (Topinka et al., 1998). Benzo[α]pyrene (BP), a prototypic PAH, is one of the most potent carcinogens found in cigarette smoke, charred foods and petroleum byproducts.

Oxidation and conjugation are two of the main reactions involved in the biotransformation of xenobiotics. Cytochrome P450s (CYP450s) constitute the primary phase I enzyme system responsible for the oxidative metabolism of a variety of xenobiotics, including carcinogens and drugs (Huang et al., 1999). The major activation pathway for the PAH is mediated by the CYP1A1 class of CYP450s, ultimately leading to the highly mutagenic BP diol epoxide (BPDE); this compound is believed to be the ultimate carcinogenic metabolite of BP (Alexandrov et al., 2002). The reactive metabolite generated by this pathway may subsequently be involved in additional metabolic processes with phase II detoxifying enzymes and may be converted to inactive product.

For phase II detoxification reactions, glutathione *S*-transferase (GST), epoxide hydrolase and quinone reductase are among the key enzymes involved. Since BPDE is a poor substrate for epoxide hydrolase, the most important mechanism of BPDE inactivation seems to be its conjugation with GSH, a reaction catalyzed by GSTs (EC 2.5.1.18) (Singh et al., 2004). In the presence of GSH, cytosolic liver GST isoenzymes reduce the binding of anti-BPDE to nuclear DNA (Hesse et al., 1980), which suggests that GSTs play a major role in the detoxification of anti-BPDE. GSTs belong to a superfamily of multifunctional isoenzymes that is divided into four major classes: α, μ, π, and θ. BPDEs are detoxified predominantly by GSTs; the μ class exhibits the highest activity towards most BPDEs, followed by GSTα and GSTπ (Sundberg et al., 2002). Antioxidant response elements (ARE) that are found in the regulatory regions of these genes control the transcription of these enzymes. The transcription factor Nrf2, which binds to the ARE, appears to be essential for the induction of phase II enzymes, including GSTs (Ramos-Gomez et al., 2001). Nrf2-deficient mice exhibited enhanced susceptibility to environmental carcinogens, including BP, through alterations in the expression of detoxifying enzymes. The activity of many chemoprotective agents, including oltipraz (Ramos-Gomez et al., 2003; Yu and Kensler, 2005), terpenoids, and isothiocyanate (McWalter et al., 2004), is also mediated by Nrf2-regulated gene activity against carcinogens.

The main objective of this study was to evaluate the protective effects of WG from Sobaeksan on BP-induced hepatotoxicity, as well as possible mechanisms for that protection. Here we report that the WG potently protects against BP-induced hepatotoxicity. This hepatoprotection by WG is regulated in part by inhibition of CYP1A1 and by induction of GST α/μ. In addition, we demonstrated for the first time the importance of Nrf2/ARE signaling in WG-mediated GST induction, which is regulated at the transcriptional level. These results suggest that WG should be considered a potential chemopreventive.

2. Materials and methods

2.1. Reagents

Anti-CYP1A1 antibody was acquired from Oxford Biomedical Research, Inc. (Oxford, MI). Anti-GSTα and anti-GSTμ antibodies were purchased from Detroit R&D (Detroit, MI). Anti-Nrf2 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-rabbit and rabbit anti-goat IgGs were purchased from Zymed Laboratories (San Francisco, CA). Benzo[α]pyrene (BP) and other reagents in the molecular studies were acquired from Sigma Chemical (St. Louis, MO).

2.2. Preparation of wild ginseng (WG) extract

Roots of WG (*Panax ginseng* C.A. Meyer) growing naturally in forested areas (Sobaeksan, Korea) were collected and identified by the Sanha Sansam Research Center (Yongju, Korea). The water extract was prepared by mixing 130 g WG with 1000 ml of pure distilled water and extracting under reflux for 5 h by repeatedly heating the formula to 100 °C three times. The extract was then filtered with filter paper (Advantec No. 2, Japan), and the filtrate was concentrated to approximately 100 ml using a rotary evaporator (Heidolph WB 2000, Germany) at −70 °C under vacuum and then freeze-dried. The yield of dry matter from the extract was approximately 12.2%. Finally, the extract was dissolved in distilled water for oral administration to Sprague–Dawley (SD) rats.

