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SPECTRAL AND METABOLIC PROPERTIES OF LIVER MICROSOMES FROM IMIDAZOLE-PRETREATED RABBITS*

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Imidazole or phenylimidazole, when administered in vivo, elevates rabbit liver microsomal cytochrome P-450 levels approximately 2.0- and 1.5-fold, respectively, as compared to controls. SDS-polyacrylamide gel electrophoresis revealed protein bands of enhanced intensity occurring at the approximate positions of LM_2, LM_3, LM_4 and possibly, LM_6. Imidazole- and phenylimidazole-induced microsomes exhibit N,N-dimethylaniline and p-nitroanisole demethylase activities which paralleled the increase in cytochrome P-450 content. Dimethylnitrosamine N-demethylase activity, however, when expressed per nmole P-450, was 2- to 8-fold greater in imidazole-induced microsomes than in control, phenobarbital-, or β-naphthoflavone-induced microsomes, and was inhibited by carbon monoxide or ethyl isocyanide. Dimethylsulfoxide inhibited dimethylnitrosamine N-demethylase activity 62% in imidazole-induced microsomes, but only 11% and 26% in phenobarbital- or β-naphthoflavone-induced microsomes, respectively. Binding of imidazole to cytochrome P-450 in imidazole-induced microsomes was monophasic, in contrast to the biphasic binding observed in phenobarbital or β-naphthoflavone-induced preparations. In addition, the spectral dissociation constant (K_d) for imidazole binding to imidazole-induced microsomes was 10- to 100-fold less (i.e. 10- to 100-fold greater affinity) than that measured in phenobarbital or β-naphthoflavone-induced preparations.

A wide variety of xenobiotics, including drugs, food additives, pesticides, and polycyclic aromatic hydrocarbons are capable of inducing the hepatic microsomal cytochrome P-450 mixed function oxidase system. Recent reports have indicated that the nitrogenous base pyrazole functions as an inducer of hepatic microsomal cytochrome P-450 in rats. Evarts et al., found that the dimethylnitrosamine N-demethylase activity in pyrazole-induced microsomes was substantially greater than that of control microsomes (1,2). Tu et al. have found comparable results

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Abbreviations: IM - imidazole, PM - phenylimidazole, DMN - N,N-dimethylnitrosamine, DMA - N,N-dimethylaniline, pNA - p-nitroanisole, PB - phenobarbital, BNF - β-naphthoflavone, TCA - trichloroacetic acid, DMSO - dimethylsulfoxide SDS - sodium dodecyl sulfate

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(3). Both reports indicate the induction of a protein believed to be an isozyme of cytochrome P-450, as shown by gel electrophoresis.

Many drugs containing an imidazole moiety appear to influence drug metabolism. The imidazole antimycotic clotrimazole, for example, has recently been found to act as an inducer of hepatic microsomal drug metabolism, whereas clotrimazole and related compounds (miconazole, ketoconazole and econazole) act acutely to inhibit microsomal drug metabolism *in vivo* in the rat (4). Other imidazole-containing drugs known to inhibit hepatic microsomal drug metabolism include the histamine H_2 receptor antagonist cimetidine (5-7).

The effects of the nitrogenous base imidazole (IM) on rabbit liver microsomal cytochrome P-450-catalyzed oxidation reactions *in vitro* and *in vivo* have been studied in our laboratory (8-11). IM acts as a noncompetitive inhibitor of N,N-dimethylaniline N-demethylase, *p*-nitroanisole O-demethylase and acetanilide *p*-hydroxylase activities in uninduced, PB- and BNF-induced rabbit liver microsomes. When administered *in vivo* to rats, a single dose of IM inhibits drug metabolism as evidenced by prolonged hexobarbital sleeping times, whereas repeated administration of IM appears to result in induction.

We have recently examined the effects of chronic administration of IM on hepatic microsomal cytochrome P-450 in rabbits. IM or PM appears to induce rabbit liver microsomal cytochrome P-450 as evidenced by increased P-450 content, SDS-polyacrylamide gel electrophoresis, and altered catalytic activity and substrate specificity. IM- and PM-induced microsomes exhibit a substantially greater DMN N-demethylase activity which is inhibited by DMSO. In addition, the binding of IM to IM-induced microsomes is monophasic, and of a greater affinity than that observed in PB- or BNF-induced preparations.

METHODS

Male New Zealand white rabbits (2 to 2.5 kg) were injected i.p. with 200 mg/kg IM or 100 mg/kg PM for 4 days then fasted 24 hr prior to sacrifice. Animals were sacrificed by injecting an overdose of pentobarbital into the marginal ear vein. Livers were removed, perfused with ice-cold saline, and microsomes were prepared as described (12,13). Protein and cytochrome P-450 content were assayed (14,15). The animals did not appear to suffer toxicity as a result of IM or PM pretreatment for 4-5 days.

