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Inhibition of Hepatic Microsomal Drug-Metabolizing Enzymes by Imperatorin*

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Abstract□The effect of imperatorin on hepatic microsomal mixed function oxidases (MFO) was investigated. On acute treatment, imperatorin (30 mg/kg, i.p.) caused a significant reduction in activities of hepatic aminopyrine N-demethylase, hexobarbital hydroxylase and aniline hydroxylase as well as cytochrome p-450 content in rats and mice. Kinetic studies on rat liver enzymes revealed that imperatorin appeared to be a competitive inhibitor of aminopyrine N-demethylase (Ki, 0.007mM), whereas a non-competitive inhibitor of hexobarbital hydroxylase (Ki, 0.0148mM). Imperatorin also inhibited non-competitively aniline metabolism (Ki, 0.2mM). Imperatorin binds to phenobarbital-induced cytochrome p-450 to give a typical type I binding spectrum (max. 388nm, min 422nm). Multiple administrations of imperatorin (30 mg/kg, i.p. daily for 7 days) to mice shortened markedly the duration of hexobarbital narcosis and increased activities of hepatic aminopyrine N-demethylase and hexobarbital hydroxylase and the level of cytochrome p-450 whereas aniline hydroxylase activity was unaffected.

Keywords□Imperatorin, Drug metabolizing enzymes, Aminopyrine N-demethylase, Hexobarbital hydroxylase, Aniline hydroxylase, Cytochrome p-450, Inhibition constants, Kinetic analysis.

Imperatorin is a linear furanocoumarin naturally occurring in many plants especially of the families Umbelliferae and Rutaceae.[2]

Unlike other furanocoumarins, imperatorin exhibits neither significant anticoagulant property[3] nor photosensitizing effect.[4,5] It was, however, reported that it had some antiinflammatory activity in mouse and rat paw edema assays[6] and elevated the hippuric acid level,[7] suggesting that it affects the detoxication mechanism. Inhibition effect on respiration and phosphorylation in isolated liver mitochondria from guinea pigs, rats and rabbits in the presence of succinate was also reported.[8,9]

It has, recently, been demonstrated that a single treatment of imperatorin caused an increase in strychnine mortality and prolongation of hexobarbital (HB)-induced sleeping time.[10] However, repeated administration of imperatorin produced a shortening effect on HB action. These results indicate that imperatorin produces biphasic alteration in the metabolism of drugs.[11]

In this paper, we report the *in vivo* and *in vitro* effect of imperatorin on hepatic microsomal enzymes using three different substrates and the nature of the observed inhibition of drug metabolism by imperatorin.

MATERIALS AND METHODS

Chemicals

Imperatorin was isolated from the roots of *Angelica koreana* (Umbelliferae). SKF-525A was a gift from Smith, Kline and French Lab., Philadelphia, PA U.S.A. Phenobarbital sodium was U.S.P. grade and NADP, glucose-6-phosphate were purchased from Sigma Chemical Co. All other chemicals were of analytical grade commercially available.

Animals and Diet

Male Sprague-Dawley (CD strain) rats weighing 150-250g and male albino dd mice weighing 17-25g were used. The animals were fed lab chows and tap water *ad lib.* Constant-temperature environments were maintained throughout the experiments.

Preparation of liver homogenates and microsomal fractions

Animals were decapitated and livers were immediately removed, weighed, chilled on ice and homogenized in phosphate buffer, pH 7.4, using a glass teflon homogenizer of the Potter-Elvehjem type to give a suspension equivalent to 250mg/ml of wet liver. The homogenates were centrifuged at 10,000g for 20 min

* Part 7 in the series: Studies on crude drugs acting on drug-metabolizing enzymes. For part 6 see ref. (1).

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and the supernatant fractions were used directly for enzyme assays.

Microsomal fractions were prepared by further centrifugation of 10,000g supernatant at 115,000g (Sorvall, Dupont, Ultracentrifuge, OTD, 65B) for 60min. Microsomal pellets were washed once with 1.15% KCl containing 10 mM EDTA by resuspension and recentrifugation. The microsomal fractions were finally suspended in the buffer used originally for tissue homogenization.

