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Activation of the cAMP/CREB/Inducible cAMP Early Repressor Pathway Suppresses Andrographolide-Induced Gene Expression of the π Class of Glutathione *S*-Transferase in Rat Primary Hepatocytes

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Andrographolide (Ap) is a bioactive compound in *Andrographis paniculata* that is a Chinese herb. The π class of glutathione *S*-transferase (GSTP) is one kind of phase II detoxification enzyme. Here we show that induction of GSTP protein and mRNA expression in rat primary hepatocytes by Ap was inhibited by forskolin and a variety of cAMP analogues. The inhibitory effect of the cAMP analogues was partially blocked by pretreatment with H89. In the presence of Ap, forskolin, or both, the expression of phospho-cAMP response element-binding protein (CREB) was increased. Ap alone had no effect on inducible cAMP early repressor (ICER) mRNA expression; however, Ap played a potentiating role in forskolin-induced ICER mRNA expression. An EMSA and immunoprecipitation assay showed that ICER binding to cAMP-response element (CRE) was increased in cells cotreated with Ap and forskolin for 3 and 8 h. Taken together, these results suggest that ICER is likely to be involved in the suppression of Ap-induced GSTP expression caused by the increase of cAMP in rat primary hepatocytes.

KEYWORDS: Andrographolide; cAMP/CREB/ICER pathway; forskolin; π class of glutathione *S*-transferase (GSTP); hepatocytes

INTRODUCTION

The biotransformation enzyme system in mammals includes phase I and phase II enzymes and phase III membrane transporters that catalyze the conversion of xenobiotics to the polar metabolites that are more readily excreted (*1*). The glutathione *S*-transferases (GSTs) are phase II detoxification enzymes and are composed of two enzyme families with distinct catalytic and noncatalytic binding properties. One enzyme family consists of membrane-bound enzymes such as microsomal GST and leukotriene C4 synthetase; the other enzyme family consists of cytosolic enzymes that are encoded by at least five distantly related gene classes, namely, α, μ, π, σ, and θ (*2*). The π class of GST (GSTP) is one of the super families of GST enzymes that catalyze the conjugation of GSH to electrophilic xenobiotics, which results in the detoxification of electrophiles such as genotoxic chemical carcinogens and cytotoxic chemotherapeutic agents (*3*−*5*).

In most normal tissues, GSTP expression is very low; however, overexpression is frequently observed in several tumor tissues, including liver, brain, prostate, and lung (*4*,*6*), and in carcinomas such as lymphomas and synovial sarcoma (*7*). The overexpression of GSTP protein has been shown to be associated not only with anticancer drug resistance but also with increased tumor grade and tumor recurrence; moreover, high GSTP protein expression may lead to poor patient survival (*8*). GSTP is highly inducible during carcinogenesis, and the expression of GSTP is regarded as being an important determinant of cancer susceptibility and a reliable marker of tumorigenesis (*6*). Thus, the high levels of GSTP expression in tumors may be either a cause or an effect of the tumorigenic process.

The mechanisms underlying the regulation of GSTP expression during malignancy are highly complicated and not well understood. The GSTP gene has an enhancer element (GPE1; GSTP enhancer 1) at 2.5 kb upstream of the cap (*9*). In GPE1, there are two phorbol-12-*O*-tetradecanoate-13-acetate responsive element (TRE)-like elements that are considered to be required for the basal and inducible expression of GSTP (*10*). The transcriptional factor activator protein-1 (AP-1) has been shown to bind to the TREs and upregulate GSTP expression (*11*). The upregulation of GSTP expression by garlic allyl sulfides is dependent on both the c-Jun NH2-terminal kinase (JNK)-AP-1 and the extracellular receptor kinase (ERK)-AP-1 signaling pathways (*12*). In addition, a highly conserved sequence known as the cAMP response element (CRE), which is present in the 5′-region

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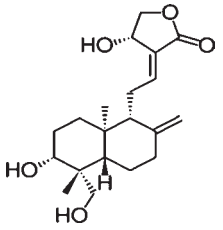

Figure 1. Chemical structure of andrographolide.

of the human GSTP gene, and cAMP- and cAMP response element-binding protein-1 (CREB-1)-mediated mechanisms are reported to be involved in the transcriptional regulation of the GSTP gene (*8*).

The CREB family belongs to the basic domain leucine zipper (bZIP) transcription factor class and comprises three members: CREB, CRE modulator (CREM), and activating transcription factor (ATF) (*13*). The CREB/CREM/ATF family members are homologous proteins that contain a basic DNA binding domain that recognizes the CRE (*14*). CREM encodes a variety of isoforms that are generated by alternative mRNA splicing. The isoforms function as either activators or repressors of cAMP-induced transcription (*15*). Inducible cAMP early repressor (ICER), an isoform of CREM, is a small protein of 120 amino acids and possesses only the bZIP domain (*16*). ICER plays a repressor role in cAMP-dependent transcription (*17*). Both CREB and ICER, members of the CREB family, are transcriptional regulators of the cAMP-mediated signaling pathway (*18*); however, they act in opposite ways. The ubiquitous transcription factor CREB binds to the CRE and activates target gene expression after the phosphorylation of its serine 133 by the cAMP-dependent PKA (*19*), and ICER competes with other bZIP proteins for the CRE and inhibits gene expression (*20*).