SDS-polyacrylamide gel electrophoresis was performed according to published procedures (16,17). Formaldehyde production from DMA and DMN was measured according to Nash (18) and pNA O-demethylation was assayed according to Shigamatsu (19). In certain cases incubation mixtures included 28 mM ethyl isocyanide, or were maintained under an atmosphere of carbon monoxide.

Difference spectra were recorded at 25° on a Cary 219 double-beam spectrophotometer. Microsomes were diluted to 0.1 mg/ml protein with potassium phosphate buffer, pH 7.5. Aliquots of IM and phosphate buffer were added to the sample and reference cuvettes, respectively, recording and measuring the $\Delta A_{430-393}$ after each addition.

RESULTS

Pretreatment of rabbits with IM resulted in a twofold increase in cytochrome P-450 levels relative to uninduced microsomes (Table 1). Cytochrome P-450 levels averaged 1.6 nmols/mg microsomal protein after IM induction, compared to values of 0.80, 3.1, and 1.9 nmoles/mg protein in control, PB-, and BNF-induced microsomal preparations, respectively. PM increased P-450 levels to 1.1 nmoles/mg protein. These data indicate that IM increased cytochrome P-450 content to a level comparable to BNF, but not as markedly as PB.

The SDS-polyacrylamide gel electrophoretic pattern of IM-induced microsomes is shown in Fig. 1, along with control, PB- and BNF-induced microsomes. For comparison, purified cytochrome P-450$_{LM_2}$ and $_{LM_4}$ are also shown. IM-induced microsomes yield protein bands of enhanced intensity at the approximate positions

Table 1

Relative induction of cytochrome P-450LM in rabbit liver microsomes

Pretreatment†	Cytochrome P-450*
Control	0.8 ± 0.1 (n = 3)
Imidazole	1.6 ± 0.3 (n = 5)
Phenylimidazole	1.1 ± 0.1 (n = 2)
β-Naphthoflavone	1.9 ± 0.7 (n = 10)
Phenobarbital	3.1 ± 0.8 (n = 10)

*Expressed as nmoles/mg microsomal protein.

†Enzyme induction was accomplished through phenobarbital pretreatment (0.1% in drinking water for 5 days) or through injection of β-naphthoflavone (80 mg/kg in corn oil, i.p., 36 hrs prior to sacrifice).

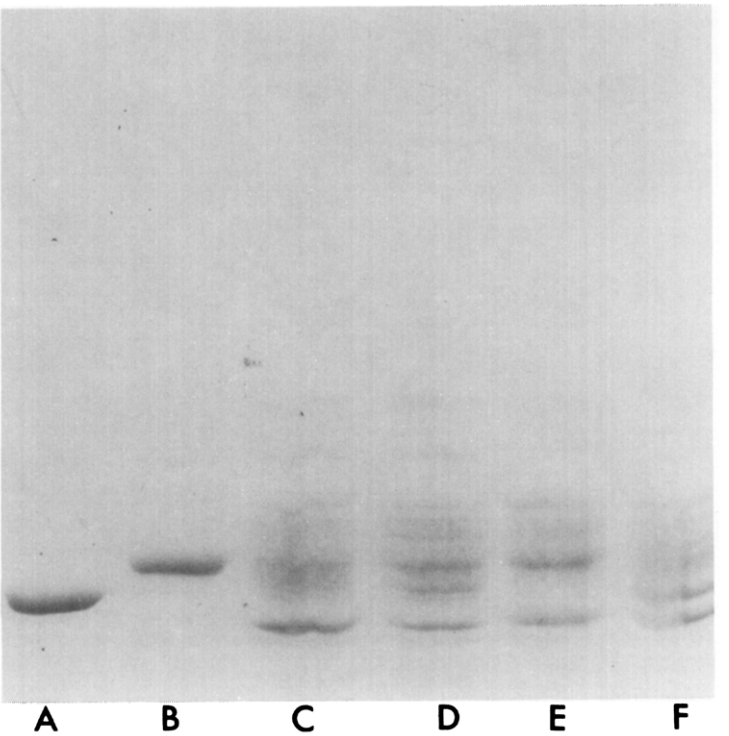

Fig. 1. SDS-polyacrylamide gel electrophoretic pattern of rabbit liver microsomes. Shown above are: A, purified cytochrome P-450$_{LM_2}$, 12.5 µg; B, purified cytochrome P-450$_{LM_4}$, 15.0 µg; C, control microsomes, 101 µg; D, IM-induced microsomes, 105 µg; E, BNF-induced microsomes, 106 µg; F, PB-induced microsomes, 122 µg. PM-induced microsomes yielded a pattern identical to that observed for IM-induced microsomes.

of P-450$_{LM_2}$, LM_3, LM_4, and possibly LM_6. Identical results were observed using PM-induced microsomes. The increased content of cytochrome P-450$_{LM}$ is paralleled by increases in the metabolic activities towards the substrates DMA and pNA in IM-induced microsomes, as shown in Table 2. The observed catalytic activities towards DMA and PNA were greatest in PB-induced microsomes, followed by IM-induced, BNF-induced, and uninduced microsomes, respectively.

