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Oral administration of naturally occurring coumarins leads to altered phase I and II enzyme activities and reduced DNA adduct formation by polycyclic aromatic hydrocarbons in various tissues of SENCAR mice

Heather E.Kleiner, Suryanarayana V.Vulimiri, Laura Miller, William H.Johnson Jr[1], Christian P.Whitman[1]and John DiGiovanni[2]

University of Texas MD Anderson Cancer Center, Science Park-Research Division, Department of Carcinogenesis, PO Box 389, Smithville, TX 78957, USA and [1]University of Texas, Division of Medicinal Chemistry, College of Pharmacy, Austin, TX 78712-1074, USA

[2]To whom correspondence should be addressed
Email: sa83017@odin.mdacc.tmc.edu

Several naturally occurring coumarins, to which humans are routinely exposed in the diet, were previously found to inhibit P450-mediated metabolism of benzo[*a*]pyrene (B[a]P) and 7,12-dimethylbenz[*a*]anthracene (DMBA) *in vitro*, block DNA adduct formation in mouse epidermis and inhibit skin tumor initiation by B[a]P and/or DMBA when applied topically to mice. The present study was designed to investigate the effects of two of these compounds, of the linear furanocoumarin type, when given orally (70 mg/kg per os, four successive daily doses), on P450 and glutathione *S*-transferase (GST) activities and DNA adduct formation by B[a]P and DMBA in various mouse tissues. Imperatorin and isopimpinellin significantly blocked ethoxyresorufin *O*-deethylase (EROD) and pentoxyresorufin *O*-dealkylase (PROD) activities in epidermis at 1 and 24 h after oral dosing. Imperatorin and isopimpinellin modestly inhibited EROD activities in lung and forestomach at 1 h and significantly inhibited PROD activities in lung and forestomach at 1 h after the final oral dose. Twenty-four hours after the final oral dose of imperatorin or isopimpinellin EROD and PROD activities remained inhibited in epidermis and lung. However, forestomach P450 activity had returned to control levels. Interestingly, imperatorin and isopimpinellin treatment inhibited liver EROD activity at 1 h, had no effect on PROD activity at this time point, but elevated both these enzyme activities at 24 h. Elevated EROD and PROD activities coincided with elevated hepatic P450 content. Imperatorin and isopimpinellin treatment also increased liver cytosolic GST activity at both 1 and 24 h after the final oral dose by 1.6-fold compared with corn oil controls. Oral administration of imperatorin and isopimpinellin also had a protective effect against DNA adduct formation by B[a]P and DMBA. Imperatorin pretreatment decreased formation of DNA adducts by DMBA in forestomach. Pretreatment with isopimpinellin led to reduced DNA adduct levels in liver (B[a]P), lung (B[a]P) and mammary epithelial cells (DMBA). These results suggest that imperatorin and isopimpinellin may have potential chemopreventive effects when administered in the diet.

Abbreviations: ARE, antioxidant response element; B[a]P, benzo[*a*]pyrene; CDNB, 1-chloro-2,4-dinitrobenzene; DAS, diallyl sulfide; DMBA, 7,12-dimethylbenz[*a*]anthracene; EROD, ethoxyresorufin *O*-dealkylase; GPEI, GST P enhancer I; GST, glutatione *S*-transferase; 8-MOP, 8-methoxypsoralen; PAH, polycyclic aromatic hydrocarbon; PB, phenobarbital; PEITC, phenethyl isothiocyanate; PROD, pentoxyresorufin *O*-dealkylase; TPA, 12-*O*-tetradecanoylylphorbol-13-acetate; XRE, xenobiotic-responsive element.

Introduction

Coumarins occur naturally in many food sources, including citrus fruits, herbs and vegetables (1,2). Coumarin itself (1,2-benzopyrone) is found in tonka beans (3). Furanocoumarins are found primarily in the Umbelliferae and Rutaceae families. The linear furanocoumarin isopimpinellin (Figure 1) is present in the rind and pulp of limes (total furanocoumarin content ~3 μg/g pulp and juice or 5 mg/g oils) and in celery (up to 1.3 p.p.m. total furanocoumarin content) (4–6), while imperatorin is present in lemon and lime oils, parsnip, parsley and many wild plants (total furanocoumarin content up to 0.003% dry weight parsley seeds) (6–8). Other coumarins found in citrus oils include isoimperatorin, bergapten (5-methoxypsoralen) and bergamottin (6). Psoralen, xanthotoxin (8-methoxypsoralen or 8-MOP) and bergapten are found in parsnip (up to 4–5 mg furanocoumarins/0.1 kg parsnip root) and are not destroyed by normal cooking procedures (9). Coriandrin, bergapten and 8-MOP have also been identified in the leaves (45 μg/g leaves) and fruit of *Coriandrum sativum* (10) and fresh coriander leaves (cilantro or Chinese parsley), which are used extensively in cooking. In addition, coriander seeds (cumin) are used in curry powder and other spices. Thus, furanocoumarins are ingested regularly as part of the human diet.

Earlier studies suggested that coumarins modulate the metabolism of polycyclic aromatic hydrocarbons (PAHs). Several 8-acyl-7-hydroxycoumarins inhibited 3-methylcholanthrene-induced rat hepatic aryl hydrocarbon hydroxylase activity and metabolism of benzo[*a*]pyrene (B[a]P) to its corresponding dihydrodiols (11). Imperatorin, isoimperatorin and other derivatives were shown to inhibit drug metabolizing enzymes (12). Imperatorin and other coumarins also blocked mutagenesis by B[a]P in *Salmonella typhimurium* in the presence of a hepatic 9000 *g* supernatant (S9) activating system (13). Coumarin was also found to induce glutathione *S*-transferase (GST) activity in the forestomach, liver and intestines of mice (14,15). Treatment of rats with coumarin and 4-methylcoumarin (1 mmol/kg body wt per os) blocked 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced mammary carcinogenesis (16). Coumarin or limettin (5,7-dimethoxycoumarin) in the diet (0.034–0.068 mmol/g diet) also inhibited DMBA-induced rat mammary tumorigenesis (17). In addition, coumarin and 6-methylcoumarin treatment inhibited B[a]P-induced forestomach neoplasia in mice (17). Thus, coumarins have been shown to exhibit antitumor initiating activity by modulating PAH metabolism.

In addition to their antitumor initiating properties, some coumarins have been shown to block tumor promotion. Imperatorin and isoimperatorin, isolated from the umbelliferous Chinese crude drugs Tang-Bai-Zai and Ashita-ba, possess

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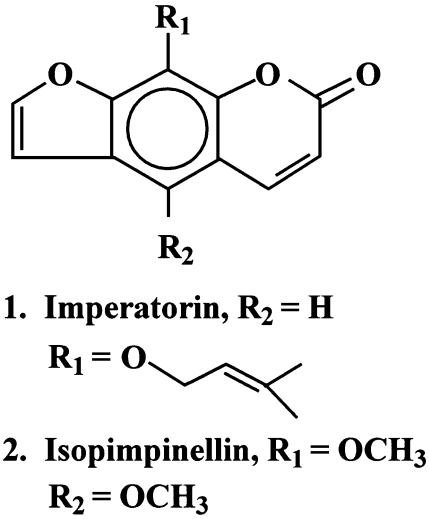

Fig. 1. Chemical structures of imperatorin and isopimpinellin.

strong antitumor promoter activity in cultured cells (18). Moreover, Pd-II [(+)anomalin, (+)praeruptorin B], a seselin-type coumarin isolated from Qian-Hu, a traditional Chinese medicine, effectively blocked 12-*O*-tetradecanoylylphorbol-13-acetate (TPA)-induced skin tumor promotion in DMBA-treated mice (19). The citrus coumarin auraptene has also been shown to have chemopreventive activities in several systems, including inhibition of azoxymethane-induced aberrant crypt foci in rat colon, prevention of 4-nitroquinoline 1-oxide-induced oral carcinogenesis in rats and attenuation of TPA-induced skin tumor promotion in mice initiated with DMBA (20–22). These studies demonstrate that, as a general class, coumarins have promising potential as chemopreventive agents and are worthy of further study.

