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IN VIVO AND *IN VITRO* EFFECTS OF HELENALIN ON MOUSE HEPATIC MICROSOMAL CYTOCHROME P450

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Abstract—Helenalin, a natural plant product with significant antitumor activities, decreased male BDF1 mouse hepatic microsomal cytochrome P450 contents *in vivo* and *in vitro*. A single i.p. dose of 25 mg helenalin/kg body weight significantly ($P < 0.05$) decreased microsomal cytochrome P450 contents and inhibited cytochrome P450-dependent mixed-function oxidase activities within 1–2 hr post-exposure. Helenalin (1.0 mM) decreased microsomal cytochrome P450 contents *in vitro* by 11% in the absence of NADPH and by 32% in the presence of NADPH. These *in vitro* and *in vivo* decreases in cytochrome P450 were accompanied by comparable decreases in total microsomal heme contents. Helenalin (1.0 mM) increased mouse hepatic microsomal oxygen consumption and NADPH utilization by 3.2 and 5.4 nmol/min/mg protein respectively. Helenalin (1.0 mM) significantly ($P < 0.05$) increased microsomal lipid peroxidation *in vitro*, and this helenalin-induced increase in lipid peroxidation was inhibited completely by the addition of 0.05 mM EDTA. However, microsomal cytochrome P450 contents were equally affected by helenalin in the presence or absence of EDTA, suggesting that lipid peroxidation did not contribute to the helenalin-induced decrease in cytochrome P450. The addition of 0.05 mM hemin to microsomes treated *in vitro* with 1.0 mM helenalin resulted in a 58% recovery of cytochrome P450 contents. This ability of hemin to reconstitute cytochrome P450 in helenalin-treated microsomes suggests that helenalin produced a selective loss of heme from the cytochrome P450 holoprotein, and that the resulting cytochrome P450 apoprotein remained intact after helenalin treatment. The increased loss of microsomal cytochrome P450 produced by helenalin in the presence of NADPH suggests that a helenalin metabolite may be responsible for heme loss and the *in vitro* destruction of cytochrome P450.

The sesquiterpene lactones are a diverse group of secondary plant metabolites that exhibit cytotoxic, antitumor, and other pharmacological activities [1]. Helenalin (Fig. 1), a pseudoguaianolide sesquiterpene lactone produced by several species of the plant genus *Helenium*, is a potent inhibitor of Walker 256 carcinosarcoma growth in rats and Ehrlich ascites carcinoma growth in mice [2, 3]. Helenalin treatment also significantly increases the life-span of P-388 lymphocytic leukemia-bearing mice [4]. The cytotoxic and antineoplastic activities of the sesquiterpene lactones are dependent on the presence of a sulfhydryl-reactive α,β-unsaturated carbonyl moiety, O=C—C=CH, and it has been suggested that the biochemical basis for sesquiterpene lactone action is the irreversible alkylation of sulfhydryl groups by Michael-addition at the carbonyl moiety [5, 6]. The *in vitro* inhibition of phosphofructokinase and glycogen synthase by sesquiterpene lactones is accompanied by a decrease in free thiol groups in these proteins [7, 8].

Previous studies in our laboratory indicated that helenalin is an effective *in vivo* inhibitor of the hepatic mixed-function oxidase system of male BDF1‖ mice [9]. Multiple i.p. helenalin exposures (25 mg/kg/day for three consecutive days) significantly inhibited microsomal mixed-function oxidase activities and decreased microsomal cytochrome P450 and b_5 contents.

Subsequent studies indicated that helenalin and alantolactone, an eremophilanolide sesquiterpene lactone, also inhibit the mouse hepatic mixed-function oxidase system *in vitro* [10]. These *in vitro* studies suggested that the sulfhydryl-reactivity of the sesquiterpene lactones was not a prerequisite for inhibition of the mixed-function oxidase system.

The present studies examined in greater detail the effects of helenalin on male BDF1 mouse hepatic microsomal cytochrome P450. We were particularly interested in the contribution of helenalin metabolism to the *in vitro* effects of helenalin on cytochrome P450. Our previous studies indicated that helenalin inhibition of microsomal enzyme activity is partially

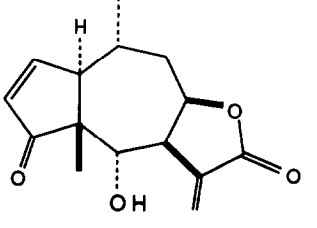

Fig. 1. Structure of helenalin.