2.3. Animals

Animal studies were conducted in accordance with institutional guidelines for the care and use of laboratory animals. Seven-week-old SD rats (160–180 g) were acquired from Orient Co. (Seoul, Korea), acclimatized for 1 week and maintained in a clean chamber. The animals were fed a standard rat diet (Purina Korea, Seoul, Korea) and water ad libitum, and were kept under pathogen-free conditions at a room temperature of 23 ± 1 °C on an alternating 12-h light and dark cycle. WG

was administered orally at a dose of 50 mg/kg/day to rats for 4 weeks through a gastric tube. Distilled water was administered to control animals using the same feeding method over the same period. After the final administration of WG, a single dose of BP was injected intraperitoneally (0.5 mg/kg dissolved in 100% corn oil). The animals were subsequently starved overnight prior to sacrifice in order to reduce variations in hepatic metabolism. Blood samples were drawn directly from the heart under ether anesthesia. The livers were removed immediately after perfusing the organs via injection of ice-cold saline solution.

2.4. Determination of AST and ALT activities

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in plasma were assayed with the ALT and AST kit (YD Diagnostics, Korea) following manufacturer's direction.

2.5. Determination of LPO levels

Lipid peroxide (LPO) was determined by the colorimetric thiobarbituric acid method (Buege and Aust, 1978). The concentration of lipid peroxides was calculated using a molar absorption coefficient of $(1.56 \times 105)\ M^{-1}\ cm^{-1}$ for malonaldehyde, a product of lipid peroxidation.

2.6. Determination of GSH levels

The glutathione (GSH) content was analyzed by the modified Ellman's method (Davies et al., 1984). The reaction mixture consisted of tissue lysate, 10 mM 5-5′-dithiobis-2-nitrobenzoic acid (DTNB) and 0.02 M EDTA–Na_2 in 0.4 M Tris–HCl buffer (pH 8.9). The reaction was initiated with 25 μl of DTNB, and conjugation of DTNB with GSH was monitored at 412 nm.

2.7. Determination of GST activity

The total activity of GST in the liver homogenate was determined by the glutathione *S*-transferase (GST) assay kit (Cayman chemical, USA) measuring the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione.

2.8. Histopathology

Hepatic morphology was assessed by light microscopy. The left lateral lobe of the liver was sliced (three slices per rat), and tissue slices were fixed in 10% buffered-neutral formalin for 24 h. Fixed liver tissue slices were processed and embedded in a paraplast automatic tissue processor, Citadel 2000 (Shandon Scientific, Cheshire, UK). Sections 5 μm in thickness were stained with hematoxylin and eosin before examination. A certified pathologist scored samples in a blinded fashion. An arbitrary score was given to each microscopic field viewed at a magnification of 200×. A minimum of five fields were scored per liver slice to obtain the mean value.

2.9. Immunohistochemistry of CYP1A1

Immunohistochemical analysis for CYP1A1 was performed using the specific anti-CYP1A1 antibody. We used a commercially available indirect avidin–biotin-complex kit (Vector Lab, CA, USA) for this analysis. In brief, tissue sections on slides were deparaffinized, hydrated at room temperature, incubated with proteinase K (20 μg/ml) and blocked with serum (10% normal goat serum). Sections were then incubated with the specific antibody for 7 days at room temperature in a humidified chamber. Slides were washed with phosphate-buffered saline (PBS) containing 0.1% Tween-20 following this incubation period with the antibody. Sections were then incubated with biotinylated goat anti-rabbit antibody for 5 days at 37 °C, followed by streptavidin–alkaline peroxidase complex for 1 h at 37 °C. Slides were washed with PBS 3–5 times after each incubation. Finally, samples on slides were incubated with 0.05% 3,3′-diaminobenzidine and 0.01% HCl in 0.05% Tris–HCl (pH 7.4) as substrates for the phosphatase reaction until adequate color was developed. In some experiments, the sections were counterstained with hematoxylin.

2.10. Cell culture

H4IIE cells (ATCC® CRL-1548), a rat hepatocyte-derived cell line, were obtained from American Type Culture Collection (Rockville, MD, USA) and maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS), 50 units/ml penicillin, and 50 μg/ml streptomycin at 37 °C in a humidified atmosphere with 5% CO_2.

2.11. Immunoblot analysis

Cells were incubated with WG (0.1–1 mg/ml) in the presence or absence of 1 μM BP for the indicated period. After washing the cells twice with PBS, the cells were scraped and sonicated for disruption. Protein samples containing microsomal or cytosolic fractions were prepared by centrifugation at $10{,}000 \times g$ for 10 min. The samples were stored at −70 °C until use. Sodium dodecylsulfate (SDS)–polyacrylamide gel electrophoresis and immunoblot analyses were performed according to published procedures (Cho et al., 2004). Microsomal and cytosolic proteins were separated by 7.5% and 12% gel electrophoresis, respectively, and transferred electrophoretically to nitrocellulose membranes. The nitrocellulose membranes were incubated with the respective specific antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibody, and developed using the ECL® chemiluminescence detection kit (Amersham Biosciences, Amersham, UK). Equal loading of proteins was verified by actin immunoblotting. Changes in the levels of protein were determined via scanning densitometry. At least three separate experiments were performed with independent lysates to confirm changes in the protein levels.

