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ARTICLE

Perilla frutescens modulates CYP1A1/2 and HO-1 and activates Nrf2 in oxidative stress-induced hepatotoxicity

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Abstract We speculated that lipid peroxidation induced by *tert*-butyl hydroperoxide (*t*-BHP) in liver is closely linked with the metabolism mediated by CYPs. In this study, we have examined the effect of *Perilla* leaf extract (PLE) on CYPs using 7-ethoxyresorufin-*O*-deethylase (EROD, indicator of CYP1A1), 7-methoxyresorufin-*O*-demethylase (MROD, indicator of CYP1A2), erythromycin *N*-demethylase (ERDM, indicator of CYP3A), and *p*-nitrophenol hydroxylase (PNPH, indicator of CYP2E1) in rat liver. Rats orally pretreated with PLE (250, 500, and 1,000 mg/kg b.w.) for 5 days were administered with a single i.p. dose of *t*-BHP (0.5 mmol/kg). Kinetic analysis of CYP1A1/2 activities in *t*-BHP-treated liver demonstrated that PLE inhibits the enzyme activities by competitive and noncompetitive inhibitions. The pretreatment with PLE decreased the expression of CYP1A1/2 mRNA and protein compared with *t*-BHP treatment alone. A Phase II enzyme, heme oxygenase-1 (HO-1), is involved in hepatoprotection against oxidative damage, and we confirmed that PLE increases the levels of HO-1 mRNA and protein, as well as its activity in *t*-BHP-induced liver damage. PLE administration resulted in enhanced nuclear translocation and ARE binding of NF-E2-related factor 2. These findings suggest that PLE protects against *t*-BHP-induced hepatotoxicity through modulated activity and expression of selective CYPs, and ARE-driven induction of HO-1 expression and its activity.

Keywords *Perilla frutescens* · Cytochrome P450s · HO-1 · Nrf2 · Oxidative stress · Hepatoprotection

Abbreviations

ARE	Antioxidant response element
CYPs	Cytochrome P450s
EROD	7-Ethoxyresorufin *O*-deethylase
ERDM	Erythromycin *N*-demethylase
HO-1	Heme oxygenase 1
MROD	7-Methoxyresorufin *O*-demethylase
NADPH	β-Nicotinamide adenine dinucleotide phosphate reduced form
Nrf2	NF-E2-related factor 2
PLE	*Perilla* leaf extract
PNPH	*p*-Nitrophenol hydroxylase
ROS	Reactive oxygen species
t-BHP	*Tert*-butyl hydroperoxide

Introduction

Perilla frutescens (L.) Britt. var. japonica (Hassk.) is an annual herbaceous plant, native to the east Asian countries. Its leaves are usually used to serve sushi, garnish soups, and wrap-cooked food, especially in Korea and Japan. By virtue of their antioxidant, anti-inflammatory, and anti-cancer activities, *Perilla* leaves are medicinally used for the treatment of food poisoning (Ueda et al. 2002; Yang

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et al. 2012). We have previously shown that aqueous *P. frutescens* extract (PLE) has a protective effect against *tert*-butyl hydroperoxide (*t*-BHP)-induced oxidative damage in rat liver (Kim et al. 2007). We found that caffeic aid (CA) in *perilla* leaves increases intracellular γ-glutamylcysteine synthetase activity in hepatocytes, leading to the elevation of glutathione (GSH) levels (Park et al. 2010). CA is a naturally occurring, dietary, non-flavonoid phenolic compound found in a large number of vegetables and medicinal herbs (Marques and Farah 2009). This phytochemical also has antioxidant, immunomodulatory, anticarcinogenic, and anti-inflammatory activities (Fesen et al. 1994; Nardini et al. 1997; Johnson et al. 2004). However, the biochemical mechanisms by which *P. frutescens* prevents oxidative damage are not well investigated.

Although cytochrome P450 enzymes (CYPs) are the most important enzymes in Phase I metabolism and play a major role in detoxification of xenobiotics, CYPs can sometimes transform a nontoxic molecule into a poisonous one (toxification) (Kappus 1993; Bernhardt 1996). *t*-BHP, which has often been used to investigate cellular and tissue damage by oxidative stress, can be metabolized to free radical intermediates by CYPs in hepatocytes, causing lipid peroxidation and consequently, liver injury (Hogberg et al. 1975; Thornalley et al. 1983; Rush et al. 1985). It has been reported that CYP1A1/2 and CYP3A/2E1 are the primary enzymes in the oxidative metabolism (Bondy and Naderi 1994; Chen et al. 1998; Barouki and Morel 2001; Nagai et al. 2004; Reed et al. 2011). This indicates that the protective activity of *P. frutescens* against *t*-BHP-induced oxidative hepatotoxicity may be associated with the known inhibition of CYPs-mediated metabolism of *t*-BHP into reactive metabolites and the resultant lipid peroxidation (Choi et al. 2006).

In addition, Phase II detoxifying enzymes are involved in a major mechanism that protects cells and tissues against oxidative stress (Droge 2002; Rushmore and Kong 2002). The induction of phase II enzymes is primarily regulated at the transcriptional level and linked to the transcription factor NF-E2-related factor 2 (Nrf2) (Alam and Cook 2003). Under normal conditions, Nrf2 exists in the cytoplasm in an inactive complex with Kelch-like ECH-associated protein 1 (Keap1) (Kang et al. 2005; Pugazhenthi et al. 2007). Upon activation by exposure to stimuli, Nrf2 dissociates from Keap1, translocates to the nucleus, and binds to the antioxidant response element (ARE) resulting in the regulation of gene expression of detoxifying/antioxidant enzymes, including heme oxygenase 1(HO-1), NAD(P)H:quinone oxidoreductase (NQO1), glutathione S-transferase (GST), and γ-glutamylcysteine ligase (GCL) (Ishii et al. 2000; McMahon et al. 2001; Talalay and Fahey 2001; Zhao et al. 2010). Among the several phase II detoxifying enzymes, HO-1 is known to have a highly responsive ARE promoter and is frequently used as an indicator of Phase II enzyme induction (Alam and Cook 2003). In addition, HO-1 has recently been shown to possess hepatoprotective roles (Kapitulnik 2004). However, it is unknown whether *P. frutescens* can upregulate detoxifying enzymes such as HO-1 or activate Nrf2 in *t*-BHP-induced hepatotoxicity. We speculated that *P. frutescens* might be involved in inducing phase II enzymes associated with the oxidative metabolism of *t*-BHP. The capacity of chemicals to both inhibit the metabolic activation by CYPs and induce chemical detoxification and antioxidant defense is worth investigating.