All the manipulations were carried out at 0-4°. Determinations of protein and cytochrome p-450 and enzyme assays were made on the day of sacrifice.

Determinations of protein and cytochrome p-450

Protein concentration was determined by the method of Lowry *et al.*,[12] using bovine serum albumin as a standard. The concentration of cytochrome p-450 in the microsomal suspension was measured from the difference in absorbance of the reduced carbon monoxide-cytochrome p-450 complex between 450 and 490 nm using an extinction coefficient of 91 mM^{-1} cm^{-1} according to Omura and Sato.[13]

Enzyme assays

The N-demethylation of aminopyrine was measured as HCHO formed and hexobarbital hydroxylase was assayed by measuring the amount of hexobarbital remaining unmetabolized as described previously.[11] The rate of aniline hydroxylation was determined by analysis of p-aminophenol formed according to the method of Imai *et al.*[14]

Spectral binding studies

The interaction of imperatorin with oxidized cytochrome p-450 was determined by differential spectrometry using liver microsomal suspensions from the rats treated with phenobarbital (80 mg/kg/3days, ip) by the method of Schenkman *et al.*[15]

RESULTS

Effect of imperatorin on MFO activities

The effect of a single treatment of animals with imperatorin on liver MFO activities and cytochrome p-450 level was shown in Table I. Intraperitoneal injection to mice and rats of 30 mg/kg of imperatorin 30 min before sacrifice resulted in a significant decrease in activities of aminopyrine N-demethylase and hexobarbital hydroxylase as well as aniline hydroxylase in liver. Inhibition of the metabolism of the type I substrates was more pronounced than inhibition of the metabolism of the type II compound. The intensity of decrease was almost comparable to that of SKF-525A, a widely used model inhibitor of MFO at the same dose level. A concomitant loss of cytochrome p-450 content was seen in the liver microsomes. It sppears that there was no species differences between rats and mice.

In contrast with the results of a single treatment, multiple treatments (30 mg/kg/day, daily for 7 days, i.p.) of mice with imperatorin caused not only a remarkable shortening of the duration of HB-induced narcosis but also a significant increase in activities of aminopyrine N-demethylase and hexobarbital hydroxylase and in cytochrome p-450 contents (Table II), which reflects induction of microsomal enzymes upon repeated treat-

Table I. Effect of a single treatment of imperatorin on MFO activities and cytochrome p-450 content in liver.

Treatment	Aminopyrine N-demethylase (μmoles/g prot./min)	Hexobarbital hydroxylase (μmoles/g prot./min)	Aniline hydroxylase (μmoles/g prot./min)	Cytochrome p-450 (nmoles/mg prot.)
Exp I (with rats)				
Control (0.5% CMC)	0.507±0.055	0.995±0.121	0.140±0.012	0.85±0.07
Imperatorin (30mg/kg, i.p.)	0.160±0.046*** (68.4)	0.533±0.108** (46.4)	0.085±0.007** (39.3)	0.61±0.05** (28.2)
SKF-525A (30mg/kg, i.p.)	0.213±0.049** (58.0)	0.426±0.136** (57.2)	0.093±0.005** (33.6)	0.54±0.05** (36.5)
Exp II (with mice)				
Control (0.5% CMC)	0.770±0.080	0.517±0.038	0.360±0.035	0.83±0.04
Imperatorin (30mg/kg, i.p.)	0.403±0.070** (47.7)	0.243±0.050** (53.0)	0.215±0.008** (40.3)	0.65±0.09* (21.7)

Animals were killed 30min after pretreatment. Each data represents mean±S.E. of 3 separate determinations. Figures in parentheses indicate % inhibition. Significantly different from control; ***$p<0.01$, **$p<0.05$, *<0.1.

Table II. Effect of repeated treatments with imperatorin on hexobarbital-induced narcosis, hepatic MFO activities and cytochrome p-450 content in mice.