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‖ Abbreviations: BDF1, (C57Bl/6 female × DBA/2 male) F_1; and K_s, spectral dissociation constant.

reversible by the subsequent addition of thiols to helenalin-treated microsomes, but this reversal of inhibition is decreased in the presence of an NADPH-generating system [10]. These results suggested that a helenalin metabolite may be responsible, in part, for the irreversible inhibition of the mixed-function oxidase system.

METHODS

Chemicals. Helenalin was isolated from *Helenium microcephalum* [11]. The identity and purity of the isolated helenalin were established by elemental analysis, thin-layer chromatography, and NMR and i.r. spectral comparisons. Helenalin was dissolved in 95% ethanol before addition to *in vitro* incubations and was dissolved in 0.05% polysorbate 80/distilled water before *in vivo* exposures. Aniline hydrochloride was obtained from the Eastman Kodak Co. (Rochester, NY). Carbon monoxide was obtained from the Union Carbide Corp. (Raleigh, NC). Carbon tetrachloride was from the Fisher Chemical Co. (Raleigh, NC) and was dissolved in 95% ethanol before addition to *in vitro* incubations. All other reagents were obtained from the Sigma Chemical Co. (St. Louis, MO) and were reagent grade or better. All concentrations are final concentrations.

Animals. Male BDF1 mice, bred from Charles River Breeding Laboratory stock (Wilmington, MA), weighing 20–27 g, were used in all studies. Mice were fed *ad lib.* and were provided with a 12-hr light–dark cycle. Mice were treated with either (a) a single i.p. injection of 25 mg helenalin/kg body weight and killed 1–16 hr post-exposure or (b) an i.p. injection of 25 mg helenalin/kg body weight on each of three consecutive days and killed 24 hr after the last exposure. Injection volumes were 0.01 mL/g body weight, and control animals received equal volumes of 0.05% polysorbate 80/water.

Preparation of microsomes. Hepatic microsomes were prepared by the procedure described previously [12]. The final microsomal pellet was covered in a minimum volume of 0.1 M Tris buffer (pH 7.7) and stored for 16 hr at −20° before use. Microsomal protein concentrations were determined as described by Lowry *et al.* [13].

Microsomal enzyme assays, heme and cytochrome measurements. Aminopyrine demethylase and aniline hydroxylase activities were determined at 37° by measurement of formaldehyde produced from 4-dimethylaminopyrine and *p*-aminophenol formed from aniline respectively [12]. Mixed-function oxidase activities were supported by an NADPH-generating system that consisted of glucose-6-phosphate (5 mM), $NADP^+$ (1 mM), $MgCl_2$ (5 mM) and yeast glucose-6-phosphate dehydrogenase (2 units/mL).

Cytochromes b_5 and P450 were measured by the procedures of Omura and Sato [14, 15]. Cytochrome difference spectra were determined at room temperature and millimolar extinction coefficients of 91 and 185 were used to calculate cytochrome P450 and cytochrome b_5 contents respectively. Total microsomal heme was measured by the pyridine hemochromagen method [14].

In vitro *effects of helenalin on microsomal cytochrome and heme contents.* Microsomes were resuspended in 0.1 M Tris buffer (pH 7.7), with or without the NADPH-generating system described above, to a final protein concentration of 1.0 to 1.5 mg/mL. Microsomes were then treated with 1.0 mM helenalin for 60 min at 22°; control samples received an equal volume of 95% ethanol. Final ethanol concentrations were less than 1% in these and all subsequent incubations. After 60 min, microsomes were reisolated by centrifugation (159,000 *g* for 30 min) and microsomal pellets were resuspended in 0.1 M Tris buffer (pH 7.7) for measurement of cytochrome P450, cytochrome b_5, and heme contents.

