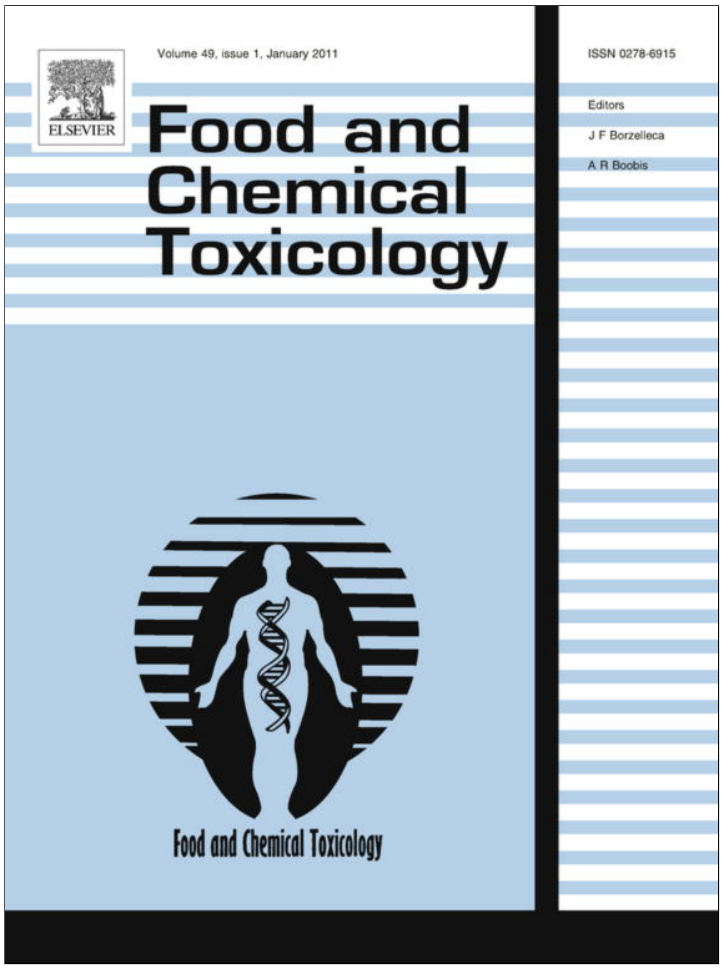

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Andrographolide-induced pi class of glutathione *S*-transferase gene expression via PI3K/Akt pathway in rat primary hepatocytes

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ABSTRACT

Andrographis paniculata is an herb widely used in China, Korea, and India for its anti-hepatotoxic, anti-viral, and anti-inflammatory effects. Andrographolide is the major bioactive diterpene lactone in *A. paniculata*. The pi class of glutathione *S*-transferase (GSTP) is one of the phase II biotransformation enzymes. Our previous study indicated that andrographolide upregulates the expression of GSTP. The aim of this study was to investigate the mechanism by which andrographolide induces GSTP gene expression in rat primary hepatocytes. In hepatocytes treated with 40 μM andrographolide, immunoblots showed maximal Akt phosphorylation at 0.5 h and maximal c-jun phosphorylation at 3 h. However, pretreatment with PI3K inhibitors, wortmannin and LY294002, or siPI3K inhibited the andrographolide-induced phosphorylation of c-jun and GSTP protein expression. EMSA showed that pretreatment with wortmannin, LY294002, or siPI3K attenuated the AP-1-DNA-binding activity caused by andrographolide. Results of immunoprecipitation indicated that nuclear c-fos/c-jun heterodimer increases with andrographolide treatment. Addition of antibodies against c-jun and c-fos decreased nuclear protein bound to the AP-1 consensus DNA sequence. In summary, andrographolide induces GSTP gene expression in rat primary hepatocytes through activation of the PI3K/Akt, phosphorylation of c-jun, nuclear accumulation of AP-1, and subsequent binding to the response element in the gene promoter region.

1. Introduction

Andrographis paniculata (Burm. f.) Nees (Acanthaceae) (*A. paniculata*) is an herbal medicine that is widely used in China, India, and other Asian countries. Clinically, *A. paniculata* is used to treat the common cold, urinary infection, diarrhea, and infectious hepatitis (World Health Organization, 2002). Andrographolide, a diterpene lactone, is the major bioactive component of *A. paniculata* and has antibacterial, anti-inflammatory, anti-oxidative, and anti-hepatotoxic properties in rodents and in cell models (Abu-Ghefreh et al., 2009; Kapil et al., 1993; Li et al., 2009; Shen et al., 2000; Visen et al., 1993).

Glutathione *S*-transferase (GST) is one of the phase II enzymes of the biotransformation system, which is composed of phase I enzymes, phase II metabolizing enzymes, and phase III transporters (van Iersel et al., 1999; Xu et al., 2005). Recent studies have shown that *A. paniculata* has effects on the biotransformation system. *A. paniculata* was demonstrated to increase the enzyme activities of cytochrome P450 (CYP450) 1A1, 2B, and GST in mouse liver (Jarukamjorn et al., 2006; Singh et al., 2001). Furthermore, crude extracts of *A. paniculata* and andrographolide were shown to induce gene expression of the pi class of GST (GSTP) in rat primary hepatocytes (Chang et al., 2008).

Akt, which is also known as protein kinase B, plays a crucial role in mammalian cellular signaling pathways involved in the regulation of cell survival, apoptosis, glucose uptake, and glycogen metabolism. Phosphoinositide 3-kinase (PI3K) phosphorylates and activates Akt (Rosseland et al., 2008; Stephens et al., 1998; Wierod et al., 2007), and the PI3K/Akt signaling pathway is affected by numerous phytochemicals (Chen and Kong, 2005; Manson et al.,

Abbreviations: AP-1, activator protein 1; CYP450, cytochrome P450; DMSO, dimethylsulfoxide; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; GPE1, GSTP enhancer 1; GST, glutathione *S*-transferase; GSTP, pi class of GST; HEPES, N-[2-hydroxythyl]piperazine-N′-[2-ethanesulfonic acid]; JNK, c-Jun NH2-terminal kinase; TRE, 12-*O*-tetradecanoate-13-acetate-responsive element.

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2007; Martin et al., 2004; Neergheen et al., 2009). A recent study showed that the PI3K/Akt pathway is blocked by apigenin in human ovarian cancer cells (Fang et al., 2005). By contrast, andrographolide was found to suppress cell apoptosis through activation of the PI3K/Akt pathway in human umbilical vein endothelial cells (Chen et al., 2004).