	Treatment		
	Control (0.5% CMC)	Phenobarbital (50mg/kg/day, i.p.)	Imperatorin (30mg/kg/day, i.p.)
Liver wt/body wt.[a]	0.053±0.001	0.080±0.005**	0.066±0.002**
Hexobarbital (100mg/kg) Sleeping time[a] (min)	51.0 ±7.0	18.9±1.6** (37.1)	28.8±2.9* (56.5)
Cytochrome p-450[b] (nmoles/mg prot.)	0.560±0.08	1.56±0.03** (278.6)	1.21±0.09** (216.1)
Aminopyrine N-demethylase[b] (μmoles/g prot./min)	0.542± 0039	1.481±0.190** (273.2)	0.887±0.046** (163.7)
Hexobarbital hydroxylase[b] (μmoles/g prot./min)	0.735±0.052	1.407±0.209* (191.4)	1.411±0.164* (192.0)
Aniline hydroxylase[b] (μmoles/g prot./min)	0.359±0.029	0.815±0.112** (227.0)	0.363±0.024 (101.1)

Mice were given drugs daily for 7 days.
All experiments were done 48hr after the last treatment
[a] Data represent mean± S.E. of 6 mice. [b] Data represent mean± S.E. of 3 separate determinations for pooled livers from two mice. Figures in parentheses indicate % of control. Significantly different from control; **p<0.01, *p<0.05.

ments. The intensity of its inducing activity was essentially comparable to that of phenobarbital.

The CO-binding spectrum of liver microsomes from imperatorin-treated mice showed an absorption maximum at 450 nm. These data indicate that imperatorin is a phenobarbital type of heme-protein inducer. But its induction patterns seem to be different from those of phenobarbital since aniline hydroxylase activity in mice treated with imperatorin remained unaffected.

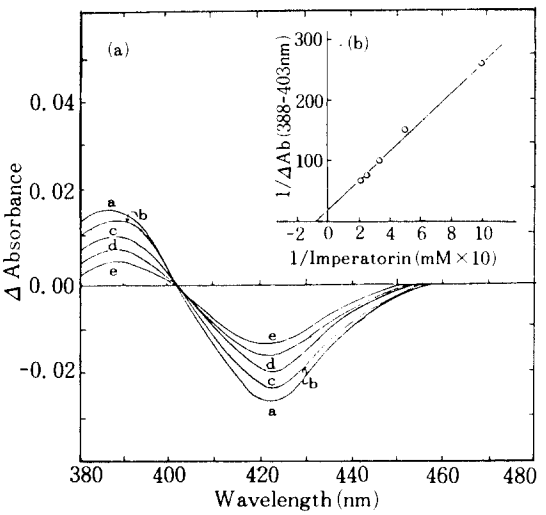

Fig. 1. Interaction of imperatorin with hepatic microsomes from rats.

(a) Difference spectra: a, 0.05; b, 0.04; c, 0.03; d, 0.02; and e, 0.01 mM of imperatorin in microsomal suspension (2 mg/ml in 0.05M tris HCl buffer, pH 7.4, containing 0.15M KCl and 0.01M $MgCl_2$).

(b) Double reciprocal plot (Ks=0.104 mM).

Binding of imperatorin to cytochrome p-450

With oxidized hepatic microsomes prepared from rats treated with phenobarbital, imperatorin was found to manifest a typical type I difference spectrum (Fig 1a) having an absorption maximum at 388 nm and a minimum at around 422 nm, indicating that imperatorin interacted with cytochrome p-450 similar to other type I compounds.

The difference in absorbance between peak and trough increased with increasing concentration of imperatorin. The apparent dissociation constant (Ks) was calculated to be 0.104 mM from the intercept on the abscissa of the double reciprocal plot of the change in absorbance at 388 nm relative to 403 nm versus the concentration of imperatorin (Fig 1b).