Andrographis paniculata (Bum. f) Nees, a Chinese herb, is a member of the Acanthaceae family. It is widely cultivated in Southeast Asia and is widely used as a traditional medicine in India, China, Thailand, and Scandinavia (*21*). *A. paniculata* was reported to have chemopreventive potential. In animal studies, administration of *A. paniculata* significantly increases hepatic activity of phase I (*22*) and phase II (*23*) enzymes. Andrographolide (Ap) (**Figure 1**) is the most abundant diterpene lactone in the leaves and stems of *A. paniculata* and has high biological activity and therapeutic potential (*24*). A clinical trial showed that Ap may inhibit HIV-induced cell cycle dysregulation and lead to a rise in $CD4^+$ lymphocyte levels in HIV-1 infected individuals (*25*). Many in vitro studies have indicated that Ap exerts anticancer activities, including antiangiogenesis (*26*), antiproliferation (*27*), and pro-apoptosis (*28*) effects. Our previous study showed that administration of ethanol or ethyl acetate extracts of *A. paniculata* and of Ap significantly increases GSTP expression in rat primary hepatocytes (*29*).

cAMP is a well-known intracellular second messenger, and it is known to play an important role in T-cell function (*30*). A variety of hormones, including glucagon, epinephrine, parathyroid, and adrenocorticotropic hormone (ACTH), were shown to increase intracellular cAMP level (*31*). In addition to its role in the immune system, cAMP has been shown to exert an inhibitory effect on the biotransformation enzyme system (*32*,*33*). In the present study, we used the rat primary hepatocyte culture system to study the role of increased cAMP as occurring in response to hormone stimulation in the modulation of Ap-induced GSTP expression which is an important component of the phase II enzyme system and the mechanisms underlying the regulation.

MATERIALS AND METHODS

Reagents and Antibodies. Cell culture medium (RPMI-1640) was purchased from Gibco-BRL (Gaithersburg, MD), collagenase type IV was obtained from Worthington Biochemical (Lakewood, NJ), Matrigel was purchased from Collaborative Biomedical Products (Bedford, MA), ITS^{TM} Premix, collagen type I, and anti-GSTP antibody were purchased from BD Biosciences (San Jose, CA), anti-CREB (48H2), and antiphospho-CREB (ser133) antibodies were purchased from Cell Signaling Technology (Boston, MA), anti-CREM (ICER) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), antimouse IgG (goat), antirabbit IgG (goat), and horseradish peroxidase-conjugated antibodies and enhanced chemiluminescence reagent were purchased from PerkinElmer Life Science, Inc. (Boston, MA), SP600125 (a JNK inhibitor) and PD98059 (an MAPK/ERK kinase inhibitor) were obtained from TOCRIS (Ellisville, MO), SB203580 (a p38 MAPK inhibitor) was purchased from Biosource (Camarillo, CA), antiactin monoclonal antibody, 8-bromo-cAMP, 8-(4-chlorophenylthio)-cAMP, dibutyryl-cAMP, forskolin, H89, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and all other chemicals were obtained from Sigma Chemical (St. Louis, MO). Taqman primer probes for GSTP, ICER, GAPDH, and Master Mixture were purchased from Applied Biosystems (Foster City, CA). Andrographolide was purchased from Calbiochem (Darmstadt, Germany).

Hepatocyte Isolation and Culture. Male Sprague−Dawley rats (weighing 250−300 g) were purchased from BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan). Hepatocytes were isolated by a modification of the two-step collagenase perfusion method described previously (*34*). After isolation, hepatocytes (3×10^6 cells/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 of penicillin/mL, and 100 μg of 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, the same medium was supplemented with Matrigel (233 mg/L), 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*.

Cell Viability. Cell viability was assessed by measuring the ability of viable cells to reduce a yellow MTT to a purple formazan by mitochondrial succinate dehydrogenase. After incubation with 40 μM andrographolide alone or in a combination of 40 μM andrographolide and various levels of forskolin for 48 h, the medium was removed, and hepatocytes were then incubated in RPMI-1640 medium containing 0.5 mg/mL MTT for an additional 3 h. The medium was then removed, and isopropyl alcohol was added to dissolve the formazan. After centrifugation at 9000*g* for 5 min, the supernatant of each sample was transferred to 96-well plates, and the absorbance was read at 570 nm in an ELISA reader. The absorbance in cultures treated with 0.1% DMSO (control) was regarded as 100% cell viability.

Intracellular cAMP Measurement. Intracellular cAMP concentrations were measured by using the cAMP EIA kit (Cayman Chemical). The cells were incubated with Ap or forskolin for 30 min, and cell extracts were prepared as described by Li et al. (*33*).

Nuclear Extracts Preparation. After each experiment, hepatocytes were washed twice with cold PBS and were then scraped from the dishes with 800 μL of PBS. Cell homogenates were centrifuged at 1800*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 extraction buffer containing 10 mM HEPES, 1 mM $MgCl_2$, 1 mM EDTA, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.5% Nonidet P-40, 4 μg/mL leupeptin, 20 μg/mL aprotinin, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). After centrifugation at 7000*g* for 15 min, the resulting supernatant was discarded, and the pellet containing nuclei was extracted by gentle mixing with 50 μL of hypertonic extraction buffer containing 10 mM HEPES, 0.4 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, 4 μg/mL leupeptin, 20 μg/mL aprotinin, 0.2 mM PMSF, and 10% glycerol 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).