Activator protein 1 (AP-1), a transcription factor, is a hetero or homodimer of the Jun and Fos families. AP-1 binds to the consensus DNA sequence including the 12-*O*-tetradecanoate-13-acetate-responsive element (TRE) and AP-1 binding motifs (Angel and Karin, 1991; Nadori et al., 1997). The 5′ upstream region of the GSTP gene promoter contains the enhancer element termed GSTP enhancer 1 (GPE1), which includes a TRE-like sequence. AP-1 was shown to bind to the GPE1 fragment in GSTP owning to the presence of the TRE-like sequence in GPE1 (Angel et al., 1987; Okuda et al., 1989; Sakai et al., 1988). The mitogen-activated protein kinases including c-jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38 are considered to activate AP-1, followed by binding to the response element in DNA, which leads to target gene expression (De Bosscher et al., 2003; Reddy and Mossman, 2002). Furthermore, Hou et al. (2009) indicated that the PI3K/Akt pathway is essential for increasing AP-1 DNA-binding activity. Those authors showed that the increased AP-1 activation was involved in the enhanced bone morphogenetic protein-2 expression caused by ultrasound in murine primary osteoblastic cells.

Our previous study showed that GSTP gene expression is upregulated by andrographolide in rat primary hepatocytes and that the PI3K inhibitor wortmannin abolishes the induction of GSTP expression by andrographolide (Chang et al., 2008). In the present study, we further investigate the transcription factor that is involved in the upregulation of GSTP expression by andrographolide and the activation of the PI3K/Akt signaling pathway in rat primary hepatocytes.

2. Materials and methods

2.1. Chemicals

Cell culture medium (RPMI-1640) and penicillin–streptomycin solution were from GIBCO-BRL (Gaithersburg, MD); collagen and ITS$^+$ (insulin, transferrin, selenium, bovine serum albumin, and linoleic acid) were from Collaborative Biomedical Products (Bedford, MA); collagenase type I was from Worthington Biochemical (Lakewood, NJ); TRIzol reagent was from Invitrogen (Carlsbad, CA); dexamethasone, HEPES, sodium bicarbonate, calcium chloride, wortmannin, and LY294002 (PI3K kinase inhibitors) were from Sigma–Aldrich, Inc. (St. Louis, MO); andrographolide was from Calbiochem (Darmstadt, Germany); anti-phospho-Akt, anti-Akt, anti-ERK1/2, anti-phospho-ERK1/2, anti-p38, and anti-phospho-p38 antibodies were from Cell Signaling Technology (Danvers, MA); anti-JNK1, anti-phospho-JNK1/2, anti-phospho-c-jun, anti-c-jun, and anti-c-fos antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); and the transfection reagent Dharmafect 1 was from Dharmacon (Lafayette, CO).

2.2. Hepatocyte isolation and culture

Male Sprague–Dawley rats (weighing 250–300 g) were purchased from the BioLASCO Taiwan Co. Ltd. (Taipei, Taiwan). Hepatocytes were isolated by a modification of the two-step collagenase perfusion method described previously (Seglen, 1976). After isolation, hepatocytes (3×10^6 cells per dish) were plated on collagen-coated 60-mm plastic tissue dishes in RPMI-1640 medium (pH 7.38) supplemented with 10 mM HEPES, 1% ITS$^+$, 1 μM dexamethasone, 100 IU penicillin/mL, and 100 μg streptomycin/mL. Cells were incubated at 37 °C in a 5% CO_2 humidified incubator. After 4 h, cells were washed with PBS to remove any unattached or dead cells, and the same medium supplemented with 1% ITS$^+$ and 0.1 μM dexamethasone was added. Thereafter, the medium was changed daily. The protocol for each experiment is described in the corresponding figure legend. The rats were treated in compliance with the *Guide for the Care and Use of Laboratory Animals*.

2.3. Western blotting

Cells were washed twice with cold PBS and were harvested in 200 μL of 20 mM potassium phosphate buffer (pH 7.0). Cell homogenates were centrifuged at 9000*g* for 30 min at 4 °C. The protein content of the supernatant was measured by using the Coomassie Plus Protein Assay Reagent Kit (Pierce Chemical Company, Rockford, IL). Sodium dodecyl sulfate polyacrylamide gels made with 7.5% polyacrylamide were prepared as described by Laemmli (1970). For Akt, and c-jun 20 μg of cellular protein was used, and for GSTP, 4 μg of cellular protein was used. After electrophoresis, the separated proteins were transferred to polyvinylidene difluoride membranes. The nonspecific binding sites in the membranes were blocked with 5% nonfat dry milk in 15 mM Tris–150 mM NaCl buffer (pH 7.4) at room temperature for 2 h. After blocking, the membrane was incubated with anti-phospho Akt, anti-phospho-JNK1/2, anti-phospho-ERK1/2, and anti-phospho-p38 and anti-phospho c-jun or anti-Akt, anti-JNK1, anti-ERK1/2, anti-p38, anti-c-jun, and anti-c-fos antibodies at 4 °C overnight. Thereafter, the membrane was incubated with the secondary peroxidase-conjugated anti-rabbit or anti-mouse IgG at 37 °C for 1 h, and the immunoreactive bands were developed by use of the Western Lightning™ Plus-ECL kit (PerkinElmer, Waltham, MA).

2.4. Gene silencing experiments

Transfection experiments with small interfering RNA (siRNA) against p85α, a regulatory subunit of PI3K, were performed using a smart-pool of four specific siRNAs (Dharmacon catalog # J-080078-05, Thermo Fisher Scientific, Lafayette, CO) targeting rat PI3K (NM_013005). The four siRNAs against the rat p85α gene are (1) ACCUAUUGCGAGGGAAA, (2) CGAGAUGCAUCCACUAAAA, (3) GAUAAUGCAUAAUCACGAU, and (4) UAUGAGGAAUACACUCGUA. A targeting control-pool siRNA was also tested. Cells were transfected with Dharmafect 1 according to the manufacturer's instructions. Cells were plated at a density of 1.2×10^6 cells in 3.5 cm^2 dishes one day before transfection with 20 nM p85α or non-targeting control-pool siRNA for 24 h. Cells were then treated with 40 μM andrographolide in RPMI-1640 medium. Cell samples were collected for Western blotting analysis and EMSA.

2.5. Preparation of nuclear extract

Rat primary hepatocytes were pretreated with or without PI3K inhibitors, wortmannin or LY294002, for 1 h and were then treated with dimethylsulfoxide (DMSO) or andrographolide for 3 h and were washed twice with cold PBS followed by scraping from the dishes with PBS. Cell homogenates were centrifuged at 2000*g* for 5 min. The supernatant was discarded, and the cell pellet was allowed to swell on ice for 15 min after the addition of 200 μL of hypotonic buffer containing 10 mM HEPES, 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, 4 μg/mL leupeptin, 20 μg/mL aprotinin, 0.5% Nonidet P-40, and 0.2 mM phenylmethylsulfonyl fluoride. After centrifugation at 6000*g* for 15 min, pellets containing crude nuclei were resuspended in 50 μL of hypertonic buffer containing 10 mM HEPES, 400 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, 4 μg/mL leupeptin, 20 μg/mL aprotinin, 10% glycerol, and 0.2 mM phenylmethylsulfonyl fluoride at 4 °C for 30 min. The samples were then centrifuged at 20,000*g* for 15 min. The supernatant containing the nuclear proteins was collected and stored at −80 °C until the electrophoretic mobility shift assay (EMSA).