Lipid peroxidation. Microsomes were resuspended in 0.1 M K_2HPO_4 buffer (pH 7.7) and the NADPH-generating system described above, with or without 0.05 mM EDTA. Final protein concentrations were 0.5 to 0.75 mg/mL. Resuspended microsomes were treated with 1.0 mM helenalin or 1.0 mM carbon tetrachloride for 60 min at 22°; control samples received an equal volume of 95% ethanol. Thiobarbituric acid-reactive products of lipid peroxidation were measured as described previously [16].

Cytochrome P450 reconstitution with hemin. Microsomes were treated with 1.0 mM helenalin for 60 min at 22° in the presence of the NADPH-generating system described above. Helenalin-treated microsomes were reisolated by centrifugation (159,000 *g* for 30 min), resuspended in 0.1 M Tris buffer (pH 7.7), and treated for an additional 30 min at 22° with 0.05 mM hemin. Hemin was dissolved in a minimal volume of 0.1 mM NaOH immediately prior to addition. The helenalin plus hemin-treated microsomes were reisolated by centrifugation (159,000 *g* for 30 min) and were resuspended in 0.1 M Tris buffer (pH 7.7) for measurement of cytochrome P450. It was assumed that the molar extinction coefficients for hemin-reconstituted and native cytochrome P450 were identical [17]. Control incubations were treated similarly but received equal volumes of 95% ethanol and 0.1 M NaOH instead of helenalin or hemin respectively. Final protein concentrations were 1.0 to 1.5 mg/mL in all incubations.

NADPH utilization and oxygen consumption. Microsomes were resuspended to a final protein concentration of 3–4 mg/mL in 0.1 M Tris buffer (pH 7.7). Substrate-stimulated increases in microsomal NADPH utilization at 22° were determined by following the disappearance of NADPH from microsomal suspensions which included 0.5 mM hexobarbital or 0.5 mM helenalin. Assays were started by the addition of NADPH (100 μM) and the decrease in absorbance at 340 nm was measured for the first 30 sec after NADPH addition. A millimolar extinction coefficient of 6.22 for NADPH at 340 nm was used to calculate the decrease in NADPH concentration. Control incubations received 95% ethanol instead of substrate. Changes in absorbance at 340 nm in the presence of substrates, but without added NADPH, were minimal.

Substrate-stimulated increases in oxygen consumption were measured in microsomes resuspended

Table 1. *In vivo* effects of helenalin on male BDF1 mouse liver microsomal proteins

Time* (hr)	P450† (nmol/mg protein)	b_5 (nmol/mg protein)	APD (nmol/min/mg protein)	ANH (nmol/min/mg protein)	APD (nmol/min/nmol P450)	ANH (nmol/min/nmol P450)
0	0.93 ± 0.08‡	0.33 ± 0.06	10.16 ± 1.08	1.12 ± 0.10	11.07 ± 1.26	1.23 ± 0.18
1	0.86 ± 0.08	0.29 ± 0.04	9.50 ± 0.90	0.72 ± 0.12§	11.04 ± 0.30	0.84 ± 0.10§
2	0.72 ± 0.06§	0.32 ± 0.04	7.18 ± 1.00§	0.67 ± 0.10§	9.90 ± 1.08	0.92 ± 0.08§
4	0.71 ± 0.04§	0.29 ± 0.04	6.83 ± 0.38§	0.64 ± 0.06§	9.64 ± 0.76	0.91 ± 0.10§
8	0.70 ± 0.08§	0.28 ± 0.04	7.01 ± 0.58§	0.66 ± 0.08§	10.22 ± 1.52	0.96 ± 0.16
16	0.68 ± 0.06§	0.28 ± 0.02	5.93 ± 0.84§	0.84 ± 0.12§	8.82 ± 1.24	1.26 ± 0.28

* Time post-exposure. Mice were given a single i.p. injection of 25 mg helenalin/kg body weight; controls (0 hr) received 0.05% polysorbate 80/water.

† P450, cytochrome P450; b_5, cytochrome b_5; APD, aminopyrine demethylase; ANH, aniline hydroxylase.

‡ Values are the means ± SD of four to eight mice.