EMSA. EMSA was carried out as described previously (*35*). Six micrograms of nuclear extract, poly (dI-dC), and biotinylated double-stranded CRE consensus oligonucleotides (5′-AGAGATTGCCT-GACGTCAGAGAGCTAG-3′) 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 27 °C for 30 min. An unlabeled double-stranded CRE oligonucleotide and a double-stranded NF-κB oligonucleotide (5′-AGTTGAGGCGACTTTCCCAGGC-3′) were used to confirm competitive binding and specific binding, respectively. The nuclear protein-DNA complex was separated by 6% Tris/boric acid/EDTA-polyacrylamide gel electrophoresis and was then transferred to Hybond N^+ membranes. 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.).

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 then incubated with 0.6 μg anti-ICER or anti-CREB antibody for 12 h at 4 °C, mixed with 4 μL protein A-Sepharose suspension (0.1 mg/mL), and incubated for an additional 1 h. Immunoprecipitates were collected by centrifugation at 16 000*g* for 2 min. The pellet was washed three times with 200 μL of IP buffer and was then subjected to Western blotting.

Western Blotting. After each experiment, hepatocytes were washed twice with cold PBS and were then scraped from the dishes with 500 μL of phosphate buffer containing 7.4 mM K_2HPO_4, 2.6 mM KH_2PO_4, and 15 M KCl (pH 7.4). Cell homogenates were centrifuged at 20 000*g* for 30 min. The resulting supernatant was used as a cellular protein for Western blot analysis. Nuclear extract proteins were prepared from rat primary hepatocytes as described above. For GSTP and actin, 4 μg of cellular proteins was used, for CREB and phospho-CREB, 30 μg of nuclear proteins was used, and for IP ICER and CREB, 60 μg of nuclear proteins was used. The protein samples were separated on 10% sodium dodecyl sulfate (SDS)−polyacrylamide gel, and the separated proteins were transferred to polyvinylidene diflouride membranes. After blocking, the membrane was incubated with anti-GSTP antibody (1:2000) and antiactin antibody (1:2000) for 45 min at room temperature, with anti-CREB antibody (1:2000) and antiphospho-CREB antibody (1:2000) for 1.5 h at room temperature, and with anti-ICER antibody (1:1000) for 12 h at 4 °C, followed by incubation with horseradish peroxidase-conjugated antibody for 1 h at room temperature. The protein bands were visualized by enhanced chemiluminescence plus Western blotting detection reagent (PerkinElmer Life Science, Inc.).

RNA Isolation and Real-Time PCR. Total RNA was isolated from rat primary hepatocytes by using TRIzol reagent (Invitrogen, Carlsbad, CA). Amounts of 0.8 μg of total RNA were reverse-transcribed with superscript II reverse transcriptase (Stratagene, Heidelberg, Germany) in a 20 μL final volume of reaction buffer, which consisted of 5 mM $MgCl_2$, 1 mM of each deoxyribonucleotide triphosphate, 2.5 U RNase inhibitor, and 2.5 mM oligo (dT). For the synthesis of cDNA, the reaction mixtures were incubated at 45 °C for 15 min; the reaction was stopped by denaturing the reverse transcriptase by heating the mixture to 99 °C for 5 min. For real-time PCR reactions, each sample was run in triplicate, and each 20 μL reaction contained 1 μL of GSTP (Rn02770492_gH), ICER (Rn00569145_m1), or GAPDH (Mm99999915_gl) Taqman primer probes and 10 μL of Taqman Master mixture, 5 μL of sample cDNA, and 4 μL of ddH_2O. Real-time PCR was performed on an SDS-7500 PCR instrument (Applied Biosystems). The real-time PCR runs consisted of first 1 cycle at 50 °C for 2 min, then 1 cycle at 95 °C for 10 min, and then 40 cycles at 95 °C for 15 s and 60 °C for 1 min. After cycling, relative quantification of mRNA against an internal control, GAPDH, was conducted according to the ΔC_T method (*36*).

Statistical Analysis. Data were analyzed by using analysis of variance (SAS Institute, Cary, NC). The significance of the difference between mean values was determined by one-way analysis of variance and Tukey's test; $p < 0.05$ was taken to be statistically significant.

RESULTS AND DISCUSSION

Cell Viability. The MTT assay was used to test whether the concentrations of Ap or Ap and forskolin used caused cell damage. There were no adverse effects on the growth of rat primary hepatocytes under these experimental conditions (**Figure 2**). In our study, the effects of Ap or Ap and forskolin observed were not due to cell viability.

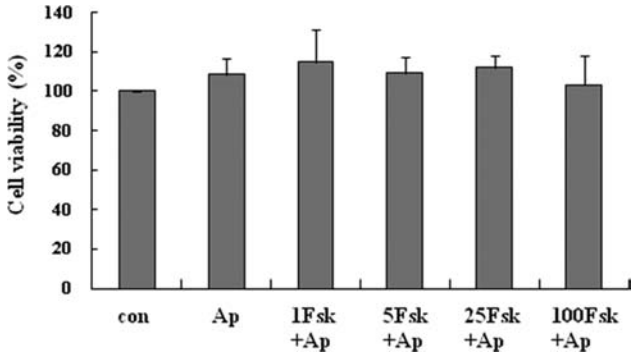

Figure 2. Effects of 40 μM Ap and of 40 μM Ap in combination with various concentrations of forskolin on cell viability. Results represent the mean ± SD (n = 3).

