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ARTICLE

# Butein Up-Regulates the Expression of the $\pi$ Class of Glutathione *S*-Transferase in Rat Primary Hepatocytes through the ERK/AP-1 Pathway

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Induction of phase II enzymes is an important mechanism of chemoprevention. Here we compared the effects of chalcones on the expression of the $\pi$ class of glutathione *S*-transferase (GSTP) in rat primary hepatocytes. Hepatocytes were treated with 10 or 25 $\mu$M of phloretin or butein for 24 h. Both butein and phloretin dose-dependently increased GSTP protein expression, and the induction potency of butein was stronger than that of phloretin. The increase in GSTP mRNA in cells treated with 25 $\mu$M of phloretin and butein was 107% and 211%, respectively ($P < 0.05$). Butein increased GST enzyme activity by 27% compared with that in the control cells ($P < 0.05$). In contrast, phloretin had no significant effect on GST enzyme activity. The pTA-luciferase reporter construct with the rat −2.7 kb GSTP promoter region was transiently transfected into Clone 9 liver cells, and the luciferase activity in butein-treated cells was 1.1-fold higher than that in control cells ($P < 0.05$). GSTP enhancer 1 (GPE1) deletion abolished the induction of reporter activity by butein. The phosphorylation of extracellular signal-regulated kinase (ERK), but not of c-Jun $NH_2$-terminal kinase (JNK) and p38, was stimulated in the presence of butein. Pretreatment with PD98059, an ERK inhibitor, alleviated the increase in activator protein-1 (AP-1)-DNA binding activity and also the activation of GSTP protein expression by butein. Moreover, c-Jun is likely to bind to the GPE1. Silencing of ERK2 by siRNA gene knockdown reduced the butein-induced expression of GSTP. In conclusion, the increased GSTP expression by butein is likely related to the ERK-AP-1 pathway.

**KEYWORDS: Butein; $\pi$ class of glutathione *S*-transferase; extracellular signal-regulated kinase; activator protein-1; rat primary hepatocytes**

## INTRODUCTION

Epidemiological studies have consistently shown an inverse association between the consumption of vegetables and fruits and the risk of human cancers (*1*, *2*). This association can be partly attributed to the high content of numerous phytochemicals, including polyphenolic compounds, carotenoids, and indoles, in vegetables and fruits (*2*−*4*). Flavonoids are members of naturally occurring polyphenolic compounds in vegetables, nuts, fruits, and herbs. Flavonoids are classified into chalcones, flavones, flavonols, flavanones, flavanols, anthocyanins, and isoflavones (*5*), and they are reported to have diverse biological activities, including antithrombotic, antiinflammatory, antioxidative, antiviral, and anticarcinogenic activities (*6*−*9*).

Flavonoids are reported to play an inhibitory role in tumorigenesis induced by various carcinogens in the skin, liver, and lung of rodents (*7*, *10*, *11*). Chalcones with two aromatic rings connected by a carbon side chain containing an $\alpha,\beta$-unsaturated ketone have been investigated for their chemopreventive potential (*12*, *13*). Supplementation with chalcones was shown to inhibit 4-nitroquinoline 1-oxide-induced oral cancer in male F344 rats (*13*). In addition, chalcones exhibited inhibitory effects on tumor promotion in skin cancer induced by 7,12-dimethylbenz[*a*]anthracene (*12*).

We hypothesized that the chemopreventive role of chalcones is partly ascribed to their modulation of phase I and phase II drug-metabolizing enzymes. Phase I and phase II biotransformation enzymes are involved in the metabolic activation and detoxification of various classes of environmental carcinogens (*14*). Chalcones have been shown to inhibit phase I enzymes (e.g., cytochromes P-450) (*15*) and to scavenge reactive oxygen species (*16*). In recent years, several studies have shown that flavonoids cause increases in the activities of phase II detoxifying enzymes such as glutathione *S*-transferases (GSTs), NAD(P)H/quinone reductases, and UDP-glucuronosyltransferases (*17*, *18*). Although chalcones are promising chemopreventive agents, their regulation of phase II drug-metabolizing enzyme is still obscure. Phase II enzyme systems catalyze the reduction or conjugation of water-soluble molecules to electrophilic xenobiotics, which results in the detoxification of electrophiles such as genotoxic chemical carcinogens and cytotoxic chemotherapeutic agents (*19*, *20*). Higher tissue levels of

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phase II detoxification enzymes correlate with lower susceptibility to carcinogen insult (*21*).

