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[Original Article]

Pulegone and Piperitone, Essential Oil Components of *Lamiaceae* Family, Enhance the Activities of Drug-Metabolizing Enzymes

Keiko SASAKI[1)], Keiji WADA[2)], and Koozi MATUOKA[1)]

The effects of pulegone (PUL) and piperitone (PIP), monoterpene ketones found in the essential oils of *Lamiaceae* family, on the activities of phase I enzymes (7-ethoxyresorufin *O*-deethylase (EROD), 7-methoxyresorufin *O*-demethylase (MROD), and 7-pentoxyresorufin *O*-depentylase (PROD)) and phase II enzymes (glutathione S-transferase (GST) and quinone reductase (QR)) were investigated. Mice were treated orally with PUL (50-200 mg/kg), and PIP (50-200 mg/kg) once a day for 7 days, and then the hepatic and extrahepatic enzyme activities were analyzed. PUL (200 mg/kg) treatment resulted in significantly higher EROD, PROD, GST, and QR activities by 1.79-fold to 6.33-fold, and PIP (200 mg/kg) treatment caused significantly higher EROD, MROD, PROD, GST, and QR activities by 1.18-fold to 3.69-fold. Moreover, PUL-treated mice showed significantly higher protein levels of GST α and PIP-treated animals had significantly higher protein levels of GST π. Similarly, PUL treatment resulted in significantly higher GST and QR activities in kidney by 1.23-fold and 1.68-fold, respectively; PIP treatment caused significantly higher kidney QR activities by 1.32-fold. Additionally, PUL and PIP also caused higher lung QR activities by 1.24-fold and 1.54-fold, respectively. Taken together, we showed that PUL and PIP are bifunctional inducers, capable of inducing both the phase I and phase II enzymes in mice.

I. INTRODUCTION

The development of cancer is closely related to environmental carcinogen exposure. Cancer chemoprevention involves prevention, delay, or reversal of the process of carcinogenesis through the application of natural or synthetic compounds[1)]. A widely accepted mechanism in cancer chemoprevention is modulation of the body's drug-metabolizing enzymes to enhance carcinogen detoxification. The normal human drug-metabolizing system is generally divided into two phases of enzymes. Phase I enzymes are involved in microsomal transformation of xenobiotic molecules via hydroxylation, oxidation or hydrolysis. This phase is catalyzed by monooxygenase cytochrome P450 (Cyt P450) to produce modified derivatives. Phase II enzymes catalyzed the conjugation of these derivatives with glutathione. The chemical modification of drugs or other xenobiotics by both phase I and II enzymes enhances their polarity and solubility, reduces their toxicity, and facilitate their excretion. It is well recognized that induction of phase II enzymes, especially the GST and QR, plays a central role in detoxifying environmental carcinogens. It has been proposed that the capacity of a compound to induce phase II enzyme expression is directly related to its ability to act as a Michael reaction acceptor[2-4)].

The natural monoterpene ketones, PUL and PIP, are major components of some essential oils of the mint family (*Lamiaceae*). Both contain α, β-unsaturated carbonyl group and thus could function as Michael reaction acceptor (Fig. 1). PUL is a major component of pennyroyal oil that has been used as a flavoring agent in foods, and as an herbal medicine to induce menstruation and abortion[5)]. PIP has been reported to have insecticidal and weak antifungal activities[6)]. However, it is not known with certainty whether PUL and PIP are responsible for modulating various drug-metabolizing enzymes. Therefore, the objective of this study was to test the hypothesis that dietary PUL and PIP increase phase I and phases II enzymes in an animal

Correspondence : Keiko Sasaki ksasaki@cis.ac.jp
1) Faculty of Pharmacy, Chiba Institute of Science
2) Health Sciences University of Hokkaido
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model under basal conditions (i.e., not following pharmacologic or carcinogen-mediated induction).

II. MATERIALS AND METHODS

1. Materials

PUL and PIP were obtained commercially from Kanto Chemical Co., Inc. (Tokyo, Japan) and Extrasynthese (Genay, France), respectively. Chemicals and reagents of analytical grade were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) or Kanto Chemical Co., Inc. (Tokyo, Japan), unless otherwise stated.