§ Significantly different from 0 hr at $P < 0.05$.

in 0.1 M Tris buffer (pH 7.7) with the NADPH-generating system described above, but without $NADP^+$. Hexobarbital (0.5 mM) or helenalin (0.5 mM) was added, and the basal rate of oxygen consumption was determined for 4 min. NADPH (100 μM) was added, and the rate of oxygen consumption was determined for an additional 4 min; basal rates of oxygen consumption were subtracted from rates of oxygen consumption after the addition of NADPH. Oxygen concentrations were measured with a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH) and a Gilson K-IC oxygraph (Gilson Medical Electronics Inc., Middleton, WI). Measurements were made at 22°; controls received 95% ethanol.

Statistical analysis. Statistical significance was established by analysis of variance followed by Student's *t*-test between means as described by Gad and Weil [18].

RESULTS

In vivo *effects of helenalin on the mixed-function oxidase system.* A single i.p. dose of 25 mg helenalin/kg body weight produced a rapid decrease in microsomal cytochrome P450 contents and enzyme activities (Table 1). Statistically significant effects were observed within 1–2 hr post-exposure. At 16 hr post-exposure, helenalin decreased microsomal cytochrome P450 contents by 27% and inhibited aminopyrine demethylase and aniline hydroxylase activities by 42 and 25% respectively. Cytochrome b_5 contents were not affected significantly by a single 25 mg/kg dose of helenalin. Helenalin inhibition of the mixed-function oxidase system was less pronounced when enzyme activities were normalized to microsomal cytochrome P450 contents instead of microsomal protein contents (Table 1). Helenalin did not affect aminopyrine demethylase activities, and helenalin inhibition of aniline hydroxylase activities was decreased, when enzyme activities were normalized to microsomal cytochrome P450 contents.

Treatment of mice with 25 mg helenalin/kg/day for three consecutive days significantly decreased microsomal heme by 47%, cytochrome P450 by 58%, and cytochrome b_5 by 13% (Table 2). Cytochromes P450 and b_5 represented 96–98% of the total microsomal heme in mouse hepatic microsomes.

Table 2. *In vivo* and *in vitro* effects of helenalin on male BDF1 mouse liver microsomal cytochrome and heme contents

	P450* (nmol/mg protein)	b_5 (nmol/mg protein)	Heme (nmol/mg protein)
In vivo†			
Control	1.06 ± 0.04‡	0.31 ± 0.01	1.40 ± 0.08
Treated	0.44 ± 0.08§	0.27 ± 0.01§	0.74 ± 0.15§
Difference	0.62	0.04	0.66
In vitro‖			
Control	0.74 ± 0.08	0.41 ± 0.04	1.24 ± 0.26
Treated	0.45 ± 0.10§	0.38 ± 0.05	0.73 ± 0.15§
Difference	0.29	0.03	0.51

* P450, cytochrome P450; b_5, cytochrome b_5; heme, total microsomal heme.

† Mice were given 25 mg helenalin/kg body weight/day i.p. for three consecutive days; controls received 0.05% polysorbate 80/water.

‡ *In vivo* and *in vitro* values are the means ± SD of three separate animals or three microsomal preparations respectively.

§ Significantly different from the appropriate control at $P < 0.05$.

‖ Microsomes were treated *in vitro* with 1.0 mM helenalin for 60 min at 22° in the presence of an NADPH-generating system; controls received 95% ethanol.

In vitro *effects of helenalin on microsomal heme and cytochrome contents.* Helenalin (1.0 mM), in the presence of an NADPH-generating system, significantly decreased microsomal heme and cytochrome P450 contents by 41 and 39% respectively (Table 2). Helenalin-induced decreases in cytochrome P450 were not accompanied by increases in cytochrome P420. Microsomal cytochrome b_5 contents were not affected significantly by helenalin *in vitro*. The amount of microsomal heme lost after helenalin treatment was greater by 0.19 nmol/mg protein than the accompanying decreases in cytochromes P450 and b_5.

Table 3. *In vitro* effects of helenalin on male BDF1 mouse liver microsomal cytochrome P450

	NADPH*	Cytochrome P450 (nmol/mg protein)	Change† (%)
Control‡	–	0.80 ± 0.04§	
Control	+	0.77 ± 0.05	−4
Helenalin	–	0.71 ± 0.04‖	−11
Helenalin	+	0.52 ± 0.03‖¶	−32

* Microsomes were incubated for 60 min at 22° with (+) or without (−) an NADPH-generating system.

