行政院國家科學委員會補助專題研究計畫成果報告

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※ 穿心蓮調控解毒代謝酵素之研究 ※

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□赴國外出差或研習心得報告一份

□赴大陸地區出差或研習心得報告一份

□出席國際學術會議心得報告及發表之論文各一份

□國際合作研究計畫國外研究報告書一份

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中文摘要

穿心蓮是亞洲地區常見的草藥，多分布於中國、韓國和印度等地，具有抗肝毒性、抗病毒、抗發炎、抗腫瘤與抗氧化等效果。穿心蓮內酯是穿心蓮成分中最具活性的雙帖類之一；研究指出，穿心蓮內酯處理下能減少四氯化碳對囓齒類肝臟所造成的損傷，達到保護肝臟的功用。Pi屬麸胱甘肽硫轉移酶 (pi class of glutathione *S*-transferase, GSTP)是生物轉換酵素系統中Phase Ⅱ酵素的一員，能代謝內生性和外生性親電性物質或自由基，以保護細胞免受毒物及致癌物傷害。本篇研究目的是想探討穿心蓮內酯誘發大鼠初代肝細胞中GSTP之表現機制。以 40 μM穿心蓮內酯處理大鼠初代肝細胞，發現處理 0.5 h會顯著增加胞內Akt的磷酸化；而處理 3 h後，會顯著活化胞內c-jun的磷酸化。接著預處理PI3K抑制劑 (wortmannin和LY294002)，或者以siPI3K knockdown PI3K，觀察到穿心蓮內酯所活化的Akt或c-jun磷酸化表現均受抑制；另外，也減少穿心蓮內酯誘發GSTP表現的作用。另外以 40 μM穿心蓮內酯處理大鼠初代肝細胞，發現處理 3 h會顯著增加細胞核內activator protein-1 (AP-1)與DNA的結合力，且細胞核內AP-1 與DNA的結合力會受到預處理PI3K抑制劑或siPI3K所抑制。更進一步以EMSA Gel Shift Assay方式證實，細胞核內AP-1 中含有磷酸化c-jun。以上結果顯示，穿心蓮內酯會透過PI3K/Akt路徑活化c-jun，進而促進核內AP-1 與DNA結合，增加初代肝細胞內GSTP蛋白質表現。

關鍵字: 穿心蓮內酯、pi 屬麸胱甘肽硫轉移酶、PI3K/Akt、activator protein-1、大鼠初代肝細胞

Abstract

Andrographis paniculata (Burm. f.) Nees 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 μmol/L 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. Electrophoretic mobility shift assays showed that pretreatment with wortmannin, LY294002, or siPI3K attenuated the AP-1-DNA binding activity caused by andrographolide. Super-shift assays revealed that the transcription factor c-jun was 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 signaling pathway, phosphorylation of c-jun, and subsequent binding to the response element in the gene promoter region.

Introduction

Andrographis paniculata (Burm. f.) Nees (Acanthaceae) 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 infections, diarrhea, and infectious hepatitis (1). 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 (2-6).

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 (7, 8). 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 (9, 10). 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 (11).

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 (12-14), and the PI3K/Akt signaling pathway is affected by numerous phytochemicals (15-18). A recent study showed that the PI3K/Akt pathway is blocked by apigenin in human ovarian cancer cells (19). By contrast, andrographolide was found to suppress cell apoptosis through activation of the PI3K/Akt pathway in human umbilical vein endothelial cells (20).

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 (21, 22). The 5’ upstream region of the GSTP gene promoter contains the enhancer element termed

GSTP enhancer I (GPEI), which includes a TRE-like sequence. AP-1 was shown to bind to the GPEI fragment in GSTP owing to the presence of the TRE-like sequence in GPEI (23-25). 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 (26, 27). On the other hand, Hou et al. (28) 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 (11). In the present study, we further investigated 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.

Materials and Methods

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, and anti-c-jun antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); and the transfection reagent Dharmafect 1 was from Dharmacon (Lafayette, CO).

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 (29). 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 mmol/L HEPES, 1% ITS^+, 1 μmol/L dexamethasone, 100 IU penicillin/mL, and 100 μg streptomycin/mL. Cells were incubated at 37℃ 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 μmol/L 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*.

