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Induction of Phenolsulfotransferase Expression by Phenolic Acids in Human Hepatoma $HepG_2$ Cells

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Phenolic acids are antioxidant phenolic compounds, widespread in plant foods, which contribute significant biological and pharmacological properties; some have demonstrated a remarkable ability to alter sulfate conjugation. However, the modulation mechanisms of antioxidant phenolic acids on phenolsulfotransferase activity have not yet been described. In the present study, the human hepatoma cell line, $HepG_2$, was used as a model to investigate the effect of antioxidant phenolic acids on enzymatic activity and expression of one of the major phase II sulfate conjugation enzymes, P-form phenolsulfotransferase (PST-P). The results showed that gallic acid, gentisic acid, *p*-hydroxybenzoic acid, and *p*-coumaric acid increased PST-P activity, in a dose-dependent manner. A maximum of 4- and 5-fold induction of PST-P activity was observed for both gallic acid and gentisic acid; however, they showed an adverse effect on cell growth at higher concentrations. A 2- or 2.5-fold increase of PST-P activity was found with either *p*-coumaric or *p*-hydroxybenzoic acid treatment, whereas no significant effect was found for ferulic acid treatment. PST-P induction, by gallic acid, was further confirmed, using reverse transcription PCR and Western blotting techniques to measure mRNA expression and protein translation. A significant correlation ($r = 0.74$, $p < 0.01$) between the expressions of PST-P mRNA and the corresponding PST-P activity was observed. Thus, gallic acid increased PST-P protein expression in $HepG_2$ cells, in a dose- and time-dependent manner. The results demonstrated that certain antioxidant phenolic acids could induce PST-P activity in $HepG_2$ cells, by promoting PST-P mRNA and protein expression, suggesting a novel mechanism by which phenolic acids may be implicated in phase II sulfate conjugation.

KEYWORDS: Sulfation; P-form phenolsulfotransferase; phenolic acids; human hepatoma cell line $HepG_2$

INTRODUCTION

Interest in food phenolics, found mainly in fruits and vegetables, has recently been increasing, owing to their role as antioxidants, with implications for the prevention of pathologies such as cancer (*1*), cardiovascular diseases (*2*), and inflammatory disorders (*3*). There has been a growing interest in the naturally occurring anticarcinogenic substances found in plant foods. Plant phenols, for example, flavonoids and phenolic acids, are currently considered one of the most promising groups of potential dietary anticarcinogens (*4*). Plant phenolic acids are of current interest, due to their important biological and pharmacological properties, especially for their anti-inflammatory (*5*), oxygen free radical scavenging (*6*), and antimutagenic and anticarcinogenic activities (*7*). The beneficial effects of phenolic acids can be traced to their antioxidant properties (*8*) and, therefore, they may play a role in both cytoprotection and chemoprevention.

Chemoprevention is one of the most promising areas in cancer research. Potential chemopreventive agents may function by a variety of mechanisms directed at all major stages of carcinogenesis. One proposed mechanism for cellular protection, against the chemical and neoplastic effects of carcinogens, involves the induction of phase II detoxification enzymes (*9*). Many carcinogens are not in their full carcinogenic forms when first encountered. They are usually metabolized to proximate carcinogens by phase I enzymes, for example, cytochromes P450, that catalyze an oxidative reaction. The oxidized metabolites of potentially carcinogenic xenobiotics are then detoxified, by phase II metabolizing enzymes, into forms that are relatively inert and more easily excreted. There is considerable evidence that induction of phase II detoxification enzymes can modulate the threshold for chemical carcinogenesis, increasing cellular resistance to carcinogen exposure (*10*). The phenolsulfotransferases (PSTs) are the main phase II sulfoconjugation enzymes for catecholamines, thyroid hormones, and drugs, thereby facilitating biliary or urinary excretion and detoxification (*11*). Sulfoconjugation plays not only an important role in xenobiotic metabolism but also a critical role in steroid biosynthesis, as well as modulating the biological activity and facilitating the inactivation and elimination of potent endogenous chemicals, including steroids, catecholamines, and thyroid hormones. Some

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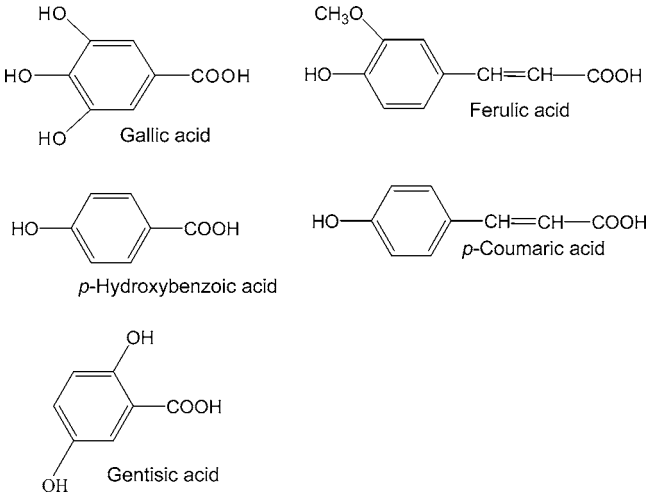

Figure 1. Chemical structures of phenolic acids.

evidence has shown that harmful substances may accumulate in the body when phenolsulfotransferase activity is inhibited (*12*). Therefore, measuring the induction of phenolsulfotransferase activity may provide an efficient approach to understanding the chemopreventive mechanisms of dietary compounds.