Ap-Induced GSTP Protein Expression Was Suppressed by Forskolin. To investigate the possible mechanisms involved in the induction of GSTP expression by Ap, we used MAPK inhibitors such as 25 μM PD98059 (an ERK1/2 inhibitor), 20 μM SP600125 (a JNK inhibitor), 20 μM SB203580 (a p38 MAPK inhibitor), and 25 μM forskolin (an adenylate cyclase activator). As shown in **Figure 3A**, 40 μM Ap significantly induced GSTP protein expression in rat primary hepatocytes and 25 μM forskolin significantly abolished this effect of Ap. The MAPK inhibitors, however, had no effect on GSTP protein expression induced by Ap (**Figure 3A**), which suggests that the ERK, JNK, and p38 MAPK pathways are not involved in the Ap-induced GSTP expression in rat primary hepatocytes. The concentrations of pharmacological inhibitors of ERK, JNK, and p38 MAPK we used were shown to be effective for their respective inhibitory functions in a previous study (*37*).

A dose−response inhibitory effect of forskolin (1−100 μM) on Ap-induced GSTP expression was further performed in rat primary hepatocytes. As shown in **Figure 3B**,**C**, forskolin suppressed Ap-induced GSTP expression in a dose-dependent manner, and a significant inhibition of both protein and mRNA levels was noted at doses ≥5 μM. These results imply that the downstream pathway of forskolin may be involved in the suppression of Ap-induced GSTP expression.

Ap-Induced GSTP Expression Was Suppressed by cAMP Analogues. Forskolin is known to increase intracellular cAMP and further activate cAMP-mediated signaling pathways (*38*). A previous study reported that treatment of hepatocytes with either forskolin or cAMP analogues results in a rapid and dramatic elevation of intracellular cAMP levels within 20 min (*39*). Increases of intracellular cAMP levels activates PKA and leads to suppression of the expression of phase I enzymes such as cytochrome P450 (CYP) 2B1 and CYP 3A1 in rat primary hepatocytes (*32*). Three highly membrane-permeable cAMP analogues, including dibutyryl-cAMP (DBT), 8-bromo-cAMP (Bromo), and 8-(4-chlorophenylthio)-cAMP (CPT), were tested to examine whether the effect of forskolin on GSTP expression was mediated by intracellular cAMP. As shown in **Figure 4A**, 1 and 10 μM DBT, 10 and 25 μM Bromo, and 0.5 and 2.5 μM CPT all significantly suppressed Ap-induced GSTP protein expression, and the inhibitory potency of the cAMP analogues was associated with their concentration. The GSTP mRNA expression pattern was similar to that of the protein (**Figure 4B**), although only the higher concentration of the cAMP analogues showed a significant effect.