† Percent change relative to appropriate control without or with NADPH.

‡ Helenalin (1.0 mM); controls received 95% ethanol.

§ Values are the means ± SD of five or six determinations.

‖ Significantly different from appropriate control, with or without NADPH, at $P < 0.05$.

¶ Significantly different from helenalin without NADPH at $P < 0.05$.

Helenalin (1.0 mM), in the absence of an NADPH-generating system, decreased microsomal cytochrome P450 contents by 11% (Table 3). There was a further 21% decrease in cytochrome P450 when microsomes were co-incubated with both helenalin and an NADPH-generating system.

Lipid peroxidation. Helenalin (1.0 mM) significantly increased the *in vitro* formation of thiobarbituric acid-reactive products in the absence of EDTA, but did not promote the formation of thiobarbituric acid-reactive products in the presence of 0.05 mM EDTA (Table 4). In the same experiments, 1.0 mM carbon tetrachloride increased the formation of thiobarbituric-acid reactive products 8-fold (0.59 ± 0.05 nmol/min/mg protein) in the absence of EDTA and 7-fold (0.21 ± 0.01 nmol/min/mg protein) in the presence of 0.05 mM EDTA (means ± SD of three microsomal preparations). Comparable increases in the formation of thiobarbituric acid-reactive products as a result of the *in vitro* treatment of rat hepatic microsomes with carbon tetrachloride have been reported [19].

Helenalin-induced decreases in microsomal cytochrome P450, cytochrome b_5 and heme contents were unaffected by the addition of 0.05 mM EDTA (Table 4). Microsomal cytochrome P450 contents were decreased 38 and 33% by helenalin in the presence and absence of EDTA respectively. Helenalin decreased microsomal heme contents by 36 and 30% in the presence and absence of EDTA respectively.

Hemin reconstitution of microsomal cytochrome P450. The addition of hemin (0.05 mM) to helenalin-treated microsomes resulted in a 58% reconstitution of cytochrome P450 contents (Table 5). Hemin treatment increased cytochrome P450 contents in control microsomes by 8%.

NADPH utilization and oxygen consumption. Helenalin (0.5 mM) and hexobarbital (0.5 mM) increased microsomal oxygen consumption and NADPH utilization by 59–109% (Table 6). Net rates of oxygen consumption after helenalin and hexobarbital addition were comparable; however, helenalin produced a 65% greater increase in net NADPH utilization. This difference in net NADPH utilization is reflected in the ratios of oxygen consumption to NADPH utilization for hexobarbital (1.03) and helenalin (0.59). The rates of oxygen consumption and NADPH utilization by mouse hepatic microsomes in the absence of substrate, i.e. controls, are comparable to reported rates of oxygen consumption and NADPH utilization by microsomes from rat liver [20, 21].

DISCUSSION

We reported previously that multiple i.p. helenalin treatments (25 mg/kg/day for three consecutive days) significantly inhibited male BDF1 mouse hepatic mixed-function oxidase activities and decreased microsomal cytochrome P450 contents [9]. Helenalin inhibition of the mixed-function

Table 4. *In vitro* effects of helenalin on male BDF1 mouse liver microsomal lipid peroxidation and heme contents

	Lipid peroxidation* (nmol/min/mg protein)	P450	b_5 (nmol/mg protein)	Heme
Without EDTA†				
Control‡	0.07 ± 0.02§	0.90 ± 0.12	0.43 ± 0.01	1.45 ± 0.14
Treated	0.16 ± 0.07‖	0.60 ± 0.06‖	0.39 ± 0.04	1.02 ± 0.10‖
Difference	0.09	0.30	0.04	0.43
With EDTA†				
Control	0.03 ± 0.01	0.96 ± 0.10	0.42 ± 0.02	1.86 ± 0.24
Treated	0.03 ± 0.02	0.60 ± 0.09‖	0.40 ± 0.01	1.19 ± 0.26‖
Difference	0.00	0.36	0.02	0.67

* Lipid peroxidation, thiobarbituric acid-reactive products; P450, cytochrome P450; b_5, cytochrome b_5; heme, total microsomal heme.