Western blotting. Cells were washed twice with cold PBS and were harvested in 200 μL of 20 mmol/L potassium phosphate buffer (pH 7.0). Cell homogenates were centrifuged at 9000 x*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 (30). 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 mmol/L Tris-150 mmol/L NaCl buffer (pH 7.4) at 4°C for 2 h. After blocking, the membrane was incubated with anti-phospho Akt, anti-phospho-JNK1/2, anti-phospho-ERK1/2, anti-phospho-p38, and anti-phospho c-jun or anti-Akt, anti-JNK1, anti-ERK1/2, anti-p38, and anti-c-jun antibodies at 4℃ overnight. Thereafter, the membrane was incubated with the secondary peroxidase-conjugated anti-rabbit or anti-mouse IgG at 37℃ for 1 h, and the immunoreactive bands were developed by use of the Western Lightning^{TM} Plus-ECL kit (PerkinElmer, Waltham, MA).

Gene silencing experiments. Transfection experiments with small interfering RNA (siRNA) against p85α, a regulatory subunit of PI3K, were performed by using a smart-pool of 4 specific siRNAs (Dharmacon catalog # J-080078-05, Thermo Fisher Scientific, Lafayette, CO) targeting rat PI3K (NM_013005). The 4 siRNAs against the rat p85α gene are (1) ACCUAUUGCGAGGGAAA, (2) CGAGAUGCAUCCACUAAAA, (3) GAUAAUGCAUAAUCACGAU, and (4) UAUGAGGAAUACACUCGUA. A nontargeting 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 x 10^6 cells in 3.5 cm^2 dishes one day before transfection with **100** nmol/L p85α or non-targeting control-pool siRNA

for 24 h. Cells were then treated with 40 μmol/L andrographolide in RPMI-1640 medium. Cell samples were collected for Western blotting analysis and EMSA.

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 x*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 mmol/L HEPES, 10 mmol/L KCl, 1 mmol/L $MgCl_2$, 1 mmol/L EDTA, 0.5 mmol/L DTT, 4 μg/mL leupeptin, 20 μg/mL aprotinin, 0.5% Nonidet P-40, and 0.2 mmol/L phenylmethylsulfonyl fluoride. After centrifugation at 6000 x*g* for 15 min, pellets containing crude nuclei were resuspended in 50 μL of hypertonic buffer containing 10 mmol/L HEPES, 400 mmol/L KCl, 1 mmol/L $MgCl_2$, 1 mmol/L EDTA, 0.5 mmol/L DTT, 4 μg/mL leupeptin, 20 μg/mL aprotinin, 10% glycerol, and 0.2 mmol/L phenylmethylsulfonyl fluoride at 4℃ for 30 min. The samples were then centrifuged at 20,000 x*g* for 15 min. The supernatant containing the nuclear proteins was collected and stored at -80℃ until the electrophoretic mobility shift assay (EMSA).

Electrophoretic mobility shift assay. EMSA was performed according to our previous study (31). 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 (32). 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.) 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.).

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.

Results

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 (11). To clarify the importance of the PI3K/Akt signaling pathway in the induction of GSTP by andrographolide, we treated rat primary hepatocytes with 40 μmol/L 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 (32, 33). However, as shown in Fig. 1C, we observed no significant phosphorylation of JNK, ERK, or p38 in rat primary hepatocytes treated with 40 μmol/L andrographolide for 0, 0.25, 0.5, 1, and 3 h.

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 (27). Our previous study showed that the upregulation of GSTP expression by diallyl disulfide and diallyl trisulfide is via an AP-1-dependent pathway (32). To determine the role of AP-1 in andrographolide-induced GSTP expression, we assayed the phosphorylation of c-jun in hepatocytes treated with 40 μmol/L 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.

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 μmol/L andrographolide for another 3 h. As shown in Fig. 3, both inhibitors significantly inhibited andrographolide-induced c-jun phosphorylation.

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 4C) 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.

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

c-Jun involvement in andrographolide-induced AP-1 nuclear protein DNA-binding complex formation. As shown in Fig. 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). To further identify whether the nuclear protein bound to the AP-1 binding site was phospho-c-jun, we performed immunoprecipitation with anti-c-jun antibody and a supershift experiment with anti-phospho-c-jun antibody. As shown in Fig. 6A, nuclear accumulation of c-jun after immunoprecipitation with anti-c-jun antibody was demonstrated in andrographolide-treated cells. In addition, anti-phospho-c-jun antibody produced a supershift in the protein-DNA binding pattern (Fig. 6B). The results implicate the importance of c-jun in andrographolide-induced AP-1 nuclear protein DNA-binding complex formation.