Previous studies in our laboratory have shown that naturally occurring coumarins (e.g. coriandrin, imperatorin and isopimpinellin) inhibited P450-mediated enzyme activities *in vitro* (23). Additional *in vitro* experiments demonstrated that certain naturally occurring coumarins are mechanism-based inactivators of P450s (e.g. coriandrin) (24). In cultured mouse epidermal keratinocytes several naturally occurring coumarins (e.g. bergamottin and coriandrin) inhibited metabolism of B[a]P and DNA adduct formation from both B[a]P and DMBA (25). *In vivo* studies showed that a series of naturally occurring coumarins inhibited covalent binding of B[a]P and/or DMBA to mouse epidermal DNA (26). Furthermore, selected naturally occurring coumarins blocked skin tumor initiation by B[a]P, DMBA or both (26). Imperatorin, an effective inhibitor of mouse skin tumor initiation by DMBA, was also found to inhibit complete carcinogenesis by DMBA in mouse skin (26).

The above studies in our laboratory were performed using topical administration of both the coumarins and the carcinogens. Since coumarins are found in the diet, the focus of the study reported in this paper was to determine whether these naturally occurring coumarins were also effective when administered orally. Our hypothesis was that coumarins capable of inhibiting P450 activity and/or enhancing GST activity could also block DNA adduct formation by B[a]P and DMBA. In order to study the mechanisms of the overall effects of coumarins, we examined the effects of imperatorin and isopimpinellin on phase I and phase II enzyme activities in liver, lung, skin epidermis and forestomach. Next, we investigated the effects of orally administered imperatorin and isopimpinellin on DNA adduct formation induced in various tissues by orally administered DMBA and B[a]P. The results suggest that linear furanocoumarins, such as imperatorin and isopimpinellin, may possess anti-carcinogenic properties when administered orally.

Materials and methods

Materials

B[a]P was purchased from Aldrich Chemical Co. (Milwaukee, WI). DMBA was purchased from Eastman Kodak Co. (Rochester, NY). [^{3}H]B[a]P (sp. act. 66–70 Ci/mmol) and [^{3}H]DMBA (sp. act. 62 Ci/mmol) were obtained from Amersham Co. (Arlington Heights, IL) and diluted with unlabeled B[a]P or DMBA to a specific activity of 5 or 2.5 Ci/mmol, respectively. Imperatorin was synthesized by a method previously described (8). Identity and purity of the product were confirmed by thin-layer chromatography, melting point and ^{1}H and ^{13}C NMR spectroscopy. Isopimpinellin was purchased from Indofine Chemical Co. (Somerville, NJ). The chemical structures of imperatorin and isopimpinellin are shown in Figure 1. Collagenase, hyaluronidase, 7-ethoxyresorufin, 7-pentoxyresorufin, resorufin, DNase I (bovine pancreas, EC 3.1.4.1), $NADP^+$, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, 1-chloro-2,4-dinitrobenzene (CDNB), snake venom phosphodiesterase (*Crotalus atrox*, EC 3.1.4.1) and alkaline phosphatase (*Escherichia coli* type III, EC 3.1.3.1) were supplied by Sigma Chemical Co. (St Louis, MO). Sep-Pak cartridges were obtained from Waters Corp. (Milford, MA). HPLC grade methanol was purchased from EM Science (Gibbstown, NJ).

Animals

Female SENCAR mice (NCI, Frederick, MD) (7–9 weeks of age) were fed AIN-76A semi-purified diet (Dyets, Bethlehem, PA) for 2 weeks prior to and during the study. Mice were randomized and divided into 3–4 mice/group. Mice were housed in disposable polystyrene cages (Lab Products, Maywood, NJ) on hardwood bedding (P.J. Murphy Inc., Montville, NJ) in an AAALAC accredited facility with a diurnal light cycle of 14 h light/10 h dark, at a temperature range of 68–74°F and relative humidity of 60–70%. Mice were allowed food and autoclaved water (reverse osmosis, pH 2.5) *ad libitum* throughout the studies.

Animals, dosing, tissue collection and tissue preparation for enzyme studies

Preparation of furanocoumarins and dosing of animals were conducted in subdued lighting. Mice were dosed with imperatorin or isopimpinellin (70 mg/kg body wt suspended in 0.1 ml corn oil) by gavage for four consecutive days. Control mice received vehicle only (0.1 ml corn oil). Mice were shaved on the dorsal side 2 days before the final dose. Mice were killed by cervical dislocation at 1 or 24 h after the final dose. Liver, lungs, forestomach and skin epidermis were collected on ice. The contents of the stomachs were removed before processing. All tissues except epidermis were first rinsed in 0.05 M Tris buffer, pH 7.5, containing 0.25 M sucrose (Tris–sucrose buffer). Then, all tissues were homogenized (1:5 w/v) in Tris–sucrose buffer using a Polytron PT10 homogenizer (three bursts of 10 s each at setting six). Epidermis and forestomach homogenates were frozen in aliquots at –80°C for later analysis. Liver and lung microsomal and cytosolic fractions were prepared by differential centrifugation as described (23). Protein concentrations were determined by the Bradford method (27) using bovine serum albumin as the standard. The study was performed three times with consistent results and data averaged together.

Enzyme assays

Ethoxyresorufin *O*-deethylase (EROD) and pentoxyresorufin *O*-dealkylase (PROD) assays were performed as described (23,28). Liver microsomes (0.25 mg/ml protein), lung microsomes (0.5 mg/ml), epidermal homogenates (1 mg/ml) and forestomach homogenates (3 mg/ml) were preincubated with a NADPH generating system (0.336 mM $NADP^+$, 0.5 mM glucose 6-phosphate, 0.15 mM $MgCl_2$ and 1 U glucose 6-phosphate dehydrogenase) in 0.05 M Tris buffer, pH 7.5 (total volume 1.0 ml) at 37°C for 5 min. Reactions were initiated with 7-ethoxy- or 7-pentoxyresorufin (5 μM) and terminated at 10 (liver) or 120 min (lung, epidermis and forestomach) using 2.5 ml ice-cold methanol. Samples were allowed to precipitate on ice and centrifuged for 10 min at 3000 *g*. Supernatants were analyzed spectrofluorometrically for product (resorufin) at an excitation of 550 nm and emission of 585 nm. Experimental values were extrapolated using a standard curve in the linear range. Background (boiled protein) was subtracted from experimental values.