2.12. RT-PCR

Total RNA was isolated from cells with TRIzol (Invitrogen). Reverse transcription-polymerase chain reaction amplification

was conducted as described by Cho et al. (2004). The number of amplification cycles was empirically determined for each primer pair to identify the logarithmic phase. The specific primer sets for CYP1A1 and GST genes including GSTA2, GSTA3, GSTA5, GSTM1 and GSTM2 were designed to have a T_m of approximately 55 °C and a GC content of ~50%; BLAST searches were used to confirm the specificity of the selected nucleotide sequences. Band intensities of the amplified DNAs were compared after visualization on an UV transilluminator.

2.13. Construction of GSTA2 promoter-luciferase constructs and luciferase assay

Firefly luciferase reporter gene constructs, pGL-1651 and pGL-ΔARE, were generated as previously described (Kang et al., 2003). To determine the promoter activity, we used a dual-luciferase reporter assay system (Promega). Briefly, cells (7×10^5 cells/well) were replated in six-well plates overnight, serum-starved for 6 h, and transiently transfected with one of the GSTA2 promoter-luciferase constructs and the pRL-SV plasmid (Renilla luciferase expression for normalization) using Lipofectamine® reagent for 3 h (Life Technologies, Gaithersburg, MD). Transfected cells were incubated in medium containing 1% FCS for 3 h, and then exposed to WG (1 mg/ml) for 18 h. Firefly and Renilla luciferase activities in cell lysates were measured using a luminometer (Luminoskan®, Thermo Labsystems, Helsinki, Finland). The relative luciferase activity was calculated by normalizing GSTA2 promoter-driven firefly luciferase activity to that of Renilla luciferase.

2.14. Statistical analysis

Scanning densitometry of the immunoblots was performed with Image Scan & Analysis System (Alpha-Innotech Corporation, San Leandro, CA). The area of each lane was integrated using AlphaEase™ Version 5.5 software, followed by background subtraction. One-way analysis of variance (ANOVA) procedures were used to assess significant differences among treatment groups. For each significant effect of treatment, the Newman–Keuls test was used for comparisons of multiple group means. The criteria for statistical significance were set at $P < 0.01$. All statistical tests were two-sided.

3. Results

3.1. Effect of WG on BP-induced plasma AST and ALT activities

We evaluated the effect of WG on BP-induced liver injury. The degree of parenchymal cell injury and loss of hepatic function were analyzed by examining conventional serum enzymes, including AST and ALT. A single dose of BP caused severe liver injury in rats, as indicated by significantly elevated plasma ALT and AST levels at 24 h (Table 1). Pretreatment of rats with WG at 50 mg/kg for 4 weeks completely abrogated increases in ALT and AST activities following challenge with BP by 85% and 88%, respectively. WG alone for 4 weeks was nontoxic. These results indicate that WG was efficacious in blocking increases in plasma AST and ALT evoked by carcinogen-induced injuries.

Table 1
Effect of WG on serum AST and ALT activities in BP-treated rats

Group	Karmen unit/ml of serum	
	AST	ALT
Control	29.4 ± 2.0	35.4 ± 5.7
BP	377.4 ± 5.1**	440.5 ± 9.3**
WG + BP	55.4 ± 0.4##	55.4 ± 0.9##
WG	25.4 ± 0.4	29.5 ± 0.1

Rats were orally pretreated with WG at 50 mg/kg for 4 weeks with or without BP treatment (0.5 mg/kg, i.p.), and the AST and ALT activities were monitored in the plasma of rats 24 h after BP administration. The values represent the mean ± S.D. from five rats. Paired Student's *t*-test was used to compare the means of two groups (significantly different from control, $^{**}P < 0.01$; significantly different from BP, $^{\#\#}P < 0.01$).

3.2. Suppression in lipid peroxidation by WG

Lipid peroxidation results in the formation of highly reactive and unstable hydroperoxides of both saturated and unsaturated lipids. LPO levels were measured directly by utilizing the redox reactions of the hydroperoxides with ferrous ions. The plasma LPO content, an index of the severity of liver cell damage, was significantly elevated by a single intraperitoneal injection of BP in rats (Table 2). Pretreatment with WG markedly decreased the plasma LPO content in rats challenged by BP. The data indicate that WG had protective effects against BP-induced oxidative injury.