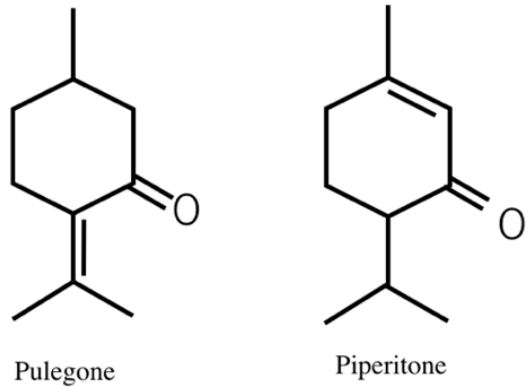

Fig. 1. Chemical structure of pulegone and piperitone

2. Animal care and treatment

Four-week-old mice (ddY strain, male) were obtained from Japan SLC, Inc. (Shizuoka, Japan). Principles in good laboratory animal carg.e were followed and animal experimentation was in compliance with the Guidelines for the Care and Use of Laboratory Animals in the Health Sciences University of Hokkaido. Mice were maintained under a controlled environment (22 ± 2°C with constant humidity of 55 ± 10% and a 12 h light/dark cycle) and provided with water and diet *ad lib*. PUL and PIP (50, 100 and 200 mg/kg/day, respectively) or a vehicle (2% gum Arabic solution) was administered orally to mice once a day for 7 successive days.

Twenty-four hours after the last treatment the mice were sacrificed by decapitation. Tissues were removed, washed with ice-cold 1.15% potassium chloride and blotted briefly. They were then weighed and subjected to the preparation of cytosolic fractions as essentially described before[7]. Briefly, livers, kidneys, and lung were homogenized with Potter-Elvehjem homogenizers in 6 volumes, 9 volumes, and 20 volumes of ice-cold physiological saline, respectively. The homogenates were first centrifuged at 9,000 x g for 20 minutes and the resultant supernatants were centrifuged at 105,000 x g for 60 minutes at 4°C. The supernatants from the second centrifugation were referred to as cytosolic fractions and stored at -80°C until use. Protein contents in the samples were determined by the Lowry-Folin method.

3. Measurement of enzyme activities

Measurement of enzyme activities were determined as described[7]. Briefly, Cytosolic GST activity with 1-chloro-2,4-dinitrobenzene (CDNB; Kanto Kagaku, Tokyo, Japan), as a substrate were determined. Cytosolic QR activity with menadione (Sigma, MO, USA) as a substrate was determined. Microsomal activities of 7-methoxyresorufin *O*-demethylase (MROD), 7-ethoxyresorufin *O*-deethylase (EROD) and 7-pentoxyresorufin *O*-dependentylase (PROD) were determined.

4. Western blot analysis of GST isozymes

Western blot analysis was performed as previously described[7]. Briefly, the cytosolic fraction from the liver was dissolved, blotted and immunoreacted by using polyclonal rabbit antiserum against GST α (PCF 403), μ (PCF408) and π (PCF401). All sera were purchased from YLEM (Rome, Italy). Signals were quantified using a densitograph (Lumino CCD Model AE-6930, ATTO, Tokyo, Japan).

5. Statistical analysis

Statistical analyses were performed by Dunnett test or Student t-test. Differences with P values < 0.05 were considered statistically significant.

III. RESULTS

1. Effects of PUL and PIP on phase I enzymes

Figure 2 summarizes our study of the effects of PUL and PIP on the activities of hepatic phase I enzymes, EROD (marker of CYP1A1), MROD (marker of CYP1A2) and PROD (marker of CYP2B) in mice. Oral administration of PUL at a dosage from 50 to 200 mg/kg enhanced the activities of EROD and PROD in a dose-dependent manner. It appears that the effect of PUL was more pronounced for PROD than EROD. Thus 200 mg/kg PUL produced a 6.33-fold increase in PROD activity, but only a 1.79-fold increase in EROD activity. However, PUL caused no change in MROD (marker of CYP1A2) activity. PIP showed a similar potency in stimulating phase I enzyme activities in

mouse liver. Thus, PIP at 200 mg/kg significantly enhanced EROD, MROD and PROD activity 1.37-fold, 1.29-fold, 3.69-fold, respectively.