Tissue GST activity was determined by the method of Habig and co-workers (29) using CDNB as substrate. Assay mixtures contained 2–50 μl of cytosol (liver and lung) or homogenate (epidermis and forestomach), 1 mM CDNB (dissolved in ethanol) and 1 mM reduced glutathione in 0.1 M potassium phosphate buffer, pH 6.5 (total volume 1.0 ml). Samples were

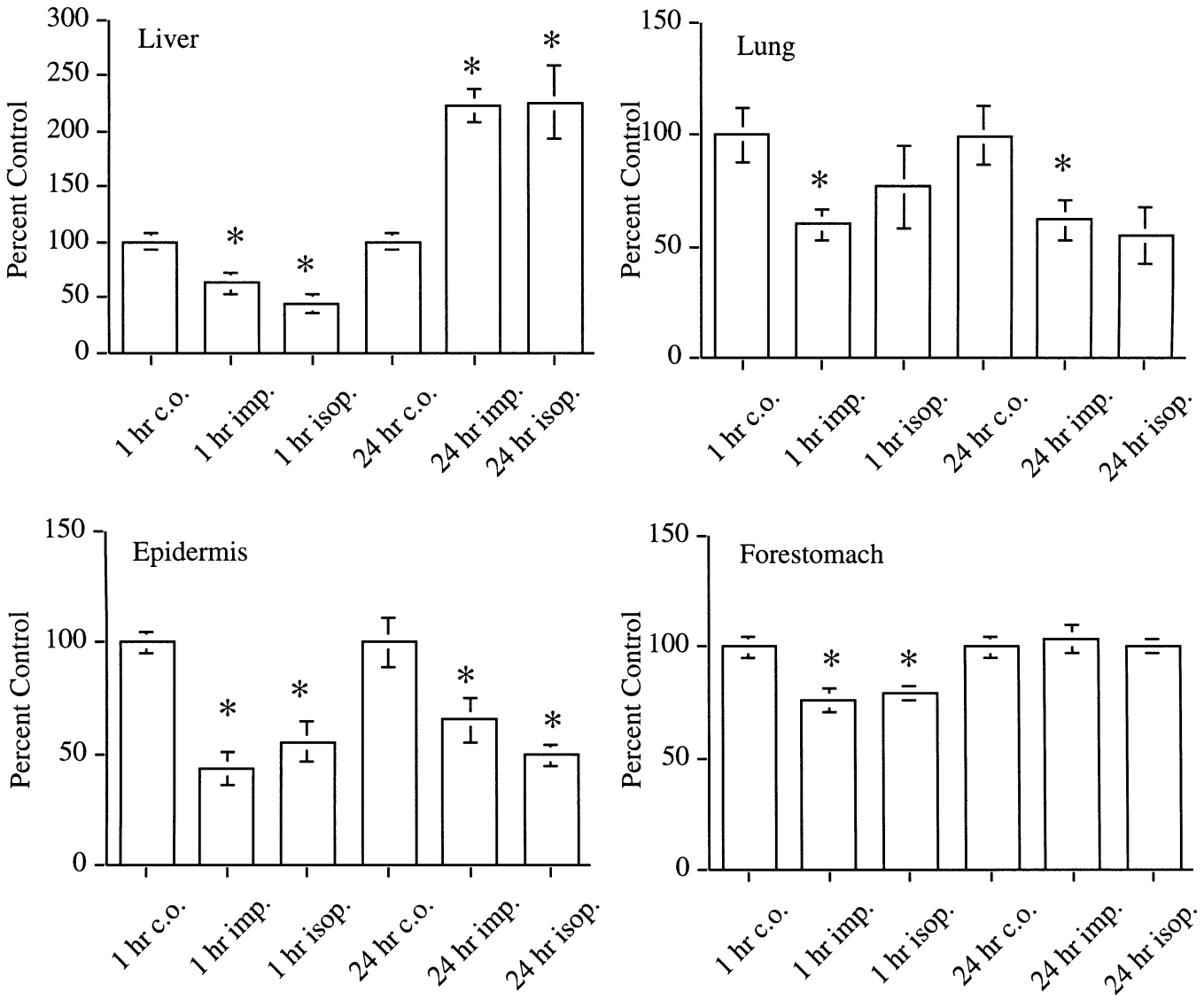

Fig. 2. Effects of orally administered imperatorin and isopimpinellin on EROD activity. Female SENCAR mice (7–9 weeks old) were maintained on AIN-76A semi-purified diet and dosed with corn oil (c.o.), imperatorin (imp.) or isopimpinellin (isop.) (70 mg/kg body wt suspended in 0.1 ml corn oil) by gavage (four consecutive daily doses). At either 1 or 24 h after the final dose mice were killed by cervical dislocation and liver, lungs, forestomach and epidermis were isolated. EROD activity was determined as described in Materials and methods. Data represent percent of corn oil controls (means ± SE, n = 3–12). Control EROD activities (pmol/mg protein) at 1 and 24 h were, respectively: 71 ± 7 and 72 ± 6 (liver); 0.37 ± 0.09 and 0.58 ± 0.19 (lung); 1.15 ± 0.13 and 1.76 ± 0.56 (epidermis); 0.098 ± 0.005 and 0.180 ± 0.041 (forestomach). *$P \leqslant 0.05$.

incubated at 25°C and monitored spectrophotometrically for changes in absorbency at 340 nm. Concentrations of product were calculated using a molar extinction coefficient of 9.6/mM/cm. Reference blanks contained the assay mixture without the tissue.

Concentrations of cytochrome P450 were determined in liver microsomes from the (dithionite + carbon monoxide) – dithionite difference spectrum as described by Omura and Sato (30). The molar extinction coefficient of 91/mM/cm was used for the absorbance change between 450 and 490 nm.

Effects of orally administered imperatorin and isopimpinellin on DNA adduct formation by orally administered B[a]P and DMBA

Preparation of compounds, treatment of animals and processing of tissues were conducted in subdued lighting. Mice were dosed with corn oil, imperatorin or isopimpinellin (70 mg/kg body wt suspended in 0.1 ml corn oil per os, four consecutive daily doses). Thirty minutes to one hour after the last pretreatment mice were dosed once with [^{3}H]B[a]P (50 μg, 5 Ci/mmol) or [^{3}H]DMBA (50 μg, 2.5 Ci/mmol) (both dissolved in corn oil). Twenty-four hours after treatment with carcinogen mice were killed by cervical dislocation and liver, lungs, dorsal skin epidermis, forestomach and abdominal and inguinal mammary glands were isolated. The mice had been shaved 2 days prior to death in order to collect epidermis. Mammary epithelial cells were isolated as described by Moon (31). DNA was hydrolyzed with DNase I and DNA adducts were determined using liquid scintillation spectroscopy as described (26). DNA concentration was estimated using diphenylamine reagent and calf thymus DNA as the standard (32). Hepatic DNA was further hydrolyzed sequentially with snake venom phosphodiesterase and alkaline phosphatase. Adducted DNA hydrolysate was then purified by Sep-Pak chromatography and radioactivity in the methanol phase was used for adduct analysis as described previously (33). Due to the limited amounts of radioactivity in the extra-hepatic tissues (lung, forestomach, epidermis and mammary gland) Sep-Pak analysis was not performed on these samples. Experiments were performed three times with consistent results and data represent an average of these experiments.

Statistical analysis

Experimental groups were compared with their respective controls using ANOVA followed by Student's t-test or Fischer's PLSD test. Differences were considered significant at $P \leqslant 0.05$.

