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MIXED-FUNCTION AMINE OXIDASE OF THE RAT HEPATOCYTE NUCLEAR ENVELOPE

DEMONSTRATION AND EFFECTS OF PHENOBARBITAL AND 3-METHYLCHOLANTHRENE

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Abstract—Mixed-function amine oxidase (EC 1.14.13.8) has been demonstrated in highly purified rat hepatocyte nuclear envelope. The enzyme was present in the nuclear envelope at a level 20 percent of that observed in microsomes. Induction studies indicated that nuclear envelope amine oxidase as well as its microsomal counterpart were refractory to the effects of phenobarbital and 3-methylcholanthrene. Phenobarbital administration increased the specific activity of the microsomal *N,N*-dimethylaniline *N*-demethylase and benzo[*a*]pyrene hydroxylase by 600 and 190 percent, respectively, but decreased the specific activity of the nuclear enzymes by 30–50 percent. In contrast, 3-methylcholanthrene increased the specific activity of benzo[*a*]pyrene hydroxylase in nuclear envelope and microsomes by 42- and 11-fold, respectively. The hydrocarbon also increased the microsomal and nuclear *N,N*-dimethylaniline *N*-demethylase by 40 and 60 percent, respectively, but the specific activity of microsomal and nuclear aniline 4-hydroxylase was decreased by 50 percent. Demonstration of amine oxidase in rat hepatocyte nuclear envelope implicates this enzyme in the toxicity and carcinogenicity of certain drugs and chemicals.

The cytochrome P-450 dependent mixed-function oxidase system is localized not only in the endoplasmic reticulum [1–4] but also in the nuclear envelope [5–8] of rat hepatocytes. In addition, epoxide hydratase and UDP glucuronosyl transferase†, two enzymes functionally associated with cytochrome P-450 dependent monooxygenases, have also been demonstrated in the two membranes [9–16]. Biochemical studies have established the immunochemical identity of nuclear and microsomal NADPH cytochrome *c* oxidoreductase [17, 18], epoxide hydratase [15, 19], and cytochrome P-448 [15, 20]. These similarities are not surprising since the nuclear envelope forms a morphological continuum with the rough endoplasmic reticulum [21], however, biochemical differences between the two membranes also exist. Phenobarbital (PB) induces microsomal epoxide hydratase and the components of the NADPH electron transport chain which include cytochrome P-450 and NADPH cytochrome *c* oxidoreductase but not the nuclear counterparts [6, 7, 12, 22, 23]. Furthermore, nuclear envelope also contains specific proteins which are not present in the endoplasmic reticulum [24–26]; this includes a unique protein kinase found only in the nuclear envelope [26].

Another enzyme reponsible for the oxidative metabolism of a variety of xenobiotics is mixed-function amine oxidase (EC 1.14.13.8), a flavoprotein present in the microsomal fraction of several porcine organ systems including liver, kidney, lung and corpus luteum [27]. Recently, this enzyme has also been demonstrated in microsomes and nuclei isolated from hamster liver [28]. Mixed-function amine oxidase mediates the NADPH and oxygen-dependent *N*-oxidation of a variety of lipid soluble secondary- and tertiary-amines [29–33] and alkylhydrazines [34] and *S*-oxidation of thioureylenes [35] and thioethers [36]. In addition, the enzyme can also catalyse the formation of disulfide bonds in proteins [37]. Purified amine oxidase from porcine liver microsomes is not sensitive to carbon monoxide or β-diethylaminoethyldiphenylpropylacetate (SKF-525A) [33] and is antigenically distinct from NADPH cytochrome *c* oxidoreductase [38]. Thus, the enzyme represents a microsomal oxidation system independent of cytochrome P-450.

As a continuation of our studies on the chemical and biochemical properties of the nuclear envelope, highly purified nuclear envelope was examined for mixed-function amine oxidase activity. Demonstration of amine oxidase in the nuclear envelope and the effects of PB and 3-methylcholanthrene (3-MC) administration on the level of amine oxidase in both rat hepatocyte nuclear envelope and microsomal membrane form the basis of this report.