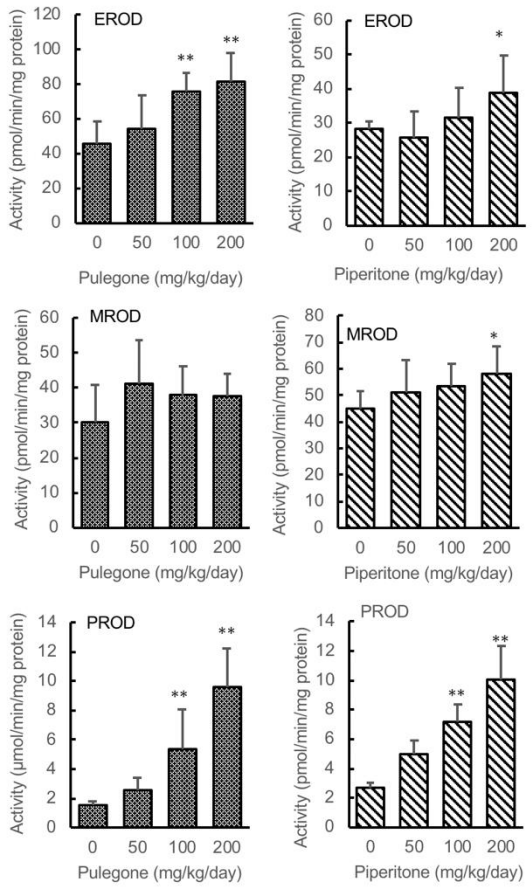

Fig. 2. Effects of PUL or PIP on the markers for CYP enzymes. Mice were treated orally with PUL (50-200 mg/kg), PIP (50-200 mg/kg) or vehicle once a day for 7 days. Date are mean ± standard deviation values of eight to ten animals. Significant differences are indicated as follows: **P* < 0.05 and ***P* < 0.01 compared with control. CYP, cytochrome P450; EROD, 7-ethoxyresorufin *O*-deethylase; MROD, 7-methoxyresorufin *O*-deethylase; PROD, 7-pentoxyresorufin *O*-depentylase.

2. Effects of PUL and PIP on phase II enzymes

Figure 3 summarizes our study of the effects of PUL and PIP on the activities of hepatic phase II enzymes GST and QR in mice liver. Oral administration of PUL (50-200 mg/kg) induced GST activity in a dose-dependent manner. At the dosage of 100 and 200 mg/kg, PUL elevated the activity 1.55-fold and 2.40-fold, respectively, compared with the control. PUL (200 mg/kg) significantly increased the QR activity by 2.73-fold. Similarly, PIP at 200 mg/kg increased the activities of GST and QR by 1.76-fold and 1.18-fold, respectively.

GST is known to possess multiple cytosolic and membrane-bond isozymes, with distinct properties. The main isozymes in mouse liver are class α, μ and π isozymes. To further explore the mechanism of effects of PUL and PIP on GST, we examined the effects of PUL and PIP (200 mg/kg, respectively) on the protein levels of three classes of GST isozymes in mouse liver by western blot analysis. Figure 4 shows the representative immunoblots. The study showed that both PUL and PIP enhanced the protein level of class α GST, whereas only PIP enhanced the protein level of class π GST.

3. Effects of PUL and PIP on the activities of GST in kidney and lung

We then investigated whether the changes in phase II enzyme activities also occur in organisms other than the liver. Specifically, we examined the effects of PUL and PIP on mouse kidney and lung. Table 1 summarized our findings: Briefly, PUL significantly elevated the QR activities in kidney and slightly the GST activity in kidney. In kidney, PUL (200 mg/kg) and PIP (200 mg/kg) enhanced QR activity by 1.68-fold and 1.32-fold, respectively.s PUL enhanced GST activity 1.32-fold as compared with the control. Both PUL and PIP significantly enhanced QR activity in lung but had only little effects on GST activity.

IV. DISCUSSION

The present study demonstrates that oral administration of PUL increases the activities of phase l enzymes, *viz*., EROD and PROD, excluding MROD, in mouse liver (Fig. 2). Similarly, PIP also significantly elevated the activities of these CYP enzymes evaluated in present study. Especially, both PUL and PIP induced higher activity of PROD, marker for a CYP2B enzyme in mouse liver than that of EROD, marker for a CYP1A. In addition, the magnitude of induction of PROD activity was higher with PUL than with equal doses of PIP. As CYP2B gene induction is known to be regulated by the constitutive androstane receptor (CAR)[8], PUL and PIP may induce certain drug-metabolizing enzymes through activation of the nuclear receptor CAR.

As for the phase II enzymes, both PUL and PIP significantly enhanced GST and QR activity in mouse liver (Fig. 3).

Moreover, these compounds significantly enhanced also QR activity in mouse kidney and lung. On the other hands, PUL and PIP did not affect GST activities in kidney and lung, except for a slightly higher activity of GST induced by PUL in kidney (Table 1). These results support the notion that the phase II enzyme induction is attributed to the presence of α,β-unsaturated carbonyl group[2-4]. GST belongs to a superfamily of multifunctional isoenzymes categorized into the three major classes, α, μ and π, and all three have overlapping substrate specificities and physiological functions[9]. The quantitative analysis of the immunoblots revealed slight difference in the action of both PUL and PIP. Thus, PUL increases mainly the protein level of class α isozyme, while PIP increases the protein level of class π (Fig. 4). It is interesting to note that class α GST isozymes are known to exhibit high catalytic efficiency toward aflatoxin B_1-8.9-epoxide, the ultimate carcinogen of the fungus mycotoxin aflatoxin B_1[10] whereas class π isozymes contribute to detoxifying reactive benzo[a]pyrene (BaP) metabolites widespread environmental pollutants in cigarette smoke and automobile exhaust and suppress BaP-induced adduct formation[11]. The reduction of electrophilic quinones by QR also is an important detoxification pathway which protects against the toxicity of quinones and their metabolic precursors, *viz.*, polycyclic aromatic hydrocarbon, benzene and reduces oxidative cycling[12].