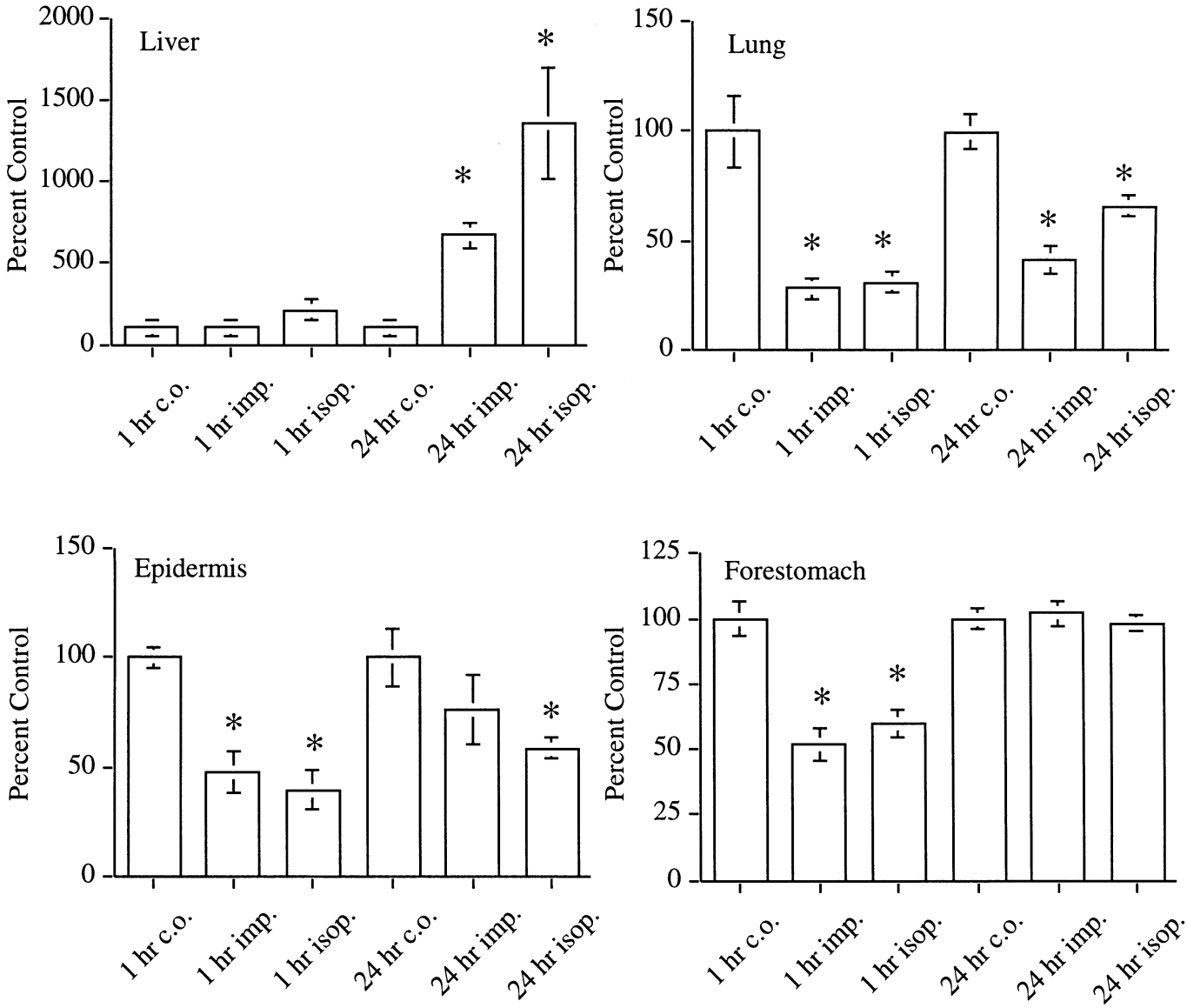

Fig. 3. Effects of orally administered imperatorin and isopimpinellin on PROD activity. Female SENCAR mice (7–9 weeks old) were maintained on AIN-76A semi-purified diet and dosed with corn oil (c.o.), imperatorin (imp.) or isopimpinellin (isop.) (70 mg/kg body wt suspended in 0.1 ml corn oil) by gavage (four consecutive daily doses). At either 1 or 24 h after the final dose mice were killed by cervical dislocation and liver, lungs, forestomach and epidermis were isolated. PROD activity was determined as described in Materials and methods. Data represent percent of corn oil controls (means ± SE, n = 3–12). Control PROD activities (pmol/mg protein) at 1 and 24 h were, respectively: 13.9 ± 1.6 and 11.8 ± 1.0 (liver); 1.98 ± 0.56 and 1.40 ± 0.26 (lung); 0.52 ± 0.12 and 0.51 ± 0.15 (epidermis); 0.084 ± 0.006 and 0.198 ± 0.048 (forestomach). *$P \leqslant 0.05$.

Results

Effects of orally administered imperatorin and isopimpinellin on phase I enzyme activities and P450 content

The effects of orally administered imperatorin and isopimpinellin on tissue EROD activities are summarized in Figure 2. One hour after the last of four consecutive daily doses imperatorin and isopimpinellin significantly inhibited EROD activity in liver by 38 and 57%, respectively. However, by 24 h after the final dose hepatic EROD activity was elevated 220% by both compounds. In lung, imperatorin and isopimpinellin inhibited EROD activity 1 h after the final dose by 40 and 23%, respectively. This inhibitory effect of imperatorin and isopimpinellin was sustained over 24 h in lung. The inhibitory effect of imperatorin was statistically significant ($P \leqslant 0.05$) at both time points. Epidermal EROD activity was also significantly inhibited (45–57%) by both coumarins at 1 h after the final dose and this effect was sustained over 24 h. In forestomach, imperatorin and isopimpinellin caused a modest but statistically significant inhibition (21–24%) of EROD activity 1 h after the final dose. Forestomach EROD activity had returned to control values by 24 h.

The effects of orally administered imperatorin and isopimpinellin on tissue PROD activities are summarized in Figure 3. Overall, the results were quite similar to the effects on EROD activity except that there was no apparent inhibition of hepatic PROD activity at 1 h after the last dose and the elevation of PROD activity in the liver at 24 h was even more pronounced than observed for EROD activity. Also, the inhibitory effects of imperatorin and isopimpinellin on lung and forestomach PROD activities at 1 h after the last dose

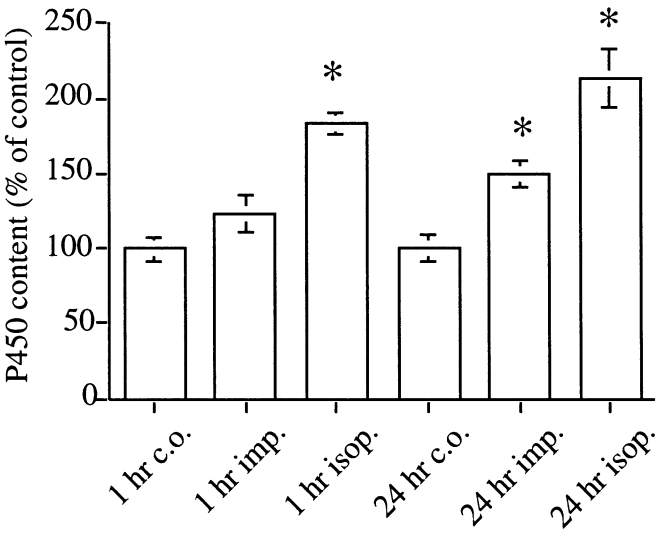

Fig. 4. Effects of orally administered imperatorin and isopimpinellin on hepatic microsomal P450 content. Female SENCAR mice (7–9 weeks old) were maintained on AIN-76A semi-purified diet and dosed with corn oil (c.o.), imperatorin (imp.) or isopimpinellin (isop.) (70 mg/kg body wt dissolved in 0.1 ml corn oil) by gavage (four consecutive daily doses). At either 1 or 24 h after the final dose mice were killed by cervical dislocation and liver microsomes were isolated. P450 content was determined as described (30). Data represent percent of corn oil controls (means ± SE, n = 3–10). Control values were 0.689 ± 0.046 and 0.682 ± 0.081 for 1 and 24 h, respectively (means ± SE). *$P \leqslant 0.001$.