2.6. Immunoprecipitation

Nuclear extracts were diluted to 1 μg/μL with IP buffer (40 mM Tris–HCl [pH 7.5], 1% NP-40, 150 mM NaCl, 5 mM EGTA, 1 mM DTT, 1 mM PMSF, 20 mM NaF, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 mM sodium vanadate). The diluted nuclear extracts (60 μg) were incubated with 0.8 μg anti-c-jun or anti-c-fos antibody for 16 h at 4 °C, then mixed with 4 μL protein A-Sepharose suspension (0.1 mg/mL), and incubated at 4 °C for an additional 1 h. Immunoprecipitates and the supernatants were collected by centrifugation at 16,000*g* for 2 min. The pellet was washed with 200 μL of IP buffer three times and was then subjected to Western blotting.

2.7. Electrophoretic mobility shift assay

EMSA was performed according to our previous study (Cheng et al., 2004). The LightShift Chemiluminescent EMSA Kit (Pierce Chemical Company, Rockford, IL) and synthetic biotin-labeled double-stranded AP-1 consensus oligonucleotides (forward: 5′-GCCTCAGCTGGTAAATGGATAA-3′; reverse: 5′-AAAGGCCCCAGAGCCAGCC-3′) were used to measure AP-1 nuclear protein–DNA-binding activity (Tsai et al., 2007). Four micrograms of nuclear extract, poly (dI–dC), and biotin-labeled double stranded AP-1 oligonucleotide were mixed with the binding buffer (LightShift EMSA Kit; Pierce Chemical Co., Rockford, IL) to a final volume of 20 μL, and the mixture was incubated at room temperature for 30 min. Unlabeled double-stranded AP-1 oligonucleotide and a mutant double-stranded oligonucleotide were used to confirm the protein-binding specificity. The nuclear protein–DNA complex was separated by electrophoresis on a 6% TBE–polyacrylamide gel and was then transferred to a Hybond–N$^+$ nylon membrane. The membranes were cross-linked by UV light for 10 min and were then treated with 20 μL of streptavidin-horseradish peroxidase for 20 min, and the nuclear protein–DNA bands were developed with a SuperSignal West Pico kit (Pierce Chemical Co.).

2.8. Determination of purity of andrographolide

For determining the purity of andrographolide, andrographolide was dissolved in acetonitrile to obtain a final concentration of 30 and 100 μg/mL, respectively. Ten microliters of acetonitrile and andrographolide solutions were injected onto a Hitachi-L7400 Series LC System (Tokyo, Japan) equipped with an ultraviolet (UV) detector set to 254 nm. An Agilent Eclipse-C8 reversed-phase column (5 μm, 150 × 3.0 mm) was used. The mobile phase consisted of acetonitrile (Solvent A) and de-ionized water (Solvent B). The flow rate was 0.5 mL/min, and the total running time was 60 min. The gradient system was 90% B to 10% B (0∼45 min), 10% B to 90% B (45∼50 min), 90% B to 90% B (50∼60 min). The column temperature was at ambient temperature (25 °C). The retention time of andrographolide was 19.8 min. The purity of andrographolide was calculated by dividing the peak area of andrographolide by the sum of peak area of all measurable peaks (without the solvent peak) × 100%.

2.9. Statistical analysis

Data were analyzed by using analysis of variance (SAS Institute, Cary, NC). The significance of the difference among mean values was determined by one-way analysis of variance followed by the Tukey's test. *P* values <0.05 were taken to be statistically significant.

3. Results

3.1. Effect of andrographolide on Akt phosphorylation in rat primary hepatocytes

In our previous study, we found that andrographolide-induced GSTP gene expression was significantly inhibited by wortmannin, a PI3K inhibitor, in rat primary hepatocytes (Chang et al., 2008). To clarify the importance of the PI3K/Akt signaling pathway in the induction of GSTP by andrographolide, we treated rat primary hepatocytes with 40 μM andrographolide for 0, 0.25, 0.5, 1 and 3 h. As shown in Fig. 1A, a significant and maximal phosphorylation of Akt was observed after 0.5 h ($P < 0.05$), and phosphorylation declined thereafter. Furthermore, Akt phosphorylation was induced by andrographolide in a dose-dependent manner (Fig. 1B). Previous studies showed that the JNK and ERK signaling pathways participate in the induction of GSTP expression mediated by diallyl disulfide and diallyl trisulfide in rat primary hepatocytes (Tsai et al., 2007, 2005). However, as shown in Fig. 1C, we observed no significant phosphorylation of JNK, ERK, or p38 in rat primary hepatocytes treated with 40 μM andrographolide for 0, 0.25, 0.5, 1, and 3 h.

3.2. Effect of andrographolide on c-jun phosphorylation

AP-1 regulates the expression of a wide variety of genes involved in various biological processes, including cell proliferation, differentiation, transformation, apoptosis, inflammation, and immune responses (Reddy and Mossman, 2002). Our previous study showed that the upregulation of GSTP expression by diallyl disulfide and diallyl trisulfide is via an AP-1-dependent pathway (Tsai et al., 2007). To determine the role of AP-1 in andrographolide-induced GSTP expression, we assayed the phosphorylation of c-jun in hepatocytes treated with 40 μM andrographolide for 0, 0.5, 1, 3, and 6 h. As shown in Fig. 2, phosphorylation of c-jun began at 0.5 h and maximal activation of c-jun was observed at 3 h and declined thereafter.

3.3. Effects of PI3K inhibitors on andrographolide-induced c-jun phosphorylation

To demonstrate the important role of the PI3K/Akt pathway in c-jun activation, hepatocytes were pretreated with PI3K inhibitors, wortmannin and LY294002, for 1 h, followed by incubation with 40 μM andrographolide for another 3 h. As shown in Fig. 3, both inhibitors significantly inhibited andrographolide-induced c-jun phosphorylation.