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 (34-36). GSTP has been associated with the carcinogenic process and with anti-neoplastic drug resistance in cancer cells (37, 38). GSTP was recognized as a marker in several rat hepatocarcinogenesis models because of its over-expression within hepatocellular foci and neoplasms (39). Several lines of study have addressed the regulation of GSTP expression, and previous studies have shown that hepatic GSTP is induced by both nutrient and non-nutrient factors (11, 33, 40-42).

Fourteen flavonoids and 13 diterpenoids have been isolated from *A. paniculata* (43, 44), and andrographolide is the most abundant diterpenoid. Andrographolide has been shown to have anti-HIV, immunostimulatory, anti-inflammatory, and anti-hepatotoxic activities (1). Li et al. (45) 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 (46). 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 (47). The effective dose of andrographolide in different biological processes varies depending on cell type. In the present study, we used 40 μmol/L andrographolide to treat rat primary hepatocytes, which was a dose we found in our previous study to cause no cell damage (11).

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 (48). Lipopolysaccharide-induced TNF-α production is suppressed by andrographolide, and this effect is via its inhibition of the ERK1/2 pathway in mouse peritoneal macrophages (49). 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 (50).

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 (51). On the plasma membrane, Akt is phosphorylated by PDKs and then translocates through the cytosol to the nucleus (51). 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} (52). Many phytochemicals present in plants, such as curcumin, resveratrol, epigallocatechin-3-gallate, indole-3-carbinol, diindolylmethane, carnosol, and andrographolide, are considered to modulate the PI3K/Akt signaling pathway (11, 15-18, 53, 54). For instance, curcumin was found to down-regulate P-glycoprotein expression by inhibiting the PI3K/Akt pathway in multidrug-resistant L1210/Adr cells (53). In another study, Roy et al. (54) 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 (17). 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 (11). 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 (11). These findings suggest that the PI3K/PDK-1/Akt signaling pathway plays a

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 (55). Neergheen et al. (18) indicated that resveratrol, gingerol, capsaicin, and ginsenosides can influence AP-1 activation. Several lines of evidence have suggested the possible participation of AP-1/Jun family members in the regulation of GSTP gene expression. In human leukemia cells, c-jun was shown to bind to the GSTP gene promoter (56). Tsai et al. (32) 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 (57).

Several studies have shown that GSTP gene expression is modulated by various phytochemicals through different signaling pathways, such as JNK, ERK, and p38 (27, 32, 58, 59). 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 (11), the downstream molecule of the PI3K/Akt pathway in this induction had not been identified. The results of Fig. 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 Fig. 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 GPEI (23-25). In the present study, we noted a dramatic increase in c-jun phosphorylation (Fig. 2) and c-jun translocation into the nucleus (Fig. 6A) as well as in the DNA binding activity of AP-1 (Fig. 6B) in cells treated with andrographolide. 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 (27, 32, 58, 59). However, the lack of change in JNK, ERK, and p38 phosphorylation in the presence of andrographolide in the present study suggests 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 (Fig. 4D and 5B). Taken 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. 7. 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.

