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INACTIVATION OF CHICK EMBRYO HEPATIC CYTOCHROME P450 1A, 2H AND 3A FOLLOWING *IN OVO* ADMINISTRATION OF 3,5-DIETHOXYCARBONYL-1,4-DIHYDRO-2,6-DIMETHYL-4-ETHYLPYRIDINE AND 3-[2-(2,4,6-TRIMETHYLPHENYL)THIOETHYL]-4-METHYLSYDNONE

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Abstract—Rat hepatic cytochrome P450 (P450) isozymes 1A1, 2C6, 2C11, 3A1 and 3A2 are targets for mechanism-based inactivation by the porphyrinogenic compound 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-ethylpyridine (4-ethyl DDC). It is of interest to determine whether similar P450 isozymes are targets of porphyrinogenic drugs in the chick embryo liver. The chick embryo expresses P450 2H1/2 isozymes, which are similar to the rat P450 2B1/2 isozymes, a polycyclic aromatic hydrocarbon-inducible P450 1A isozyme, and a pregnenolone 16α-carbonitrile-inducible P450 3A isozyme. We have found previously that chick embryo hepatic P450 1A and 3A isozymes are targeted for *in vitro* mechanism-based inactivation by 4-ethyl DDC and by the sydnone 3-[2-(2,4,6-trimethylphenyl)thioethyl]-4-methylsydnone (TTMS). Marked differences have been observed between the *in vitro* and *in vivo* effects of porphyrinogenic drugs on P450 isozymes. Thus, the first objective of this study was to determine whether chick embryo hepatic P450 1A and 3A isozymes are subject to *in ovo* inactivation by these porphyrinogenic compounds. Our second objective was to determine whether the chick embryo hepatic P450 2H isozyme(s) was subject to *in ovo* and *in vitro* inactivation by 4-ethyl DDC and TTMS. Using hepatic microsomes prepared from β-naphthoflavone-, dexamethasone-, phenobarbital-, and glutethimide-induced 19-day-old chick embryos, we found that total P450 content was decreased significantly in microsomes prepared from all treatment groups following *in ovo* administration of 4-ethyl DDC and TTMS. Moreover, *in ovo* administration of both 4-ethyl DDC and TTMS caused a significant decrease of 7-ethoxyresorufin *O*-deethylase, erythromycin *N*-demethylase, and benzphetamine *N*-demethylase activities, which are selective catalytic markers for chick embryo hepatic P450 1A, 3A and 2H isozymes, respectively. In addition, *in vitro* administration of 4-ethyl DDC and TTMS caused mechanism-based inactivation of benzphetamine *N*-demethylase activity in microsomes from phenobarbital- and glutethimide-treated chick embryos, showing that the chick embryo hepatic P450 2H isozyme is a target for mechanism-based inactivation. Therefore, it was concluded that the chick embryo hepatic P450 1A, 2H and 3A isozymes serve as targets for both *in ovo* and *in vitro* mechanism-based inactivation by 4-ethyl DDC and TTMS.

Key words: porphyria, drug-induced; chick embryo liver; cytochrome P450 isozymes; dihydropyridine; sydnone; mechanism-based inactivation

Xenobiotics, which disrupt heme biosynthesis and cause porphyrin accumulation, are referred to as porphyrinogenic compounds. The dihydropyridine 4-ethyl DDC† (Fig. 1a) [1–11], and the sydnone TTMS (Fig. 1b) [12–15], are porphyrinogenic compounds that cause mechanism-based inactivation of P450 (EC 1.14.14.1). The inactivation of P450 isozymes by these compounds can result from one or a combination of three distinct mechanisms: (i) prosthetic heme N-alkylation, (ii) apocytochrome P450 alkylation, and (iii) conversion of prosthetic heme to a product or products that irreversibly bind the apocytochrome P450. However, the porphyrinogenic effect of 4-ethyl DDC and TTMS is believed to result from prosthetic heme N-alkylation of P450 by alkyl radicals generated during the metabolism of these xenobiotics by P450 isozymes [16].

Following heme N-alkylation, iron is released from *N*-alkylheme to form a mixture of four regioisomers of *N*-alkylPP. *N*-AlkylPPs are potent inhibitors of ferrochelatase (EC 4.99.1.1) [17, 18], the terminal enzyme in heme biosynthesis. Thus, the formation of *N*-alkylPPs decreases heme production, leading to a depletion of regulatory

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† Abbreviations: 4-ethyl DDC, 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-ethylpyridine; TTMS, 3-[2-(2,4,6-trimethylphenyl)thioethyl]-4-methylsydnone; P450, cytochrome P450; *N*-alkylPP, *N*-alkylprotoporphyrin IX; ALAS, 5-aminolevulinic acid synthase; EROD, 7-ethoxyresorufin *O*-deethylase; ERND, erythromycin *N*-demethylase; PB, sodium phenobarbital; BZND, benzphetamine *N*-demethylase; βNF, β-naphthoflavone; BSA, bovine serum albumin; DEX, dexamethasone; GLUT, glutethimide; HCHO, formaldehyde; UT, untreated; and 3MC, 3-methylcholanthrene.