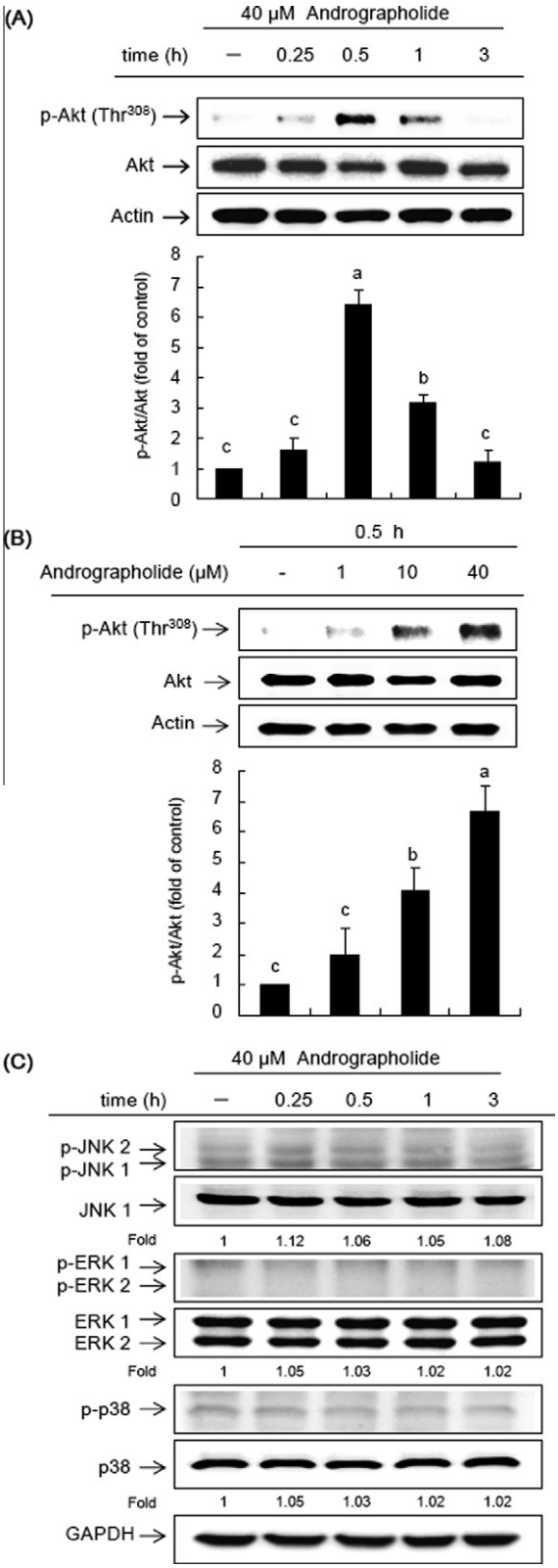

Fig. 1. Effect of andrographolide on Akt phosphorylation and MAPKs activation in rat primary hepatocytes. After attachment, (A) hepatocytes were treated with 40 μM andrographolide for various time periods and Akt phosphorylation was determined, (B) hepatocytes were exposed to various concentrations of andrographolide and Akt phosphorylation was determined, or (C) hepatocytes were treated with 40 μM andrographolide for various time periods and activation of various MAPKs was determined. Aliquots of total protein (20 μg) were used for Western blot analysis. Values are mean ± SD, $n = 3$. The levels in control cells were set at 1. Values not sharing the same letter are significantly different ($P < 0.05$).

3.4. Effect of PI3K siRNA on the andrographolide-induced phosphorylation of Akt and c-jun and GSTP expression

To further demonstrate the role of the PI3K signaling pathway in the regulation of andrographolide-induced GSTP gene expression, we transfected siRNA of p85α (siPI3K), a regulatory subunit of PI3K, in rat primary hepatocytes to knock down p85α. The efficiency of the siRNA SMARTpool system to knock down PI3K was assayed by Western blot (Fig. 4A). siPI3K abolished the andrographolide-induced phosphorylation of Akt and c-jun (Fig. 4B and C) and GSTP protein expression (Fig. 4D). These data suggest that PI3K/Akt-mediated c-jun activation is involved in the andrographolide-induced GSTP gene expression.

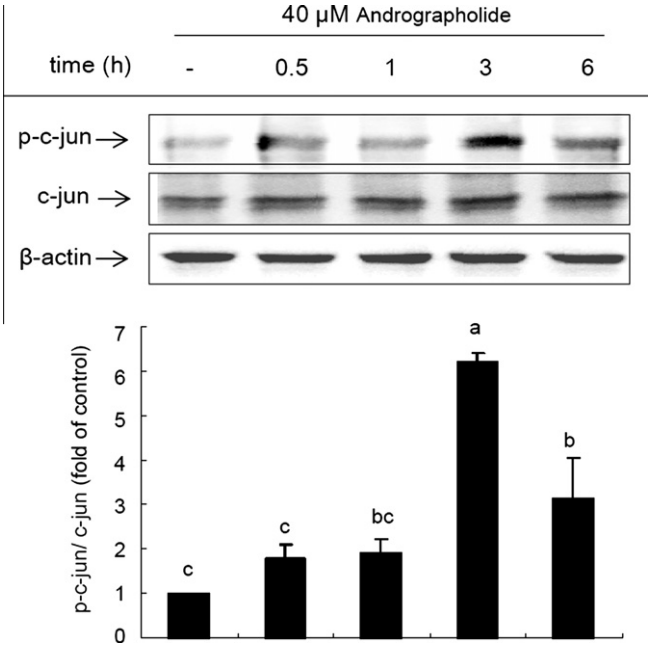

Fig. 2. Effect of andrographolide on c-jun phosphorylation in rat primary hepatocytes. After attachment, hepatocytes were treated with 40 μM andrographolide for various time periods. Aliquots of total protein (20 μg) were used for Western blot analysis. Values are mean ± SD, $n = 3$. The levels in control cells were set at 1. Values not sharing the same letter are significantly different ($P < 0.05$).

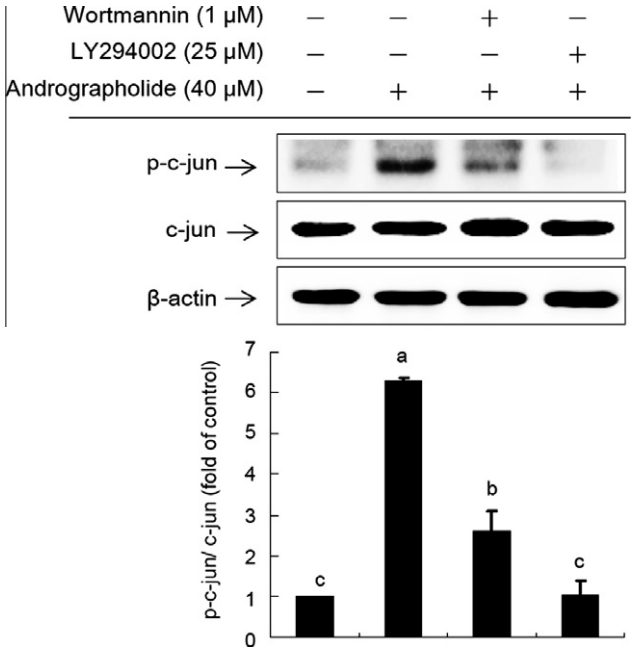

Fig. 3. Effects of PI3K inhibitors on andrographolide-induced c-jun phosphorylation in rat primary hepatocytes. After attachment, hepatocytes were pretreated with PI3K inhibitors, wortmannin and LY294002, for 1 h, and then treated with 40 μM andrographolide for another 3 h. Aliquots of total protein (20 μg) were used for Western blot analysis. Values are mean ± SD, $n = 3$. The levels in control cells were set at 1. Values not sharing the same letter are significantly different ($P < 0.05$).