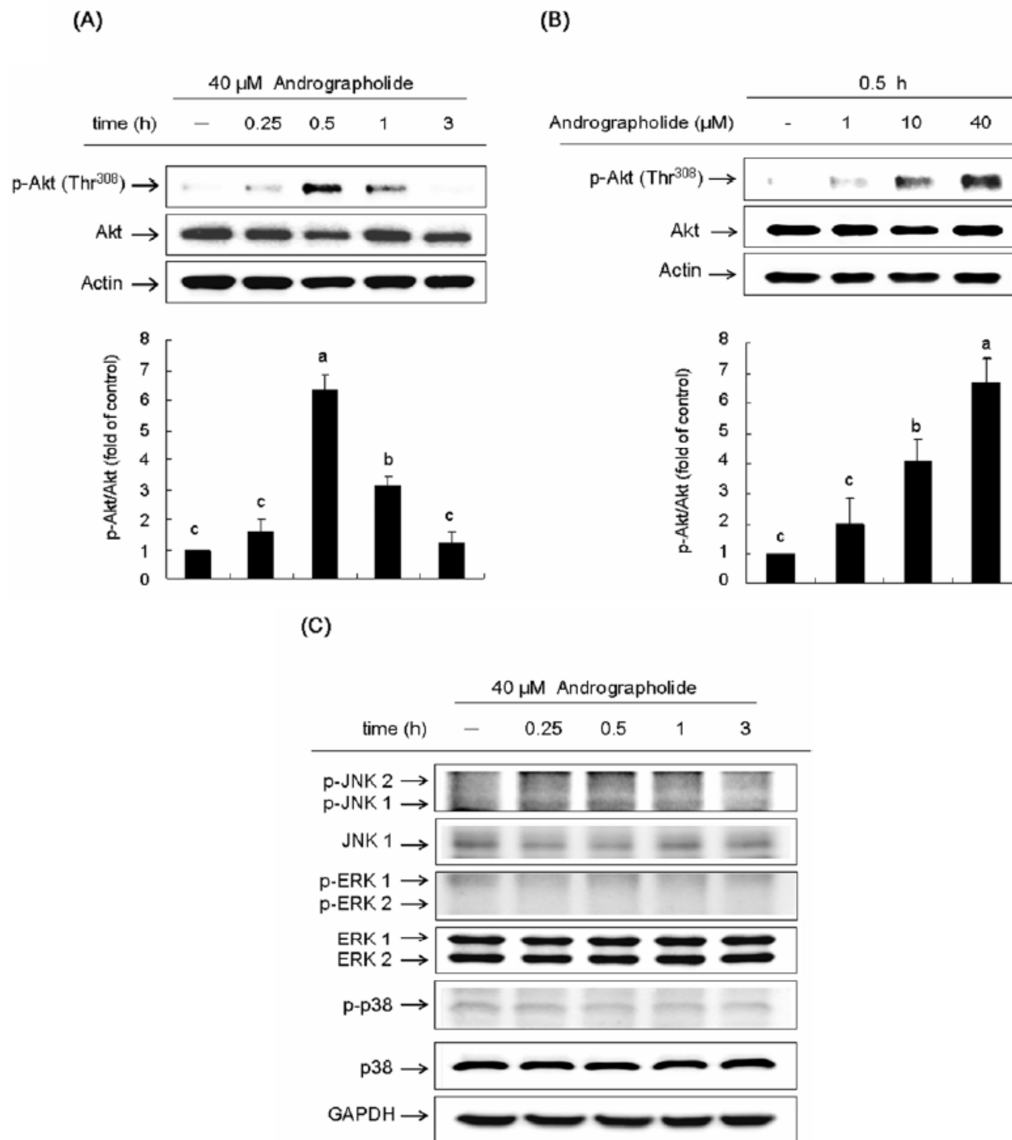

FIGURE 1 Effect of andrographolide on Akt phosphorylation and MAPK activation in rat primary hepatocytes. After attachment, (A) hepatocytes were treated with 40 μmol/L andrographolide for various time periods and Akt phosphorylation was determined, (B) hepatocytes were exposed to various concentrations of andrographolide and Akt phosphorylation was determined, or (C) hepatocytes were treated with 40 μmol/L 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 means ± SD, *n*=3. The levels in control cells were set at 1. Means without a common letter differ, $P<0.05$.

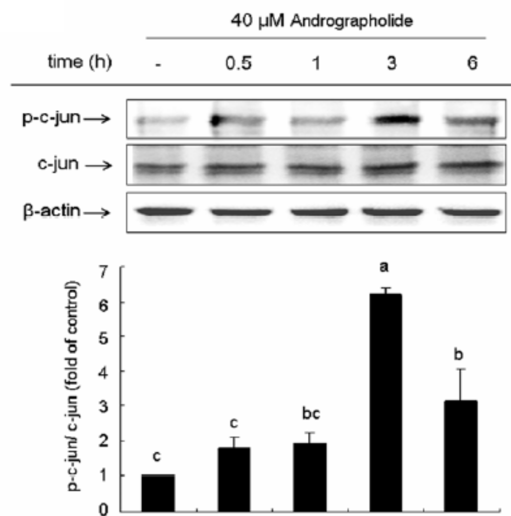

FIGURE 2 Effect of andrographolide on c-jun phosphorylation in rat primary hepatocytes. After attachment, hepatocytes were treated with 40 μmol/L andrographolide for various time periods. Aliquots of total protein (20 μg) were used for Western blot analysis. Values are means ± SD, n=3. The levels in control cells were set at 1. Means without a common letter differ, P<0.05.