GSTs are phase II detoxification enzymes that catalyze the conjugation of glutathione (GSH) with a variety of electrophilic xenobiotics and facilitate their excretion. The cytosolic GSTs in mammalian tissues are classified into eight classes that exhibit overlapping yet distinct substrate specificities: A (alpha), M (mu), O (omega), P (pi), S (sigma), T (theta), Z (zeta), and K (kappa). The induction of the pi class of GST (GSTP) may be a reliable marker for evaluating the efficacy of potential inhibitors of chemical-induced cancer (*22*). GSTP has been shown to be highly efficient in the GSH conjugation of (+)-7,8-dihydroxy-9,10-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (*23*). A strong enhancer named GSTP enhancer 1 (GPE1), which has two phorbol-12-*O*-tetradecanoate-13-acetate responsive element (TRE)-like elements in the 5′ upstream region (*24*), is essential for GSTP induction. Multiple transcriptional factors, mainly activator protein-1 (AP-1), that bind to the TRE are responsible for the up-regulation of GSTP expression (*25*). AP-1 activity is regulated by various signaling pathways, including extracellular signal-regulated kinase (ERK), c-Jun $NH_2$-terminal kinase (JNK), and p38 kinase (*26*).

To determine the importance of chalcones in chemoprevention, we first examined the effects of phloretin and butein on the GSTP mRNA and protein expression in rat primary hepatocytes and then identified the role of GPE1 in GSTP expression. Moreover, we examined the signaling pathways involved in GSTP expression.

## MATERIALS AND METHODS

**Materials.** Phloretin, butein, ethacrynic acid, wortmannin, 1-chloro-2,4-dinitrobenzene (CDNB), dexamethasone, poly(dI-dC), β-mercaptoethanol, bovine serum albumin, deoxynucleotide triphosphate, and HEPES were obtained from Sigma Chemical Company (St. Louis, MO). $ITS^+$ (insulin, transferrin, selenium, bovine serum albumin, and linoleic acid) and type I rat tail collagen were purchased from BD Biosciences (San Jose, CA). RPMI-1640 medium and penicillin-streptomycin solution were obtained from Gibco Laboratory (Gaithersburg, MD). Fetal bovine serum was purchased from Hyclone (Logan, UT). RNase inhibitor, oligo dT, and Moloney murine leukemia virus reverse transcriptase were purchased from Promega Company (Madison, WI). GSTP and GAPDH primer were obtained from Applied Biosystems (Foster City, CA). Trizol and lipofectamine were ordered from Invitrogen (Carlsbad, CA). Collagenase was purchased from Worthington Biochemical (Lakewood, NJ). SP600125, SB203580, and PD98059 were purchased from TOCRIS (Bristol, U.K.). Antibodies against c-Jun, JNK, and phospho-JNK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against ERK, phospho-ERK (Thr202/Tyr204), p38, and phospho-p38 (Thr180/Tyr182) were purchased from Cell Signaling Technology (Beverly, MA).

**Cell Isolation and Culture.** Male Sprague-Dawley rats were purchased from LASCO (BioLASCO Taiwan Co., Ltd.) and were used for hepatocyte isolation when aged 7 to 8 weeks old. Rats were treated in compliance with the Guide for the Care and Use of Laboratory Animals (*27*). Hepatocytes were isolated by a two-step collagenase perfusion method as described previously (*28*). Cell viability was >90% as determined by trypan blue exclusion. The isolated hepatocytes were suspended in RPMI-1640 medium containing 10 mM HEPES, $1 \times 10^5$ unit/L penicillin, 100 mg/L streptomycin, 0.1 mM dexamethasone, and 1% $ITS^+$. The cells were plated in 35-mm plastic tissue culture dishes (Falcon, Franklin Lakes, NJ) precoated with rat tail collagen I at a density of $1 \times 10^6$ cells per dish and were incubated at 37 °C in a humidified atmosphere of 5% $CO_2$ and 95% air. Cell attachment on the culture dish was achieved 48 h after plating; fresh culture medium containing 10 or 25 μM of phloretin or butein (**Figure 1**) was then added for an additional 24 h. Inhibition of kinase activity by SP600125 (20 μM), SB203580 (20 μM), and PD98059 (20 μM) was performed 1 h before butein treatment. Cells treated with 0.1% dimethylsulfoxide (DMSO) alone were regarded as the control.

**SDS-PAGE and Western Blot.** Hepatocytes were washed twice with cold phosphate-buffered saline and were then harvested in 300 μL of 20 mM potassium phosphate buffer (pH 7.0). The homogenates were centrifuged at 10,000*g* for 30 min at 4 °C. Protein concentrations were determined with the Bradford Protein Assay Kit (OZ Biosciences, Marseille, France). Four micrograms of cellular proteins from each sample was applied to 10% SDS-polyacrylamide gels and electrophoretically transferred to polyvinylidene fluoride membranes. The membranes were blocked at 4 °C overnight with 50 g/L nonfat dry milk solution and were then incubated with antibodies against GSTP (BD Biosciences Pharmingen, Bedford, MA) or actin (Sigma Chemical, St. Louis, MO) for 70 min at room temperature and were subsequently incubated with horseradish peroxidase-conjugated secondary antibody. For the detection of MAPKs, the membranes were incubated overnight at 4 °C with anti-JNK1, anti-ERK1/2, and anti-p38 or antiphospho-JNK1, ERK1/2, and p38 antibodies. The bands were visualized by using an enhanced chemiluminescence kit (Perkin-Elmer Life Science, Boston, MA).