3.3. Effect of WG on GSH content and GST activity

In this study, intracellular GSH levels were measured. The hepatic GSH level was decreased to 21.6 μmol/g tissue after treatment with BP (Table 2). Thus, BP caused a 57% reduction in hepatic GSH levels relative to control. The hepatic GSH level was recovered by WG treatment for 4 weeks in rats, resulting in levels that were 196% of BP-treated rats; however, treatment with WG alone resulted in no change in GSH levels.

We next evaluated GST activity to determine the capacity for GSH conjugation. BP treatment significantly decreased total

Table 2
Effect of WG on the LPO or GSH contents and GST activities in BP treated rat liver

Group	LPO content (nmol/g of tissue)	GSH content (μmol/g of tissue)	GST activity (nmol/g of tissue)
Control	22.7 ± 1.6	50.3 ± 0.1	380.1 ± 6.6
BP	310.2 ± 2.9**	21.6 ± 2.4**	184.7 ± 8.1**
WG + BP	41.3 ± 4.2##	42.3 ± 0.0##	306.1 ± 0.2##
WG	11.9 ± 0.8	48.1 ± 0.2	344.4 ± 3.2

Rats were treated as described in Table 1. The LPO or GST content and GST activities were monitored in the plasma of rats 24 h after BP administration. The values represent the mean ± S.D. from five rats. Paired Student's *t*-test was used to compare the means of two groups (significantly different from control, $^{**}P < 0.01$; significantly different from BP, $^{\#\#}P < 0.01$).

GST activity in the cytosol compared to control (Table 2). The decrease in the cytosolic GST activity by BP was abolished by pretreatment with WG for 4 weeks. WG alone had no effects. Taken together, these results imply WG is efficacious in detoxifying BP-induced metabolic reactive intermediates.

3.4. Effects of WG on histopathology

In subsequent experiments, the effects of WG on the acute liver toxicities induced by BP were assessed by histopathological examinations. Healthy control rats showed no pathological changes (Fig. 1A). WG treatment alone also resulted in no change in hepatic morphology (Fig. 1B). In contrast, treatment of rats with BP resulted in focal nodular necrosis and degeneration around the central veins and infiltration of inflammatory cells in the periportal area (Fig. 1C). However, rats that had been pretreated daily with WG at 50 mg/kg for 4 weeks exhibited marked improvements in the liver histopathology induced by BP (Fig. 1D). Multiple analyses showed that WG weakened central necrosis and hepatocyte degeneration induced by BP from severe to moderate (Table 3).

Table 3
Morphological activity index scores in the livers of BP treated rats with or without WG

Parameters	Control	BP	WG + BP	WG
Necrosis	0	++++	++	0
Degeneration	0	++++	++	0
Hepatitis	0	++	+	0

Rats were treated as described in Table 1. The liver morphology was assessed microscopically 24 h after the last treatment. Living hepatocytes were defined as the cells with well demarcated nuclear membranes and with prominent, round central nucleoli after hematoxylin and eosin staining. Severity of liver lesions was classified as follows, based on the extent of cytoplasmic necrosis and degeneration of hepatocytes: no lesion, 0; mild (+), 25% or less; moderate (++), 25–50%; severe (+++), 50–75%; and extensive (++++), 75% or more. Multiple analyses of four independent samples yielded reproducible and comparable data.

3.5. Immunohistochemistry of CYP1A1

Carcinogens are detoxified via activation or inactivation of hepatic enzymes. BP is primarily bioactivated by the catalysis of CYP1A1 (Huang et al., 1999). Furthermore, BP can induce expression of CYP1A1 via the aryl hydrocarbon receptor (Houser et al., 1992; Huang et al., 1999). We immunohistochemically examined the extent of CYP1A1 expression in rat liver after 4 weeks of WG treatment with or without BP. Healthy control and WG alone-treated rats showed no immunohistochemical changes (Fig. 2A and B). However, exposure of rats to BP resulted in notable changes in CYP1A1 expression (Fig. 2C). The intensities of CYP1A1 were weakly positive in rats treated with WG + BP compared to those treated by BP alone (Fig. 2D), implying that WG might decrease direct BPDE–DNA adduct levels by BP.