Our previous studies revealed that *p*-hydroxybenzoic acid, gentisic acid, ferulic acid, gallic acid, and *p*-coumaric acid can increase the activities of both P-form and M-form phenolsulfotransferases. These phenolic acids were also found to possess antioxidant capacity in the ORAC and TEAC assays (*13*). Moreover, the activity of PST-P could be promoted by combinations of all of these phenolic acids (*14*). The overall effects of phenolic acids, on the activities of PST-P, are highly correlated to their ORAC values, suggesting that phenolic acids may alter sulfate conjugation. However, the molecular mechanisms by which phenolic acids regulate the activities of phase II sulfoconjugation enzymes have not yet been elucidated.

The hepatoma cell line, $HepG_2$, not only resembles morphologically normal hepatocytes (*15*) but has also been shown to retain many of the enzymes involved in xenobiotic metabolism, including a functional aryl hydrocarbon receptor (AhR) (*16*), an inducible sulfotransferase (*17*), and an inducible NAD(P)H quinone oxidoreductase (*18*). In this cell line, phenolsulfotransferase is also inducible, the predominant isoforms, present in the control cells, being the phenol (P) and monoamine (M) forms of human phenolsulfotransferases (*19*). To experimentally test the hypothesis that phenolic acids may induce the activity of phase II sulfoconjugation enzymes, we used this highly differentiated human hepatoma cell line, $HepG_2$, as a model to assess the effects of five phenolic acids (**Figure 1**) on the induction of PST-P activity. In addition, the mRNA expression and protein translation of the PST-P, by reverse transcription PCR and Western blotting techniques were developed and performed to further confirm a possible regulatory mechanism.

MATERIALS AND METHODS

Cell Culture and Chemicals. Human hepatoma cells ($HepG_2$ cells) were obtained from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsin Chu, Taiwan). Cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (v/v) fetal bovine serum (Gibco BRL, Grand Island, NY), 100 units/mL penicillin, 100 μg/mL streptomycin, 0.37% (w/v) $NaHCO_3$, 0.1 mM NEAA, 1 mM sodium pyruvate, and 0.03% L-glutamine at 37 °C, in a humidified atmosphere of 95% air and 5% CO_2. The culture medium was renewed each day. Cells were detached weekly for transfer with 0.1% trypsin and 10 μM EDTA in phosphate-buffered saline (PBS). DMEM, fetal bovine serum (FBS), and trypsin−EDTA (T/E) were obtained from Gibco BRL; *p*-hydroxybenzoic acid, gentisic acid (2,5-dihydroxybenzoic acid, 98% purity), ferulic acid (*trans*-4-hydroxy-3-methoxycinnamic acid, 99% purity), gallic acid (3,4,5-trihydroxybenzoic acid, 98% purity), *p*-coumaric acid (*trans*-4-hydroxycinnamic acid), dopamine, sucrose, and Na_2EDTA were obtained from Sigma Chemical Co. (St. Louis, MO); ^{35}S-labeled 3′-phosphoadenosine-5′-phosphosulfate ($PAPS^{35}$) (1.0−1.5 Ci/mmol) was obtained from DuPont New England Nuclear (Boston, MA); [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) and the anti-rabbit IgG polyclonal antibody conjugated to peroxidase were obtained from Sigma Chemical Co.; anti-PST-P antibody was obtained from Calbiochem-Novabiochem Co. (San Diego, CA); anti-β-actin antibodies were obtained from Cell Signaling Technology (Beverly, MA); molecular mass markers for proteins were obtained from Pharmacia Biotech (Saclay, France); polyvinyldifluoride (PVDF) membrane for Western blotting was obtained from Millipore (Bedford, MA); a TRIzol RNA isolation kit was obtained from Life Technologies (Rockville, MD); and primers for RT-PCR, dNTP, reverse transcriptase, and Taq polymerase were obtained from Gibco BRL (Cergy Pontoise, France). All other chemicals used were of the highest pure grade available.

Cell Proliferation Assays. The cell proliferation and cytotoxicity were determined with the MTT and lactate dehydrogenase (LDH) leakage assays. $HepG_2$ cells were seeded onto 96-well plates at a concentration of 1×10^6 cells/well in DMEM plus 10% FBS. After incubation for 24 h, the cells were treated with various concentrations of phenolic acids in 0.1% dimethyl sulfoxide (DMSO) for 24 h. The controls were treated with 0.1% DMSO alone. Dye solution (10 μL), specific for the MTT assay, was added to each well for an additional 4 h of incubation at 37 °C. After the addition of DMSO (100 μL/well), the absorbance at 570 nm (formation of formazan) and that at 630 nm (reference) were recorded with a Fluostar Galaxy plate reader (BMG Lab Technology, GmbH, Offenburg, Germany). The percent viability of the treated cells was calculated as follows: $(A_{570nm} - A_{630nm})_{sample}/(A_{570nm} - A_{630nm})_{control} \times 100$. LDH leakage was measured as an another index of cytotoxicity. $HepG_2$ cells were seeded onto 12-well plates at a concentration of 1×10^6 cells/well in DMEM plus 10% FBS. Cells were incubated with various phenolic acids for 24 h and then analyzed for LDH leakage into the culture media by using the commercial kit (Sigma Chemical Co.). The total LDH activity was determined after cells were thoroughly disrupted by sonication. The percentage of LDH leakage was then calculated to determine membrane integrity. The LDH leakage was expressed as a percentage of total activity: (activity in the medium)/(activity in the medium + activity of the cells) × 100.