Kinetic inhibition experiments

To define the role of imperatorin as a MFO inhibitor, a series of classical inhibition studies was carried out in which various concentrations of imperatorin were incubated with 10,000g supernatant fractions of untreated rat liver in the presence of various concentrations of model substrates (hexobarbital, aminopyrine and aniline) for the enzyme.

Kinetics for type I substrates followed simple monophasic Michaelis-Menten behavior and could be

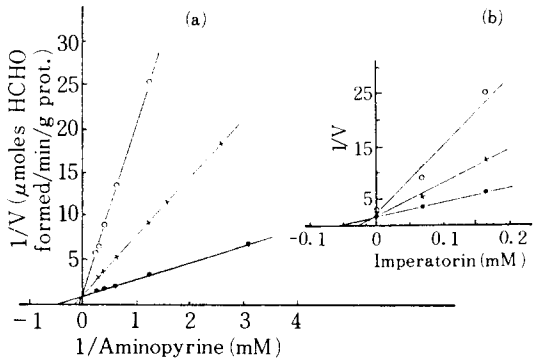

Fig. 2. Effect of imperatorin on hepatic aminopyrine N-demethylase in rats.

(a) Lineweaver-Burk plot (Km=2.22 mM). imperatorin concentrations; -●- none; -X-, 0.07 mM and -○-, 0.16 mM.

(b) Dixon plot (Ki=0.007 mM). aminopyrine concentrations; -○- 0.82mM; -X-, 1.64 mM; and -●-, 3.22 mM.

analyzed from Lineweaver-Burk plots. Imperatorin inhibited aminopyrine N-demethylase competitively (Km = 2.22 mM, Ki = 0.007 mM) (Fig 2), while the inhibition of hexobarbital hydroxylase was non-competitive (Km = 0.42 mM, Ki = 0.0148 mM) (Fig 3).

However, as shown in Fig 4, Lineweaver-Burk plot for aniline hydroxylase activity gave nonlinear at high concentrations of the substrate while a straight line was obtained at low concentrations. A similar biphasic kinetic has been reported for rat and mouse liver enzymes by Wada *et al.*,[16] for rabbit liver and lung enzymes by Bend *et al.*[17] and for sheep liver enzyme by Arinc and Iscan[18]. These observations led to the suggestion that the enzyme appears to exist in at least two forms differing in their substrate affinities, a high affinity

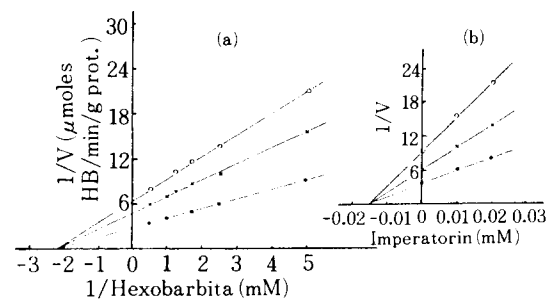

Fig. 3. Effect of imperatorin on hepatic hexobarbital hydroxylase in rats. (HB; hexobarbital)

(a) Lineweaver-Burk plot (Km=0.42 mM). imperatorin concentrations; -●-, none; -X-, 0.01 mM; and -○-, 0.02 mM.

(b) Dixon plot (Ki=0.0148 mM). hexobarbital concentrations; -○-, 0.20 mM; -X-, 0.40 mM; and -●-, 2.0 mM.

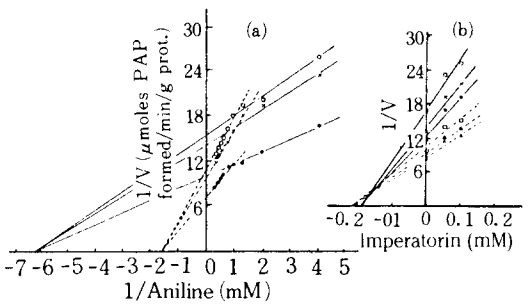

Fig. 4. Effect of imperatorin on hepatic aniline hydroxylase in rats. (PAP; p-aminophenol)

(a) Lineweaver-Burk plot (Km= 0.160 mM for high affinity form and 0.667 mM for low affinity form).
imperatorin concentrations; -•-, none; -X-, 0.05 mM; and -o-, 0.1 mM.