Therefore, this study was primarily aimed at investigating whether *P. frutescens* provides protective effects against *t*-BHP-induced hepatotoxicity by modulating CYPs and HO-1 enzyme. To this end, we first examined whether *P. frutescens* is involved in inhibition of specific CYPs in vivo. Subsequently, we also studied the upregulation of the Phase II enzyme, HO-1, through the activation of Nrf2 by *P. frutescens*.

Materials and methods

Materials and chemicals

Formaldehyde, *t*-BHP, resorufin, 7-ethoxyresorufin, 7-methoxyresorufin, erythromycin, N-nitrophenol, 4-nitrocatechol, β-nicotinamide adenine dinucleotide phosphate reduced form (NADPH), glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase (G6PDH), and hemin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-CYP1A1 (sc-48432), anti-CYP1A2 (sc-30085), anti-HO-1 (sc-1797), and anti-Nrf2 (sc-722) antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Bicinchoninic acid (BCA) protein assay kit was purchased from Thermo Scientific (Rockford, IL).

Plant material and preparation of extract

Leaves of *perilla* were obtained from Miryang city Agricultural Technology Center (Miryang, Korea). The *perilla* plants were cultivated and treated with 175 mM (6 %) of sucrose. For the treatments, 800 L of water containing 175 mM sucrose (6 %) was used twice a week via irrigation. After 7 days, the fresh leaves were washed, immediately stored in a freezer at −70 °C, and freeze dried for 7 days. The dried *perilla* leaves were ground and soaked in distilled water (30 g/L), followed by refluxing at 100 °C for 3 h. Undissolved material was removed by filtration through a Whatman filter paper (Clifton, NJ, USA) followed by a membrane filter with 0.45-μm pore size

(Millipore, Billerica, MA, USA). The filtrate was freeze dried and stored in a deep freezer (−60 °C) until further analysis.

Animals and treatment

Male Sprague–Dawley rats were purchased from Samtako Bio Korea Co. (Gyeonggi, Korea) and provided free access to standard diet (Samyang Feed Co. Ltd., Incheon, Korea) and tap water. The animals were maintained according to the guidelines set by the Committee for Ethical Usage of Experimental Animals (KUIACUC-20100319-2) in Korea University. The rats were divided into five groups containing seven rats each. To investigate the effect of PLE on CYPs, Phase II enzyme (HO-1) and Nrf2 activities against *t*-BHP-induced oxidative stress, 250, 500, and 1,000 mg/kg of PLE were administered to the animals daily by gavage for five consecutive days, while the control rats received oral dosage with vehicle only. Three hours after the final treatment, 0.5 mmol/kg b.w. of *t*-BHP was i.p. injected into each animal, and 18 h later, the rats were sacrificed.

Isolation of liver microsomes

After sacrifice, liver tissues were immediately stored in liquid nitrogen until the microsomes were obtained. The liver was homogenized with five volumes of lysis buffer (50 mM Tris–HCl, 150 mM KCl, 2 mM EDTA) followed by centrifugation at 1,200×*g* for 10 min. The supernatant obtained was centrifuged at 10,000×*g* for 30 min, and the resulting pellet contained crude mitochondria. This supernatant in turn was centrifuged at 100,000×*g* for 60 min, and the resulting rough microsomal pellet was suspended in 0.1 M potassium phosphate buffer containing 0.1 mM EDTA(depending on the assay to be performed) and stored at −60 °C.

Phase I enzyme activity assay

Ethoxyresorufin-*O*-deethylase (EROD) and methoxyresorufin-*O*-demethylase (MROD) activities were measured as reported previously, with a slight modification using a spectrofluorophotometer, with an excitation wavelength of 530 nm and emission wavelength of 590 nm (Pohl and Fouts 1980). We used 1 mL of reaction mixture consisting of 0.1 M potassium phosphate buffer (pH 7.4) containing 3 mM MgCl, 1 mM EDTA, 5 mM G6P, 5 μM G6PDH, 2.5 μM 7-ethoxy/methoxy resorufin, and microsomes. The reaction was initiated by the addition of 5 μM NADPH, and the change in fluorescence intensity due to product formation was measured after 3 min. The amount of resorufin product formed in the microsomes was calculated from a standard curve, and the protein concentration was measured by BCA protein assay kit using BSA as the standard.

Erythromycin *N*-demethylase (ERDM) was determined by measuring the amounts of formaldehyde formed as described by Nash (1953). Briefly, the reaction mixture (500 μL) included 50 mM potassium phosphate buffer (pH 7.4) containing 15 mM MgCl, 1 mM erythromycin, and microsomes. The reaction was started by the addition of the 1 mM β-NADPH, maintained at 37 °C for 10 min, and stopped by the addition of 500 μL of 12.5 % trichloroacetic acid. The solution was centrifuged at 5,000 rpm for 10 min at 4 °C and 500 μL of the supernatant was mixed with 500 μL of Nash reagent. The mixture was boiled at 50 °C for 30 min, cooled at room temperature, and its absorbance was measured spectrophotometrically at 425 nm. The protein concentration was measured by BCA protein assay kit using BSA as the standard.

p-Nitrophenol hydroxylase (PNPH) activity was determined as described by Koop (1986). The reaction mixture (500 μL) included 50 mM potassium phosphate buffer (pH 7.4) containing 5 mM *p*-nitrophenol, 10 mM β-NADPH, and microsomes. The reaction was started by adding 1 mM NADPH and after 30 min of incubation at 37 °C, it was stopped by adding 100 μL of 20 % trichloroacetic acid. The solution was centrifuged at 5,000 rpm for 10 min at 4 °C, and the amount of 4-nitrocatechol formed was determined spectrophotometrically in 500 μL of supernatant at 535 nm, following the addition of 250 μL of 2 N NaOH. The protein concentration was measured by BCA protein assay kit using BSA as the standard.