Assay of Phenolsulfotransferase Activity. The induced phenolsulfotransferase activity, by phenolic acids, was determined using the phenolsulfotransferase assay (*13*). The cells were grown in 12-well plates (Costar 3524, Corning Inc., Corning, NY) for 24 h and then induced for another 24 h by exposure to fresh medium, containing serial dilutions of each tested compound. Each test compound, at concentrations from 10 to 50 μM, was supplemented with culture medium. All antioxidant phenolic acids were solubilized in DMSO, and the final solvent concentrations, within all compounds containing cultures and the appropriate vehicle control cultures, were adjusted to 0.1%. Treated cells were scraped off, washed, and suspended in ice-cold 5 mM potassium phosphate buffer, pH 7.4, before homogenization to produce a cell homogenate. Aliquots of the cell homogenates were collected and immediately tested for phenolsulfotransferase activity. The incubation mixture contained 100 μL of 0.1 M potassium phosphate buffer (pH 7.0), 20 μL of the $HepG_2$ cell homogenates, and 20 μL of the substrate; 20 μL of ^{35}S-labeled PAPS (final concentration = 6.7 μM) was added, at successive intervals, to tubes at 37 °C in a water bath, and the reaction was terminated after 20 min by the addition of 0.1 M barium acetate (200 μL). Any unreacted PAPS, free sulfate, or protein was precipitated by two additions of 0.1 M barium hydroxide (200 μL), followed by 0.1 M zinc sulfate (200 μL). After centrifugation (11500*g* for 3 min), 500 μL of the supernatant was thoroughly mixed with 4 mL of scintillant, and the radioactivity was measured by liquid scintillation spectrometry. The protein content of the cell homogenates

was determined using a Bio-Rad protein assay kit, and PST-P acitivity was expressed as picomoles per minute per milligram of protein. All samples were assayed, in triplicate, in three independent experiments.

RNA Extraction and RT-PCR. RT-PCR was performed to determine the level of PST-P gene expression. $HepG_2$ cells (1×10^6 in 10 mL of medium) were plated in 100 mm tissue culture dishes. After preincubation for 24 h, $HepG_2$ cells were subjected to a dose and time course, using phenolic acids in 0.1% DMSO, as described for the phenolsulfotransferase activity assay. Cellular RNA was extracted with a TRIzol RNA isolation kit (Life Technologies) as described in the manufacturer's manual. The 987 bp target in SULT1A1 cDNA (GenBank accession no. L10819) was amplified, using the sense primer (5′-ATGGAGCTGATCCAGGACAC-3′) at positions 39−58 and the anti-sense primer (5′-TGACCTACCGTCCCAGGCCC-3′) at positions 1006−1025. As a housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (*20*) was amplified, using the sense primer (5′GACCCCTTCATTGACCTCAAC) at positions 143−162 and the anti-sense primer (5′-CATACCAGGAAATGAGCTTG) at positions 965−984. Briefly, from each sample, 250 ng of RNA was reverse-transcribed, using 200 units of Superscript II reverse transcriptase, 20 units of RNase inhibitor, 0.6 mM dNTP, and 0.5 μg/μL of oligo(dT) 12−18. Then, PCR analyses were performed on the aliquots of the cDNA preparations to detect PST-P and GAPDH (as an internal standard) gene expression, using the FailSafe PCR system (Epicenter Technologies, Madison, WI). The reactions took place in a volume of 50 μL, containing (final concentrations) 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MnCl_2$, 0.2 mM dNTP, 2 units of Taq DNA polymerase, and 50 pmol of 5′ and 3′ primers. After initial denaturation for 2 min at 95 °C, 30 cycles of amplification (at 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1.5 min) were performed, followed by a 7 min extension at 72 °C.

Analysis of PCR Products. A 10 μL aliquot from each PCR reaction was electrophoresed in a 1.8% agarose gel, containing 0.2 μg/mL ethidium bromide. The gel was then photographed under ultraviolet transillumination. For quantification, the PCR bands on the photograph of the gel were scanned using a densitometer, linked to a computer analysis system. We normalized the PST-P signal, relative to the corresponding GAPDH signal, from the same sample, expressing the data as the PST-P/GAPDH ratio.