were even greater than observed for EROD activity at the same time point. Imperatorin and isopimpinellin inhibited PROD activity in lung by 72 and 69%, respectively, in epidermis by 52 and 61%, respectively, and in forestomach by 48 and 40%, respectively, at 1 h after the last dose. In lung and epidermis, the inhibition of PROD activity was sustained at 24 h, whereas in forestomach, PROD activity had returned to control levels.

In the light of the increased EROD and PROD activities observed in hepatic microsomes at 24 h post-dosing with imperatorin and isopimpinellin, hepatic microsomal P450 content was determined (Figure 4). P450 content was significantly elevated by isopimpinellin at 1 h after the last treatment and by both imperatorin (49%) and isopimpinellin (113%) at 24 h after the last treatment. The elevated hepatic P450 content may explain the elevated hepatic EROD and PROD activities observed at 24 h after the last dose of imperatorin and isopimpinellin. This may also explain why there was no inhibitory effect of either compound on hepatic PROD activity at 1 h or on hepatic EROD or PROD activity at 24 h post-dosing.

Effects of orally administered imperatorin and isopimpinellin on GST activity

As part of this study we also examined the effect of imperatorin and isopimpinellin on tissue GST activity (using CDNB as substrate). These results are summarized in Figure 5. Imperatorin and isopimpinellin significantly increased liver cytosolic GST activity 1.6-fold over control levels at both 1 and 24 h after the final dose. The maximum increase was observed at 24 h after the last dose of isopimpinellin in liver. There was a slight elevation of cytosolic GST activity in lungs 1 h following treatment (~1.25-fold) with imperatorin, which was statistically significant. This slight elevation in lung GST activity was not observed at 24 h post-dosing. Although the results were not statistically significant, there was a slight elevation in forestomach GST activity after isopimpinellin treatment. Finally, there were no significant changes in epidermal GST activity induced by either coumarin.

Effects of orally administered imperatorin and isopimpinellin on PAH DNA adducts

The effects of orally administered imperatorin and isopimpinellin on DNA adduct formation in liver, lung, skin epidermis, forestomach and mammary gland (isopimpinellin only) were evaluated using orally administered carcinogens. As shown in Figure 6, imperatorin significantly inhibited formation of [^{3}H]DMBA DNA adducts in mouse forestomach by 49% (Figure 6D) compared with mice that received corn oil. At the dose tested (70 mg/kg body wt) imperatorin did not significantly inhibit formation of [^{3}H]DMBA DNA adducts in any of the other tissues examined (Figure 6A–C). In addition, imperatorin dosing did not significantly reduce [^{3}H]B[a]P DNA adduct formation in any of the tissues examined (see Figure 6A–D). In contrast, isopimpinellin significantly inhibited formation of [^{3}H]B[a]P DNA adducts in liver by 43% and in lung by 26% (Figure 7A and B). Furthermore, isopimpinellin significantly ($P \leqslant 0.05$) inhibited formation of [^{3}H]DMBA DNA adducts by 28 ± 2% (mean ± SE, n = 4) in mammary gland compared with the corn oil control (data not shown). At the dose tested isopimpinellin did not significantly inhibit [^{3}H]B[a]P DNA adduct formation in mammary epithelial cells (data not shown).

Discussion

The focus of the current study was to evaluate the effects of naturally occurring linear furanocoumarins when administered orally on several carcinogen metabolizing enzymes and on the formation of DNA adducts induced by orally administered PAHs in various tissues. Our results demonstrate that orally administered imperatorin and isopimpinellin could modulate phase I and II enzyme activities in several tissues, including tissues that are targets for PAH carcinogenesis in the mouse. These results also confirmed that orally administered imperatorin and isopimpinellin were absorbed and distributed to various tissues.

The effects of orally administered imperatorin and isopimpinellin on hepatic P450 activities were very interesting. The lack of an overall inhibitory effect of isopimpinellin on PROD activity was likely due to the elevated P450 content observed at 1 h post-dosing with this compound. By 24 h post-dosing, clearance of the furanocoumarins from the liver would have eliminated their inhibitory effects, thus allowing more pronounced elevated enzyme activities. Previous studies with the related compound 8-MOP have shown that when administered systemically (10 mg/kg i.v.) to rats it is widely distributed (34). In addition, a single injection of 8-MOP in rats (27 mg/kg i.p.) decreased clearance from the blood of theophylline, caffeine, phenytoin and hexobarbital and inhibited 7-ethoxycoumarin deethylase activity in liver, presumably by inhibition of P450-mediated drug metabolism (35–37). Similar to the effects seen with imperatorin and isopimpinellin, 8-MOP (0.8 mg/kg/day for six consecutive days) significantly induced P450 activity in rat and mouse liver but not in rat skin (35). In addition, multiple administrations (27–50 mg/kg i.p. or per os, three daily doses) of 8-MOP also resulted in elevated drug metabolism in rats, as indicated by increased clearance of caffeine and theophylline (37,38) and increased 7-ethoxycoumarin deethylase activity