Kinetic parameters and type of inhibition

The kinetics of EROD and MROD reactions was evaluated using *t*-BHP-induced microsomes and determined as previously reported by Lubet et al. (1985). Four concentrations of ethoxyresorufin and methoxyresorufin (0.3, 0.6, 1.2, and 2.5 μM) and the liver microsomes treated with 250, 500 and 1,000 mg/kg b.w. of PLE extract were used. The kinetic parameters were calculated using the Lineweaver–Burk plot, and the type of inhibition was inferred from the changes produced by PLE in the values of K_m and V_{max}.

Determination of HO-1 activity

HO activity in rat liver microsomes was determined by measuring bilirubin generation (Raju and Maines 1996). The microsomal supernatant (200 μL) was added to the reaction mixture (200 μL), which contained 2 mg/mL of rat liver cytosol (as a source of biliverdin reductase), 10 μM hemin, 0.2 mM G6P, 0.2 units of G6PDH, and 0.8 mM NADPH. The reaction was carried out for 1 h at

37 °C in the dark, and the samples were placed in an ice bath to terminate the reaction. The extracted bilirubin was measured by the difference in absorbance between 464 and 530 nm (extinction coefficient = 40 mM^{-1} cm^{-1}), and the protein concentration was measured by BCA protein assay kit using BSA as the standard.

RNA extraction, reverse transcriptase PCR, and quantitative real-time PCR

Total RNA was extracted from a pool of three individual rat livers in each group using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to manufacturer's instructions. First-strand cDNA primed by the oligoprimer was prepared from the total RNA (2 μg) using the SuperScriptIII First-Strand Synthesis System (LeGene Biosciences, San Diego, CA). The following primers (for conventional RT-PCR) were designed based on the published cDNA sequences: CYP1A1 primer: forward, 5′-CTG GCT CTG GAT ACC CAG CTG-3′; reverse, 5′-CCT AGG GTT GGT TAC CAG-3′ and CYP1A2 primer: forward, 5′-ATC CCC GAG GAG AAG ATT GT-3′; reverse, 5′-TTG GTA AGA AAC CGC TCT GG-3′. β-actin mRNA was analyzed as a normalization control. Additionally, the following primers for rat β-actin were designed based on the published cDNA sequences: forward, 5′-GAG AGG GAA ATC GTG CGT GAC-3′; reverse, 5′-CAT CTG CTG GAA GGT GGA CA-3′. PCR was conducted in a 20 μL solution containing Dream Taq polymerase (0.2 μL), template (5 μL), water (10.4 μL), primers (0.8 μL), and dNTP (1.6 μL) (Koma Biotech, Seoul, Korea) under the conditions set at 94 °C for 1 min, followed by 35 cycles of 94 °C for 10 s, 60 °C for 30 s, and 72 °C for 20 s. The amplification products were electrophoresed on 1.5 % agarose gel and visualized by G-red staining (Koma Biotech, Seoul, Korea). In addition, the following primers (for quantitative real-time PCR) were designed based on the published cDNA sequences: HO-1 primer: forward, 5′-GAG CCA GCC TGA ACT AGC-3′; reverse, 5′-GAT GTG CAC CTC CTT GGT-3′. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was analyzed as a normalization control. Additionally, the following primers for human GAPDH were designed based on the published cDNA sequences: forward, 5′-ACT GCC AGC CTC GTC TCA TAG-3′; reverse, 5′-CCT TGA CTG TGC CGT TGA ACT-3′. The quantitative real-time PCR was performed using the real-time SYBR Green method on a BioRad iQ-5 thermal cycler, and PCR was conducted in a 20 μL solution containing template (2 μL), water (6 μL), primers (2 μL), and SYBR green PCR Master Mix (10 μL) with the PCR conditions set at 94 °C for 1 min, followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. The values for HO-1 mRNAs were divided by the corresponding values for GAPDH mRNA.

Preparation of nuclear fractions

The nuclear extract from rat livers was prepared as described previously (Han et al. 2001; Chun et al. 2003). Briefly, liver samples were homogenized in 1 mL of lysis buffer [10 mM HEPES (pH 7.8), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/mL of aprotinin, and 5 μg/mL of leupeptin]. The homogenate was treated with 60 μL of 10 % Nonidet P-40 (NP-40) solution and placed on ice for 15 min. The mixture was then centrifuged for 5 min at 12,000×*g*, and the supernatant was collected as a cytosolic fraction. The precipitated nuclei were washed twice with 500 μL of lysis buffer plus 30 μL of 10 % NP-40, centrifuged, resuspended in 200 μL of extract buffer [50 mM HEPES (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 5 μg/mL of aprotinin, 5 μg/mL of leupeptin, and 20 % glycerol], and centrifuged for 10 min at 14,000×*g*. The supernatant containing nuclear proteins was collected and stored at −70 °C after determination of protein concentrations.

Western blot analysis

Livers were homogenized in 1 mL RIPA buffer containing 25 mM Tris–Cl (pH 7.4), 1 % Triton X-100, 0.1 % SDS, 0.5 % Deoxycholic acid, 10 % Glycerol, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF (1/200), 5 μg/mL of aprotinin (1/1000), leupeptin (1/1000), and pepstatin A (1/1000). Samples were sonicated, the lysates were centrifuged at 12,000×*g* for 30 min, and their protein concentration was measured using the BCA protein assay kit with BSA as the standard. Diluted samples containing equal amounts of protein (30 μg) were prepared in 5× sample buffer [60 mM Tris–Cl (pH 6.8), 25 % glycerol, 2 % β-mercaptoethanol, 0.025 % bromophenol blue] and boiled for 5 min at 100 °C. Proteins were resolved on a 10 % SDS–polyacrylamide gel in running buffer containing 25 mM Tris, 192 mM glycine, and 0.1 % SDS (pH 8.3). After electrophoresis, proteins were transferred to a PVDF membrane for 120 min at 300 mA using transfer buffer containing 25 mM Tris, 192 mM glycine, and 20 % methanol (pH 8.3), and the blots were blocked with 5 % fat-free dry milk in TBST buffer [25 mM Tris–Cl (pH 7.4), 150 mM NaCl, and 0.1 % Tween-20] for 1 h at room temperature. The membranes were incubated overnight in primary antibodies [CYP1A1 (1:100), CYP1A2 (1:400) and HO-1 (1:200)] at 4 °C. Blots were washed five times with TBST at 10-min intervals followed by incubation with

1:5000 diluted secondary antibodies conjugated to HRP for 1 h and washed again in TBST five times. The transferred proteins were visualized with an ECL chemiluminescence detection kit (Abclon, Seoul, Korea). Anti-Nrf2 (1:200) antibodies were used as markers for the cytosolic and nuclear extracts, respectively. Membranes were probed for GAPDH (1:4000) or PCNA (1:200) to verify equal loading of proteins. Normalization of Western Blot bands was performed using GAPDH or PCNA, and bands were quantified using a scanner and accompanying software.