Western Blotting. The phenolic acid-treated and -untreated cells were rinsed twice with phosphate-buffered saline (pH 7.0) and the total proteins extracted by adding 200 μL of cold lysis buffer (50 mM Tris-HCl, pH 7.4; 1 mM NaF; 150 mM NaCl; 1 mM EGTA; 1 mM phenylmethanesulfonyl fluoride; 1% NP-40; and 10 μg/mL leupeptin) to the cell pellets, on ice, for 30 min; this was followed by centrifugation at 10000*g* for 30 min at 4 °C. Western blotting was performed according to the method of Maiti and Chen (*21*). The cytosolic fraction (supernatant) proteins were measured by Bradford assay, with bovine serum albumin as the standard. The samples (50 μg of protein) were mixed with 5× sample buffer containing 0.3 M Tris-HCl (pH 6.8), 25% 2-mercaptoethanol, 12% sodium dodecyl sulfate (SDS), 25 mM EDTA, 20% glycerol, and 0.1% bromophenol blue. The mixtures were boiled at 95 °C for 5 min and subjected to 12% SDS−polyacrylamide minigels, at a constant current of 20 mA. Electrophoresis was ordinarily carried out on SDS−polyacrylamide gels (SDS-PAGE). Following electrophoresis, proteins on the gel were electrotransferred onto an immobile membrane (PVDF; Millipore Corp., Bedford, MA) with transfer buffer composed of 25 mM Tris-HCl (pH 8.9), 192 mM glycine, and 20% methanol. The membrane was blocked in 5% bovine serum albumin solution for 1 h at room temperature and then incubated overnight at 4 °C with primary antibodies (1:1000 dilutions). After hybridization with primary antibodies, the membrane was washed with Tris-buffered saline Tween-20 (TBST) three times, then incubated with HRP-labeled secondary antibody for 45 min at room temperature, and washed with TBST three times. Final detection was performed with enhanced chemiluminescence (ECL) Western blotting reagents (Amersham Pharmacia Biotech, Buckinghamshire, U.K.).

Statistical Analysis. Correlation and regression analyses and principal component analysis (PCA) were performed using the SigmaPlot scientific graph system. The component loading included relative PST-P mRNA levels and PST-P activities. Each experiment was performed in triplicate and repeated three times. The results were expressed as means ± SD. Statistical comparisons were made by means of one-way analysis of variance (ANOVA), followed by a Duncan multiple-comparison test. Differences were considered to be significant when the P values were <0.05.

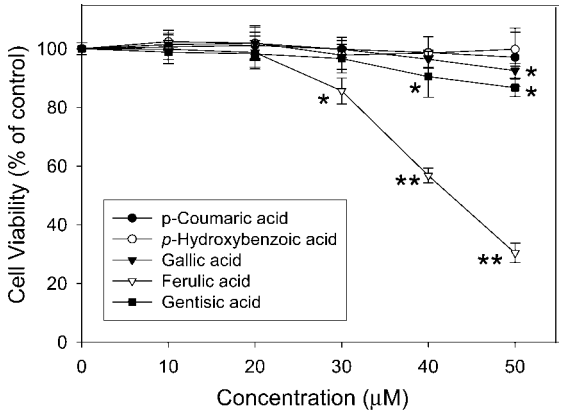

Figure 2. Cytotoxicity of phenolic acids on $HepG_2$ cells. $HepG_2$ cells were cultured for 24 h and then exposed to the indicated concentrations of phenolic acids for 24 h. The cell viability was assessed by MTT assay as described under Materials and Methods. Each data point represents the mean ± SD of three experiments. *, $p < 0.05$, and **, $p < 0.01$, versus vehicle control.

RESULTS

Growth Inhibitory Effect of Phenolic Acids on $HepG_2$ Cells. The chemical structures of phenolic acids are illustrated in **Figure 1**. To determine the appropriate concentration range of phenolic acids required to induce PST-P activity, the cytotoxicity of phenolic acids on $HepG_2$ cells was investigated by MTT assay. As presented in **Figure 2**, no significant effect on the cell growth of $HepG_2$ cells was observed in treatments below 30 μM phenolic acids. However, significant inhibitory effects were observed when the cells were treated with >40 μM phenolic acids. Ferulic acid, gentisic acid, and gallic acid were found to have growth inhibitory effects at higher concentrations. At a concentration of 50 μM, the maximum inhibitions were found with 70% ferulic acid, 13% gentisic acid, and 6% gallic acid, when compared to the vehicle control. No significant effects on cell growth were observed in treatments with *p*-hydroxybenzoic acid or *p*-coumaric acid, at any concentration. The LDH leakages after treatment with 50 μM of each phenolic acids in $HepG_2$ cells are shown in **Figure 3**. Ferulic acid and gentisic acid caused significant LDH leakage ($p < 0.05$) as compared with the control.

Induction of PST-P Activity in $HepG_2$ Cells by Phenolic Acids. To investigate the induction ability of phenolic acids on PST-P activity, a concentration-dependent experiment was performed in the $HepG_2$ cells. The result demonstrated that PST-P activity of the control (without phenolic acids) was 22 ± 3 pmol/min/mg of protein. Moreover, the addition of phenolic acids was found to affect PST-P activity. The induction of PST-P activity, after a 24 h of exposure to various concentrations (between 10 and 50 μM) of each phenolic acid, is shown in **Figure 4**. Gallic acid, gentisic acid, *p*-coumaric acid, and *p*-hydroxybenzoic acid were found to increase PST-P activity. The greatest proportionate induction of PST-P activity was observed in treatments with either gallic acid or gentisic acid, both inducing PST-P activity, in a concentration-dependent manner, in concentrations of 10−50 μM, with a maximum of 4−5-fold induction. Both *p*-coumaric acid and *p*-hydroxybenzoic