IM-induced microsomes also display an altered catalytic activity towards the carcinogen DMN. As shown in Table 3, IM- and PM-induced microsomes produce 2.9 and 2.1 nmoles formaldehyde per minute per milligram microsomal protein, compared to 0.7, 0.6, and 0.6 nmoles/min/mg protein in control, BNF- and PB-induced microsomes, respectively. When expressed per nanomole P-450$_{LM}$ to account for the relative levels of induction among the various microsomal preparations, an enhancement of two to eightfold is observed in the IM-induced microsomes relative to uninduced,

Table 2

Relative metabolism of N,N-dimethylaniline and para-nitroanisole in rabbit liver microsomes

Pretreatment*	Dimethylaniline† N-demethylase activity (nmoles HCHO/min/mg protein)	para-nitroanisole† O-demethylase activity (nmoles p-aminophenol/min/mg protein)
Control	4.0 ± 0.6	0.8 ± 0.1
Imidazole	6.5 ± 0.8	1.9 ± 0.1
β-Naphthoflavone	4.3 ± 0.6	1.0 ± 0.2
Phenobarbital	19.0 ± 1.9	3.4 ± 0.4

*As described in Methods and the legend to Table 1.

†Incubations contained 1 mg/ml microsomal protein, substrate (1.0 mM DMA; 0.1 mM pNA), NADPH (1 mM) and 0.1 M phosphate buffer, pH 7.5, in a total volume of 1.0 ml. Reactions were initiated with NADPH after a 2-min preincubation period and terminated with 0.3 ml cold 20% TCA. Results are expressed as the mean ± standard error for 12 determinations. For this series of experiments the cytochrome P-450 content was 3.0, 2.0, 1.8, and 0.8 nmoles/mg microsomal protein for the PB-, IM-, and BNF-induced and uninduced preparations, respectively.

PB-, or BNF-induced microsomes. The DMN N-demethylase activity of IM-induced microsomes was inhibited approximately 80% and 70%, by either carbon monoxide or ethyl isocyanide, respectively.

Table 3

Dimethylnitrosamine N-demethylase activities in rabbit liver microsomes

Pretreatment*	Activity† (nmoles HCHO/min/mg protein)	Activity (nmoles HCHO/min/nmole P-450)
Control	0.7 ± 0.2	0.9 ± 0.2
Imidazole	2.9 ± 0.9	2.3 ± 0.7
Phenylimidazole	2.1 ± 0.4	2.3 ± 0.4
β-Naphthoflavone	0.6 ± 0.2	0.4 ± 0.2
Phenobarbital	0.6 ± 0.1	0.2 ± 0.1

*As described in Methods and in the legend to Table 1.

†Incubations were performed using the conditions described in Methods and contained 1 mg/ml microsomal protein, substrate (5.0 mM DMN), 1.0 mM NADPH and 0.1 M phosphate buffer, pH 7.5 in a total volume of 1.0 ml. Reactions were initiated with NADPH after a 2-minute preincubation period and terminated with 0.3 ml cold 20% TCA. Results are expressed as the mean ± standard error for 12 determinations.

Table 4

Effect of DMSO on Dimethylnitrosamine N-demethylase activities in rabbit liver microsomes

Pretreatment*	[DMN] mM	[DMSO] mM	Activity†,§ nmoles HCOH/min/mg protein	% Inhibition
Imidazole	5	-	2.9 ± 0.9	
	5	28	1.1 ± 0.2	62
β-Naphthoflavone	10	-	0.9 ± 0.4	
	10	56	0.7 ± 0.1	26
Phenobarbital	10	-	0.9 ± 0.1	
	10	56	0.8 ± 0.2	11

*As described in Methods and in the legend to Table 1.

†Incubations were performed using IM-induced microsomes at 1 mg/ml, or BNF- or PB-induced microsomes at 2 mg/ml. DMSO was present at 28 mM in IM-induced microsomes and 56 mM in BNF- or PB-induced microsomes (i.e. the DMSO/protein concentration ratio was constant for all incubations). The increased protein concentration for BNF- or PB-induced microsomal preparations was required because of the low metabolic activities of these microsomes towards DMN and because of the relatively small inhibitory effect of DMSO. Product formation was linear up to 4 mg/ml microsomal protein.

§Results are expressed as the mean ± standard error for 6-12 determinations.