Statistical analysis

The statistical analysis was performed using SAS version 9.3 (SAS institute Inc., Cary, NC, USA). Values were expressed as mean ± standard deviation (SD). Differences among groups were evaluated by one-way analysis of variance and Duncan's studentized range tests.

Results

Effects of PLE on phase I enzyme activities in t-BHP-treated liver microsomes

The activities of CYPs on EROD (as indicator of CYP1A1), MROD (as indicator of CYP1A2), ERDM (as indicator of CYP3A), and PNPH (as indicator of CYP2E1) in microsomes prepared from livers of *t*-BHP-treated rats are shown in Fig. 1. EROD and MROD activities were highly enhanced in livers of rats treated with *t*-BHP alone. The pretreatment of 250, 500, and 1,000 mg/kg b.w. of PLE significantly inhibited EROD activity in *t*-BHP-treated microsomes (CYP1Al) by 2.5-, 3.3-, and 4.0-fold, respectively (Fig. 1a), as well as the MROD activity (CYP1A2) by 2.7-, 3.7- and 3.9-fold, respectively (Fig. 1b). In contrast, ERDM (CYP3A) and PNPH (CYP2E1) activities were not significantly different

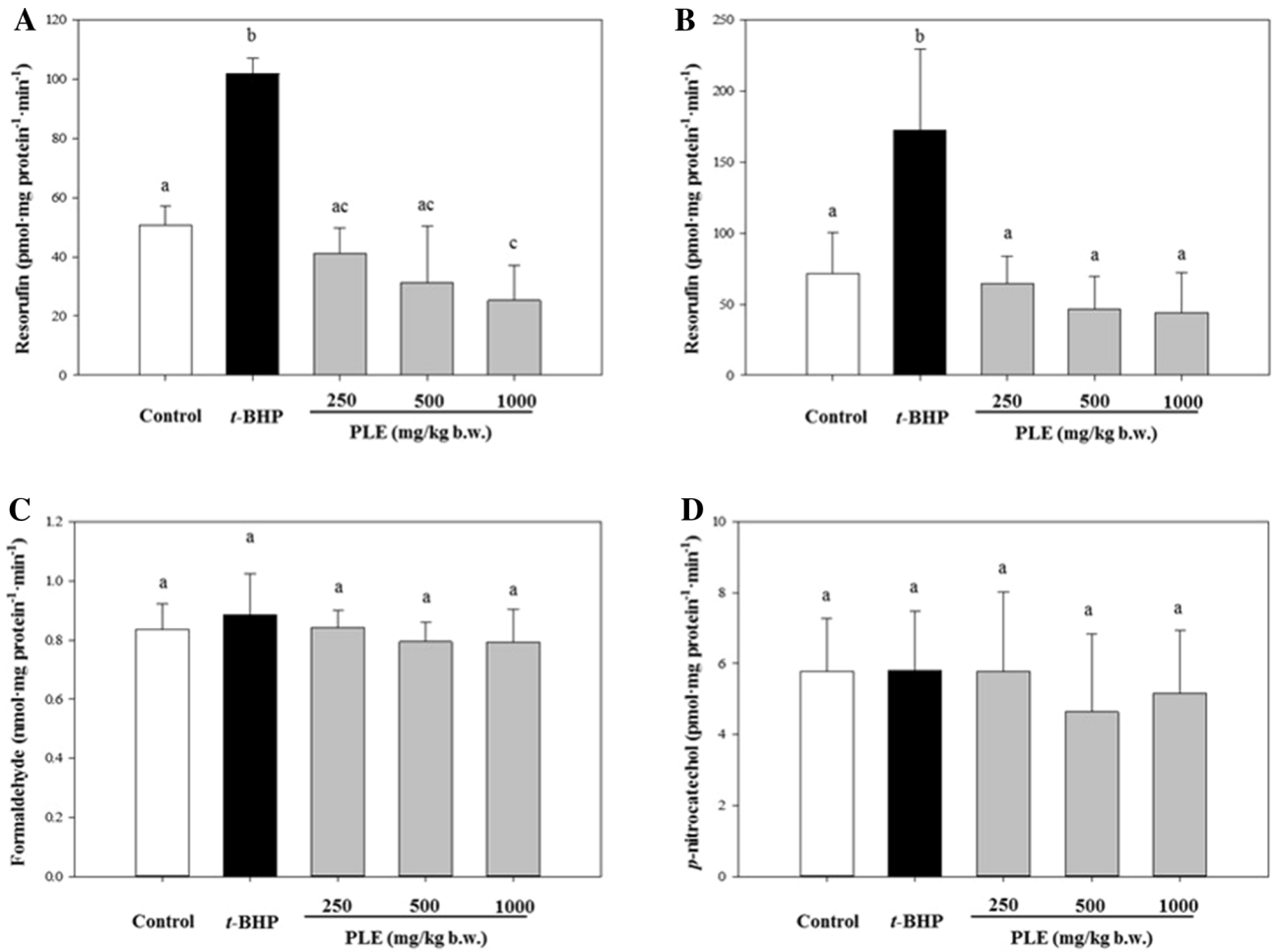

Fig. 1 Effect of PLE on in vivo hepatic microsomes isolated from rats treated with *t*-BHP on monooxygenase activities. SD rats were untreated or pretreated with multiple concentrations of PLE (250, 500, 1,000 mg/kg) and then injected (i.p.) with *t*-BHP to induce oxidative stress. The rat liver microsomes are used to measure CYPs enzyme activities including **a** EROD (CYP1A1), **b** MROD (CYP1A2), **c** ERDM (CYP3A), and **d** PNPH (CYP2E1). The results represent mean ± SD ($n = 7$). *Bars with different letters* indicate significant difference with $p < 0.05$ by Duncan's studentized range tests