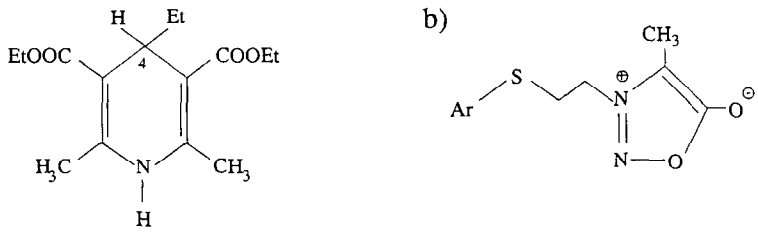

Fig. 1. Structures of: (a) 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethyl-4-ethylpyridine (4-ethyl DDC), Et = ethyl; and (b) 3-[2-(2,4,6-trimethylphenyl)thioethyl]-4-methylsydnone (TTMS), Ar = aryl.

"free" heme that acts to repress the rate-controlling enzyme in heme biosynthesis, namely ALAS. "Free" heme refers to heme that is either very recently synthesized and not yet bound to protein, or heme that has just been released from hemoproteins. The induction of ALAS activity, together with inhibition of ferrochelatase activity, leads to an accumulation of the final precursor of heme biosynthesis, namely protoporphyrin IX.

Several rat hepatic P450 isozymes have been assayed for their susceptibility to both *in vitro* mechanism-based inactivation and *in vivo* inactivation by a series of dihydropyridines and methylsydnones. Rat hepatic P450 1A1, 2C6, 2C11, 3A1 and 3A2 were found to be targets for mechanism-based inactivation by 4-ethyl DDC [4, 6, 8–11], while P450 1A and 3A are targets for mechanism-based inactivation by TTMS [14]*. To isolate *N*-alkylPPs for purification, structural identification, and regioisomer separation, the rat has been used as the experimental animal of choice, as sufficient liver tissue was required for these purposes. However, studies of porphyrin accumulation and ferrochelatase inhibition were more conveniently performed on the chick embryo *in ovo* or in culture, as the chick embryo liver is more sensitive to most porphyrinogenic compounds than is the rat liver [20]. One factor that contributes to the enhanced sensitivity of the chick embryo has been identified. Thus, De Matteis [21] has shown that the ferrochelatase activity of adult chicken liver is 25% the level found in rat liver, while Cole *et al.* [22] have shown that the level of ferrochelatase in chick embryo liver is 15% of that found in rat liver. It has been generally assumed that porphyrinogenic xenobiotics behave similarly in both the rat and chick embryo. However, little is known of the chick embryo hepatic P450 isozyme profile or about which chick embryo hepatic P450 isozymes are targeted for inactivation by porphyrinogenic compounds. P450 isozymes can have completely different substrate specificities following a single amino acid substitution [23]. Therefore, it cannot be assumed that P450 isozymes shown to be targets for xenobiotic-induced mechanism-based inactivation in the rat liver are also targets for inactivation in the chick embryo liver. For this reason, it is important to elucidate the P450 isozyme profile in chick embryo liver and to determine which P450 isozymes are targets for mechanism-based inactivation by 4-ethyl DDC and TTMS.

Several studies have shown that the chick embryo liver contains P450 1A and 3A isozymes [24–29]. Throughout this manuscript, we refer to these isozymes as P450 1A and 3A, with the understanding that further work is necessary to delineate completely the nature of these isozymes. We have demonstrated that the P450 1A and 3A isozymes of the chick embryo share the ability of rat liver P450 1A1 and 3A1/2 isozymes to serve as targets for *in vitro* mechanism-based inactivation by 4-ethyl DDC and TTMS [14]. However, marked differences have been shown to exist between the *in vivo* and *in vitro* effects of porphyrinogenic xenobiotics on selected P450 isozymes [6, 8–11, 30]. An example of a previously observed difference between *in vivo* and *in vitro* effects of porphyrinogenic xenobiotics on selected P450 isozymes is the following: Rat hepatic P450 2B1/2, two major isozymes that are induced following PB treatment, undergo *in vivo* but not *in vitro* inactivation by 4-ethyl DDC. *In vivo*, 4-ethyl DDC causes N-alkylation of the heme moieties of several P450 enzymes other than P450 2B1/2. As a result, total hepatic heme levels drop and heme is removed from other P450 isozymes, such as P450 2B1/2, to replenish the pool of heme. The process of removal of heme from the apo-P450 proteins is referred to as "heme stripping." The phenomenon of "heme stripping" explains the *in vivo* loss of P450 2B1/2 activity. Therefore, because of previously observed *in vivo/in vitro* differences in inactivation by porphyrinogenic xenobiotics, the first objective of the current study was to determine whether 4-ethyl DDC and TTMS were able to cause *in ovo* inactivation of chick embryo hepatic microsomal P450 1A and 3A, in addition to the previously observed *in vitro* inactivation.