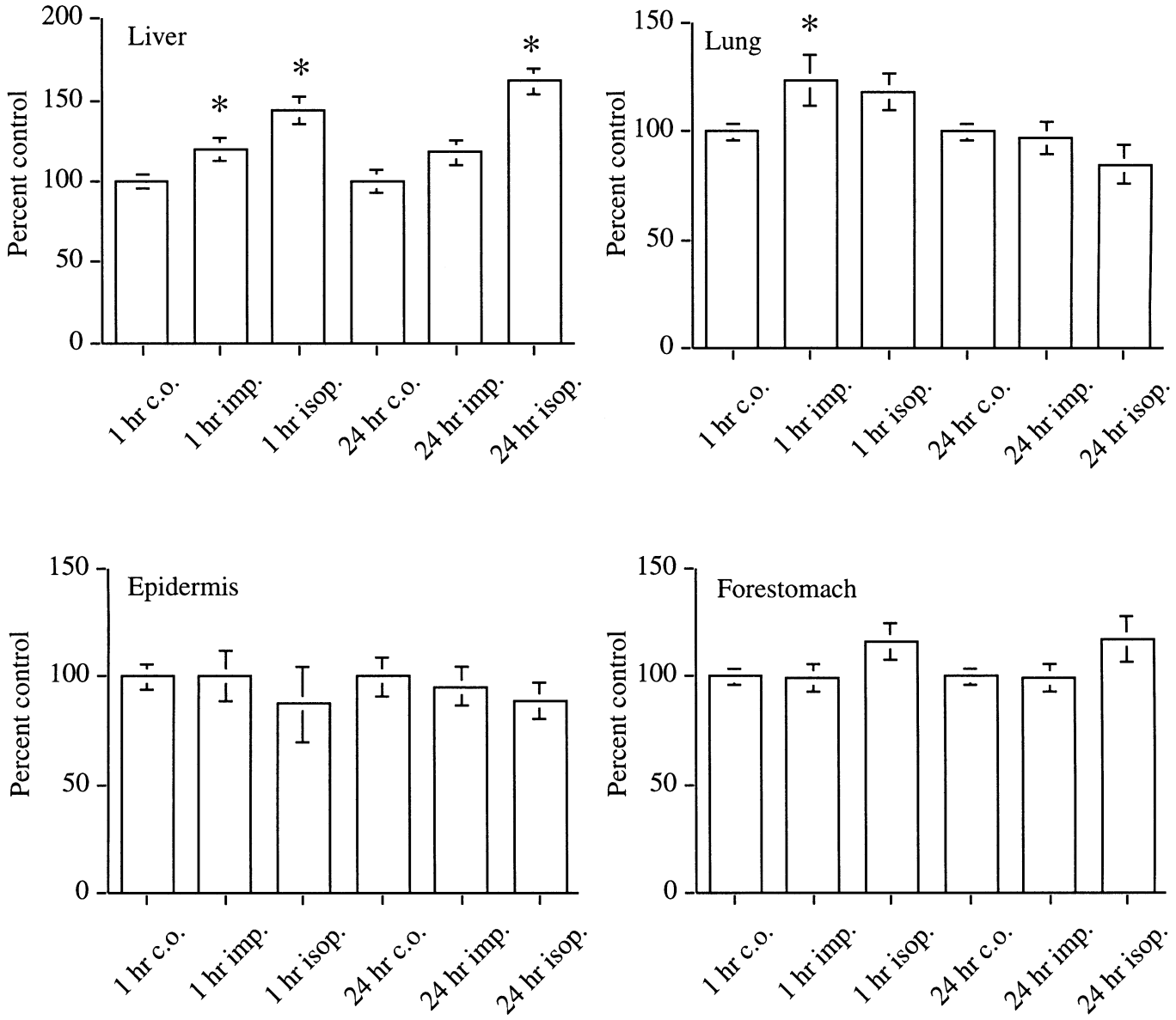

Fig. 5. Effects of orally administered imperatorin and isopimpinellin on GST activity. Female SENCAR mice (7–9 weeks old) were maintained on AIN-76A semi-purified diet and dosed with corn oil (c.o.), imperatorin (imp.) or isopimpinellin (isop.) (70 mg/kg body wt dissolved in 0.1 ml corn oil) by gavage (four consecutive daily doses). At either 1 or 24 h after the final dose mice were killed by cervical dislocation and liver, lungs, forestomach, and epidermis were isolated. GST activity was determined as described in Materials and methods. Data represent percent of corn oil controls (means ± SE, n = 3–12). Control GST activities (μmol/min/mg protein) at 1 and 24 h were, respectively: 2.0 ± 0.2 and 2.0 ± 0.2 (liver); 0.28 ± 0.03 and 0.30 ± 0.02 (lung); 0.12 ± 0.02 and 0.16 ± 0.03 (epidermis); 0.50 ± 0.06 and 0.56 ± 0.08 (forestomach). *$P \leq 0.05$.

in liver (39). The short-term inhibition of P450 in liver by 8-MOP observed following acute administration is also likely to be due to presence of the compound and its direct effect on the P450 enzyme (36), similar to our results with imperatorin and isopimpinellin. In terms of the induction of hepatic P450 by these compounds, there are several possible mechanisms.

One possible mechanism for the elevation of hepatic EROD and PROD activities and P450 content by linear furanocoumarins may be a compensatory mechanism in response to their P450 inactivating effects (24,36). Alternatively, these effects may be similar to the pleiotropic effects observed after phenobarbital (PB) treatment. In this regard, PB causes a 50- to 100-fold induction of hepatic CYP2B levels and a modest induction of other cytochrome(s) P450 and phase II metabolic enzymes in the rat (40,41). In our studies, the enhancement of hepatic PROD activity was much greater than EROD activity for both imperatorin and isopimpinellin. Thus, the elevated P450 content observed in liver 24 h after the last of four doses of imperatorin or isopimpinellin may primarily be P450 2B1/2B2. Interestingly, the fragrance ingredient musk xylene (1,3,5-trinitro-2-t-butylxylene) also produces a PB-like induction of hepatic enzymes, but it also inhibits P450 2B enzyme activity *in vitro* (42). Similarly, imperatorin and isopimpinellin inhibited EROD and PROD activity *in vitro* in mouse liver microsomal incubations (23). Therefore, imperatorin and isopimpinellin may act similarly to musk xylene in that they inhibit EROD and PROD activity (and the corresponding P450s mediating these activities), but with consecutive treatments cause up-regulation of hepatic expression of the enzyme. Another compound that appears to have a similar spectrum of activities