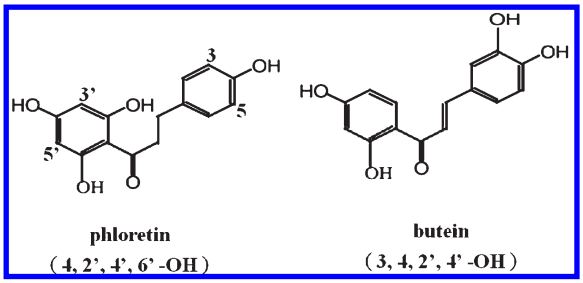


**Figure 1.** Structures of phloretin and butein.

**Real-Time PCR.** Total RNA was extracted by using Trizol reagent. Briefly, 0.2 μg of total RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase in a 20 μL of final volume of the reaction buffer containing 25 mM Tris-HCl (pH 8.3), 50 mM $(NH_4)_2SO_4$, 0.3% β-mercaptoethanol, 0.1 g/L bovine serum albumin, 5 mM $MgCl_2$, 1 mM each of deoxynucleotide triphosphate, 2.5 units RNase inhibitor, and 2.5 mM oligo dT. For the synthesis of cDNA, reaction mixtures were incubated for 15 min at 45 °C and the reaction was stopped by denaturing the reverse transcriptase at 99 °C for 5 min. Real-time PCR was carried out in an ABI Prism 7000 sequence detector (Applied Biosystems, Foster City, CA) by adding 5 μL of cDNA, 10 μL of Master Mixture, 5 μL of $ddH_2O$, and 1 μL of GSTP (Rn02770492_gh) and GAPDH (Mm99999915_gl) primers to each microwell. The reaction was run with the following program: 1 cycle at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The delta Ct (threshold cycle) method was used for quantification of the amplified gene targets according to the manufacturer's protocol (Applied Biosystems, Foster City, CA).

**GST Activity Assay.** GST activity was measured by using CDNB as the substrate. Briefly, the reaction mixture in a final volume of 1 mL contained 100 mM potassium phosphate buffer (pH 6.5), 1 mM glutathione, 50 mM CDNB, and an appropriate amount of the total proteins. The CDNB-glutathione conjugate formed was measured at 340 nm.

**Expression and Reporter Constructs.** The pTA-GSTP Luc reporter with the GSTP gene promoter region was constructed as described previously (*29*). A 2.7-kb fragment of the gene for GSTP was inserted into the MluI and NheI sites of the pTA-SEAP/Luc vector (Clontech, Palo Alto, CA). In addition to the full-length construct (pTA-2713), two constructs with deletions from −2713 to −2605 bp (pTA-2604) and from −2713 to −2376 bp (pTA-2375) were generated.

**Transient Transfection and Luciferase Activity Assay.** Clone 9 cells, which were derived from normal rat livers, were obtained from the Bioresources Collection and Research Center (BCRC, Taiwan). They were grown in RPMI-1640 medium supplemented with 10 mM HEPES, $1 \times 10^5$ unit/L penicillin, 100 mg/L streptomycin, and 10% fetal bovine serum at 37 °C in a humidified atmosphere of 5% $CO_2$ and 95% air. A total of $4 \times 10^5$ cells were plated on each 35-mm plastic tissue culture dish (Nuck, Roskilde, Denmark), and the dishes were incubated until 80% confluence was reached. Cells were transiently transfected for 7 h with 0.1 μg of the Luc-2713 vectors by lipofectamine reagent and were then exposed to phloretin or butein for an additional 24 h. Cells were then washed twice with phosphate-buffered saline and were lysed in 100 μL of lysis buffer. Luciferase activity was measured by using luciferase assay reagent (Clontech, Palo Alto, CA) according to the manufacturer's instructions.

The luciferase activity of each sample was corrected on the basis of β-galactosidase activity, which was measured at 420 nm with *O*-nitrophenyl β-D-galactopyranoside as a substrate. The value for cells treated with 0.1% DMSO vehicle was regarded as 1.

**Electromobility Gel Shift Assay.** Hepatocytes were treated with butein for 1 h and were then washed twice with cold phosphate-buffered saline and scraped from the dishes with phosphate-buffered saline. Crude nuclear extracts were prepared according to the method described previously (*30*). The LightShift Chemiluminescent electromobility gel shift assay (EMSA) kit (Pierce Chemical Company, Rockford, IL) and synthetic biotin-labeled double-stranded AP-1 consensus oligonucleotides (5′-CGCTTGATGACTCAGCCGGAA-3′) or GPE1 oligonucleotides (5′-AGTAGTCAGTCACTATGATTCAGCAAC-3′) were used to measure the effect of butein on AP-1 and GPE1 binding activity. Unlabeled double-stranded oligonucleotides (200 ng) and mutant double-stranded oligonucleotides were also used to confirm specific binding. Two micrograms of nuclear protein, poly(dI-dC), and biotin-labeled double-stranded AP-1 or GPE1 oligonucleotides were mixed with the binding buffer to a final volume of 20 μL and were incubated at room temperature for 30 min. The nuclear protein−DNA complex was separated by electrophoresis on a 6% Tris−boric acid−EDTA−polyacrylamide gel and was then electrotransferred to a Hybond-$N^+$ nylon membrane (GE Healthcare, Buckinghamshire, U.K.). The membrane was treated with streptavidin-horseradish peroxidase, and the nuclear protein−DNA bands were developed by using an enhanced chemiluminescence kit.