Dimethylsulfoxide, an inhibitor of ethanol oxidation by purified cytochrome P-450$_{LM_{3_a}}$ (20) was found to be a potent inhibitor of DMN N-demethylase activity in IM-induced microsomes, as seen in Table 4. DMSO (28 mM) inhibited DMN metabolism by more than 60% in IM-induced microsomes, whereas an equivalent concentration ratio of DMSO to protein resulted in only an 11% inhibition of DMN N-demethylase activity in PB-induced microsomes or a 26% inhibition of activity in BNF-induced microsomes.

Additional studies examined the binding of IM to IM-induced microsomes using UV-visible difference spectroscopy. When added to IM-induced microsomes, IM causes a type II difference spectrum with the magnitude of the absorbance change dependent upon imidazole concentration, as shown in Fig. 2. Eadie-Scatchard analysis of the concentration-dependent absorbance changes, Fig. 2 (inset), reveals

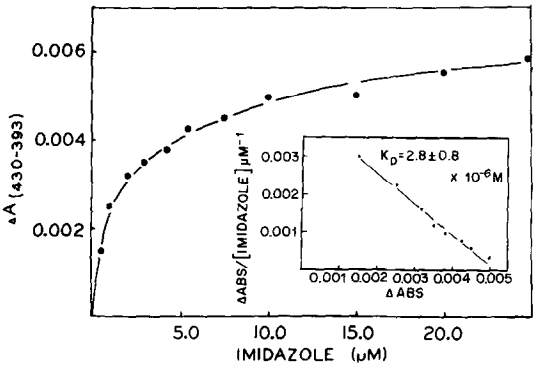

Fig. 2. The concentration-dependent absorbance changes in the type II difference spectrum caused by IM in IM-induced microsomes (0.1 mg/ml). Eadie-Scatchard analysis (inset) reveals monophasic binding with a spectral dissociation constant value of $2.8 \pm 0.8 \times 10^{-6}$ M.

monophasic binding of IM to cytochrome P-450 in IM-induced microsomes, with a K_d value of 2.8×10^{-6} M.

DISCUSSION

IM, which acts as an inhibitor of cytochrome P-450-catalyzed drug metabolism both *in vivo* and *in vitro*, also induces rabbit liver microsomal cytochrome P-450. Pretreatment of rabbits with IM or PM increased cytochrome P-450 levels, with the anticipated parallel increase in the metabolism of DMN and pNA. Gel electrophoresis reveals protein bands of enhanced intensity that differ from those observed in PB- or BNF-induced microsomes; in particular, bands at the approximate positions of LM_3 and LM_6 were observed, in addition to those at the positions of LM_2 and LM_4.

These results, in conjunction with the enhanced DMN N-demethylase activity observed in IM- and PM-induced microsomes relative to control, PB-, and BNF-induced microsomes, suggest that IM and PM induce an isozyme (or isozymes) of P-450LM with an altered catalytic activity towards various substrates. Involvement of the hemeprotein in the metabolism of DMN is confirmed by the elimination of approximately 75-80% of catalytic activity when incubations are carried out in the presence of a carbon monoxide atmosphere or ethyl isocyanide. Additional support for the involvement of P-450 in DMN metabolism is provided by Evarts *et al.* (*1*), in which it was demonstrated that NADPH was a requirement for metabolic activity. DMSO markedly inhibits DMN N-demethylase activity in IM-induced micro-

somes; in contrast, DMSO produces only a small decrease in such activity in PB- or BNF-induced microsomes, suggesting the participation of a unique isozyme (or isozymes) of cytochrome P-450$_{LM}$ in the metabolism of DMN.

IM binds to IM-induced microsomes monophasically and with high affinity, as demonstrated by UV-visible difference spectroscopy. The monophasic binding of IM to IM-induced microsomes is in direct contrast to the biphasic binding observed in PB- or BNF-induced microsomal suspensions or to the purified isozymes P-450$_{LM_2}$ and $_{LM_4}$ (11). The resultant spectral dissociation constant value is one-to-two orders of magnitude smaller than that measured in PB- or BNF-induced microsomal suspensions, indicating a substantially greater affinity of IM-induced microsomes for IM.

The above results suggest that induction of rabbit liver microsomal cytochrome P-450 by IM or PM encompasses not only increased cytochrome P-450 content, but also the induction of an isozyme or isozymes of the cytochrome which have altered affinity and catalytic activity towards various substrates. The enhanced N-demethylase activity towards DMN could potentiate the carcinogenic effect of this compound *in vivo*. In addition, the metabolism of other drugs may be altered as a result of induction by imidazole. Perhaps a wide variety of IM-containing drugs and chemicals act similarly to induce isozyme(s) of cytochrome P-450 having unique substrate specificities.

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