MATERIALS AND METHODS

Materials. All reagents used in this study were of the highest chemical grade commercially available and were used without further purification. *N,N*-Dimethylaniline *N*-oxide was synthesized as previously described [39]; 2,4-dichloro-6-phenylphenoxyethylamine was obtained from Dr. Robert

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† J. Gorski and C. B. Kasper, unpublished data.

McMahon, Eli Lilly & Co., Indianapolis, IN, and 3-hydroxybenzo[*a*]pyrene was a gift from Dr. Harry Gelboin of the National Cancer Institute.

Animals. Male Sprague–Dawley rats (Madison, WI) weighing 50–60 g were used for all studies. Induction of xenobiotic metabolizing enzymes was accomplished by daily intraperitoneal injection of PB in 0.9% NaCl (100 mg/kg body wt) for 5 successive days or by a single intraperitoneal injection of 3-MC in trioctanoin (40 mg/kg body wt). Control animals received only the vehicle.

Preparation of nuclear envelope and microsomal membranes. Animals were starved for a 24-hr period prior to being decapitated. Nuclear envelope was isolated and prepared according to Kasper [25], and microsomal membranes were isolated and prepared according to Blackburn *et al.* [40]. The purified membranes were used immediately after isolation.

Assays. Mixed-function amine oxidase activity was determined by a modification of the procedure described by Ziegler and Pettit [41]. Nuclear and microsomal suspensions (3–4 mg protein for control and induced membranes) were incubated in a total volume of 3 ml of 0.1 M glycine, 0.025 M pyrophosphate buffer, pH 8.4, containing 1.5 μmoles $NADP^+$, 0.5 μmole glucose-6-phosphate, 15 μmoles $MgCl_2$, 2 I.U. of glucose-6-phosphate dehydrogenase, 3 μmoles 2,4-dichloro-6-phenylphenoxyethylamine (DPEA), and 3 μmoles *N,N*-dimethylaniline (DMA). DPEA was added to inhibit cytochrome P-450 dependent *N*-oxide *N*-dealkylase [41, 42]. The reaction mixture minus substrate was incubated for 5 min at 37° prior to addition of DMA. After 5 min, the reaction was terminated by addition of 1.5 ml of 0.9 N trichloroacetic acid. Denatured protein was sedimented and 3.0 ml of the acid supernatant fraction was transferred to 15 ml culture tubes. Then 0.18 ml of 6 N KOH was added and unreacted DMA was quantitatively extracted by 3 × 3.5 ml of diethyl ether. An aliquot (2.5 ml) of the aqueous phase was removed, acidified by adding 0.25 ml of 1.0 N HCl and 0.25 ml of 0.11 N $NaNO_2$, and heated for 5 min at 60°. After cooling to room temperature, the product was quantitated by measuring the absorbance at 420 nm.

DMA *N*-demethylase activity was determined according to Cho and Miwa [43] and the formaldehyde was assayed according to Nash [44]. Aniline 4-hydroxylase activity was determined according to Kato and Gillette [45]. Benzo[*a*]pyrene hydroxylase activity was determined according to Nebert and Gelboin [46] as modified by Poland and Glover [47] using 3-hydroxybenzo[*a*]pyrene as the standard.

RESULTS

Results obtained with highly purified nuclear envelope, using DMA as substrate, indicated that the specific activity of the nuclear enzyme was about 20 percent of that observed in microsomes. The enzymatic activity in both membranes showed an absolute dependence on NADPH, and the activity was destroyed by heating the membranes in a 90° waterbath for 5 min (Table 1).

The effects of PB on mixed-function amine oxidase and other xenobiotic metabolizing activities present in the nuclear envelope and microsomal membrane are shown in Table 2. PB increased the specific activity of microsomal DMA *N*-demethylase and benzo[*a*]pyrene hydroxylase by 600 and 190 percent, respectively, but decreased the specific activity of DMA *N*-oxidase by 60 percent. The barbiturate had little or no effect on the microsomal aniline 4-hydroxylase. Xenobiotic metabolizing activities in nuclear envelope were completely refractory to the inductive effects of PB. In fact, specific activities for DMA *N*-oxidase, DMA *N*-demethylase, aniline 4-hydroxylase, and benzo[*a*]pyrene hydroxylase in the nuclear envelope were decreased 30–40 percent when compared to saline control.