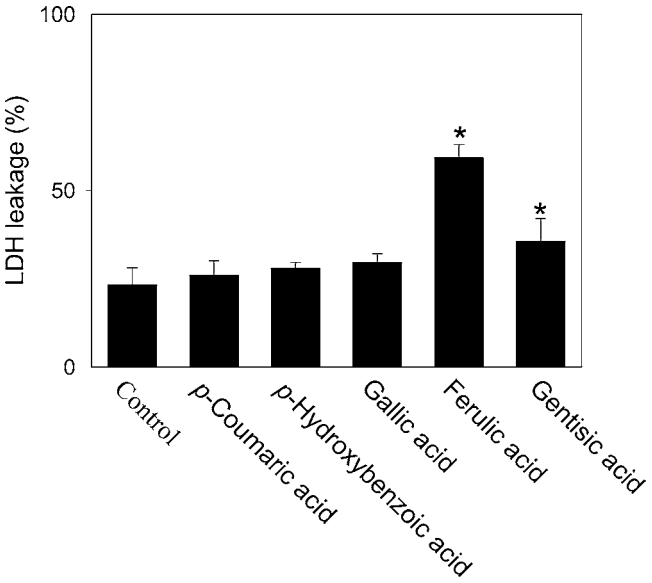

Figure 3. LDH leakage in $HepG_2$ cells treated with phenolic acids. $HepG_2$ cells were incubated with and without phenolic acids for 24 h. LDH leakage was determined. The concentration of each phenolic acid was 50 μM. Each column represents the mean ± SD of three experiments. *, $p < 0.05$, significantly different to control.

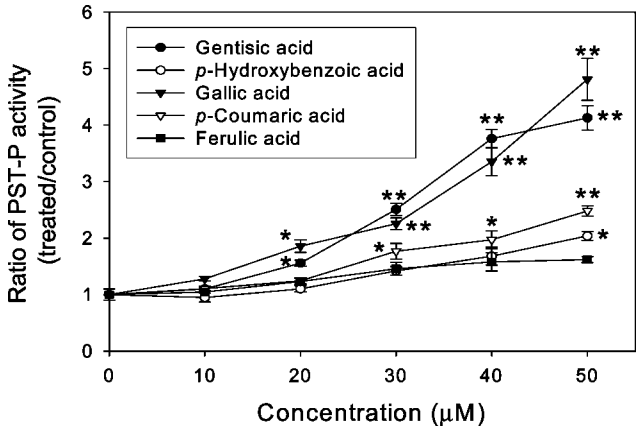

Figure 4. Induction of PST-P activity in $HepG_2$ cells by five phenolic acids. $HepG_2$ cells were cocultured with each indicated compound at concentrations between 10 and 50 μM for 24 h. The activity of PST-P was measured as described under Materials and Methods. The results are represented as the ratios of PST-P activities in the treated cells to vehicle control. Each value of the ratio of PST-P activity represents the mean ± SD of three independent experiments. *, $p < 0.05$, and **, $p < 0.01$, versus vehicle control. The mean uninduced activity in the vehicle control cells was 22 ± 3 pmol/min/mg of protein.

acid showed a moderate increase of PST-P activity at concentrations of 10−50 μM, with a maximum of 2−2.5-fold. However, no significant ($p < 0.05$) effect was found in treatments with ferulic acid in this in vitro model. Gallic acid, a trihydroxybenzoic acid, has been found to cause a significant increase in the activity of PST-P; thus, we proceeded in using gallic acid, the strongest inducer, to elucidate its molecular mechanism on induction of PST-P.

Transcriptional Induction of the PST-P Gene by Gallic Acid. To further investigate the induction of PST-P mRNA by gallic acid, the $HepG_2$ cells were exposed to the concentration range of gallic acid indicated for 24 h; RNA was then extracted for RT-PCR. As shown in **Figure 5A**, PST-P mRNA expression was induced markedly by gallic acid, in a dose-responsive manner. A maximum of 4.6-fold mRNA expression was

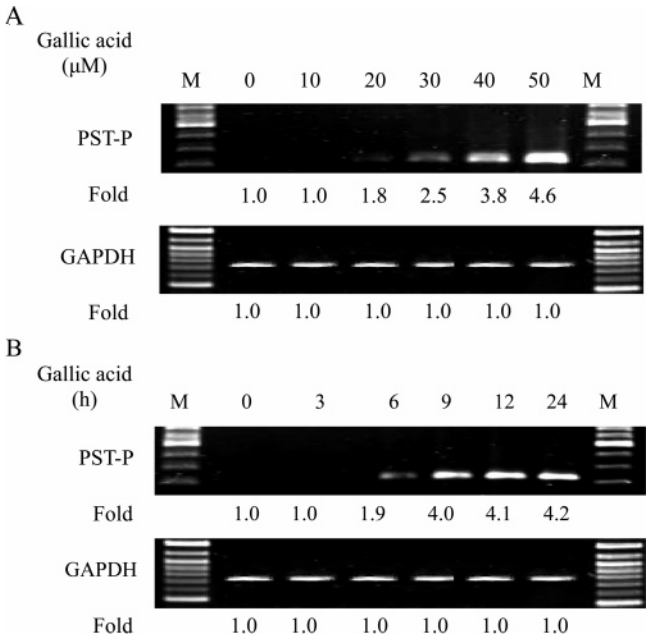

Figure 5. Induction of PST-P mRNA expression in $HepG_2$ cells by gallic acid. $HepG_2$ cells were exposed to the indicated concentrations of gallic acid for 24 h in a dose–response experiment (**A**) or to 50 μM gallic acid for 3–24 h in a time course experiment (**B**). Cellular RNA was extracted with an Invitrogen RNA isolation kit, and RT-PCR was performed as described under Materials and Methods. The RT-PCR products were separated on 1.8% agarose gel and digitally imaged after staining with ethidium bromide. GAPDH, the housekeeping gene, was used as an internal control. The results are presented as the ratios of PST-P mRNA in treated cells to those of control.