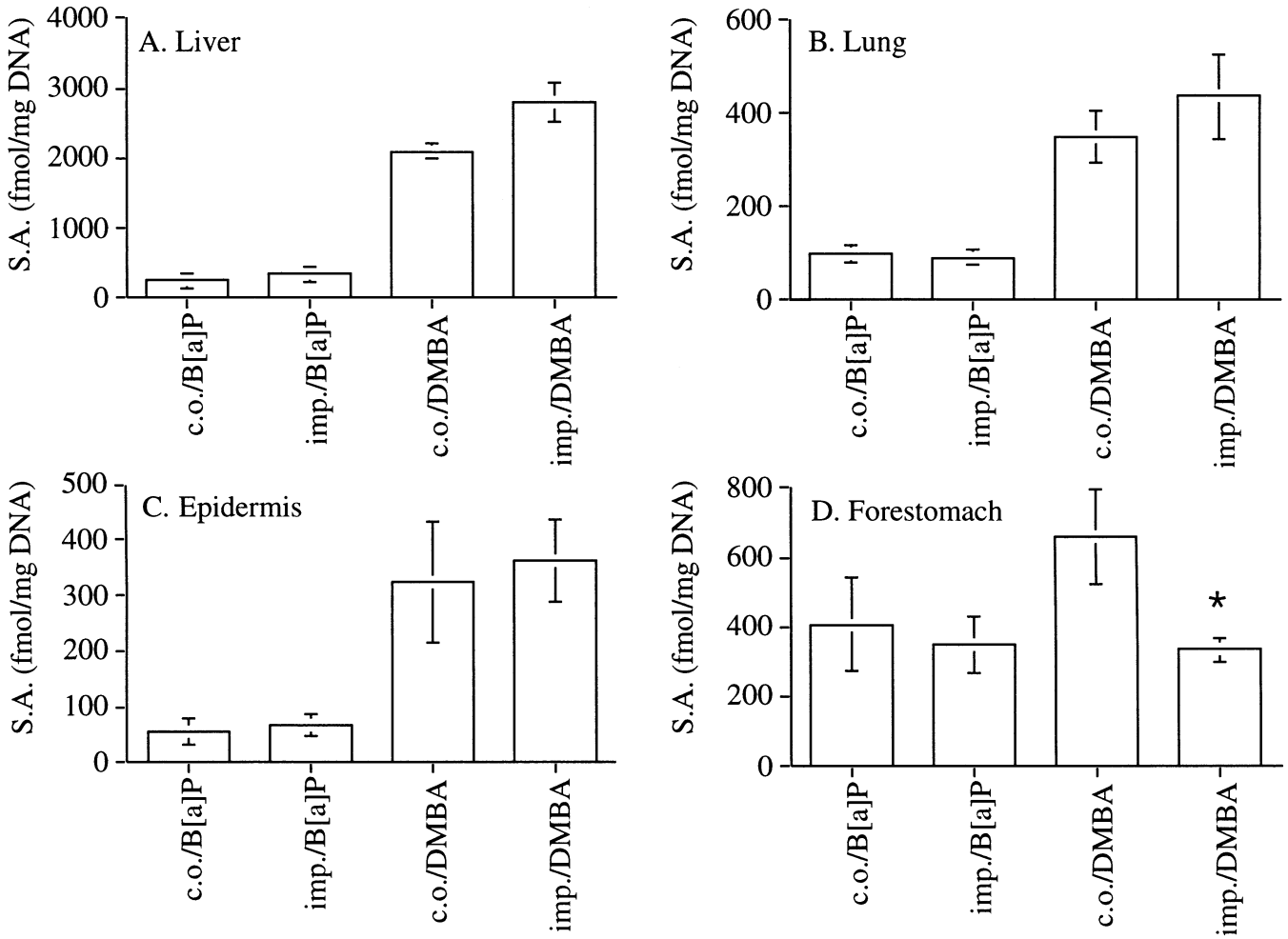

Fig. 6. Effects of orally administered imperatorin on DNA adduct formation induced by orally administered [^{3}H]PAH. Female SENCAR mice (7–9 weeks old) were maintained on AIN-76A semi-purified diet before and during the study. Mice were dosed with corn oil (c.o.) or imperatorin (imp.) (70 mg/kg body wt suspended in corn oil) by gavage for four consecutive days. Thirty minutes after the last pretreatment mice were dosed with [^{3}H]B[a]P (50 μg) or [^{3}H]DMBA (50 μg) (both dissolved in corn oil). Twenty-four hours after treatment with carcinogen mice were killed by cervical dislocation and liver, lungs, epidermis and forestomach were isolated. DNA adducts were determined as described in Materials and methods. Figures represent specific activity (fmol carcinogen equiv. bound/mg DNA; means ± SE). *$P \leq 0.05$.

is phenethyl isothiocyanate (PEITC). After a single oral administration to male F344 rats, PEITC caused an initial inhibition of hepatic EROD activity, followed by a significant elevation in EROD and PROD activity at 24 h (43). PEITC also induced GST activity in liver (43). Immunoblot analysis showed that PEITC caused a time-dependent increase in P450 2B1/2B2 immunoreactive proteins (43). Therefore, linear furanocoumarins may induce certain P450s (especially those of the 2B family) in liver by a PB-like mechanism.

A third possible mechanism for the observation that imperatorin and isopimpinellin induce hepatic P450 content and elevated EROD and PROD activities involves interaction with a xenobiotic-responsive element (XRE). Recent studies with the plant-eating insects *Papilio polyxenes* and *Papilio glaucus* have shown that a xanthotoxin response element exists and is responsible for both basal and inducible transcription of members of the CYP6B family in this species (44). These P450s metabolize a variety of linear furanocoumarins to which the insect larvae are exposed in the diet, including isopimpinellin, imperatorin, bergapten, xanthotoxin and psoralen. These data suggest the possibility that an XRE, which may be evolutionarily conserved across species, mediates the induction of certain P450s by linear furanocoumarins.

Another interesting finding in the current study was that orally administered imperatorin and isopimpinellin elevated hepatic cytosolic GST activity 1.5- to 2-fold after four consecutive daily doses. As noted in the Introduction, coumarin was found to induce GST activity when administered orally in forestomach, liver and intestine in mice (15,16). Xenobiotics induce GST by a variety of mechanisms, including transcriptional activation through the antioxidant response element (ARE), specific XREs or GST P enhancer I (GPEI) (45). More recently, Hayes and co-workers (46) have shown that coumarin is a potent inducer of aflatoxin B1-aldehyde reductase, the GST A5 and P1 subunits and NAD(P)H:quinone oxidoreductase in rat liver. Based on their data it was postulated that coumarin induced these enzymes via a mechanism involving an ARE/GPEI. To our knowledge, the current study is the first report of GST induction by linear furanocoumarins. The double bond in the furano ring is a site for epoxidation by cytochrome P450 and this metabolite can further generate an α,β-unsaturated aldehyde (35,36), possibly generating a Michael reaction