**Immunoprecipitation.** Nuclear proteins were first incubated with anti-c-Jun antibody overnight at 4 °C. The cells were mixed with 0.1 g/L of Protein A-Sepharose beads for 1 h at 4 °C. Immunoprecipitated complexes were pelleted by centrifugation at 16000*g* for 2 min at 4 °C. The supernatant was then subjected to electrophoresis followed by EMSA.

**Transient Transfection of Small RNA Interference.** Hepatocytes were seeded at a density of $3 \times 10^6$ cells/dish in a 60-mm plastic tissue culture dish. Twenty-four hours after attachment, for ERK2 small interfering RNA (siRNA) transfection, the cells were transfected with ERK2-siRNA (100 nM) or nontargeting control siRNA by using DharmaFECT siRNA transfection reagent according to the manufacturer's instructions (all from Thermo Fisher Scientific, Lafayette, CO) for 24 h. The sense sequences of these ERK2 siRNAs were as follows: (1) 5′-ACACUAAUCUCUCGUACAU-3′; (2) 5′-AAAAUAAGGUGCCGUGGAA-3′; (3) 5′-UAUACCAAGUCCAUUGAUA-3′; and (4) 5′-UCGAGUUGCUAUCAAGAAA-3′. Twenty-four hours after transfection, the cells were treated with 25 μM butein for 24 h and protein expression was examined by Western blot analysis.

**Statistical Analysis.** Statistical analysis was performed with commercially available software (SAS Institute Inc., Cary, NC). Data were analyzed by means of one-way ANOVA, and the significant difference among treatment means was assessed by use of Tukey's test. A value of $P < 0.05$ was considered to be significant.

## RESULTS

**GSTP Protein and mRNA Expression and Enzyme Activity.** The study of cell viability by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method showed that neither phloretin nor butein at 25 μM caused cell toxicity in rat primary hepatocytes (supplemental file). The immunoblot assay showed that phloretin and butein dose-dependently increased GSTP protein levels in rat primary hepatocytes, and the increase in expression caused by butein was higher than that caused by phloretin (**Figure 2A**). Phloretin and butein at 25 μM increased the GSTP protein level by 2.7- and 5.9-fold, respectively, compared with that of the control cells (**Figure 2B**). By real-time PCR, mRNA levels were greater in cells exposed to phloretin or butein than in control cells (**Figure 3A**). Additionally, we used CDNB as a substrate to measure GSTP activity. Compared with that of the control cells, enzyme activity was increased by butein treatment ($P < 0.05$) (**Figure 3B**). Phloretin, however, had no significant effect on GSTP enzyme activity.

**GSTP Promoter Activity.** Clone 9 cells were used to measure pTA-GSTP luciferase reporter activity, and the response of GSTP to phloretin and butein was examined. The induction of GSTP

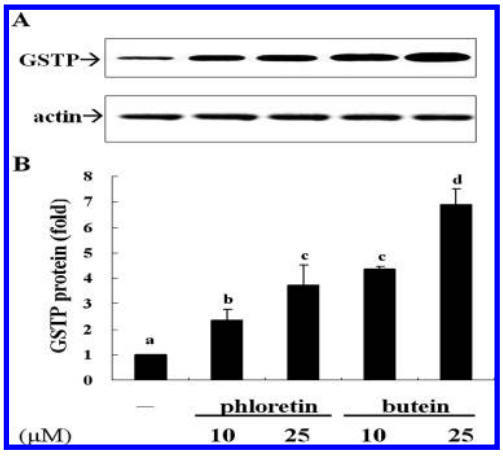


**Figure 2.** Protein levels of the π class of glutathione *S*-transferase (GSTP) induced by chalcones in rat primary hepatocytes. After 48 h of plating, cells were cultured with 0.1% dimethylsulfoxide (DMSO) alone (−) or with 10 or 25 μM of phloretin or butein for an additional 24 h. (**A**) GSTP protein was determined by immunoblot assay. (**B**) Changes in GSTP protein expression were measured by densitometry. The level with DMSO treatment was set at 1. Values are means ± SD, $n = 3-4$. Groups not sharing a common letter differ significantly, $P < 0.05$.