observed at 50 μM. To detect the induction time of PST-P mRNA expression by gallic acid, the $HepG_2$ cells were exposed to 50 μM gallic acid for 3, 6, 9, 12, and 24 h, and PST-P mRNA was then detected by RT-PCR. As shown in **Figure 5B**, a marked induction of PST-P mRNA expression, by gallic acid, was observed after 6 h of exposure; maximum induction of PST-P mRNA expression was observed at 12 h after the addition of 50 μM gallic acid. To elucidate the association of induced PST-P mRNA expression, with PST-P activity, we calculated the correlation coefficient, using the relative PST-P mRNA levels; this was plotted against the corresponding ratio of PST-P specific activity (pmol/min/mg of protein), for each of the phenolic acids, tested at the effective concentrations, between 10 and 50 μM. As shown in **Figure 6**, a significant ($N = 24$, $r = 0.74$, $p < 0.01$) linear correlation was observed. Thus, the induction of PST-P activity, by gallic acid, appears to be mediated by the enhancement of PST-P gene expression and/or the stabilization of mRNA levels.

Induction of PST-P Protein Expression in $HepG_2$ Cells by Gallic Acid. To further explore the induction of PST-P protein by gallic acid, dose- and time-dependent experiments were performed with the $HepG_2$ cells, using Western blotting techniques. As shown in **Figure 7A**, PST-P protein was markedly induced by gallic acid, in a dose−response manner, at concentrations of 10−50 μM. A maximum of 3.5-fold induction was observed at 50 μM. To determine the induction time of PST-P protein by gallic acid, a time course study was also undertaken. Significant ($p < 0.05$) induction of PST-P protein was observed 24 h after the addition of 50 μM gallic acid (**Figure 7B**). The increased PST-P gene transcription, observed in $HepG_2$ cells after exposure to gallic acid, corresponded to increased protein expression (**Figures 5** and **7**).

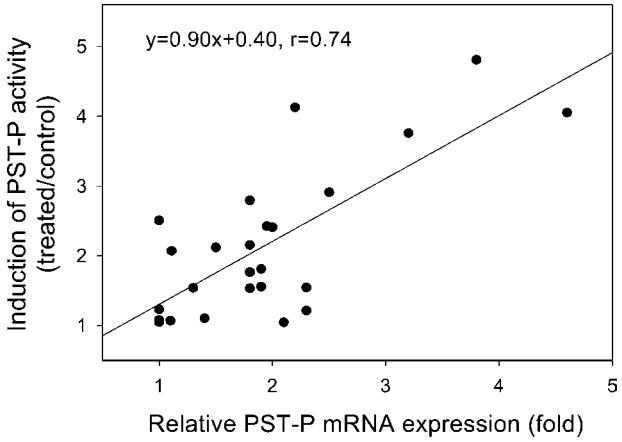

Figure 6. Correlation between the induction of PST-P activity and the relative expression of PST-P mRNA. $HepG_2$ cells were cocultured with various phenolic acids at concentrations between 10 and 50 μM for 24 h. Both PST-P specific activity and mRNA levels were measured simultaneously as described under Materials and Methods. The correlation coefficient (r) was determined between logarithms of relative PST-P mRNA expression and PST-P activity in each of the phenolic acids tested at effective concentrations between 10 and 50 μM ($N = 24$, $r = 0.74$, $p < 0.01$).

The enhanced PST-P protein expression in $HepG_2$ cells, after treatment with gallic acid, corresponded to the induction of PST-P activity (**Figures 4** and **7**), suggesting that the observed induction of PST-P activity, by gallic acid, is due to increased PST-P protein synthesis, through activation of gene transcription (**Figures 4**, **5**, and **7**).

DISCUSSION

A large body of evidence, based on preclinical and clinical researches, indicates that modulation of the body's drug-metabolizing enzyme could provide an effective approach for cancer prevention (*22*). Understanding the molecular mechanism of drug-metabolizing enzyme modulation is critically important in designing rational cancer preventive agent. Many naturally occurring cancer compounds have shown robust effects on drug metabolism enzyme modulation; however, the underlying molecular mechanisms are not fully understood. In the present study, five phenolic acids were used to investigate the regulatory mechanism on the induction of PST-P in human $HepG_2$ cells. Our data revealed that ferulic acid, gentisic acid, and gallic acid possessed inhibitory effects upon cell growth at higher concentrations. Ferulic acid and gentisic acid at a concentration of 50 μM significantly affected the viability of $HepG_2$ cells, suggesting the observed growth inhibition was caused by a cytotoxic effect rather than a cytostatic effect of phenolic acids. This shows a close agreement with prior work, done by Kampa et al. (*23*), showing that phenolic acids, especially ferulic acid, significantly suppressed the growth of cultured cells, derived from human breast cells (T47D cells). Inoue et al. (*24*) reported that gallic acid had been shown to induce cell death in cancer lines. Gallic acid derivates with a lipophilic group (hydrogenated farnesyl gallate, lauryl gallate, gallic acid laurylamide, and cholesteryl gallate) have also been shown to induce apoptosis in human monoblastic leukemia U937 cells (*25*). Our results indicated that phenolic acids, at higher concentrations, generally showed higher growth inhibition.