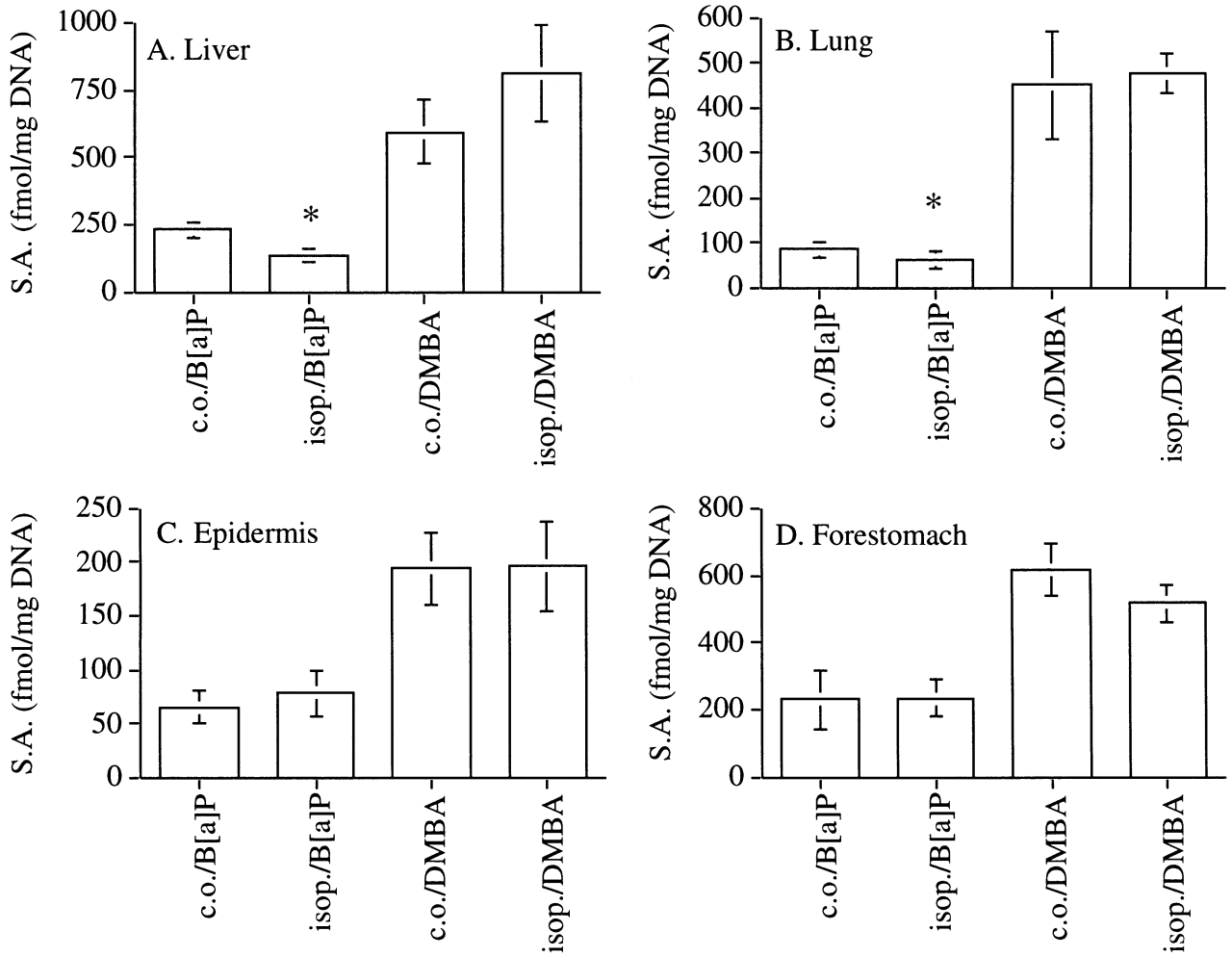

Fig. 7. Effects of orally administered isopimpinellin on DNA adduct formation induced by orally administered [^{3}H]PAH. Female SENCAR mice (7–9 weeks old) were maintained on AIN-76A semi-purified diet before and during the study. Mice were dosed with corn oil (c.o.) or isopimpinellin (isop.) (70 mg/kg body wt suspended in corn oil) by gavage for four consecutive days. Thirty minutes to one hour after the last pretreatment mice were dosed once with [^{3}H]B[a]P (50 μg) or [^{3}H]DMBA (50 μg) (both dissolved in corn oil). Twenty-four hours after treatment with carcinogen mice were killed by cervical dislocation and liver, lungs, epidermis and forestomach were isolated. Figures represent specific activity (fmol carcinogen equiv. bound/mg DNA; means ± SE). *$P \leq 0.05$.

acceptor. Thus, induction of GST by linear furanocoumarins might occur via an XRE or, alternatively, via an ARE/GPEI mechanism as hypothesized for coumarin. Further work is in progress to address these questions.

As an initial attempt to examine the potential anticarcinogenic activities of orally administered imperatorin and isopimpinellin, we analyzed their effects on DNA adduct formation by B[a]P and DMBA. In some tissues (liver, lung and forestomach), suppression of P450 activity corresponded with the abilities of the coumarins to inhibit DNA adduct formation by orally administered carcinogens. The inhibitory effects of isopimpinellin on hepatic B[a]P DNA adducts occurred despite the apparent elevation of P450 activity in liver at 24 h. Imperatorin, which also elevated hepatic P450 content and enzyme activities at 24 h after the last dose, did not increase DNA adduct levels in this tissue. This may be due to the fact that imperatorin, like isopimpinellin, also elevated hepatic GST activity. Our current data suggest that imperatorin and isopimpinellin, when given orally, may produce anticarcinogenic effects in several tissues, depending on the carcinogen. It is also important to stress that the current studies used only a single dose of the coumarin and higher doses may produce greater inhibitory effects on PAH DNA adduct formation. These experiments are in progress.

Other potential anticarcinogenic agents have been shown to modulate phase I or phase II enzyme activities simultaneously. Dietary neem flowers and Thai bitter gourd both inhibit P450 activities and enhance GST activity in rat liver, whereas Chinese bitter gourd inhibits P450 activity without affecting hepatic GST activity (47). However, all three agents inhibit the metabolic activation of aflatoxin B1 and B[a]P into mutagenic metabolites (47). Isothiocyanates, when administered orally, inhibit lung and forestomach tumorigenesis in A/J mice by B[a]P (48). Benzyl isothiocyanate inhibits lung tumorigenesis while PEITC inhibits forestomach tumorigenesis (47). Isothiocyanates are selective inhibitors of P450 activity in lung and nasal muscosa and also induce hepatic GST activity (43,49). Oral administration of diallyl sulfide (DAS), a component of garlic, significantly inhibits B[a]P-induced forestomach tumors and pulmonary adenomas in A/J mice (50). DAS also inhibits 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced lung tumors in A/J mice (51) and 1,2-dimethylhydrazine-induced colon carcinogenesis in mice (52). One proposed mechanism for the anticarcinogenic effects of

garlic components such as DAS is induction of GST activity (53). DAS induces GST activity in liver and forestomach in mice (50). Modulation of P450 activity by DAS may also play a role in its anticarcinogenic action, since it inhibits P450 2E1 activity in rat liver microsomes (54). The balance between the abilities of these compounds to inhibit P450s and induce GST may be critical for their ability to function as anticarcinogenic agents. Certain linear furanocoumarins, such as imperatorin and isopimpinellin, may fit into this type or class of potential anticarcinogenic agents.

Since humans ingest furanocoumarins regularly in the diet, it is important to understand the biological activities of these compounds. Although the levels of simple coumarins from dietary sources in humans is estimated to be ~0.06 mg/kg/day (55), humans also ingest furanocoumarins in the diet from various sources, so the total levels of exposure to both coumarins and furanocoumarins may be even higher. While the exposure levels to imperatorin and isopimpinellin (70 mg/kg/day) and to B[a]P (2 mg/kg) used in the current study are higher than dietary or environmental exposures, the critical issue for human exposure is the ratio of furanocoumarin to carcinogen levels. It is highly likely that humans consume much higher levels of furanocoumarins and related compounds in the diet than their exposure to carcinogens such as PAH. It is noteworthy that dietary levels of furanocoumarins found in grapefruit juice (~2.7 mg in an 8 ounce glass of reconstituted juice) are enough to cause a 47% decrease in intestinal P450 3A4 in a healthy volunteer (56,57). Hollenberg (56,57) has shown that bergamottin and 6',7'-dihydroxybergamottin, related furanocoumarins, are the principal components of grapefruit juice which are metabolized to reactive intermediates that inhibit human P450 3A4 by mechanism-based inactivation. Furthermore, acute administration of 8-MOP to rats (15 mg/kg i.v.) or humans (1.2 mg/kg per os) leads to inhibition of theophyllin metabolism (38). In addition, 8-MOP (1.2 mg/kg per os) decreases clearance of caffeine in human volunteers with psoriasis (58), by covalent binding and inactivation of P450 (36,59). Furanocoumarins, which are metabolically activated by epoxidation of the double bond on the furan ring, form reactive intermediates that act as mechanism-based inactivators of P450 by binding to the active site (36,39,59). In summary, it is highly likely that dietary levels of furanocoumarins are biologically relevant and humans can tolerate pharmacological doses of these compounds.

In conclusion, the results of our studies demonstrate that imperatorin and isopimpinellin are absorbed and distributed extensively to various tissues after oral administration and that they can modulate phase I and phase II enzyme activities involved in PAH metabolism. We propose that the mechanism for inhibition of PAH DNA adduct formation by orally administered imperatorin and isopimpinellin in specific tissues is a result of both inhibition of P450-mediated bioactivation and elevation of certain hepatic P450 and GST activities leading to greater detoxification in this tissue. These results suggest that imperatorin and isopimpinellin have potential as anticarcinogenic agents when administered orally. Future studies will further evaluate their anticarcinogenic potential after oral administration.

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