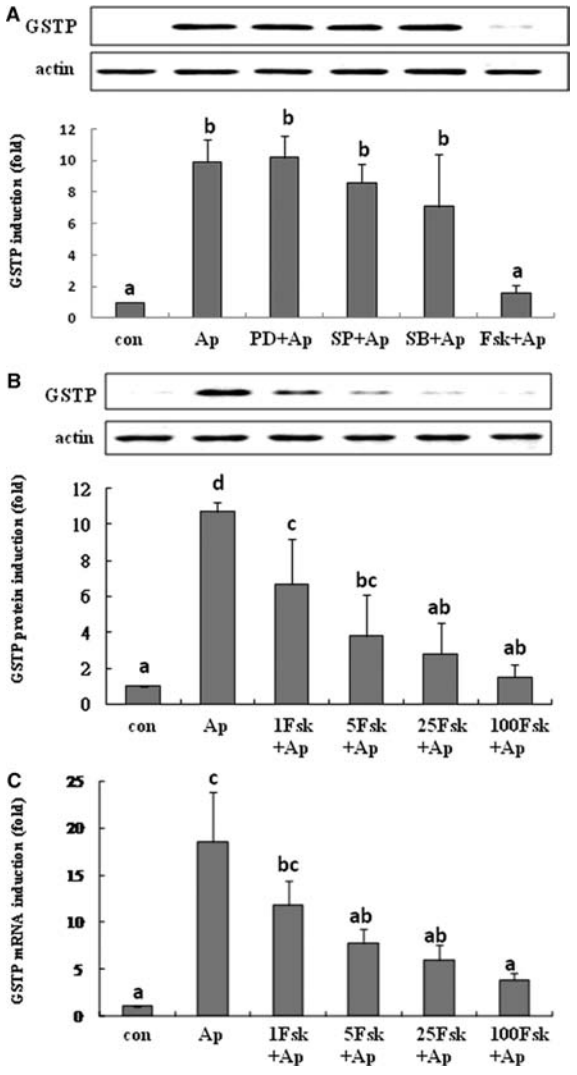

Figure 3. Effects of MAPK inhibitors and various concentrations of forskolin on Ap-induced GSTP expression. (**A**) Twenty-four hours after attachment, hepatocytes were pretreated with or without 25 μM PD98059 (PD), 20 μM SP600125 (SP), 20 μM SB203580 (SB), and 25 μM forskolin (Fsk) for 1 h before the addition of Ap. Cells were then treated with Ap for an additional 48 h. (**B**) Twenty-four hours after attachment, hepatocytes were pretreated with or without 1, 5, 25, or 100 μM forskolin (Fsk) for 1 h and were then incubated with 40 μM Ap for another 48 h. Aliquots of total protein (4 μg) were used for Western blot analysis. One representative experiment out of three independent experiments is shown (upper panel). Results represent the mean $\pm$ SD of three independent experiments (lower panel). Bars with different letters are significantly different ($p < 0.05$). (**C**) Aliquots of total RNA (0.8 μg) isolated from hepatocytes were subjected to real-time PCR analysis. Values of GSTP mRNA induction were normalized to the GAPDH mRNA level. Results represent the mean $\pm$ SD ($n = 3$). Bars with different letters are significantly different ($p < 0.05$).

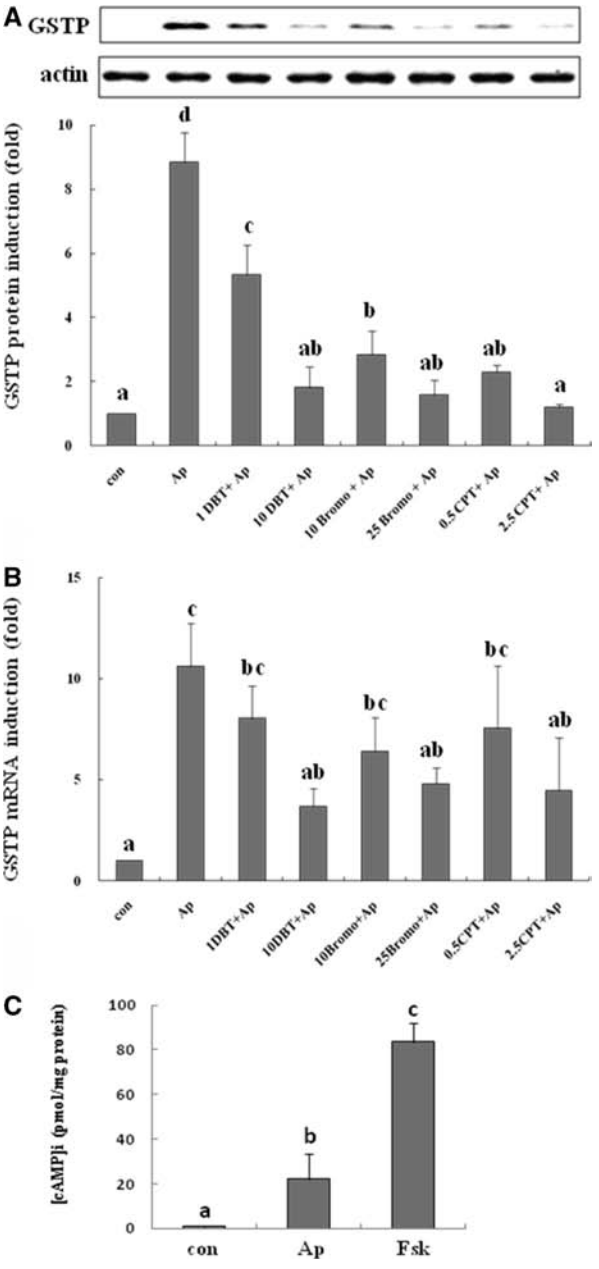

Figure 4. Effects of cell-permeable cAMP analogues on Ap-induced GSTP protein and mRNA expression and the changes of Ap and forskolin on the intracellular cAMP concentration. Twenty-four hours after attachment, hepatocytes were pretreated with or without 1 and 10 μM dibutyryl-cAMP (DBT), 10 and 25 μM 8-bromo-cAMP (Bromo), and 0.5 and 2.5 μM 8-(4-chlorophenylthio)-cAMP (CPT) for 1 h and were then incubated with 40 μM Ap for another 48 h. Cells were prepared for (**A**) GSTP protein and (**B**) GSTP mRNA determination. Values of GSTP mRNA induction were normalized to the GAPDH mRNA level. (**C**) Intracellular cAMP concentration of cells treated with 40 μM Ap or 25 μM forskolin. Results represent the mean $\pm$ SD ($n = 3$). Bars with different letters are significantly different ($p < 0.05$).

The changes of intracellular cAMP concentration following Ap and forskolin treatments were monitored, and results showed that both Ap and forskolin significantly increased intracellular cAMP concentrations in comparison with the control group (**Figure 4C**). The intracellular cAMP concentration was 82-fold that of the control with forskolin treatment. An increase of cAMP concentration was also noted in Ap-treated cells, and the intracellular cAMP level was 22-fold that of the control.