Phenolic acids, especially hydroxycinnamic acids and hydroxybenzoic acid, are secondary plant products, commonly found in plant-derived foodstuffs. Ferulic acid and *p*-coumaric

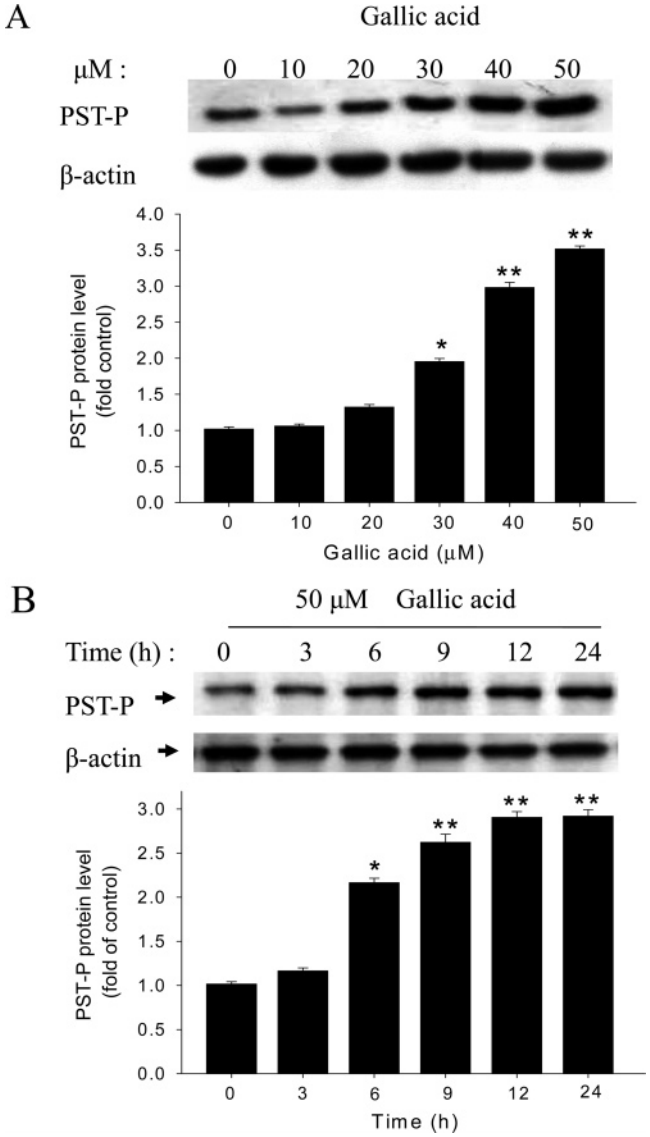

Figure 7. Gallic acid induced PST-P protein expression in $HepG_2$ cells: (**A**) to test the dose–response, the cells were exposed to the indicated concentrations of gallic acid for 24 h; (**B**) to investigate the time course, the cells were exposed to 50 μM gallic acid for 2–24 h. Protein expressions were detected by Western blot. The upper part of each figure indicates an original blot and the lower part, results of densitometric analyses. Data are means ± SD from three independent experiments. *, $p < 0.05$, and **, $p < 0.01$, versus vehicle control.

acid have been reported to act as scavengers of thiol free radicals (*26*). *p*-Coumaric acid also possesses potent antioxidant activity, in enhancing the resistance of low-density lipoprotein to oxidation (*27*). As well, *p*-coumaric acid (50 mg/kg of body weight) significantly decreased the basal level of oxidative damage in rat colonic mucosa (*28*), whereas gentisic acid has been reported to have an inhibitory action in the myeloperoxidase system and was able to impair tyrosyl radical catalyzed low-density lipoprotein peroxidation (*29*). In addition, gallic acid is a potent natural antioxidant, exhibiting antimutagenic and anticarcinogenic activity, and is expected to reduce the risk of disease and bring health benefits through daily intake (*30*). In the present study, we examined the PST-P activity-inducing potency of phenolic acid and its molecular mechanism in human $HepG_2$ cells. The result demonstrated a concentration-dependent 4−5-fold induction in PST-P activity, with the addition of either gallic acid or gentisic acid at 10−50 μM, and a 2−2.5-fold

induction, with either *p*-hydroxybenzoic acid or *p*-coumaric acid at 10−50 μM. No significant induction was found for ferulic acid. These results are in agreement with our previous findings (*14*), in which gallic acid, gentisic acid, *p*-hydroxybenzoic acid, and *p*-coumaric acid enhanced the activity of PST-P. These phenolic compounds, especially gallic acid, gentisic acid, *p*-hydroxybenzoic acid, and *p*-coumaric acid, exhibited strong antioxidant activity in the oxygen radical absorbance capacity ($ORAC_{ROO^{\bullet}}$) assay and the Trolox equivalent antioxidant capacity (TEAC) assay. Thus, a significant correlation ($r = 0.71$, $p < 0.01$) was found between the promotion effect in PST-P activity and the antioxidant capacity of phenolic compounds (*13*). The observed effects of phenolic acids in the present study on PST-P activity suggest the potential for this abundant dietary polyphenol to alter detoxification of carcinogens. Indeed, numerous animal and cell culture studies have shown naturally occurring polyphenols to exert anticarcinogenic and cytoprotective activities against diverse chemical carcinogens and mutagens via induction of phase II detoxification enzymes (*31*). On the basis of the induction potency of PST-P activity, by five phenolic acids in cultured human $HepG_2$ cells, gallic acid, showing the strongest potent induction, was used to investigate the molecular mechanisms of phenolic acids on PST-P activity in $HepG_2$ cells.