Administration of 3-MC produced a different spectrum of responses (Table 3). This xenobiotic dramatically increased the specific activity of microsomal benzo[*a*]pyrene hydroxylase 11-fold while increasing DMA *N*-demethylase activity by only 40 percent. Furthermore, the specific activities of microsomal DMA *N*-oxidase and aniline 4-hydroxylase were decreased by 30 and 50 percent, respectively. In contrast to PB, 3-MC increased the specific activity of nuclear benzo[*a*]pyrene hydroxylase 42-fold and also increased the specific activity of DMA *N*-demethylase by 50 percent. However, in the case of nuclear DMA *N*-oxidase and aniline 4-hydroxylase, 3-MC administration produced a decrease in specific activity of 25 and 50 percent, respectively.

Table 1. Rate of formation of DMA *N*-oxide by nuclear and microsomal mixed-function amine oxidase*

Incubation conditions	*N*-oxide [nmoles · (mg protein)$^{-1}$ · min^{-1}]
Microsomes + cofactors†	5.0 ± 0.05
Microsomes − cofactors	0
Heat-treated microsomes‡ + cofactors	0
Nuclear envelope + cofactors	0.93 ± 0.05
Nuclear envelope − cofactors	0
Heat-treated nuclear envelope‡ + cofactors	0

* Results are means ± S.D. of two to six groups of animals. Incubations were carried out as described in Materials and Methods.

† Cofactors refer to an NAPH-generating system described in Materials and Methods.

‡ Nuclear envelope or microsomes were heated at 90° for 5 min in a waterbath.

Table 2. Effects of phenobarbital on nuclear envelope and microsomal membrane xenobiotic metabolizing enzyme systems*

	Nuclear envelope			Microsomal membrane		
			PB			PB
	Saline	PB	Saline	Saline	PB	Saline
DMA *N*-oxidase†	0.93 ± 0.05	0.61 ± 0.04	0.66	5.02 ± 0.47	2.15 ± 0.34	0.43
DMA *N*-demethylase‡	1.12 ± 0.06	0.86 ± 0.07	0.77	3.45 ± 0.08	20.75 ± 0.25	6.01
Aniline 4-hydroxylase§	1.80 ± 0.09	0.98 ± 0.07	0.54	4.05 ± 0.12	5.06 ± 0.08	1.25
Benzo[*a*]pyrene hydroxylase‖	8.10 ± 0.30	5.39 ± 0.31	0.67	276 ± 36	524 ± 62	1.90

* Results are means ± S.D. of two to six groups of animals. Incubations were carried out as described in Materials and Methods.
† Activity is expressed as nmoles DMA *N*-oxide · (mg protein)$^{-1}$ · min^{-1}.
‡ Activity is expressed as nmoles HCHO · (mg protein)$^{-1}$ · min^{-1}.
§ Activity is expressed as nmoles 4-hydroxyaniline · (mg protein)$^{-1}$ · min^{-1}.
‖ Activity is expressed as pmoles hydroxylated benzo[*a*]pyrene · (mg protein)$^{-1}$ · min^{-1}.

DISCUSSION

The endoplasmic reticulum of hepatocytes contains three flavoproteins: NADPH cytochrome *c* (P-450) oxidoreductase [48–50] which transfers reducing equivalents from NADPH to cytochrome P-450, NADH cytochrome b_5 oxidoreductase [51, 52] which transfers reducing equivalents from NADH to cytochrome b_5, and mixed-function amine oxidase [29, 38, 53] which mediates the *N*-oxidation of secondary and tertiary amines and hydrazines. NADPH cytochrome *c* oxidoreductase [5, 6, 17, 18] and NADH cytochrome b_5 oxidoreductase [6, 54, 55] have already been demonstrated in the nuclear envelope, and results obtained in this study establish that mixed-function amine oxidase is also an intrinsic nuclear envelope enzyme. Hence, the metabolic capacity of the nuclear envelope is qualitatively similar to that of the endoplasmic reticulum in terms of the membrane-associated mixed-function oxidation systems.