PKA Inhibitor Reversed the Suppression of cAMP analogues on Ap-Induced GSTP Expression. The cAMP-dependent PKA pathway is involved in the regulation of the expression of genes such as those encoding CYP 1A1 phase I enzyme (*40*), nuclear receptors, such as glucocorticoid receptor-interacting protein 1 (*41*), and phase III transport systems, such as organic anion transporting polypeptide 2 (*42*). In the present study, we used H89, a PKA inhibitor, to test whether the suppression of GSTP expression by forskolin is mediated through a cAMP-dependent PKA pathway. As shown in **Figure 5**, H89 significantly attenuated the suppression of GSTP expression by 10 μM DBT and 25 μM Bromo in the

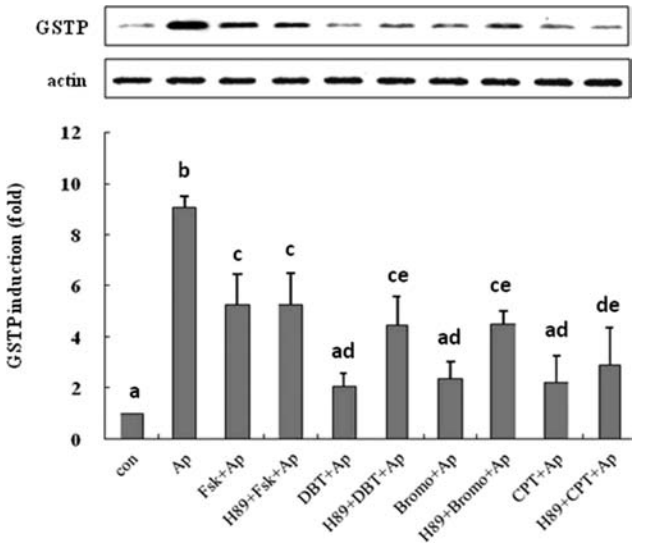

Figure 5. Effect of PKA inhibitor H89 on the suppression of GSTP protein expression by forskolin and cAMP analogues in the presence of Ap. Twenty-four hours after attachment, hepatocytes were pretreated with or without 7.5 μM H89 for 1 h and the cells were then treated with 5 μM forskolin (Fsk), 10 μM dibutyryl-cAMP (DBT), 25 μM 8-bromo-cAMP (Bromo), and 2.5 μM 8-(4-chlorophenylthio)-cAMP (CPT) for 1 h before the addition of Ap. Cells were then incubated with 40 μM Ap for another 48 h. One representative experiment out of three independent experiments is shown. Results in the lower panel represent the mean ± SD (n = 3). Bars with different letters are significantly different ($p < 0.05$).

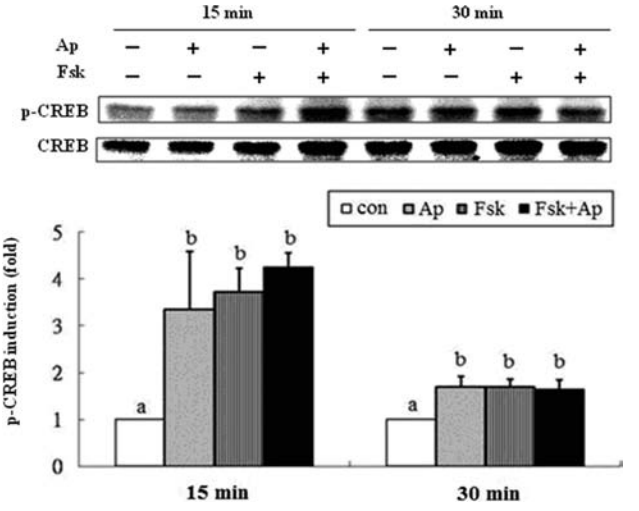

Figure 6. Effects of Ap and forskolin (Fsk) on CREB phosphorylation. Forty-eight hours after attachment, cells were treated with 40 μM Ap or 25 μM forskolin or cotreated with both Ap and forskolin for 15 or 30 min. After harvesting, aliquots of total protein (30 μg) were used for Western blot analysis. Results represent the mean ± SD (n = 3). Bars with different letters are significantly different within the group ($p < 0.05$).

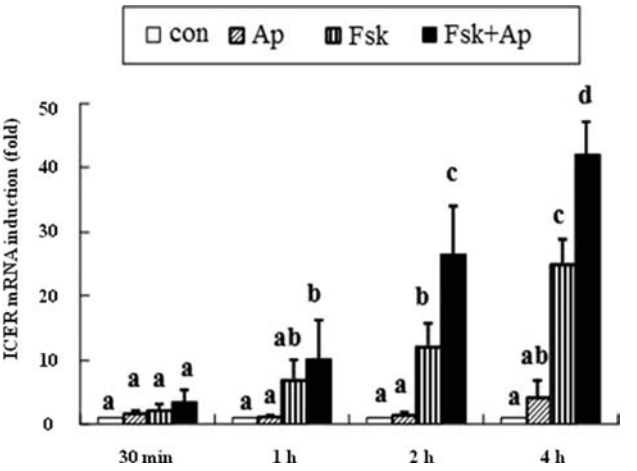

Figure 7. Effects of Ap and forskolin (Fsk) on the ICER mRNA level. Forty-eight hours after attachment, cells were treated with 40 μM Ap or 25 μM forskolin or cotreated with Ap and forskolin for 30 min or 1, 2, or 4 h. After harvesting, 0.8 μg of total RNA isolated from hepatocytes was subjected to real-time PCR analysis. Values of ICER mRNA induction were normalized to the GAPDH mRNA level. Results represent the mean ± SD (n = 3). Bars with different letters are significantly different ($p < 0.05$).

presence of Ap, which suggests that the cAMP-dependent PKA pathway is involved in this suppression by cAMP. However, H89 only showed a partial effect in the 2.5 μM CPT group and did not reverse the inhibition of Ap-induced GSTP protein expression by 5 μM forskolin, which may be attributed to the ineffective concentration of H89 used.