Several flavonoids and dietary phenolics strongly inhibited neoplastic transformation in mammary organ cultures or epithelial cells, inhibited benzo[*a*]pyrene DNA adduct formation, and induced the phase II metabolizing enzymes GST, NQO, and UGT (*32*, *33*). Phase II enzyme induction may explain the chemopreventive effect of polyphenolic compounds in inhibiting heterocyclic amine-induced colonic aberrant crypt foci formation in the rat (*34*). The synthetic flavonoid 4′-bromoflavone was the most potent in vivo inducer of NQO and GSH synthesis enzymes and prevented mammary carcinogenesis in rats induced by polycyclic aromatic hydrocarbons (*35*). Dietary flavonoid/phenolic-mediated induction of UGT may be important for the glucuronidation and detoxification of colon and other carcinogens, as well as for the metabolism of therapeutic drugs (*36*). Studies of the mechanisms by which polyphenolc compounds are involved in chemoprevention constitute an increasingly active area of research. The effects of polyphenolic compounds on phase I enzymes, such as cytochromes P450, or on phase II enzymes, such as GST and NQO1, appear to involve multiple mechanisms (*37*). Gallic acid, and its catechin derivatives, has demonstrated excellent chemopreventive effects in many target organs challenged with various carcinogens. A number of studies indicate that gallic acid is a potent inducer of phase II drug metabolism enzymes; this molecular mechanism is thought to involve transcriptional up-regulation of phase II genes (*38*). Therefore, the observed induction of PST-P, by phenolic acids, appears to be through an increase in PST-P mRNA expression and/or stabilization of mRNA levels. Next, we tested the ability of phenolic acids to increase PST-P protein expression. To our knowledge, this is the first reported Western analysis of human PST-P protein expression in response to in vitro exposure to phenolic acids. The enhanced PST-P protein expression in $HepG_2$ cells, after treatment with phenolic acids, corresponded to the induction of PST-P activity, suggesting that the observed induction of PST-P activity was due to increased PST-P protein synthesis, through activation of gene transcription. These data are consistent with previous studies in which treatment with phenolic acids resulted in a maximum 2-fold induction of PST-P activity (*13*, *14*).

Various polyphenol or flavone compounds, natural and synthetic, produce effects similar to those of gallic acid in the increase of phase II activity. Such agents have been classified as monofunctional (phase II) or bifunctional inducers with capacity to increase both phase I and phase II enzymes (*39*). Recent studies demonstrated that gallic acid has mixed aryl hydrocarbon receptor (AhR) agonist/antagonist activities in that it can bind to the AhR and induce CYP1A1 transcription (*40*) but can also inhibit 3-methylcholanthrene-induction of cytochrome P450 1A1 expression (*41*). These studies suggest that gallic acid could be a natural dietary ligand of the AhR affecting phase I gene transcription. The ability to induce both cytochrome P450 1A1 and PST-P enzymes suggests the likelihood that gallic acid can operate as a bifucntional inducer. A recent study demonstrated that plasma concentrations of gallic acid, in humans given a single dose of gallic acid (two acidum gallicum tablets), could reach 52.3 μM (*42*). The effect of increased gallic acid levels in plasma on PST-P remains to be proven. In the present study, the ability of this phenolic acid to modulate PST-P was found at concentrations that may well be achievable in human plasma. Furthermore, the different regulatory abilities of phenolic acids tested in this study may be associated with their chemical structures. The difference in induction effects among the phenolic acids on PST-P activity can be attributed to the variations in the hydroxyl groups in the A-ring and their antioxidant activities (*14*). Among the dietary polyphenols, gallic acid and *p*-coumaric acid are widely distributed in many foods, the main sources of *p*-coumaric acid acid including wine and tea (1−24 mg/L), spinach, Brussels sprouts, and cereal brans (2−60 mg/kg), and apples and berries (69−1700 mg/L). Because of their ubiquitous presence in vegetables, the estimated range of human consumption is 25 mg−1 g a day, depending on diet (*43*).

The results shown in the present study indicate an induction response of PST-P activity to various phenolic acids in human hepatoma cells. Because phenolsulfotransferase is a key enzyme in drug metabolism, bile acid detoxification, and the regulation of intratissue active hormone levels, this increased gene expression of phenolsulfotransferase will, therefore, promote the efficiency of detoxification. Therefore, the significant induction of PST-P by the phenolic acids used in this study appears to be important. The biological implications of these findings offer some understanding of the antioxidant properties of phenolic acids, which show great potential in the induction of phase II chemopreventive enzymes.

ABBREVIATIONS USED

AhR, aryl hydrocarbon receptor; DMSO, dimethyl sulfoxide; ECL, enhanced chemiluminescence; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GST, glutathione *S*-transfersase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NQO-1, NAD(P)H:quinine oxidoreductase 1; $ORAC_{ROO^{\bullet}}$, oxygen radical absorbance capacity; PSTs, phenosulfotransferases; RT-PCR, reverse transcription polymerase chain rection; PST-P, P-form phenolsulfotransferase; SD, standard deviation; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gels; TEAC, Trolox equivalent antioxidant capacity; TBST, Tris-buffered saline Tween-20; UGT, UDP-glucuronosyltransferasese; VSMCs, vascular smooth muscle cells.

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