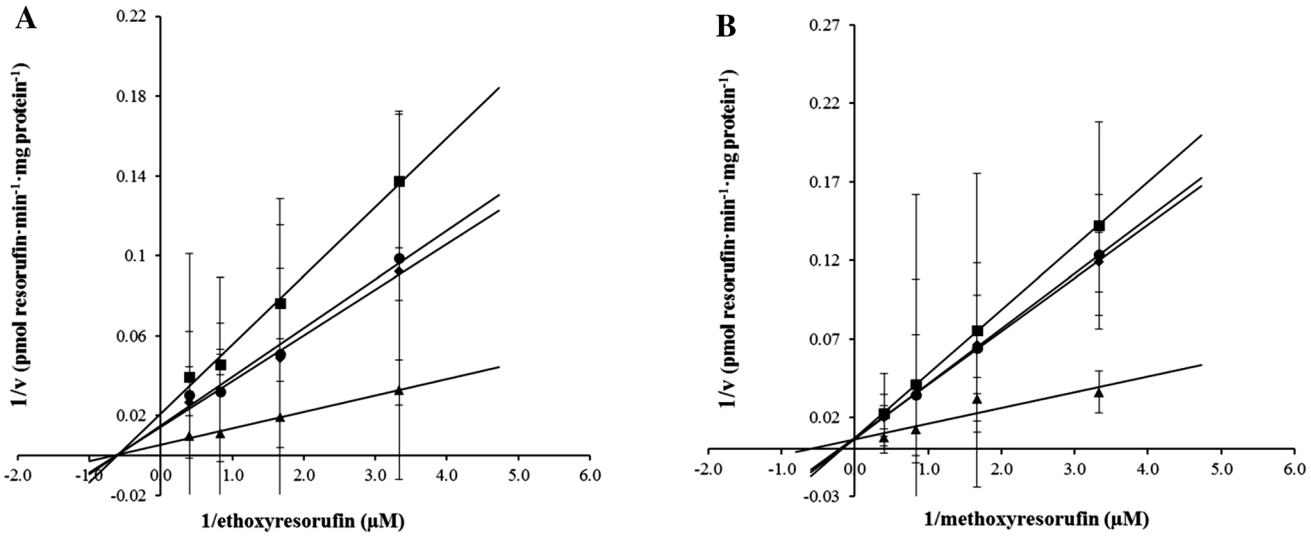

Fig. 2 Lineweaver–Burk plots of CYPs enzyme activities in liver microsomes of PLE-pretreated *t*-BHP-treated rats. **a** EROD and **b** MROD activities were measured in the absence of (*t*-BHP; *filled triangle*) or in the presence of PLE extract (*filled diamond*; 250 mg/kg, *filled circle*; 500 mg/kg, *filled square*; 1,000 mg/kg). The results represent mean ± SD ($n = 7$)

between the group treated with *t*-BHP alone and the groups pretreated with PLE followed by *t*-BHP treatment (Fig. 1c and d).

Lineweaver–Burk plots of CYP1A1/2 enzyme activities in t-BHP-treated liver microsomes

We investigated the inhibition mode of PLE-treated liver microsomes using Lineweaver–Burk plots. Figure 2a shows the Lineweaver–Burk plot for EROD activity (CYP1A1) and the inhibition by PLE extract in *t*-BHP-treated liver microsomes. CYP1A1 reaction was characterized with K_m (1.85 ± 0.27 μM) and V_{max} (195.77 ± 85.75 pmol/mg protein/min) without PLE extract. In the presence of PLE, the K_m remained unchanged. However, V_{max} was significantly decreased to 89.28 ± 8.42 pmol/mg protein/min, 71.83 ± 52.14 pmol/mg protein/min, and 54.14 ± 33.06 pmol/mg protein/min with PLE pretreatments of 250, 500, and 1,000 mg/kg b.w., respectively (Fig. 2a). Because the apparent K_m was unchanged in non-PLE and PLE extracts in *t*-BHP-treated liver, and the values for V_{max} decreased with PLE, the type of inhibition of the CYP1A1 activity by PLE is a noncompetitive inhibition. On the other hand, Fig. 2b shows the Lineweaver–Burk plot for MROD activity (CYP1A2), and the inhibition by PLE in *t*-BHP-treated liver microsomes. In the absence and presence of the PLE extract, V_{max} (172.52 ± 18.20 pmol/mg protein/min) remained unchanged; however, with 250, 500, and 1,000 mg/kg b.w. treatment of PLE, K_m increased to 5.30 ± 1.26, 5.93 ± 0.55, and 6.06 ± 2.41 μM, respectively, compared with the untreated control (2.96 ± 1.22 μM). Therefore, this result indicates that PLE is a competitive inhibitor of the CYP1A2 activity.

Effects of PLE on the expression of CYP1A1/2 mRNA and protein

We confirmed that CYP1A1/2 enzyme activities were increased in rat livers with *t*-BHP injections, while the PLE extract inhibited CYP1A1/2 activities. Next, we investigated the effect of PLE on the expression of CYP1A1/2 mRNA and protein induction. As seen in Fig. 3a and b, the expression of CYP1A1 mRNA increased in the group with *t*-BHP treatment alone compared with the control group, whereas this expression was significantly down-regulated in *t*-BHP group pretreated with PLE (1,000 mg/kg b.w.), compared to the group with *t*-BHP treatment alone. In addition, CYP1A2 mRNA expression in the *t*-BHP-treated liver of rats was higher than in the untreated liver, and the expression level significantly decreased with 1,000 mg/kg b.w. of PLE (Fig. 3c). On the other hand, CYP1A1 protein expression was decreased in the PLE-pretreated *t*-BHP treatment group compared with the *t*-BHP-treatment alone (Fig. 4a and b), although this reduction was not statistically significant. In addition, the PLE pretreatment caused a dose-dependent decrease in CYP1A2 protein levels in *t*-BHP-treated rat livers (Fig. 4c).