† Microsomes were incubated without EDTA or with 0.05 mM EDTA.

‡ Microsomes were treated with 1.0 mM helenalin for 60 min at 22° in the presence of an NADPH-generating system; controls received 95% ethanol.

§ Values are the means ± SD of three separate microsomal preparations.

‖ Significantly different from the appropriate control, without or with EDTA, at $P < 0.05$.

Table 5. Reconstitution of microsomal cytochrome P450 by hemin after *in vitro* treatment with helenalin

	Cytochrome P450 (nmol/mg protein)	Change* (%)
Without hemin†		
Control‡	0.80 ± 0.15§	
Helenalin	0.49 ± 0.08	−39
With hemin†		
Control	0.86 ± 0.06	+8
Helenalin	0.73 ± 0.09‖	−12

* Percent change relative to appropriate control with or without hemin.

† Helenalin-treated microsomes were incubated with or without 0.05 mM hemin for 30 min at 22°.

‡ Microsomes were treated with 1.0 mM helenalin for 60 min at 22° in the presence of an NADPH-generating system; controls received 95% ethanol.

§ Values are the means ± SD of six separate microsomal preparations.

‖ Significantly different from cytochrome P450 content in helenalin-treated microsomes without added hemin at $P < 0.05$.

oxidase system was tentatively attributed to the decrease in cytochrome P450, since enzyme activities normalized to microsomal cytochrome P450 contents instead of microsomal protein contents were not decreased [9]. However, it was unclear from these studies whether helenalin affected microsomal cytochrome P450 directly or decreased cytochrome P450 contents secondarily, as a result of the systemic toxicity associated with multiple helenalin exposures. In the present studies, helenalin significantly decreased microsomal cytochrome P450 contents within 2 hr after a single *in vivo* exposure and significantly decreased microsomal cytochrome P450 contents *in vitro*. These results suggest that helenalin is capable of directly affecting microsomal cytochrome P450.

Helenalin decreased microsomal cytochrome P450 contents *in vitro* to a significantly greater extent in the presence of NADPH, than in the absence of NADPH. Comparable results have been reported for the *in vitro* effects of the sesquiterpene lactone, alantolactone, on rat hepatic microsomal cytochrome P450 [22]. Alantolactone (1.0 mM) had no effect on cytochrome P450 in the absence of NADPH, but decreased cytochrome P450 contents by 57% when NADPH was present. One possible interpretation of these results is that sesquiterpene lactone metabolites produced by the mixed-function oxidase system are responsible for the decrease in microsomal cytochrome P450 contents. Previous studies in our laboratory indicated that no change in microsomal cytochrome P450 contents occurs when 1.0 mM helenalin is incubated with mouse hepatic microsomes saturated with carbon monoxide [10]. These results appear to be contradictory to the *in vitro* decreases in microsomal cytochrome P450 produced by helenalin in the present studies. However, carbon monoxide is an effective inhibitor of the mixed-function oxidase system [23], and the present studies suggest that the mixed-function oxidase system may contribute significantly to the *in vitro* effects of helenalin on cytochrome P450.

The effects of microsomal lipid peroxidation on the breakdown and loss of cytochrome P450 are well-established. The *in vitro* formation of lipid peroxidation products (malondialdehyde) concomitant with a decrease in cytochrome P450-dependent enzyme activities has been observed in rat hepatic microsomes treated with carbon tetrachloride [24]. Lipid peroxidation *in vitro* can be inhibited by EDTA which may act by chelating reduced iron and decreasing the production of the active perferryl ion responsible for lipid peroxidation [25]. Helenalin significantly increased lipid peroxidation in mouse hepatic microsomes, but these increases were relatively modest when compared to the effects of carbon tetrachloride. In addition, under conditions in which no helenalin-induced lipid peroxidation occurred, i.e. in the presence of 0.5 mM EDTA, helenalin continued to decrease microsomal heme and cytochrome P450 contents. This suggests that lipid peroxidation does not contribute to the *in vitro* decrease in cytochrome P450 produced by helenalin.