In general, it is recognized that main function of phase I metabolism is to prepare a compound for phase II metabolism and not to prepare the drug for excretion. Phase II is usually the true detoxification of drugs and xenobiotics which gives products that are generally water soluble and can be easily excreted. The action of phase II enzymes on the substrates generated by the action of phase I enzymes on innocuous and/or hazardous chemicals, lead to their solubilization and excretion[10]. Compounds that both phase I and phase II enzymes are known as bifunctional inducers. It has been shown that bifunctional inducers such as oltipraz are effective chemopreventive agents[14-16]. Here we showed that PUL and PIP are indeed bifunctional inducers. As such, their effects on drug-metabolizing enzymes should be responsible, at least partly, for the chemopreventive action of these monoterpene ketones.

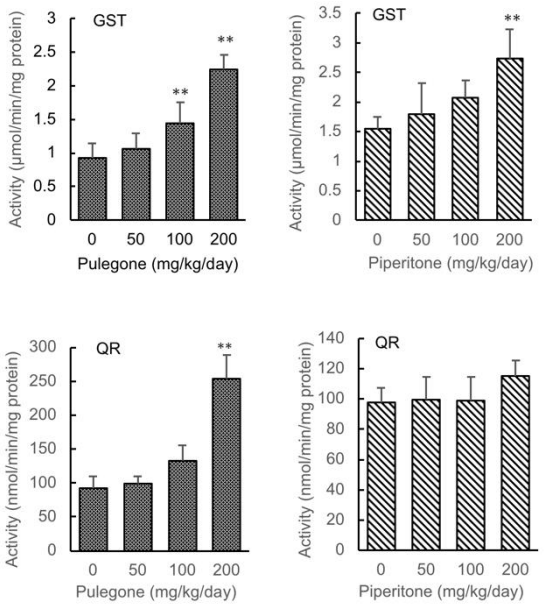

Fig. 3. Effects of PUL or PIP on the activities of GST and QR in mouse liver. Mice were treated orally with PUL (50-200 mg/kg) or PIP (50-200 mg/kg) or vehicle once a day for 7 days. Data are mean ± standard deviation values of three to six animals. Significant differences are indicated as follows: $*P < 0.05$ and $**P < 0.01$ compared with control.

Table 1 Effects of pulegone and piperitone on activities of GST and QR in mouse kidney and lung

	Kidney			Lung		
	Weight (g)	GST (μmol/min/mg protein)	QR (nmol/min/mg protein)	Weight (g)	GST (μmol/min/mg protein)	QR (nmol/min/mg protein)
Control	0.94 ± 0.05	0.42 ± 0.02	291.1 ± 21.1	0.48 ± 0.03	0.19 ± 0.04	43.88 ± 0.67
Pulegone	0.95 ± 0.02	0.52 ± 0.05**	490.5 ± 51.5***	0.41 ± 0.03*	0.23 ± 0.04	54.62 ± 4.62**
Control	1.01 ± 0.02	0.46 ± 0.03	285.3 ± 27.9	0.43 ± 0.01	0.23 ± 0.06	41.77 ± 5.99
Piperitone	1.06 ± 0.01***	0.47 ± 0.06	375.5 ± 68.7*	0.39 ± 0.00**	0.28 ± 0.02	64.31 ± 7.08***

Mice were treated orally pulegone (200 mg/kg), piperitone (200 mg/kg), or vehicle once a day for 7 days. Data are mean ± standard deviation values of five animals. Significant differences are indicated as follows: $*P < 0.05$ and $**P < 0.01$ compared with control.

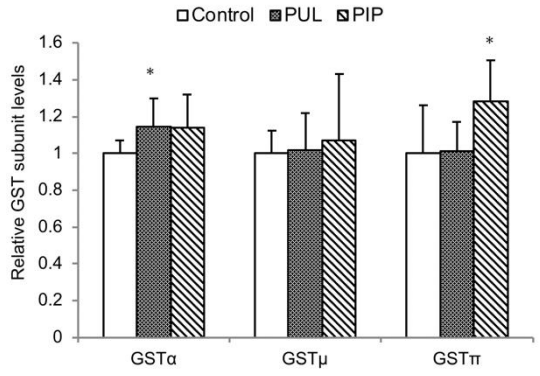

Fig. 4. Western blot analysis of the protein levels of GST isoenzymes. Cytosol fractions were prepared from the liver of mice treated with PUL (200 mg/kg, *p.o.* for 7 days), PIP (200 mg/kg, *p.o.* for 7 days) or vehicle and analyzed by immunoblotting. The protein levels were quantified by densitometry, and the level in the control was set at 1. Data are mean ± standard deviation values of eight to twelve animals. Significant differences are indicated as follows: $*P < 0.05$ compared with control

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