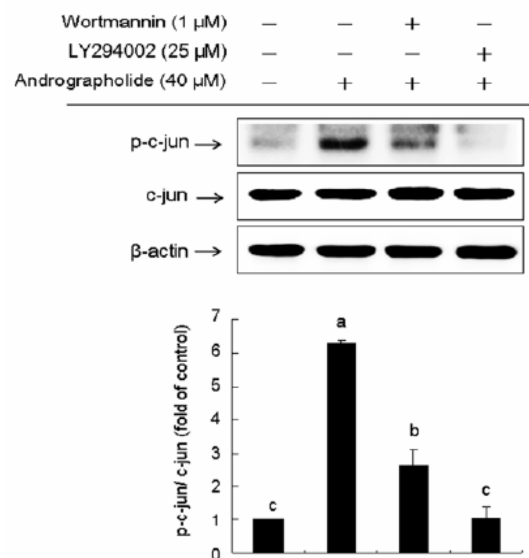

FIGURE 3 Effects of PI3K inhibitors on andrographolide-induced c-jun phosphorylation in rat primary hepatocytes. After attachment, hepatocytes were pretreated with PI3K inhibitors, wortmannin and LY294002, for 1 h, and were then treated with 40 μmol/L andrographolide for another 3 h. Aliquots of total protein (20 μg) were used for Western blot analysis. Values are means ± SD, n=3. The levels in control cells were set at 1. Means without a common letter differ, P<0.05.

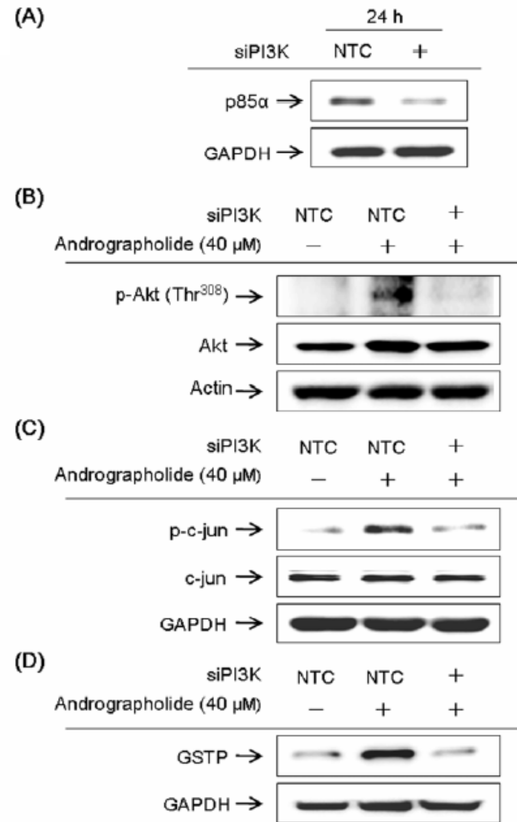

FIGURE 4 Effect of PI3K siRNA on the andrographolide-induced phosphorylation of Akt and c-jun and GSTP expression in rat primary hepatocytes. After attachment, hepatocytes were transiently transfected with non-targeting control siRNA or siPI3K for 24 h, followed by treatment with or without 40 μmol/L andrographolide. Akt phosphorylation was observed at 0.5 h, c-jun phosphorylation was observed at 3 h, and PGST expression was observed at 48 h. Aliquots of total protein (20 μg) were used for Western blot analysis. (A) Cells were transfected with 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.