(b) Dixon plot (Ki = 0.186 mM for high affinity form and 0.211 mM for low affinity form).
aniline concentrations; -▲-, 2.44 mM; -△-, 1.95 mM; -□-, 1.22 mM; -•-, 0.73 mM; -X-, 0.49 mM; and -o-, 0.244 mM.

form (Km = 0.160 mM) and a low affinity form (Km = 0.667 mM).

Imperatorin also inhibited aniline hydroxylase but to a much lesser extent than was its effect on type I substrates. The inhibition was noncompetitive and Ki values of 0.186 mM and 0.211 mM were calculated for the high affinity form and for the low affinity form, respectively.

DISCUSSION

In previous papers it was demonstrated that imperatorin caused a marked prolongation of the duration of hexobarbital-induced narcosis and a significant elevation of serum hexobarbital level when estimated 30 min after a single administration in mice[10,11]. Imperatorin also exhibited a significant inhibition on hexobarbital and aminopyrine metabolism when incubated with direct addition in untreated rat microsomal fractions *in vitro*[11].

In combination with those findings, the decrease in hepatic microsomal MFO activities in animals treated with a single administration in the present report suggested that imperatorin was an inhibitor of oxidative microsomal enzymes. Concomitant decrease in the level of cytochrome p-450 was also observed, suggesting that decreased activity of microsomal enzymes could be due to the reduced concentration of cytochrome p-450 caused by exposure to imperatorin.

In vitro experiments described here, imperatorin proved to be a competitive inhibitor of aminopyrine N-demethylation and noncompetitive inhibitor of hexobarbital hydroxylation and aniline hydroxylation.

As expected, imperatorin binds to cytochrome p-450, resulting in spectral change of Type I binding according to the classification given by Schenkman[15].

Imperatorin was compared to SKF 525-A that binds with cytochrome p-450 according to the spectral type I[19], in order to characterize its capability to inhibit the *in vitro* biotransformation of substrates of MFO. When aminopyrine was used as a substrate, Ki of imperatorin was approximately two times greater than that of SKF 525-A (Ki = 0.004 mM)[20]. Inhibition of aniline hydroxylase was similar in type and magnitude to the inhibition produced by SKF 525-A (Ki = 0.2 mM)[20]. All these data are consistent with the contention that imperatorin is a potent inhibitor of MFO, acting primarily by interaction at or near the type I binding site of cytochrome p-450.

Drugs that initially inhibit the activity of the microsomal MFO when given acutely often stimulate the biotransformation of other drugs when administered chronically[20]. Table II shows that imperatorin will also act as an inducer when given chronically for 7 days, like a typical enzyme inducer, phenobarbital. It was previously reported that after seven daily oral doses of 1mmole/kg of imperatorin, coumarin 3-hydroxylase activity was increased 14 fold[21]. Increases in microsomal protein and cytochrome p-450 were also seen (Table II). However, aromatic hydroxylation of aniline was unaffected. This observation is in agreement with the result that 8-methoxypsoralen interacted with liver microsomes similar to other type I compounds and its chronic administration caused significant increases in hepatic cytochrome p-450 levels and in drug metabolizing enzyme activities but had no effect on aniline hydroxylase[22]. Interestingly, Emerole *et al.* in contrast to our findings observed a decrease of 30% in aniline hydroxylase activity by administration of 50 mg/kg of imperatorin for 3 days[3]. These discrepant findings are not explainable at present.

The pharmacological and clinical significance of the potent inhibition or induction of biotransformation of drugs in animals produced by imperatorin and related furanocoumarins have not been fully understood. However, the present results suggest that continued ingestion of medicinal plants or spices containing such furanocoumarins could play a part in the modification of the metabolism and the therapeutic effects or toxicity of concomitantly administered drugs.

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