3.5. Andrographolide induces AP-1 nuclear protein DNA-binding activity via the PI3K/Akt pathway in rat primary hepatocytes

As described above, activation of c-jun via the PI3K/Akt pathway is involved in andrographolide-induced GSTP expression in rat primary hepatocytes. We next performed an EMSA to determine whether the PI3K/Akt pathway plays an important role in andrographolide-mediated AP-1 DNA-binding activity. As shown in Fig. 5A, both PI3K inhibitors suppressed andrographolide-induced AP-1 nuclear protein DNA-binding complex formation. In

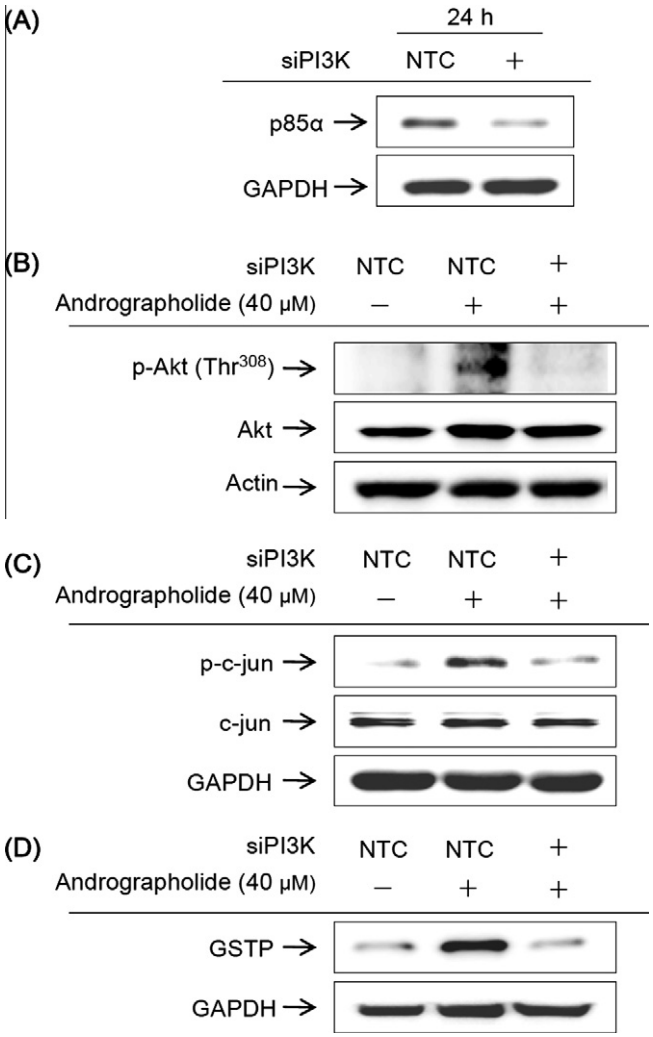

Fig. 4. Effect of PI3K siRNA on the andrographolide-induced phosphorylation of Akt and c-jun and GSTP expression in rat primary hepatocytes. After attachment, hepatocytes were transiently transfected with non-targeting control siRNA (NTC) or siPI3K for 24 h, followed by treatment with or without 40 μM andrographolide. Akt phosphorylation was observed at 0.5 h, c-jun phosphorylation was observed at 3 h, and GSTP expression was observed at 48 h. Aliquots of total protein (20 μg) were used for Western blot analysis. (A) Cells were transfected with non-targeting control siRNA or siPI3K for 24 h. (B) The blot was probed with anti-phospho-Akt (Thr^{308}) antibody. (C) The blot was probed with anti-phospho-c-jun antibody. (D) The blot was probed with anti-GSTP antibody. One representative immunoblot out of three independent experiments is shown.

addition, transient transfection with siPI3K abolished AP-1 nuclear protein DNA-binding complex formation (Fig. 5B).

3.6. c-Jun and c-fos involvement in andrographolide-induced AP-1 nuclear protein DNA-binding complex formation

As shown in Figs. 3A and 4C, the andrographolide-induced phosphorylation of c-jun was significantly inhibited by both PI3K inhibitors and siPI3K. Additionally, andrographolide-induced AP-1 nuclear protein DNA-binding complex formation was attenuated by both PI3K inhibitors and siPI3K (Fig. 5). AP-1 is a transcription factor mainly composed of Jun–Jun homodimer or Jun–Fos heterodimer (Reddy and Mossman, 2002). To further identify whether the nuclear proteins bound to the AP-1 binding site were c-jun and c-fos, we performed immunoprecipitation experiments with anti-c-jun or anti-c-fos antibody and a supershift experiment with anti-c-jun or anti-c-fos antibody, respectively. As shown in Fig. 6A, nuclear accumulation of c-fos after immunoprecipitation with anti-c-jun antibody and nuclear accumulation of c-jun after immunoprecipitation with anti-c-fos antibody were demonstrated in andrographolide-treated cells. This result suggested that nuclear

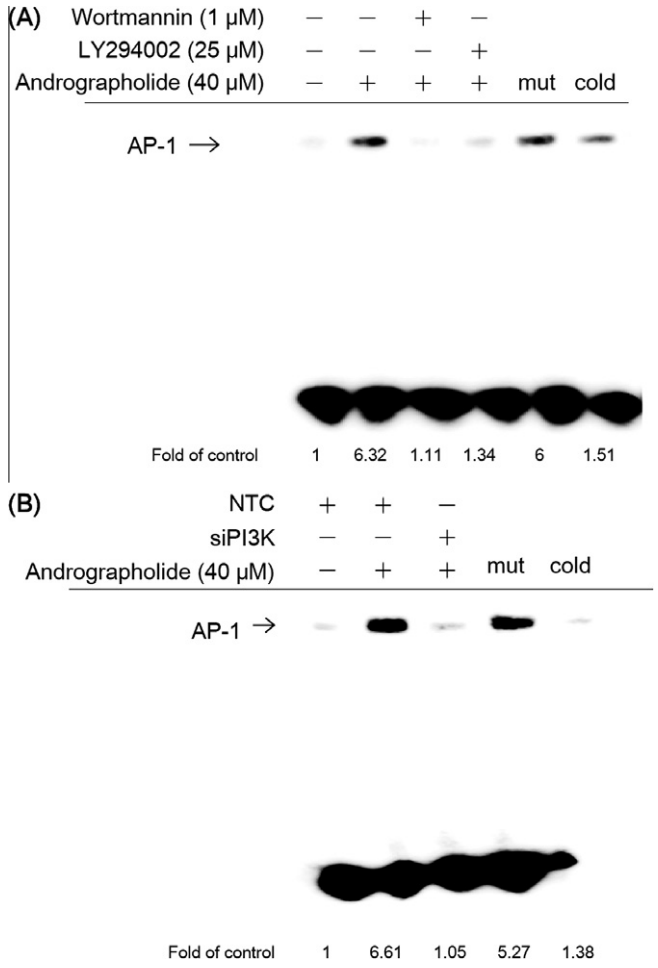

Fig. 5. Andrographolide induces AP-1 nuclear protein DNA-binding activity via the PI3K/Akt pathway in rat primary hepatocytes. After attachment, (A) hepatocytes were pretreated with PI3K inhibitors, wortmannin and LY294002, for 1 h or (B) were transiently transfected with non-targeting control siRNA or siPI3K for 24 h. After PI3K inhibitors or siRNA transfection, hepatocytes were treated with 40 μM andrographolide for an additional 3 h. Aliquots of nuclear extracts (4 μg) were used for EMSA. To confirm the specificity of the nucleotide, 600-fold cold probe (biotin-unlabeled AP-1 binding site) and biotin-labeled double-stranded mutant AP-1 oligonucleotide (2 ng) were included in the EMSA. One representative experiment out of three independent experiments is shown.