Earlier studies on the effect of PB on porcine [38] and 3-MC on hamster [56] hepatic microsomes indicated that microsomal amine oxidase was refractory to the inducing effects of these two compounds. Our results using rat liver microsomes support these earlier findings and, furthermore, establish that amine oxidase present in the nuclear envelope is also refractory to the effects of PB and 3-MC. Studies in rodents, however, have shown that the activity of microsomal amine oxidase can be altered by prednisolone [57], corticosterone [58], and steroid sex hormones [59].

Our finding that PB had no effect on nuclear amine oxidase is in agreement with the lack of effect of PB on epoxide hydratase [12] and cytochrome P-450

Table 3. Effects of 3-methylcholanthrene on nuclear envelope and microsomal membrane xenobiotic metabolizing enzyme systems*

	Nuclear envelope			Microsomal membrane		
			3-MC			3-MC
	Trioctanoin	3-MC	Trioctanoin	Trioctanoin	3-MC	Trioctanoin
DMA *N*-oxidase†	1.20 ± 0.03	0.88 ± 0.01	0.73	4.22 ± 0.27	2.85 ± 0.16	0.68
DMA *N*-demethylase‡	0.98 ± 0.02	1.55 ± 0.03	1.58	2.69 ± 0.15	3.73 ± 0.11	1.39
Aniline 4-hydroxylase§	1.75 ± 0.05	0.89 ± 0.02	0.51	4.57 ± 0.17	2.39 ± 0.20	0.52
Benzo[*a*]pyrene hydroxylase‖	6.3 ± 0.1	264 ± 3	41.9	250 ± 14	2805 ± 460	11.2

* Results are means ± S.D. of two to six groups of animals. Incubations were carried out as described in Materials and Methods.
† Activity is expressed as nmoles DMA *N*-oxide · (mg protein)$^{-1}$ · min^{-1}.
‡ Activity is expressed as nmoles HCHO · (mg protein)$^{-1}$ · min^{-1}.
§ Activity is expressed as nmoles 4-hydroxyaniline · (mg protein)$^{-1}$ · min^{-1}.
‖ Activity is expressed as pmoles hydroxylated benzo[*a*]pyrene · (mg protein)$^{-1}$ · min^{-1}.

dependent mixed-function oxidase systems [6, 7, 12, 22, 23] present in the nuclear envelope. Xenobiotic metabolizing systems in the nuclear envelope are not, however, totally refractory to the effects of inducing agents. 3-MC treatment increased the specific activity of nuclear benzo[*a*]pyrene hydroxylase 10- to 60-fold whereas PB decreased the specific activity [7, 12, 22, 23]. Furthermore, results in this study showed that 3-MC increased the specific activity of nuclear DMA *N*-demethylase while PB had no effect.

Most chemical carcinogens require metabolic activation forming reactive electrophiles which interact with DNA, RNA, and protein [60–62]. The instability of these ultimate carcinogens suggests that the proximity of the site of generation of these reactive metabolites to DNA may determine the extent of DNA alteration. Thus, formation of mutagenic and/or carcinogenic metabolites by monooxygenases in nuclear envelope is more likely to result in interactions with DNA, despite the minor contribution of the nuclear enzymes to the total cellular metabolism of chemical carcinogens.

Existence of mixed-function amine oxidase in nuclear envelope of hepatocytes may implicate the enzyme in the carcinogenicity and toxicity of xenobiotics, especially chemicals containing a hydrazine moiety. Purified amine oxidase has been shown to *N*-oxidize alkyl hydrazines including 1,2-dimethylhydrazine [34], a potent carcinogen in laboratory animals [63]. Recently, two hydrazine-containing drugs, isoniazid and iproniazid, have been shown to cause liver damage at therapeutic levels [64, 65]. The toxic effects of these drugs appear to be dependent on *N*-oxidation of the hydrazine portion by liver [66–71]. Nuclear metabolic activation of hydrazine derivatives to reactive metabolites by mixed-function amine oxidase could result in covalent binding of these metabolites to DNA, RNA, and protein. Modification of DNA may lead to perturbation of normal cellular processes resulting in clinically observed toxicity [64, 65] and tumors in laboratory animals [63, 69].

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