3.6. Effect of WG on CYP1A1 gene expression

Next, we evaluated effects of WG on BP-induced CYP1A1 gene expression in an *in vitro* cell culture model. CYP1A1 levels were monitored in H4IIE cells, a hepatocyte derived cell

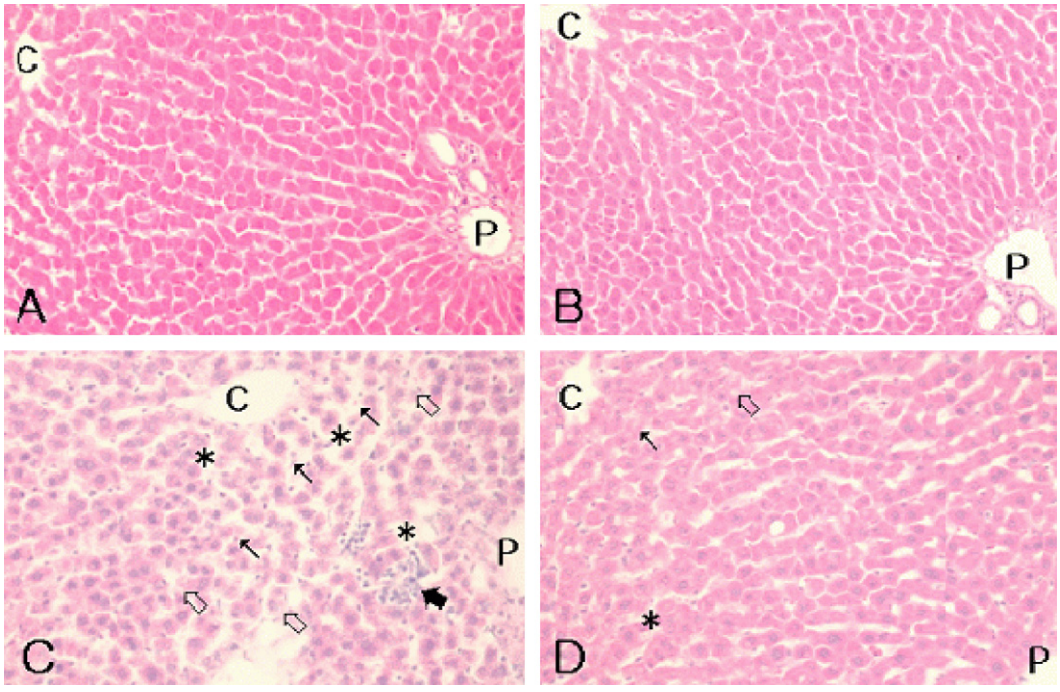

Fig. 1. Representative hepatic histopathology (100×): the liver sections from control rats (A); or rats treated with WG (50 mg/kg/day, 4 weeks) (B); BP (0.5 mg/kg) alone (C); or WG (50 mg/kg/day, 4 weeks) + BP (0.5 mg/kg) (D) were stained with hematoxylin and eosin. Photomicrographs show various views of the liver sections: central vein (c); portal triad (p); severe necrosis (black arrow); degeneration of hepatocytes (open arrow); disappearance of hepatic plate (*) and hepatitis (arrow) were indicated.