Effect of PLE on HO-1 mRNA and protein expression in t-BHP-treated rat livers

To determine whether the protective effect of PLE against *t*-BHP-induced hepatotoxicity was mediated through the

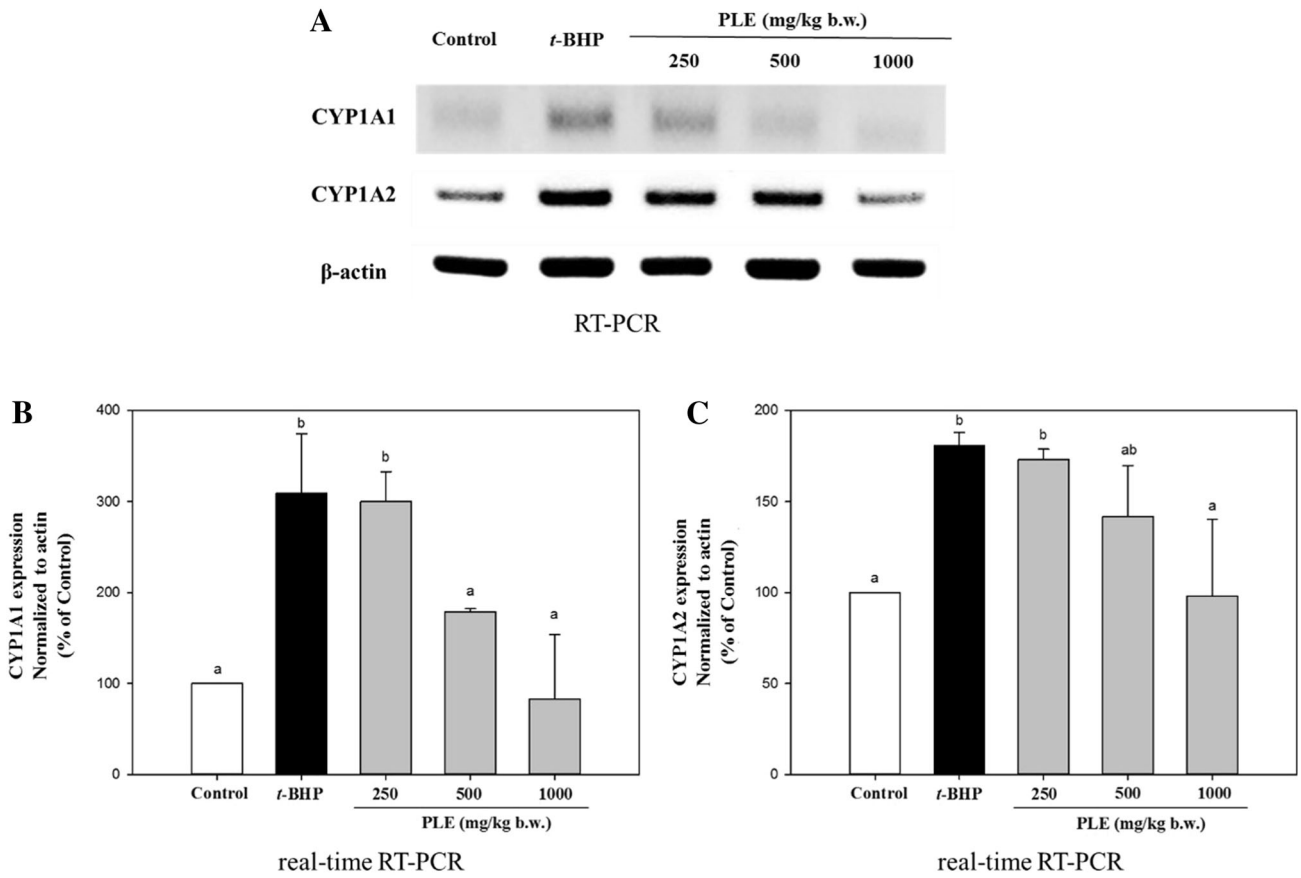

Fig. 3 Effect of PLE on the expression of CYP1A1/2 mRNA in the rat livers. **a** mRNA expression of CYP1A1/2 by RT-PCR. **b** Relative CYP1A1 mRNA level. **c** Relative CYP1A2 mRNA level. These levels are normalized to the β-actin mRNA level and the results represent mean ± SD ($n = 7$). *Bars with different letters* indicate significant difference with $p < 0.05$ by Duncan's studentized range tests

upregulation of HO-1, a Phase II enzyme, we examined the expression of HO-1 mRNA and protein in *t*-BHP-treated liver (Fig. 5). While mRNA transcript level of HO-1 decreased in the liver treated with *t*-BHP alone, PLE pretreatment was found to increase the expression of HO-1 mRNA reaching a peak at 1,000 mg/kg b.w of PLE. HO-1 protein expression in the *t*-BHP-treatment alone group also significantly decreased, while the PLE-pretreated groups showed a significant increase in HO-1 protein level compared with the *t*-BHP-treated group (Fig. 6). A maximal induction of HO-1 expression was achieved with the oral administration of PLE at 1,000 mg/kg b.w.

Effect of PLE on HO-1 enzyme activity in t-BHP-treated rat livers

To further examine the increased HO-1 activity in the PLE-pretreated groups with *t*-BHP treatment, we prepared microsomal fractions from rat livers and assayed them for HO-1 activity by measuring the bilirubin level. As shown in Fig. 7, HO-1 activity in the *t*-BHP-treated group was not different from that in the control group. However, PLE pretreatment increased the HO-1 activity, and 1,000 mg/kg b.w. of PLE significantly increased the activity by 1.2-fold, compared with *t*-BHP-treated alone group.

Expression of Nrf2 protein in the nuclear fraction of rat livers

The nuclear translocation of Nrf2 activates the up-stream regulator of HO-1 expression. Therefore, to verify whether PLE induces the translocation of Nrf2 to the nucleus, nuclear fractions were extracted for Western Blot analysis. As shown in Fig. 8a and b, nuclear fractions of rat livers showed that Nrf2 expression in the PLE-pretreated group, specifically with 1,000 mg/kg b.w. of PLE, significantly increased compared with the group with *t*-BHP treatment alone.