The chick embryo liver is also known to contain two PB-inducible P450 isozymes that express more than 93% amino acid sequence homology to one another [31, 32]. These chicken hepatic P450 isozymes share approximately 43% amino acid sequence homology with rat hepatic P450 2B isozymes [19], and have been designated P450 2H1 and 2H2. Both P450 2H1 and 2H2 catalyze BZND activity to the same extent [33], and BZND is used as a functional marker of these two isozymes. Since P450 2H1/2 are the major PB-inducible isozymes in

* The cytochrome P450 nomenclature used is that outlined by Nebert *et al.* [19].

the chick embryo, the second objective of this study was to test whether 4-ethyl DDC and TTMS cause *in ovo* inactivation and *in vitro* mechanism-based inactivation of chick embryo hepatic P450 2H, using BZND activity as a functional marker for these isozymes.

MATERIALS AND METHODS

Source of chemicals

TTMS and 4-ethyl DDC were obtained from Colour Your Enzyme (Bath, Ontario), and benzphetamine was obtained from the Bureau of Drug Research, Health Protection Branch, Canada. Chemicals were purchased from the following sources: PB (British Drug Houses Inc., Toronto, Ontario); resorufin and 7-ethoxyresorufin (Molecular Probes Inc., Eugene, OR); βNF, BSA, DEX, GLUT, NADPH and erythromycin (Sigma Chemical Co., St. Louis, MO).

Source of animals

Fertilized White Leghorn chicken eggs were obtained from Archer's Poultry Farm (Brighton, Ontario). Eggs were stored at 4° for up to 9 days prior to incubation at 37°, humidity 86%, in a Humidaire Incubator (New Madison, OH). Embryo age was determined by the number of days from the onset of incubation.

Induction of chick embryo hepatic cytochrome P450 isozymes, in ovo administration of 4-ethyl DDC and TTMS, and preparation of hepatic microsomes

Chick embryos received no treatment or were injected through a small hole in the top of the egg shell above the air sac using a 1 in. 20-gauge needle and a 1-mL Tuberculin syringe, as described previously [14]. PB was administered in 0.1 mL deionized water (6 mg/egg/day for 2 days), βNF in 0.1 mL DMSO (2 mg/egg for 1 day), DEX in 0.1 mL DMSO (5 mg/egg for 1 day), and GLUT in 0.1 mL DMSO (4 mg/egg for 1 day) such that the chick embryos had been incubated for 19 days at the end of the treatment period. Following inducer administration, the hole in the egg shell was sealed with cellotape and the eggs were returned to the incubator. The porphyrinogenic compounds, 4-ethyl DDC and TTMS, were administered to the chick embryos 24 hr following the final inducer treatment. Chick embryos received either no treatment, 0.1 mL DMSO, 4-ethyl DDC (4 mg/egg) in 0.1 ml DMSO, or TTMS (4 mg/egg) in 0.1 mL DMSO. The 19-day-old chick embryos were killed by decapitation 4 hr later, their livers were removed, and microsomes were prepared by differential centrifugation, as described previously [1, 8]. Final microsomal pellets were kept frozen at −70° and stored for less than 2 weeks before use [34].

Cytochrome P450 determination

Microsomes were resuspended in 0.1 M potassium phosphate (K_2HPO_4) buffer (pH 7.4) containing 1.5 mM EDTA, to yield a protein concentration of approximately 1 mg/mL. All samples were kept on ice until cytochrome P450 content was determined by the spectrophotometric method of Estabrook *et al.* [35]. Protein content was assayed by the method of Lowry *et al.* [36].