c-fos/c-jun heterodimer increases with andrographolide treatment. Although no supershift data observed, anti-c-jun and anti-c-fos antibodies decreased nuclear protein binding to the AP-1 oligonucleotide (Fig. 6B), and this implicated the components in nuclear protein binding to AP-1 oligonucleotide were c-jun and c-fos.

4. Discussion

GST, which is one of the phase II drug metabolizing enzymes, plays an important role in the biotransformation system. GST catalyzes the conjugation of glutathione with xenobiotic-derived and endogenous electrophiles and facilitates the excretion of conjugates. In mammalian cells, GST is classified into Alpha, Mu, Pi, Theta, Sigma, Kappa, and Omega classes (Lu, 1999; Sheehan et al., 2001; Zablotowicz et al., 1995). GSTP has been associated with the carcinogenic process and with anti-neoplastic drug resistance in cancer cells (Hayes and Pulford, 1995; Henderson et al., 1998). GSTP was recognized as a marker in several rat hepatocarcinogenesis models because of its over-expression within hepatocellular foci and neoplasms (Perez-Carreon, 2009). Several studies have addressed the regulation of GSTP expression, and previous studies have shown that hepatic GSTP is induced by both nutrient and non-nutrient factors (Chang et al., 2008; Chen et al., 2001; Liu et al., 2009; Milbury et al., 2007; Tsai et al., 2005).

Fourteen flavonoids and 13 diterpenoids have been isolated from *A. paniculata* (Chen et al., 2006a,b), and andrographolide is the most abundant diterpenoid. To determine the storage stability of andrographolide, the purity of androgarpholide used was determined by HPLC. The results showed the purity of andrographolide crystalline is 98% (Fig. 7). Andrographolide has been shown to have anti-HIV, immunostimulatory, anti-inflammatory, and anti-hepatotoxic activities (World Health Organization, 2002). Li et al. (2007) indicated that andrographolide can induce cell cycle arrest at the G2/M phase and cell death in HepG2 cells. A recent study showed that andrographolide can inhibit the migration and invasion of human non-small-cell lung cancer cells (Lee et al., 2010). Such evidence suggests that andrographolide has anti-tumor cell growth properties and inhibits tumor progression. Andrographolide was also shown to have antioxidant activity via the upregulation of a large number of antioxidant enzymes, such as the γ-glutamate cysteine ligase catalytic subunit and modifier subunit (Woo et al., 2008). The effective dose of andrographolide in different biological processes varies depending on cell type. In the present study, we used 40 μM andrographolide to treat rat primary hepatocytes, which was a dose we found in our previous study not to inhibit cell growth (Yang et al., 2010). The tested compound andrographolide dissolves well in culture medium. We do not know whether 40 μM andrographolide is the physiologically achievable level, however, 1–40 μM andrographolide shows a dose-response effect on Akt phosphorylation (Fig. 1B). In our previous study, andrographolide (2.5–7.5 μM) was found to inhibit the TNF-α-induced expression of ICAM-1 in a dose-dependent manner and result in a decrease in HL-60 cell adhesion to EA.hy926 cells (Yu et al., 2010). We will try the lower concentration of andrographolide for a longer time period in our future study to determine the function of physiologically achievable level of andrographolide.

Andrographolide regulates the expression of different genes through distinct signal transduction pathways. In human cancer cells, andrographolide inhibits the Janus-activated kinase/signal transducers and activator of transcription 3 (JAK-STAT3) pathway and reduces its downstream protein expression, including Bcl-xL, Mcl-1, and cyclin D1 (Zhou et al., 2010). Lipopolysaccharide-induced TNF-α production is suppressed by andrographolide, and this effect is via its inhibition of the ERK1/2 pathway in mouse