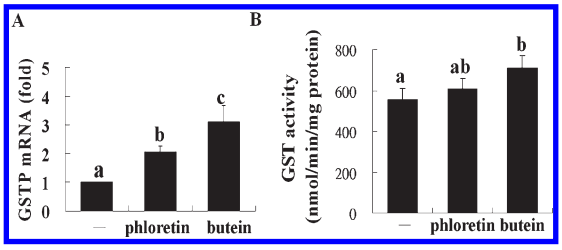


**Figure 3.** mRNA expression and enzyme activity of the π class of glutathione *S*-transferase (GSTP) induced by chalcones in rat primary hepatocytes. After 48 h of plating, cells were cultured with 0.1% dimethylsulfoxide (DMSO) alone (−) or with 25 μM of phloretin or butein for an additional 24 h. (**A**) Real-time PCR of GSTP mRNA expression. The GSTP mRNA level in the control cells was regarded as 1. (**B**) The activity of GST was determined by using CDNB as a substrate. Values are means ± SD, $n = 3-4$. Means not sharing a common letter differ significantly, $P < 0.05$.

protein expression by butein was similar to that noted in rat primary hepatocytes (**Figure 4A**). In the Clone 9 cells, butein increased the GSTP protein level by 5.2-fold. However, no significant change was noted in cells treated with phloretin. Three pTA-GSTP luciferase reporters were constructed (**Figure 4B**). The pTA-2713 reporter construct included both GPE1 and GPE2, whereas the pTA-2604 reporter construct contained only GPE2. Neither enhancer was present in the pTA-2375 construct. After transient transfection of Luc-2713 reporter into Clone 9 cells, butein caused a 1.1-fold increase in luciferase activity compared with that of the control cells. In contrast, luciferase activity was not significantly changed by phloretin. This induction of reporter activity was completely abolished when the −2713 to −2604 bp region of the GSTP promoter was deleted. The induction of reporter activity was also absent in cells transfected with the pTA-2375 reporter construct. These results indicate that the sequences between −2713 bp and −2604 bp of the promoter region are responsible for the up-regulation of GSTP transcription by butein.

**Butein and MAPK Kinases.** MAPK signaling pathways, including JNK, ERK, and p38, are upstream mediators of AP-1. The activation of individual MAPK kinases at different time points was determined. Immunoblot analysis showed that butein

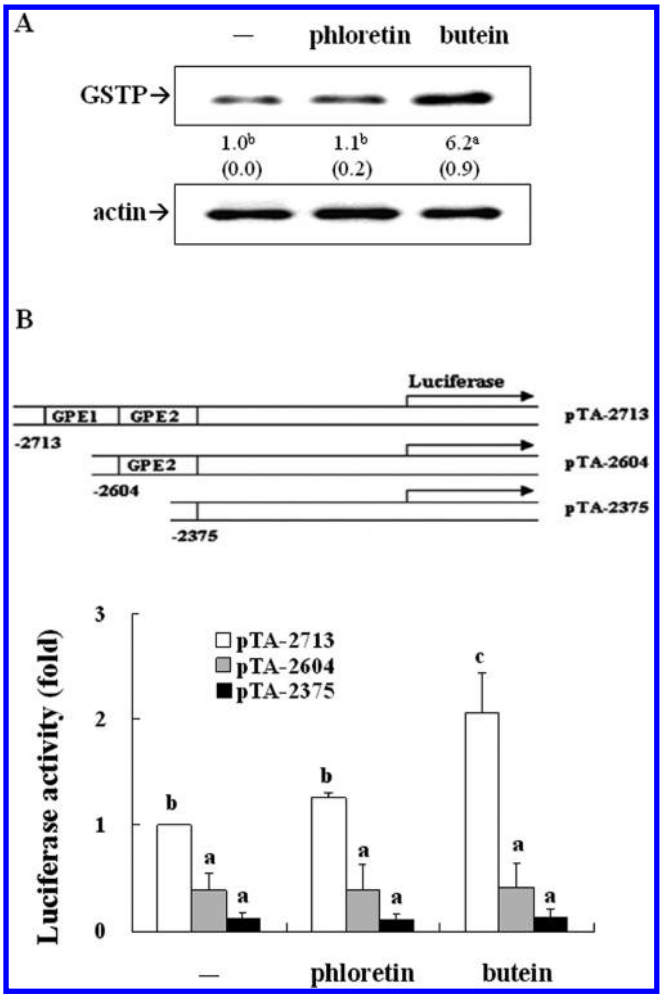


**Figure 4.** GPE1 is required for the up-regulation of GSTP by butein. (**A**) Protein levels of GSTP induced by phloretin or butein in Clone 9 cells. Cells were treated with 0.1% dimethylsulfoxide (DMSO) alone (−) or with 10 $\mu$M of phloretin or butein for 24 h. The protein was quantified by densitometry, and the level in the DMSO group was set at 1. Values are means (SD), $n = 3$. (**B**) Changes in GSTP promoter activity. Serial deletions of the pTA-GSTP Luc DNA constructs (pTA-2713, pTA-2604, and pTA-2375) were transfected into Clone 9 cells for 24 h. The activity of cells transfected with pTA-2713 and treated with DMSO alone was set at 1. Values are means ± SD, $n = 3$. Means not sharing a common letter differ significantly, $P < 0.05$.