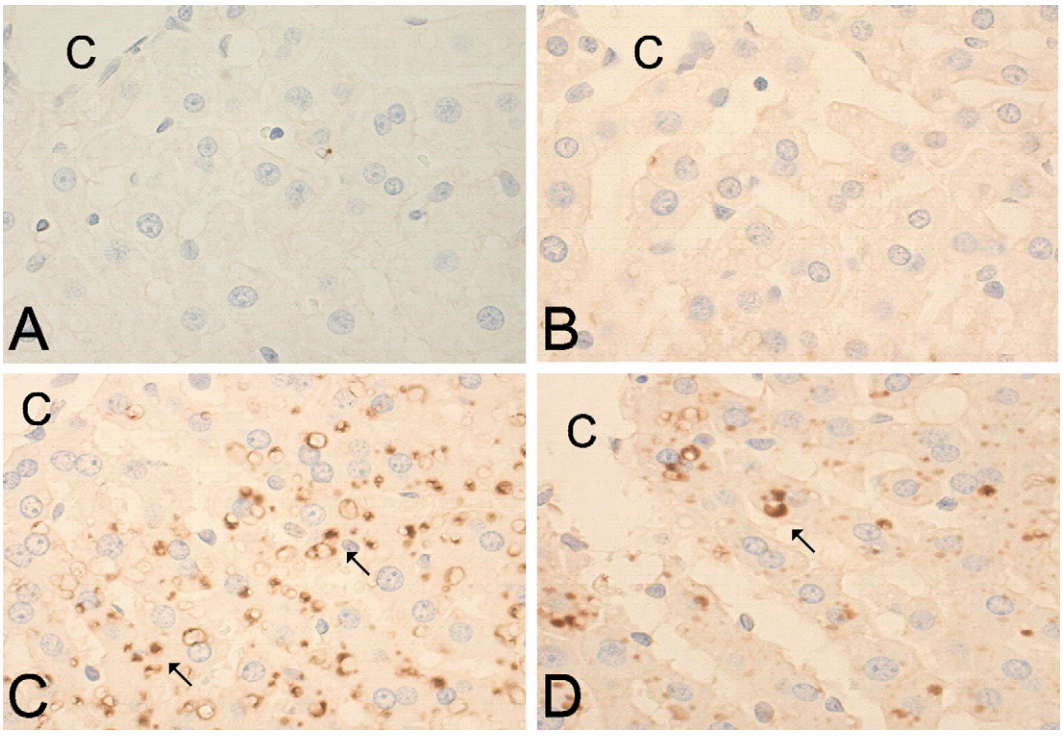

Fig. 2. Immunohistochemical labeling of CYP1A1 in rats treated with WG and/or BP (200×). Rats were treated as described in the legend to Fig. 1. CYP1A1 positive (arrow) is indicated in the hepatic section.

line, treated with WG with or without BP (Fig. 3). Quantitative RT-PCR showed that CYP1A1 mRNA was significantly increased by BP treatment (1 μM) for 12 h (Fig. 3A). Treatment of the cells with WG (0.1–1 mg/ml) for 1 h following BP exposure blocked the increase in CYP1A1 mRNA by BP. Equal mRNA loading in the lanes was confirmed by GAPDH. Western blot analysis revealed that BP treatment resulted in the induction of CYP1A1. WG pretreatment caused 60% sup-

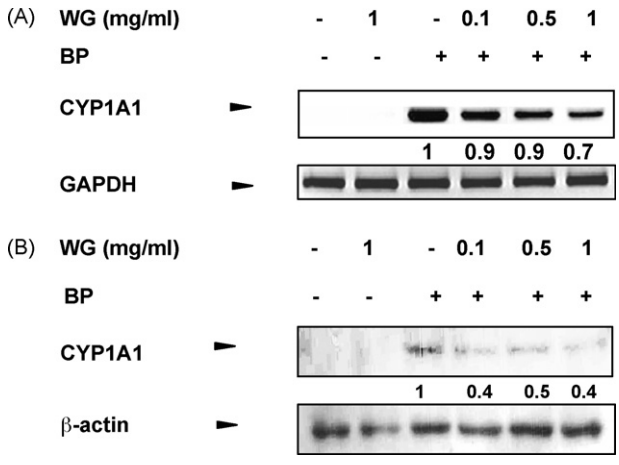

Fig. 3. The effects of WG on BP-induced CYP1A1 expression: (A) the level of CYP1A1 mRNA was assessed by RT-PCR. Cells were pretreated with WG (0.1–1 mg/ml) for 1 h, followed by BP treatment for 6 h. Results were confirmed by repeated experiments. Values indicate the relative densitometric intensities (relative level in untreated control = 1) and (B) the level of CYP1A1 was assessed by immunoblotting. Cells were treated with WG (0.1–1 mg/ml) in the presence of BP for 12 h.

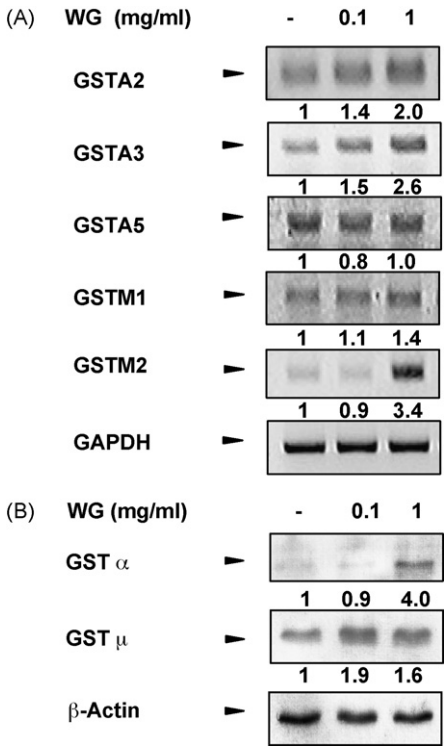

Fig. 4. The effects of WG on GST expression: (A) the levels of GSTA2/3/5 and GSTM1/2 were assessed by RT-PCR. Cells were treated with WG (0.1, 1 mg/ml) for 6 h. Results were confirmed by repeated experiments. Values indicate the relative densitometric intensities (relative level in untreated control = 1) and (B) the levels of GSTα and μ were assessed by immunoblotting. Cells were treated with WG (0.1, 1 mg/ml) for 12 h.

pression of BP-induced CYP1A1 protein expression (Fig. 3B). These results were consistent with those of the RT-PCR analyses.