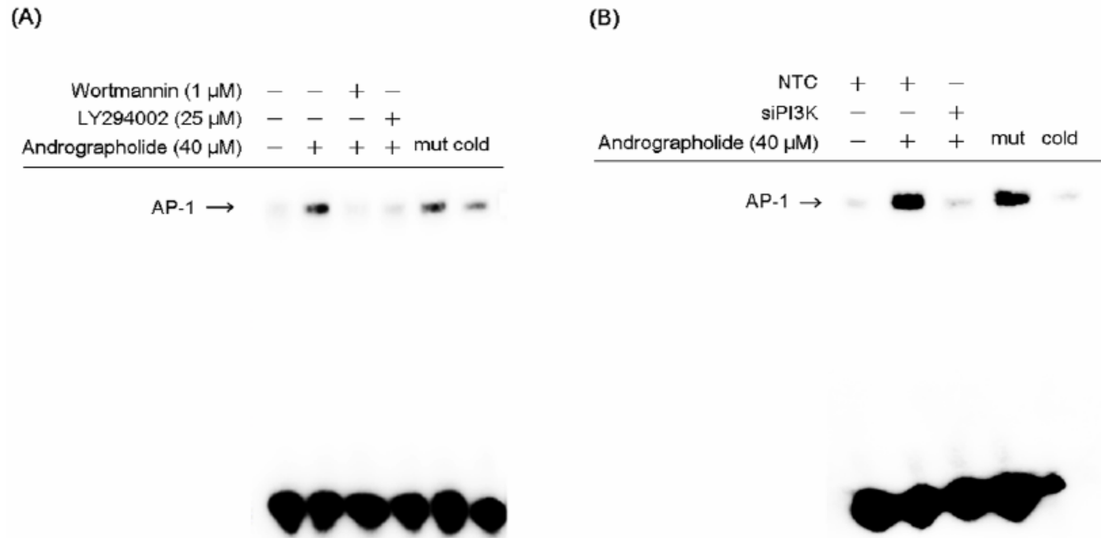

FIGURE 5 Andrographolide induces AP-1 nuclear protein DNA-binding activity via the PI3K/Akt pathway in rat primary hepatocytes. After attachment, (A) hepatocytes were pretreated with PI3K inhibitors, wortmannin and LY294002, for 1 h (B) or were transiently transfected with non-targeting control siRNA or siPI3K for 24 h. After PI3K inhibitors or siRNA transfection, hepatocytes were treated with 40 μmol/L 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.

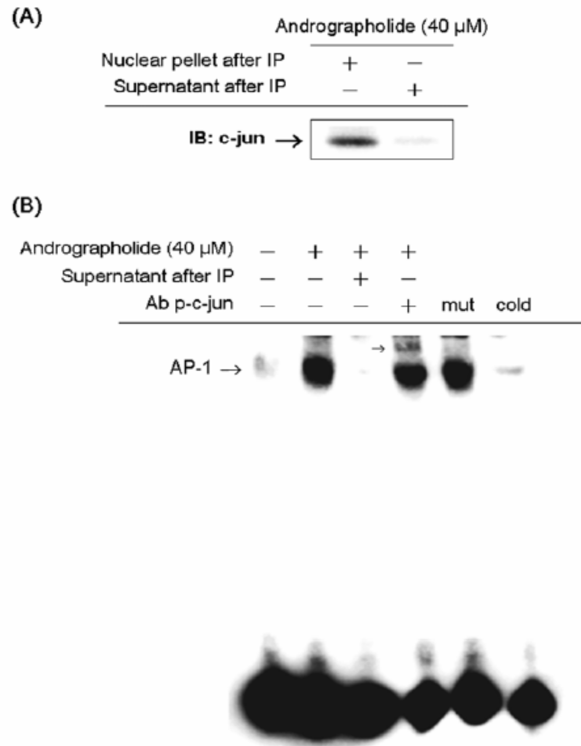

FIGURE 6 Andrographolide-induced abundant constitutive c-jun/AP-1 DNA-binding activity in rat primary hepatocytes. After attachment, hepatocytes were treated with or without 40 μmol/L andrographolide for 3 h. (A) Nuclear extracts were subjected to immunoprecipitation with anti-c-jun antibody. Aliquots of pellet and supernatant after immunoprecipitation(20 μg) were used for Western blot analysis with anti-c-jun antibody. (B) Aliquots of nuclear extracts (8 μg) and the supernatant after immunoprecipitation with anti-c-jun were used for EMSA. To identify the protein bound to the AP-1 binding site, anti-phospho-c-jun antibody was 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.

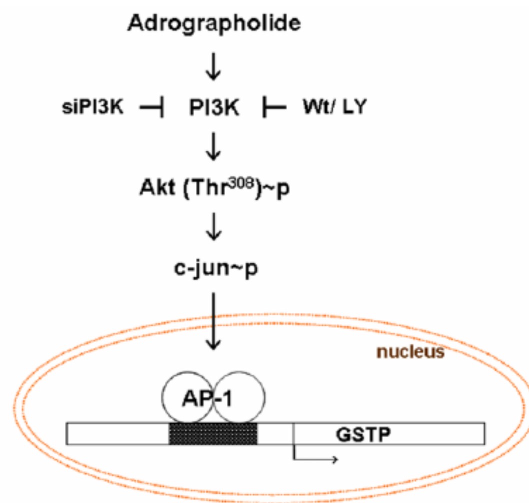

FIGURE 7 Model showing the pathways that mediate andrographolide-induced GSTP expression in rat primary hepatocytes.

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