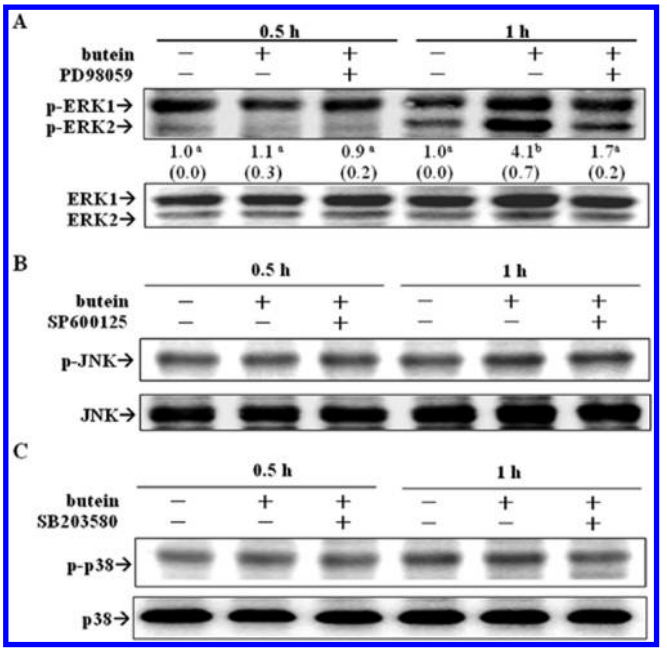


**Figure 5.** Activation of ERK1/2 by butein in rat primary hepatocytes. After 48 h of plating, cells were cultured with 0.1% dimethylsulfoxide (DMSO) alone (−) or with 25 $\mu$M of butein for the indicated time points in the presence or absence of PD98059 (**A**), SP600125 (**B**), and SB203580 (**C**), which were added to cells 1 h before butein treatment. The protein was quantified by densitometry, and the level in the control cells was set at 1. Values are the pooled results of both ERK1 and ERK2 and are expressed as means (SD), $n = 3$. Means at the same time not sharing a common letter differ significantly, $P < 0.05$.

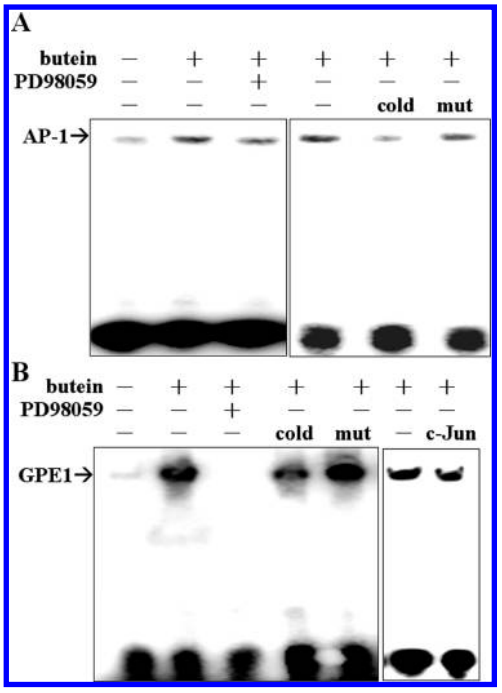


**Figure 6.** Activation of AP-1 and GPE1 binding activity by butein in rat primary hepatocytes. After 48 h of plating, cells were treated with 0.1% dimethylsulfoxide (DMSO) alone (−) or with 25 $\mu$M of butein for 1 h in the presence or absence of PD98059, and nuclear extracts were then prepared to measure AP-1 (A) or GPE1 (B) binding activity by electromobility gel shift assay. The supernatant after immunoprecipitation with anti-c-Jun antibody was used for EMSA. Unlabeled double-stranded oligonucleotides (cold) and mutant double-stranded oligonucleotides (mut) were added for the specificity assay.

increased ERK phosphorylation at 60 min (**Figure 5A**), but not the phosphorylation of JNK or p38 (**Figure 5B** and **C**). Pretreatment of cells with PD98059, an ERK inhibitor, suppressed the activation of ERK by butein.

**Protein Binding Activity on AP-1 by EMSA.** The activation of nuclear AP-1 and GPE1 by butein was measured by EMSA. As indicated, the DNA binding activity of AP-1 (**Figure 6A**) and GPE1 (**Figure 6B**) was increased in cells treated with butein for 1 h. The specificity of the DNA−protein interaction for AP-1 or GPE1 was demonstrated by a competitive assay with a 200-fold excess of unlabeled double-stranded oligonucleotides (cold) and also with mutant double-stranded oligonucleotides (mut). In the presence of PD98059, the activation of AP-1 and GPE1 by butein was attenuated. In addition, an immunoprecipitation with anti-c-Jun antibody was performed before EMSA. As noted, anti-c-Jun antibody diminished the binding of nuclear proteins to GPE1 oligonucleotides.