3.7. Effect of WG on GST gene expression

Studies were extended to determine the effects of WG on GST gene expression. The mRNA levels for the major GSTs were monitored in H4IIE cells (Fig. 4A). First, the mRNA levels were determined in cells treated with WG (0.1, 1 mg/ml). WG treatment caused significant induction in GSTA2, GSTA3 and GSTM2 mRNA in a dose-dependent manner by two-, two-, and three-fold, respectively, compared to the control (Fig. 4A). GSTM1 was moderately induced by WG, whereas GSTA5 was not changed.

To determine whether changes in the levels of the major GST mRNA paralleled those of GST subunits, western blot analysis was also performed. The GSTα protein expression was four-fold induced by WG (1 mg/ml) relative to control (Fig. 4B). The levels for GSTμ subunits were ∼90% induced in WG-treated (0.1 mg/ml) cells. Hence, the major GST subunits were significantly induced by WG.

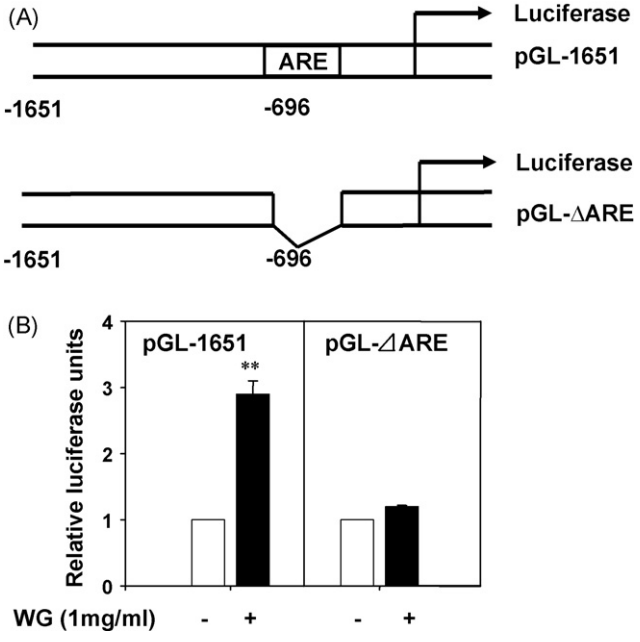

Fig. 5. GSTA2 promoter luciferase reporter gene analyses: (A) the GSTA2 chimeric gene constructs in the flanking insert and (B) induction of luciferase activity by WG in H4IIE cells transiently transfected with GSTA2 chimeric gene construct pGL-1651, which contains the ARE binding site of the GSTA2 promoter, or pGL-ΔARE, in which the ARE is deleted. H4IIE cells were co-transfected with the GSTA2-luciferase gene construct pGL-1651 or ARE-deleted pGL-1651 (firefly luciferase) and pRL-SV (Renilla luciferase) at a ratio of 200:1 and subsequently treated with WG (1 mg/ml) for 12 h; lysates from these cells were used for dual luciferase reporter assays. Activation of the reporter gene was calculated as a change in the ratio of firefly luciferase activity to Renilla luciferase activity (control level = 1). One-way analysis of variance was used for comparisons of means of multiple groups followed by Newman–Keuls test (significant compared to control, $^{**}P < 0.01$).

3.8. Analysis of the ARE in the GSTA2 promoter

A previous study showed that activation of ARE is involved in the induction of GSTA2 expression (Kang et al., 2003). Given the role of ARE in GSTA2 expression, we examined whether WG might transcriptionally activate GSTA2 expression via the ARE binding site. Reporter gene assays were therefore performed using H4IIE cells transfected with the mammalian cell expression vector pGL-1651, which contained the luciferase structural gene downstream of the −1.65 kb GSTA2 promoter region (Fig. 5). Exposure of transiently transfected cells to WG resulted in a three-fold increase in luciferase activity. No WG-mediated luciferase activity was induced in cells transfected with the pGL-ΔARE gene construct, which lacks the ARE. Promoter deletion experiments demonstrated that the location of the GSTA2 regulatory region that confers WG responsiveness included the ARE binding site.