Effects of Ap and Forskolin on the Phosphorylation of CREB. To investigate whether Ap and forskolin induced the phosphorylation of CREB in hepatocytes, we assayed the effect of 40 μM Ap, 25 μM forskolin, or both on CREB phosphorylation at 15 and 30 min. The results showed that the phosphorylation of CREB was significantly increased in rat primary hepatocytes treated with forskolin or Ap or both for 15 and 30 min (**Figure 6**). The phosphorylation of CREB by Ap and forskolin is quick response. This result is in line with that of a previous study, which showed that the phosphorylation of CREB by forskolin occurred rapidly and the peak in phosphorylation was achieved within 15 min, decay began in 30 min, and phosphorylation returned to the basal level after 8 h in H35 hepatoma cells (*43*).

Effects of Ap and Forskolin on ICER mRNA Level. ICER is a transcriptional repressor (*17*), and its competition with other bZIP proteins, such as CREB and ATF (*17*, *44*), accounts for its inhibitory effect on the expression of several genes, including CYP 19, CYP 51, Rab3a, Rab27 GTPase, Granuphilin/S1p4, and Noc2 (*45*−*47*). It was reported that the cAMP-mediated ICER expression depends on CREB phosphorylation (*48*). In this study, both Ap and forskolin were shown to significantly increase the intracellular cAMP levels (**Figure 4C**) and the phosphorylation of CREB (**Figure 6**). To investigate the role of ICER involved in the suppression of GSTP expression, we studied the effects of Ap and forskolin on ICER mRNA expression in rat primary hepatocytes. As indicated in **Figure 7**, ICER mRNA expression was increased by forskolin in a time-dependent manner. It was significantly increased after 2 h, and the increase was further enhanced after 4 h (**Figure 7**). With Ap alone, ICER mRNA was not changed up to 4 h. However, in cells cotreated with forskolin and Ap, the expression of ICER mRNA was even greater than that of cells treated with forskolin alone. This finding suggests that there is a potentiating effect of Ap on forskolin-induced ICER mRNA expression and the inhibition of Ap-induced GSTP expression by forskolin in rat primary hepatocytes is likely related to the induction of ICER expression by forskolin.

ICER, a product of the CREM gene, is reported to be involved in an autoregulatory feedback loop of transcription that governs the suppression of early response genes, such as the proto-oncogene c-*fos* (*17*). Intriguingly, ICER mRNA expression was not induced by Ap; however, the induction of ICER mRNA expression was greater by Ap and forskolin cotreatment than by forskolin treatment alone (**Figure 7**). These results suggest that an

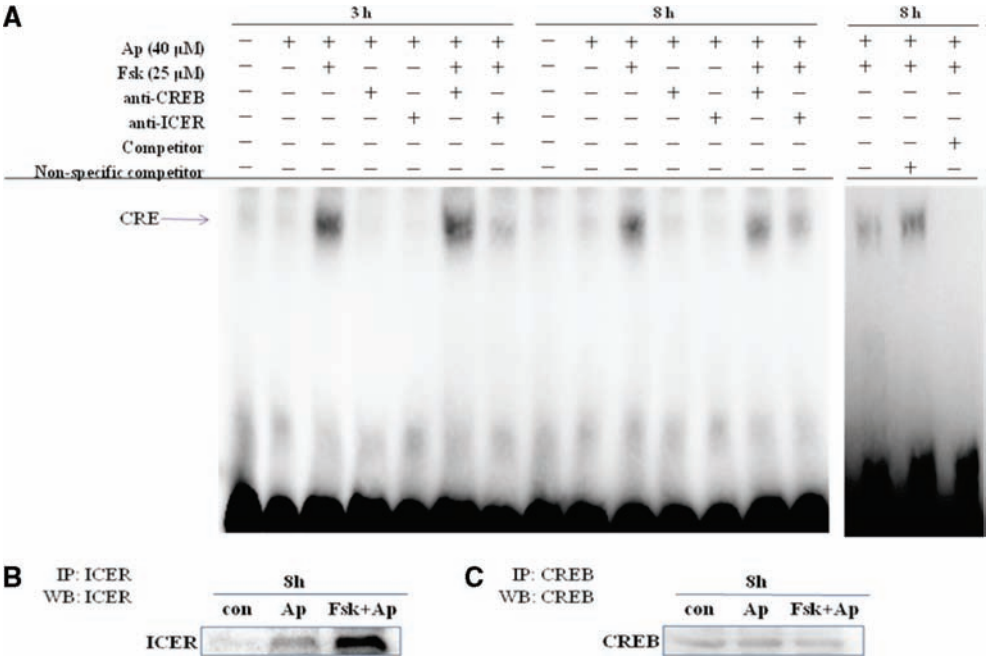

Figure 8. Identification of the component in the CRE complex by EMSA and immunoprecipitation (IP). Forty-eight hours after attachment, cells were treated with 40 μM Ap alone or cotreated with 40 μM Ap and 25 μM forskolin for 3 or 8 h. (**A**) Aliquots of total hepatocyte nuclear proteins (6 μg) were used for EMSA. To identify the protein bound to the CRE complex, either anti-CREB or anti-ICER antibody was added. To confirm the specificity of the nucleotides, 100-fold cold probe (biotin-unlabeled CRE) and NF-κB probe were included in the EMSA. One representative experiment out of three independent experiments is shown. The nuclear immunoprecipitates following antibody addition were subjected to Western blotting to confirm the presence of (**B**) ICER or (**C**) CREB in nuclear extracts.