**Butein Activates GSTP via ERK.** Finally, we determined whether the up-regulation of GSTP protein expression by butein

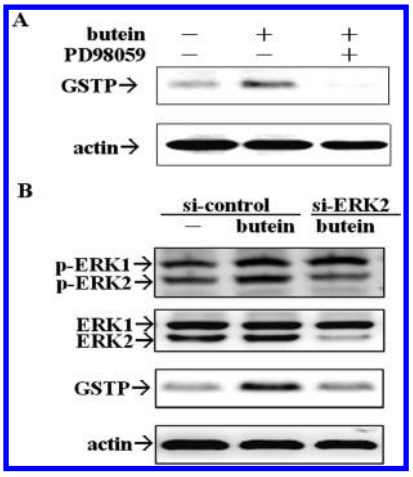


**Figure 7.** PD98059 and ERK2-siRNA suppress butein-induced GSTP protein expression in rat primary hepatocytes. (**A**) After 48 h attachment, cells were cultured with 0.1% dimethylsulfoxide (DMSO) alone (−) or with 25 $\mu$M butein for 24 h in the presence or absence of PD98059, which was added to cells 1 h before butein treatment. (**B**) Cells were transfected with ERK2-siRNA (si-ERK2) or nontargeting control siRNA (si-control) for 24 h. The transfected cells were then treated with 25 $\mu$M of butein for 24 h and the expression of GSTP protein was measured by Western blot. The activation of ERK in hepatocytes treated with butein for 1 h is shown. One representative immunoblot out of three independent experiments is shown.

is mediated by ERK. As noted, PD98059 suppressed the expression of this phase II detoxification enzyme by butein (**Figure 7A**). To further confirm these observations, cells were transfected with siERK2. ERK2 but not ERK1 plays a key role in rat hepatocyte replication (*31*). It can fulfill most ERK1 functions in several cells (*32*). The butein-induced GSTP protein was alleviated by silencing ERK2 expression with specific siRNA, whereas transfection of the cells with the same amount of negative control siRNA was not effective (**Figure 7B**). These results indicate that ERK is required for the induction of GSTP expression by butein.

## DISCUSSION

Flavonoids have been promoted as health-promoting, disease-preventing, and cancer-preventing agents (*7*, *10*, *11*). One of the major mechanisms of the anticarcinogenic effects of flavonoids is their activation of phase II detoxifying enzymes, such as GSTs, which results in the detoxification of carcinogens. The importance of GSTP in cancer prevention is supported by the finding that mice lacking this detoxification enzyme show a significantly greater incidence of benzo[*a*]pyrene-induced lung cancer (*19*). A point mutation in the GSTP gene leading to a decrease in enzyme activity has also been reported to be associated with increased cancer risk of the oral cavity, bladder, lung, testicles, larynx, and breast (*33*). Because GSTP can be induced by numerous dietary factors, it is accepted that enhancement of GSTP expression and activity through a dietary regimen is a practical means of cancer chemoprevention. Dietary administration of flavone may exert chemopreventive effects in the stomach, small intestine, and liver by enhancing the GSTP detoxification enzyme (*34*). The results of the present study indicate that two chalcones, phloretin and butein, differentially up-regulate GSTP expression in rat primary hepatocytes. Moreover, we further showed that such up-regulation by butein is likely ERK-dependent.

In the present study, butein and phloretin increased GSTP protein and mRNA expression in rat primary hepatocytes and the effects of the chalcones were in the order butein > phloretin (**Figures 2** and **3**). GST enzyme activity was also increased by butein treatment, whereas phloretin had no significant effect. Butein with the hydroxylation at C3 in the B-ring and a C2═C3 double bond in the C-ring was a more potent inducer than phloretin, which implies that these structural features of butein may be associated with its stronger induction capability. In addition, in human hepatocellular carcinoma HepG2 cells, Yang et al. (*35*) also showed that butein is a more potent inhibitor of epidermal growth factor receptor tyrosine kinase activity than phloretin, and this could be attributed to the neighbor hydroxylation at C3 and C4 on the B-ring. Structure−function relationship study has been widely used to examine the relative biological activity of structurally related flavonoids (*36*,*37*). The order of their potency at suppressing human liver HepG2 cancer cells is chalcones > flavones > isoflavones > flavanones. An analysis of structure−activity relationship showed that the unique backbone structure of chalcones with an open C-ring, the 2′-OH in the B-ring of chalcones, and the 3′-OH in the B-ring of flavones are required for their inhibitory effect (*38*). The structural features of flavonoids that are important for their cell transformation−inhibitory activity are the 3′- and 4′-OH on the B-ring, the 3-OH on the C-ring, and the C2═C3 double bond in the C-ring (*36*). Several structural features, including the C2═C3 double bond and the 3-OH group in the C-ring and the presence of both the 5-OH and 7-OH groups in the A-ring, were found to be important to confer the high antioxidant activity of flavonoids toward hypobromous acid (*37*).