4. Discussion

Panax ginseng C.A. Meyer (Araliaceae) is a valuable herb in East Asia that has also gained popularity in the West because of its pharmacological properties (Zhang et al., 1996). Ginsenosides, such as Rg3, Rg5 and Rh2, are active anti-carcinogenic components in red ginseng, and they prevent cancer either singularly or synergistically (Yun et al., 2001b). White ginseng may have inhibitory effects on the progression stage of rat intestinal carcinogenesis (Ichihara et al., 2002). In the present study, wild ginseng, which grows naturally in Sobaeksan, potently prevented the BP-induced hepatotoxicity in rats, as assessed by blood biochemical and histopathological analyses. BP is an effective carcinogen that interacts with membrane lipids and, consequently, induces free radical formation (Garcon et al., 2001). Free radicals react further with lipids causing peroxidation, resulting in the release of products such as malondialdehyde, hydroperoxides and hydroxyl radicals. Free radicals also participate in carcinogenesis as measured by BP-induced hepatic 8-hydroxyguanine production (Urios and Blanco, 1996; Garcon et al., 2001). Our results demonstrate that WG also reduced BP-induced lipid peroxidation, which may in turn block formation of free radical-mediated DNA adducts.

GSH, a substrate for GST, plays an important role in detoxifying reactive metabolites of electrophilic carcinogens. BP resulted in decrease of the hepatic glutathione content, which would enhance oxidative stress and stimulate serious damage to hepatocytes. Srivastava et al. (2000) reported that D,L-buthionine-*S*,*R*-sulfoximine (BSO)-mediated tissue GSH depletion caused an increase in BP-induced tumor multiplicity. As shown in Table 2, WG reversed BP-mediated GSH decreases to the control level in hepatic tissues. In addition, BP decreased GST activity, which catalyzes GSH conjugation with BP metabolites. However, WG pretreatment returned GST activity to control levels. The effects of WG on GSH/GST levels suggest that it offers potential protection against carcinogenic risks associated with BP.

The hepatotoxic potential of BP not only varies between species, but also depends on several drug metabolizing enzymes

and the antioxidant defense system (Alexandrov et al., 2002). CYP1A1 and GSTM1 genotypes are critical determinants of BP DNA adduct formation (Alexandrov et al., 2002). Thus, this protective effect of WG may be associated with metabolic regulation of BP. In general, CYP450-related enzymes activate BP by producing highly mutagenic DNA-damaging metabolites called BPDE (Melendez-Colon et al., 1999). Elevated CYP1A1 activity correlates with BPDE-associated DNA adduction, one of the first critical events in tumor initiation (Rojas et al., 1992). This study showed that WG inhibited BP-induced CYP1A1 expression in both rats and H4IIE cells. It appears likely that the inhibitory effects of WG may partly prevent the BP-induced tumor initiation and DNA binding.

GST enzymes are involved in the metabolism of a wide variety of electrophilic carcinogens (Alexandrov et al., 2002; Singh et al., 2004). The GSTs are a family of enzymes that assist in the excretion of carcinogens by making them soluble via conjugation with GSH (Hesse et al., 1980; Sundberg et al., 2002). Thus, the GSTs are used as a biomarker to investigate the chemopreventive effects of agents (Manson et al., 1997). GSTA1-1 conjugates metabolites derived from dibenzo[α]pyrene very efficiently (Sundberg et al., 2002). Moreover, the GST M1 null genotype in humans has also been shown to be a risk factor in BP-induced cancer (Alexandrov et al., 2002). This study showed that WG may hinder carcinogenesis by aiding the excretion of electrophilic carcinogens in the early stage of cancer development, via inducing GSH and GST α/μ. Here, we found for the first time that WG significantly increases GSTA2, GSTA3 and GSTM2 mRNA and moderately increases GST M1 mRNA. These mRNA increases are consistent with protein expression as well. These results suggest that WG-mediated increases in GST detoxify metabolites of BP by conjugating them with GSH.

Nrf2-deficient mice exhibit increased sensitivity to carcinogens, which supports the concept that Nrf2-mediated gene transcription is essential for the prevention of chemical carcinogenesis by chemoprotective agents. Here, we reported that WG increased GST promoter activity, but failed to increase this activity when the ARE binding site was deleted from the promoter. These results provide evidence that Nrf2 transactivation is functionally involved in WG-mediated increases in GSTA2 expression.

Taken together, the above results suggest that WG inhibits BP-induced hepatotoxicity and modulates the metabolizing enzymes, reducing the P450-mediated metabolic activation and enhancing detoxification through induction of phase II enzymes. Thus, WG appears to have the potential to defend against BP-induced carcinogenic risk.

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