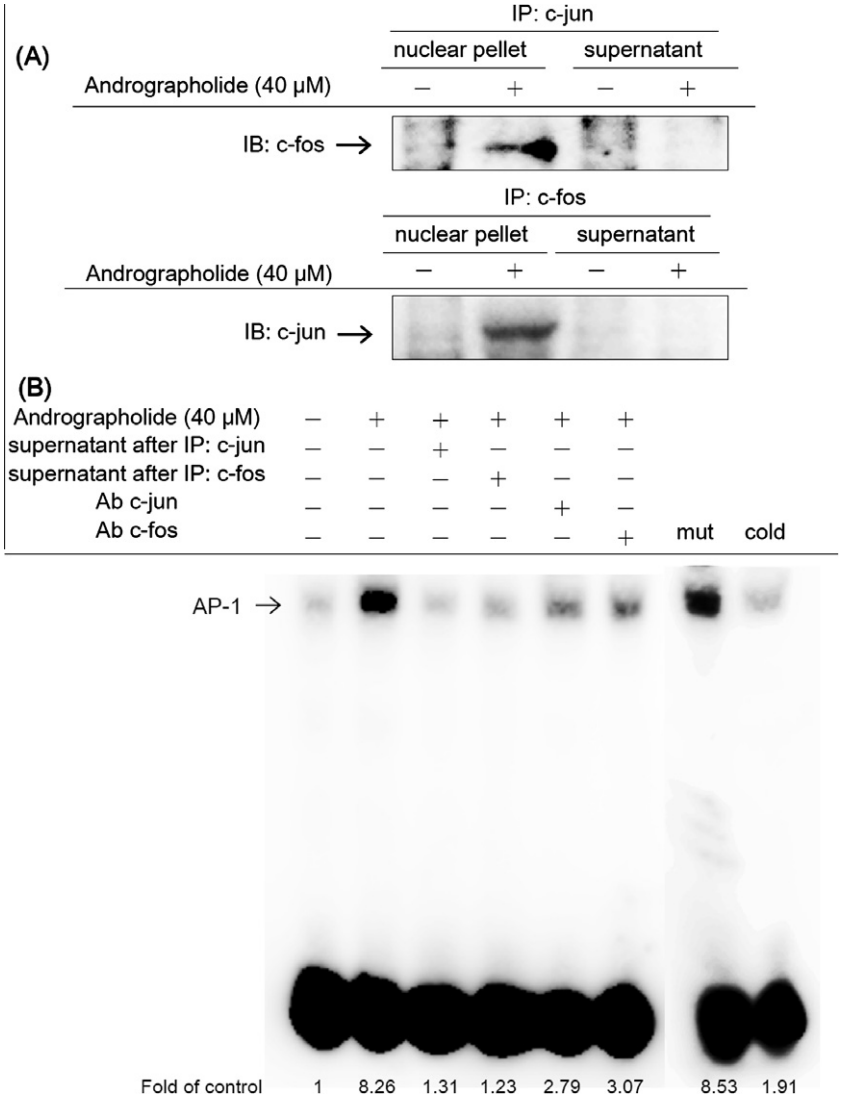

Fig. 6. Andrographolide-induced abundant constitutive c-jun and c-fos/AP-1 DNA-binding activity in rat primary hepatocytes. After attachment, hepatocytes were treated with or without 40 μM andrographolide for 3 h. (A) Immunoprecipitation of c-jun and c-fos in the nuclear extracts of andrographolide-treated cells. Western blot analysis was used to detect c-fos and c-jun protein in the pellets and supernatants which were pulled down by anti-c-jun and anti-c-fos antibodies, respectively. (B) Aliquots of nuclear extracts (8 μg) and the supernatants after immunoprecipitation with anti-c-jun or anti-c-fos antibodies were used for EMSA. To identify the proteins bound to the AP-1 binding site, anti-c-jun and anti-c-fos antibodies were used and EMSA supershift assay was performed. To confirm the specificity of the nucleotide, 600-fold cold probe (biotin-unlabeled AP-1 binding site) and biotin-labeled double-stranded mutant AP-1 oligonucleotide (2 ng) were included in the EMSA. One representative experiment out of three independent experiments is shown.

peritoneal macrophages (Qin et al., 2006). In addition, andrographolide inhibits the TNF-α-induced NF-κB signaling pathway and reduces the expression of the cell adhesion molecule E-selectin in human umbilical vein endothelial cells (Xia et al., 2004).

PI3K was found to activate phosphoinositide-dependent protein kinase-1 (PDK-1) and PDK-2 upon phosphorylation. Akt is a cytosolic protein that is recruited to the plasma membrane upon activation of PI3K (Andjelkovic et al., 1997). On the plasma membrane, Akt is phosphorylated by PDKs and then translocates through the cytosol to the nucleus (Andjelkovic et al., 1997). Akt has two phosphorylation residues, Thr^{308} and Ser^{473}. PDK-1 is identified to phosphorylate Thr^{308} of Akt, and PDK-2 phosphorylates Ser^{473} (Alessi et al., 1997). Many phytochemicals, such as curcumin, resveratrol, epigallocatechin-3-gallate, indole-3-carbinol, diindolylmethane, carnosol, and andrographolide, are considered to modulate the PI3K/Akt signaling pathway (Chang et al., 2008; Chen and Kong, 2005; Choi et al., 2008; Manson et al., 2007; Martin et al., 2004; Neergheen et al., 2009; Roy et al., 2009). For instance, curcumin was found to down-regulate P-glycoprotein expression by inhibiting the PI3K/Akt pathway in multidrug-resistant L1210/Adr cells (Choi et al., 2008). In another study, Roy et al. (2009) showed that resveratrol suppresses 7,12-dimethylbenz(*a*)anthracene-induced mouse skin tumorigenesis by abolishing the PI3K/Akt pathway. In addition, carnosol was found to upregulate HO-1 gene expression by activating the PI3K/Akt pathway (Martin et al., 2004). In our previous study, we showed that *A. paniculata* and its bioactive diterpene andrographolide upregulate GSTP gene expression in rat primary hepatocytes via the PI3K/Akt signaling pathway (Chang et al., 2008). Although the same result was found in the present study, we presented a new finding that PI3K/Akt activates AP-1 which is involved in andrographolide-induced GSTP expression (Fig. 5). In the present study, we found that andrographolide significantly induced phosphorylation of Thr^{308} of Akt (Fig. 1) and that wortmannin suppressed the andrographolide-induced GSTP expression, as reported previously (Chang et al., 2008). These findings suggest that the PI3K/PDK-1/Akt signaling pathway plays a

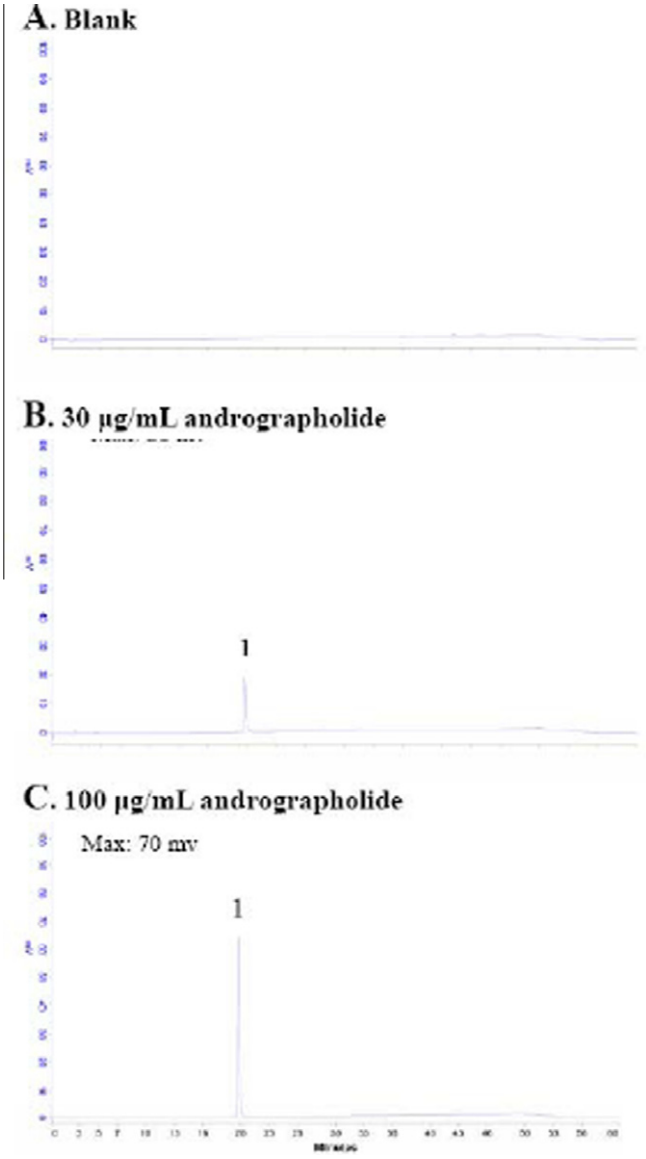

Fig. 7. HPLC/UV chromatograms of (A) blank (acetonitrile), (B) 30 μg/mL andrographolide, and (C) 100 μg/mL andrographolide. The retention time of peak 1 (andrographolide) was 19.8 min.

critical role in andrographolide-induced GSTP expression in rat primary hepatocytes.