interaction takes place between Ap and forskolin. Surprisingly, both Ap and forskolin significantly increased the intracellular cAMP level (**Figure 4C**), although forskolin showed a more potent effect and their effects on ICER formation were not consistent (**Figure 7**).

In a previous study (*49*), glucagon, glucagon-like peptide 1, and pituitary adenylyl cyclase-activating peptide all stimulate the formation of cAMP to a comparable extent in rat pancreatic islets, but only glucagon activates the expression of ICER. It was proposed that glucagon-like peptide 1 and pituitary adenylyl cyclase-activating peptide activate additional signal pathways that inhibit cAMP-mediated induction of ICER expression. For ICER formation, Ap may act in a manner similar to that for glucagon-like peptide 1 and pituitary adenylyl cyclase-activating peptide.

Effects of Ap and Forskolin on Nuclear CRE Complex Formation. In the present study, EMSA was performed to confirm whether the suppression of Ap-induced GSTP expression by forskolin was mediated by transcriptional factors binding to the CRE. As shown in **Figure 8A**, CRE complex formation was significantly increased in hepatocytes cotreated with Ap and forskolin for 3 and 8 h. To identify whether the component in the CRE complex was CREB or ICER, either anti-CREB or anti-ICER antibody was added to study their effects on EMSA. Results indicated that anti-ICER antibody apparently decreases nuclear protein binding to the CRE of cells cotreated with Ap and forskolin for 3 and 8 h (**Figure 8A**). In contrast, the binding of nuclear protein to the CRE was not affected by anti-CREB antibody. To further confirm the component in the CRE complex was ICER, the immunoprecipitates from nuclear extracts were thereafter subjected to SDS-PAGE and Western blotting. As shown, the amount of ICER in the nuclear immunoprecipitates was increased in cells cotreated with Ap and forskolin (**Figure 8B**); however, no effects of Ap and forskolin on CREB in the nuclear

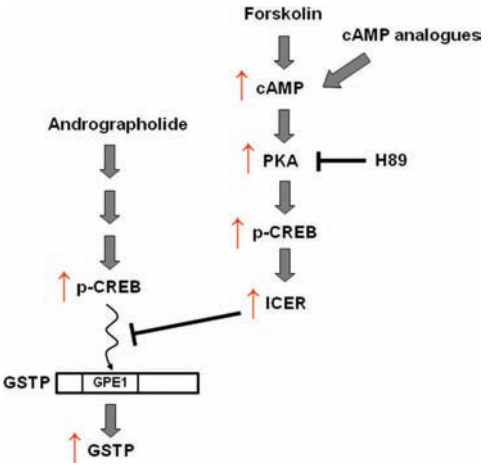

Figure 9. Model showing pathways that mediate cAMP suppresses Ap-induced GSTP expression in rat primary hepatocytes.

immunoprecipitates was found (**Figure 8C**). These results indicated that cotreatment with Ap and forskolin significantly increased nuclear ICER and subsequent binding to the CRE.

Although the intracellular cAMP level and the phosphorylation of CREB were increased by both Ap and forskolin (**Figure 4C**, **6**), the increased expression of ICER was only noted in cells treated with forskolin (**Figure 7**). Interestingly, the increase of ICER expression was even more significant in Ap- and forskolin-cotreated cells than in forskolin-treated cells. This raised the possibility that ICER or phospho-CREB/ICER protein ratio is likely to play a critical role in the suppression of Ap-induced GSTP expression by forskolin. The importance of phospho-CREB/ICER protein ratio in the transcriptional regulation of arylalkylamine *N*-acetyltransferase by norepinephrine has been reported in the rodent pineal gland (*50*).

The findings of the present study are schematically presented in **Figure 9**. This figure shows that Ap-induced expression of GSTP in rat primary hepatocytes is suppressed by an increase in cAMP level caused by forskolin, activation of cAMP/PKA pathway, phosphorylation of CREB, enhancement of ICER formation, binding of ICER to the CRE in the GSTP promoter, and concomitant attenuation of the CREB binding to the GPE1.

ABBREVIATIONS USED

Ap, andrographolide; AP-1, activator protein-1; ATF, activating transcription factor; ACTH, adrenocorticotropic hormone; bZIP, basic domain leucine zipper transcription factor; cAMP, 3,5′-cyclic adenosine monophosphate; CRE, cAMP-response element; CREB, cAMP response element-binding protein; CREM, CRE modulator; ERK, extracellular receptor kinase; ICER, inducible cAMP early repressor; JNK, c-Jun NH2-terminal kinase; GPE1, GSTP enhancer 1; GSTP, π class of glutathione *S*-transferase; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; TRE, phorbol-12-*O*-tetradecanoate-13-acetate responsive element.

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