We previously demonstrated the essentiality of GPE1 for GSTP induction by allyl sulfides in garlic (*29*). In the present study, pTA-GSTP Luc DNA constructs as used for the garlic allyl sulfides study were transfected into Clone 9 liver cells to study the role of GPE1 and GPE2 in the induction of GSTP by butein. Clone 9 liver cells, which are a permanently growing, nontransformed rat liver cell line (*39*), are derived from normal rat liver and retain an epithelial morphology. The cell line has been widely used as a model to study the function of hepatocytes, including the expression of GSTP (*29*). By constructing Luc-reporters through serial deletion of the 5′-flanking region of the GSTP gene, we clearly showed that the section from −2713 bp to −2605 bp, which contains an enhancing element named GPE1, is required for the induction of GSTP expression by butein in Clone 9 cells (**Figure 4**). However, the second enhancer GPE2 (−2604 bp to −2376 bp), which is adjacent to the GPE1, had no influence on the induction of the GSTP gene. These findings agree with reports by others that the highly inducible characteristic of GSTP is attributed mostly to GPE1 and not to GPE2 (*40*).

GPE1 contains two TRE-like elements, and both elements are required for the basal and inducible expression of GSTP (*24*). Deletion of the TRE abolishes the induction of GSTP transcription by 3,4,5,3′,4′-penta-chlorinated biphenyl in primary hepatocytes (*41*). Several transcriptional factors, particularly AP-1, have been shown to participate in the transcriptional activation of the enhancer of the GSTP gene (*42*). AP-1 motifs commonly compose either Jun homodimers or Jun/Fos heterodimers. In our previous study, we implicated AP-1 in the up-regulation of GSTP expression by garlic allyl sulfides in Clone 9 liver cells (*30*). To verify whether c-Jun binds to the GPE1 by butein, we performed an assay combining immunoprecipitation and EMSA. Our results clearly showed that c-Jun is likely to bind to the GPE1. In addition to AP-1, nuclear factor erythroid-2 related factor 2 (Nrf2) is a possible transcriptional factor that may up-regulate GSTP expression, because the TRE-like elements on GPE1 share sequence similarity with the antioxidant response element (ARE). The binding of Nrf2 to the ARE is well-known to up-regulate the transcription of several phase II detoxification enzymes, including NAD(P)H/quinone oxidoreductase and heme oxygenase (*43*). Evidence has shown that the Nrf2/Mafk heterodimer can bind to

GPE1 and up-regulate GSTP transcription in the early hepatocarcinogenesis stage of rat H4IIE hepatoma cells (*20*). Moreover, the induction of GSTP by 6-methylsulfinylhexyl isothiocyanate of wasabi and oltipraz is completely abrogated in Nrf2-deficient mice (*44*). Taken together, these data suggest that Nrf2 is a possible candidate to regulate the induction of GSTP expression by butein. Future study is warranted to answer this question.

The signaling pathways involved in AP-1 and Nrf2 activation are not fully clear. The ERK, JNK, and p38 kinase pathways are known to be a common signal mediating AP-1 and Nrf2 activity (*26*, *45*). In recent years, evidence has indicated that activation of the MAPKs is closely associated with the biological effects of chalcones. Pretreatment of human intestinal epithelial HT-29 cells with 2′,4′,6′-tris(methoxymethoxy)chalcone increases the phosphorylation of ERK and p38 kinase, which leads to the nuclear translocation of Nrf2 and consequent heme oxygenase 1 expression (*46*). The synthetic 2′-methoxy-4′6′-bis(methoxymethoxy)chalcone inhibits nitric oxide production in lipopolysaccharide-stimulated RAW 264.7 macrophages via down-regulation of p38 and JNK and induction of Nrf2 (*47*). In the present study, ERK inhibitors and ERK2 siRNA decreased the effect on butein-stimulated AP-1 activation and GSTP induction (**Figures 5**−**7**). In contrast, the lack of effect by SP600125 and SB203580 suggests that JNK and P38 are not involved in the action of butein.

In conclusion, our results suggest that the effectiveness of butein on GSTP expression is likely related to the ERK signaling pathway. Moreover, butein enhances the binding of AP-1 to GPE1.

## ABBREVIATIONS

AP-1, activator protein-1; ARE, antioxidant response element; DMSO, dimethylsulfoxide; EMSA, electromobility gel shift assay; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSTs, glutathione *S*-transferases; GSTP, $\pi$ class of GST; GPE1, GSTP enhancer 1; GPE2, GSTP enhancer 2; JNK, c-Jun $NH_2$-terminal kinase; MAPKs, mitogen-activated protein kinases; Nrf2, nuclear factor erythroid-2 related factor 2; siRNA, small interfering RNA; TRE, phorbol-12-*O*-tetradecanoate-13-acetate responsive element.

**Supporting Information Available:** Chart of effect of chalcones on cell viability in primary rat hepatocytes. This material is available free of charge via the Internet at http://pubs.acs.org.

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