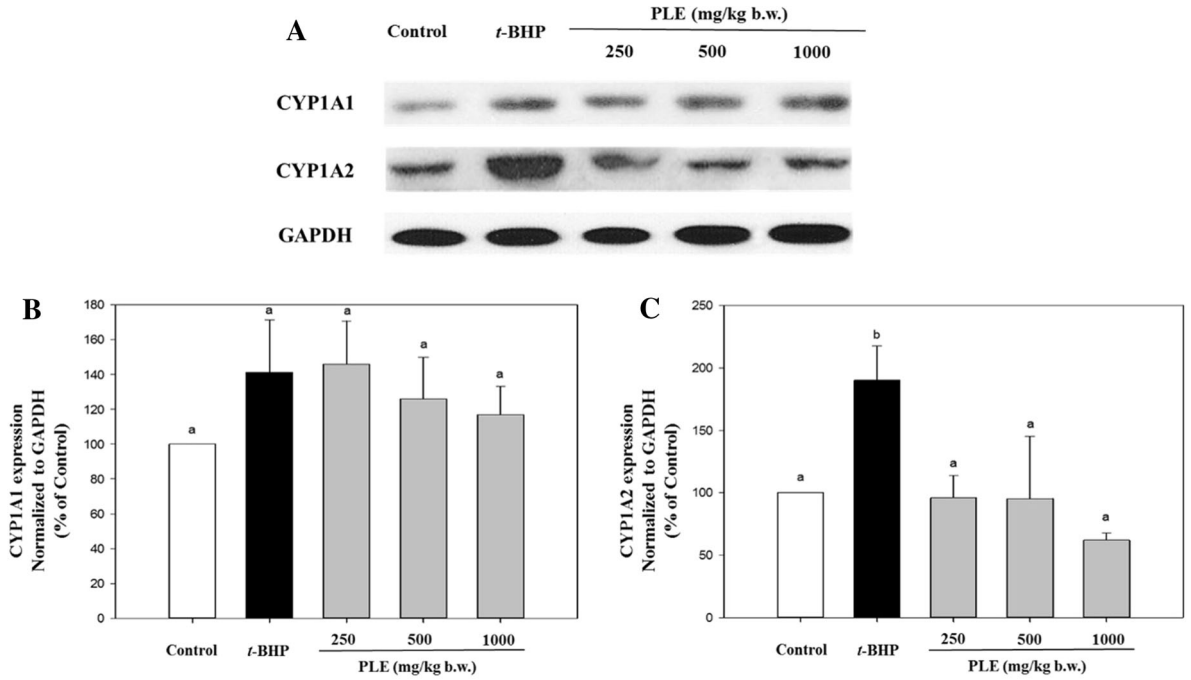

Fig. 4 Effect of PLE on the expression of CYP1A1/2 proteins in the rat livers. **a** Protein expression of CYP1A1/2 by Western Blot. **b** Relative CYP1A1 protein level. **c** Relative CYP1A2 protein level. These levels are normalized to the GAPDH expression level and the results represent mean ± SD ($n = 7$). *Bars with different letters* indicate significant difference with $p < 0.05$ by Duncan's studentized range tests

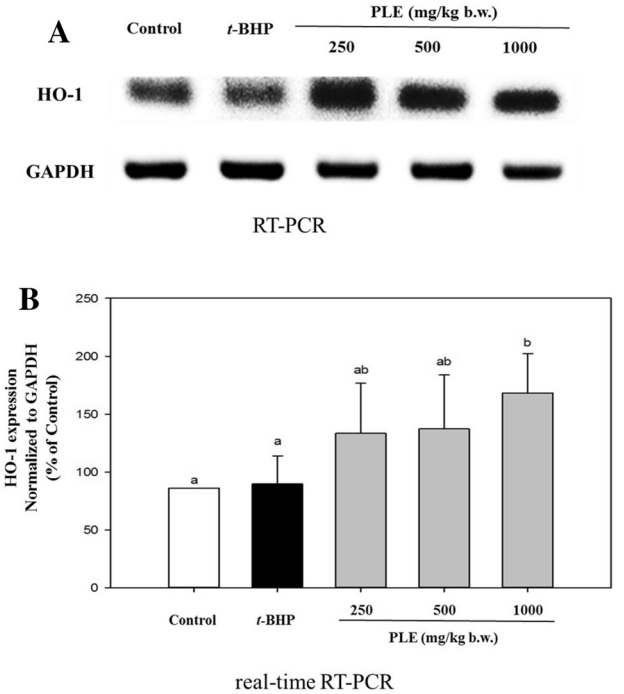

Fig. 5 Effect of PLE on the expression of HO-1 mRNA in the rat livers. **a** mRNA expression of HO-1 by RT-PCR. **b** Relative HO-1 mRNA level. These levels are quantified with real-time PCR and normalized to the expression of GAPDH. The results represent mean ± SD ($n = 7$). *Bars with different letters* indicate significant difference with $p < 0.05$ by Duncan's studentized range tests

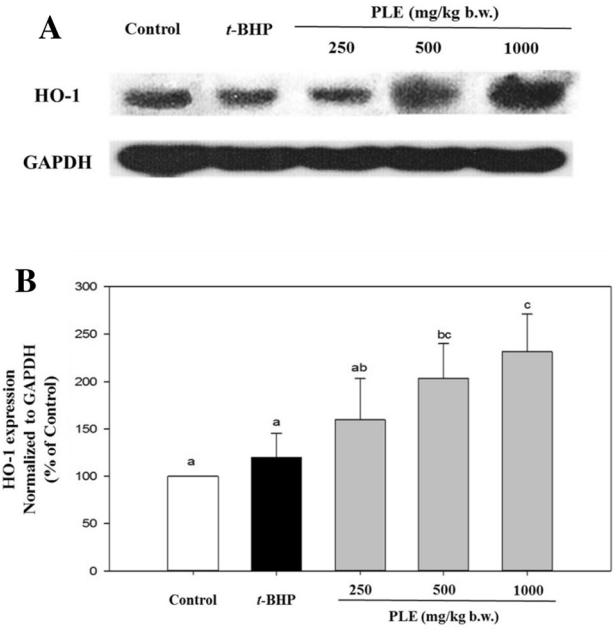

Fig. 6 Effect of PLE on the expression of HO-1 protein in the rat livers. **a** Protein expression of HO-1 by Western Blot. **b** Relative HO-1 protein level. These levels are normalized to the expression of GAPDH. The results represent mean ± SD (n = 7). *Bars with different letters* indicate significant difference with $p < 0.05$ by Duncan's studentized range tests