Helenalin-induced decreases in microsomal heme *in vivo* and *in vitro* were accompanied by comparable decreases in cytochrome P450 content. In contrast, helenalin had no effect on cytochrome b_5 *in vitro* and did not affect cytochrome b_5 after a single *in vivo* exposure. These results suggest that the effects of helenalin on microsomal heme are selective for cytochrome P450. There was some discrepancy

Table 6. Substrate-induced utilization of oxygen and NADPH by male BDF1 mouse liver microsomes

	Oxygen consumption (nmol/min/mg protein)	Net oxygen consumption* (nmol/min/mg protein)	NADPH utilization (nmol/min/mg protein)	Net NADPH utilization* (nmol/min/mg protein)	Ratio†
Control‡	5.40 ± 0.77§		4.97 ± 0.84		
Hexobarbital	8.80 ± 0.72	3.40	8.26 ± 0.86	3.29	1.03
Helenalin	8.60 ± 1.56	3.20	10.41 ± 0.65	5.44	0.59

* Difference between control and substrate-induced increases in oxygen consumption or NADPH utilization.

† Ratio of net oxygen to net NADPH utilization.

‡ Hexobarbital and helenalin concentrations: 0.5 mM; control received 95% ethanol.

§ Values are the means ± SD of six to twelve determinations.

between the amount of cytochrome P450 lost from microsomes treated *in vitro* with helenalin and the decrease in total microsomal heme contents. Since cytochromes P450 and b_5 constitute 96–98% of the total microsomal heme in mouse hepatic microsomes (Table 2), this discrepancy cannot result from the loss of microsomal hemoproteins other than cytochromes P450 and b_5. Helenalin did not interfere with the pyridine hemochromagen assay for heme, and the reason for the difference between the amounts of heme and cytochrome P450 lost from microsomes *in vitro* is unknown.

Alkylation of cytochrome P450 heme and subsequent loss of the heme moiety from the cytochrome P450 holoprotein occurs after *in vivo* or *in vitro* exposure to a variety of compounds, e.g. allylisopropylacetamide [26]. A portion of the cytochrome P450 apoprotein which remains intact and associated with the microsomal membrane after loss of the heme moiety can be reconstituted with exogenous heme, i.e. hemin [17]. The reconstitution by hemin of cytochrome P450 in helenalin-treated microsomes suggests that helenalin produces a similar loss of the cytochrome P450 heme moiety. Alkylation and loss of cytochrome P450 heme is generally associated with the metabolic activation of the causative agent [26]. Of particular interest is the observation that allylisopropylacetamide alkylation of heme proceeds via oxygen transfer from cytochrome P450 and activation of the terminal π-bond in allylisopropylacetamide [27]. Helenalin also contains a terminal π-bond in the exocyclic double bond of the α-methylene-γ-lactone moiety, and this π-bond may be a target for activation by cytochrome P450 in a manner analogous to that described for allylisopropylacetamide.

Helenalin stimulation of microsomal NADPH utilization and oxygen consumption suggests that helenalin may be metabolized by the mixed-function oxidase system. However, it should be stressed that these results provide only circumstantial evidence for helenalin metabolism, and NADPH utilization and oxygen consumption can be stimulated by compounds which bind to cytochrome P450, but are not metabolized by the mixed-function oxidase system [28, 29]. Previous studies in our laboratory indicated that helenalin binds to cytochrome P450 and produces a type I binding spectrum similar to that produced by other mixed-function oxidase substrates [10]. The spectral dissociation constant (K_s) for the interaction of helenalin with cytochrome P450 (161 μM) was comparable to K_s values reported for other cytochrome P450 substrates (e.g. 330 μM for aminopyrine and 80 μM for hexobarbital) [30]. Sesquiterpene lactone metabolism has received little previous attention, probably because of the assumed dependence of sesquiterpene lactone biological effects on the sulfhydryl-reactivity of the unsaturated lactone moiety. However, rat bile and urinary metabolites of the sesquiterpene lactone, hymenoxon, were identified as glucuronide conjugates, rather than the mercapturic acid derivatives expected if hymenoxon reacted nonenzymatically with glutathione [31]. Our studies suggest that helenalin may be metabolized by the mixed-function oxidase system, possibly resulting in the production of a metabolite capable of decreasing microsomal cytochrome P450 contents and irreversibly inhibiting the mixed-function oxidase system.

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