AP-1 is one kind of transcription factor that modulates the expression of numerous genes involved in cellular adaptation, differentiation, and proliferation (Surh, 2003). Neergheen et al. (2009) indicated that resveratrol, gingerol, capsaicin, and ginsenosides can influence AP-1 activation. In human leukemia cells, c-jun was shown to bind to the GSTP gene promoter (Borde-Chiche et al., 2001). Tsai et al. (2007) suggested that AP-1 is an important transcription factor for GSTP gene expression by diallyl disulfide and diallyl trisulfide. In addition, sulforaphane, alpha-lipoic acid, and dihydrolipoic acid were shown to induce enzyme activity and gene expression of GSTP, and this induction was associated with the AP-1 and Nrf2 transcription factors (Lii et al., 2010).

Several studies have shown that GSTP gene expression is modulated by various phytochemicals through different signaling pathways, such as JNK, ERK, and p38 (Bergelson et al., 1994; Duvoix et al., 2004; Reddy and Mossman, 2002; Tsai et al., 2007). However, those pathways were not involved in the andrographolide-induced GSTP expression in our cell model system. Although the role of the PI3K/Akt signaling pathway in andrographolide-induced GSTP expression has been explored in rat primary hepatocytes (Chang et al., 2008), the downstream molecule of the PI3K/Akt pathway in this induction had not been identified. The results of Figs. 1 and 2 show that andrographolide can induce Akt and c-jun phosphorylation. These results suggest that AP-1 activation by andrographolide is possibly through the PI3K/Akt pathway rather than the MAPK pathway.

To further demonstrate the critical role of PI3K in andrographolide-induced GSTP gene expression, we used siPI3K to knock down the expression of PI3K and reduce its downstream molecule activation and the expression of its target genes. As shown in Figs. 4 and 5, siPI3K acted like the PI3K inhibitors wortmannin and LY294002 and suppressed the andrographolide-induced phosphorylation of Akt and c-jun, AP-1 DNA-binding activity, and GSTP expression. These data implicate the PI3K/Akt signaling pathway and the AP-1 transcription factor in andrographolide-induced GSTP gene expression.

We were also interested in investigating which transcription factor is the main target of the PI3K/Akt signaling pathway in the andrographolide induction of GSTP. Among the possible candidates, AP-1 seemed to be the most attractive because several lines of evidence have suggested the possible participation of AP-1/Jun family members in the regulation of GSTP gene expression by binding to the enhancer element GPE1 (Angel et al., 1987; Okuda et al., 1989; Sakai et al., 1988). In the present study, we noted a dramatic increase in c-jun phosphorylation (Fig. 2) and AP-1 translocation into the nucleus (Fig. 6A) as well as in the DNA-binding activity of AP-1 (Fig. 6B) in cells treated with andrographolide. c-Fos and c-jun are components of transcription factor AP-1, and fos/jun heterodimers are the most active. To identify whether the nuclear proteins bound to the AP-1 response element were c-jun and c-fos, we performed immunoprecipitation with anti-c-jun or anti-c-fos antibody and a supershift experiment with anti-c-jun or anti-c-fos antibody, respectively. As shown in Fig. 6A, nuclear accumulation of c-fos after immunoprecipitation with anti-c-jun antibody and nuclear accumulation of c-jun after immunoprecipitation with anti-c-fos antibody were demonstrated in andrographolide-treated cells. This result suggested that nuclear c-fos/c-jun heterodimer increases with andrographolide treatment. Anti-c-jun and anti-c-fos antibodies were used to perform the supershift experiment. Although no supershift data observed, anti-c-jun and anti-c-fos antibodies decreased nuclear protein binding to the AP-1 oligonucleotide (Fig. 6B). It might be because the anti-c-jun and anti-c-fos antibodies compete with AP-1 oligonucleotide for the same binding site on c-jun and c-fos. The results implicate the importance of c-jun and c-fos in andrographolide-induced AP-1 nuclear protein DNA-binding complex formation. Thus, we showed that AP-1 likely participates in upregulating GSTP transcription.

MAPKs are known to be the upstream activator of AP-1, and several studies have reported that GSTP gene expression is modulated by various phytochemicals through the MAPKs-AP-1 signaling pathway (Reddy and Mossman, 2002; Tsai et al., 2007; Bergelson et al., 1994; Duvoix et al., 2004). However, the lack of change in JNK, ERK, and p38 phosphorylation in the presence of andrographolide in the present study (Fig. 1C), therefore, the possible involvement of MAPK signaling pathways in p-cJun was excluded and no MAPK inhibitors were tested. The results suggest that AP-1 activation is not likely to be via the MAPK pathway (Fig. 1C). Instead, we found that wortmannin and LY294002 suppressed the andrographolide-induced phosphorylation of Akt, which resulted in abolishing c-jun activation (Fig. 3) and AP-1 DNA-binding activity (Fig. 5A). Furthermore, the experiment with siPI3K clearly showed that knock down of PI3K expression abolishes the DNA-binding activity of AP-1 and also the induction of GSTP expression by andrographolide (Figs. 4D and 5B). Taken

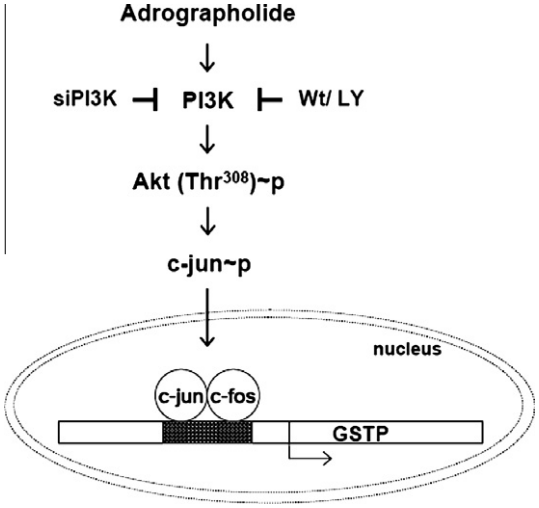

Fig. 8. Model showing the pathways that mediate andrographolide-induced GSTP expression in rat primary hepatocytes.

together, these data implicate the PI3K/Akt-AP-1 and not the MAPK-AP-1 signaling pathway in the andrographolide induction of GSTP gene expression.

The findings of the present study are summarized schematically in Fig. 8. We propose that andrographolide-induced GSTP expression is mediated by the PI3K/Akt signaling pathway and the AP-1 transcription factor in rat primary hepatocytes.

Conflict of Interest

The authors declare that they have no conflicts of interest associated with this work.

Acknowledgment

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