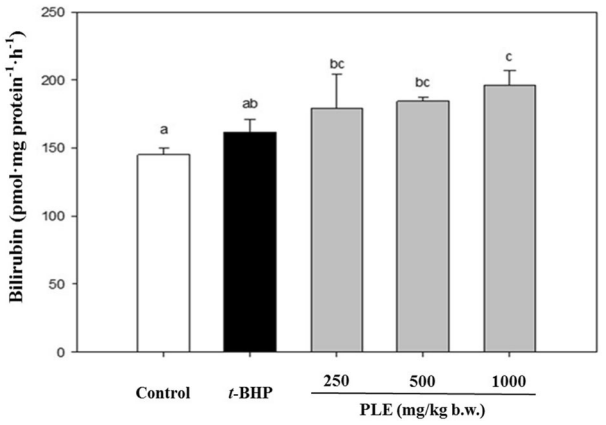

Fig. 7 Effect of PLE on the HO-1 enzyme activity in rats treated with *t*-BHP. The results represent mean ± SD ($n = 7$). *Bars with different letters* indicate significant difference with $p < 0.05$ by Duncan's studentized range tests

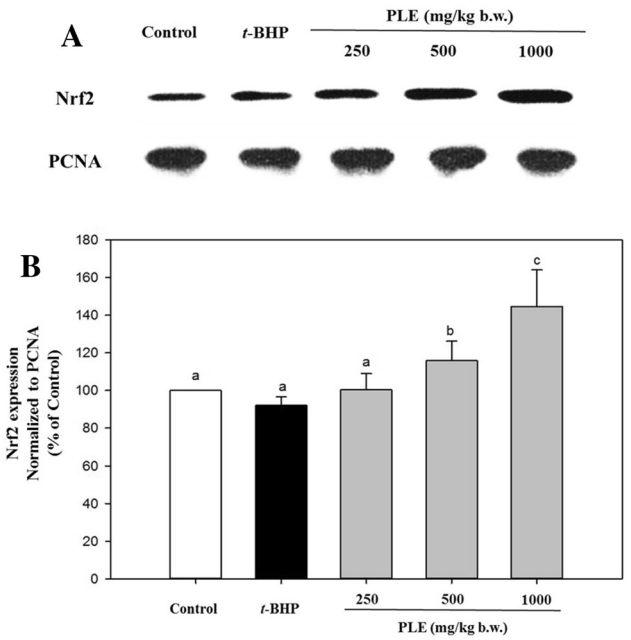

Fig. 8 Effect of PLE on the expression of Nrf2 protein in the nuclear fraction of rat livers. **a** Protein expression of Nrf2 by Western Blot. **b** Relative Nrf2 protein level. These levels are normalized to the expression of PCNA. The results represent mean ± SD ($n = 7$). *Bars with different letters* indicate significant difference with $p < 0.05$ by Duncan's studentized range tests

Discussion

Our previous study showed that PLE has antioxidant and hepatoprotective activities against *t*-BHP-induced oxidative damage (Kim et al. 2007; Yang et al. 2012). *t*-BHP, a well-known oxidant that is often used as a model agent inducing oxidative stress during in vitro and in vivo studies (Rush et al. 1985; Liu et al. 2002; Lee et al. 2004; Valentao et al. 2004; Yen et al. 2004), is metabolized to free radical intermediates in hepatocytes by CYPs, leading to the formation of peroxyl and alkoxyl radicals by lipid peroxidation (Hogberg et al. 1975). However, the identity of the specific CYPs modulated in *t*-BHP-induced oxidative stress is unknown. It has been reported that the *t*-BHP metabolism by CYPs primarily generates oxidative stress (Harman 1985). We observed expression of CYP1A1/2 and inhibition of their activity by PLE, which contributes to liver protection against *t*-BHP. In our study, the inhibition type of EROD was noncompetitive whereas that of MROD was competitive, suggesting that components in PLE possible bind not only to the substrate-binding site but also to an additional site, thus causing loss of enzyme activity (Gyamfi et al. 2000). Our results suggest that *t*-BHP is metabolized to free radical intermediates by the selective induction of CYP1A1/2, and PLE acts as a protective factor against oxidative stress by virtue of its specific inhibitory activity toward CYP1A1/2 as well as their reduced expression of CYP1A1/2 mRNA and protein.

In addition, activity of free radicals is decreased by Phase II enzymes including HO-1, which is a rate-limiting enzyme in heme catabolism and produces carbon monoxide (CO), biliverdin, and free ions (Fe^{2+}) as reaction products (Morse and Choi 2002). Specifically, biliverdin is converted to bilirubin by the action of biliverdin reductase, which mediates the protective effect of HO-1 by providing a strong antioxidant activity. HO-1 is known to have anti-inflammatory, antioxidant, and active anti-apoptotic effects in a number of tissues, and has therefore been suggested to be a novel therapeutic target for the management of oxidative and inflammatory processes in various diseases (Chan et al. 2011). We showed that pretreatment with PLE increased the expression of HO-1 mRNA/protein and its activity compared with *t*-BHP treatment alone. We also showed that 1,000 mg/kg b.w. of PLE significantly increased Nrf2 levels in rat livers treated with *t*-BHP compared with ones treated with *t*-BHP alone. This suggests that PLE-induced HO-1 expression may occur via the Nrf2 signaling pathway and thus upregulate the expression of antioxidant enzymes in rat liver to *t*-BHP-induced oxidative injury. This pathway is thought to be important to the intracellular antioxidant defense mechanism mediated by PLE. Although we did not include an experimental design regarding what chemical compound (s) in PLE is (are) responsible for the observed effects in this study, we previously confirmed that caffeic acid and rosmarinic acid are major compounds accounting for 74 % of PLE (Yang et al. 2013). In our preliminary study, the treatment of caffeic acid increased the expression of HO-1 in vitro (data not shown). Further investigation of the detailed mechanisms involved in the above modulations of CYPs and Phase II

enzymes by pretreatment with these major compounds of PLE in liver is needed.

In summary, we demonstrated that *P. frutescens* has protective effects against *t*-BHP-induced hepatotoxicity in rat liver through the modulated activity and expression of CYP1A1/2 and HO-1 as well as the activation of Nrf2, suggesting that PLE could be beneficial in treating several diseases associated with oxidative stress, through its enhanced antioxidant mechanisms, and may also have therapeutic applications in liver diseases involving oxidative damage.

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