Enzyme assays

EROD. Hepatic microsomes from untreated (UT) and βNF-treated chick embryo hepatic microsomes were assayed for EROD activity according to the method of Burke *et al.* [37]. Microsomal pellets were rehomogenized in 0.1 M Na/K-PO_4 buffer (pH 7.6) containing 1.5 mM EDTA, and resulted in a homogenate containing approximately 0.5 to 2.0 mg of protein/mL. The microsomal suspension (2.97 mL) was incubated for 2 min at 37° in the presence of 7-ethoxyresorufin (added as 15 μL of a 1.0 mM stock solution in DMSO). The reaction was initiated by the addition of 15 μL of 50 mM NADPH, which gave a final concentration of NADPH in the reaction mixture of 0.25 mM. Formation of the reaction product, resorufin, was monitored fluorometrically over time (2 min), using a Perkin Elmer LS-5B luminescence spectrometer (excitation, 535 nm; emission, 580 nm; slits, 5 nm). The initial rate of reaction was measured and resorufin formation was determined by reference to a standard curve of fluorometric intensity versus resorufin (0 to 0.2 nmol/mL).

ERND. ERND activity was assayed in hepatic microsomes prepared from UT, PB- and DEX-treated chick embryos according to a modified method of Wrighton *et al.* [38], as described previously [14]. Microsomal pellets were rehomogenized in 0.1 M potassium phosphate (K_2HPO_4) buffer (pH 7.4) containing 1.5 mM EDTA, to yield a homogenate with a protein concentration of approximately 3–5 mg/mL. The microsomal suspension (0.5 mL) was added to a 25-mL Erlenmeyer flask containing 0.5 mL of a 5.0 mM solution of erythromycin in phosphate buffer (pH 7.4) and allowed to incubate for 2 min at 37° in a shaking water bath. Enzymatic activity was initiated by the addition of 1.5 mL of a cofactor solution (2.0 mM NADPH, 5.0 mM $MgCl_2$), and allowed to proceed for 10 min before the reaction was halted by the addition of 1.0 mL of 15% (w/v) $ZnSO_4$. The reaction product, HCHO, was measured by the method of Nash [39] using a Hewlett–Packard 8451A diode array spectrophotometer (415 nm). Quantitation of product formation was determined by comparison with an HCHO standard curve (0–200 nmol/mL).

BZND. BZND activity was assayed by a modified method of Hewick and Fouts [40]. Hepatic microsomes from UT, PB- and GLUT-treated chick embryos were resuspended separately in 0.1 M potassium phosphate (K_2HPO_4) buffer (pH 7.4) containing 1.5 mM EDTA, to yield a homogenate containing 0.5 to 1.5 mg protein/mL. Benzphetamine (0.5 mL of a 5 mM solution) was added to each of the microsomal suspensions (0.5 mL) to achieve a concentration of 1.0 mM in the final reaction volume (2.5 mL). All samples were incubated for 2 min at 37° prior to the initiation of enzymatic activity by the addition of 1.5 mL of the cofactor solution (2.0 mM NADPH, 5.0 mM $MgCl_2$). The reaction was allowed to proceed for 10 min before it was halted by the addition of 0.5 mL of 15% (w/v) $ZnSO_4$. The reaction product, HCHO, was measured

by the method of Nash [39], as described above for the ERND assay, and quantitated using an HCHO standard curve (0–200 nmol/mL).

In vitro *incubation of hepatic microsomes with 4-ethyl DDC and TTMS*

Microsomes used for *in vitro* experiments were prepared as described for *in ovo* experiments with the exception that 4-ethyl DDC and TTMS were not administered to chick embryos, but were added to the rehomogenized microsomes. Microsomal incubations with 45, 150 and 450 μM 4-ethyl DDC and TTMS were performed as previously described [14]. The reaction was initiated by the addition of 2 mL of 0.1 M potassium phosphate (K_2HPO_4) buffer (pH 7.4) containing 1.5 mM EDTA and 50 mM NADPH. Controls received the same solution minus 50 mM NADPH. After 30 min, the reaction was halted by placing the samples on ice. All samples (4 mL) were then diluted with 4 mL of 2% BSA in phosphate buffer and centrifuged at 106,000 *g* for 1 hr to remove residual unmetabolized 4-ethyl DDC and TTMS. The microsomal pellets were immediately rehomogenized and used for BZND determinations, as described above.

Protein determinations for all enzymatic assays were performed according to the method of Lowry *et al.* [36]. Appropriate experimental controls for all enzymatic assays and P450 determinations were performed both in the absence and presence of vehicle and were not significantly different from one another. The reaction rates for all enzymatic assays were linear over the range of protein and time used to determine the rate of reaction (results not shown).

Statistical analysis

A repeated-measures design one-way analysis of variance was used to determine whether two means in the *in vitro* BZND experiments were significantly different from one another ($P \leq 0.05$). If a significant F ratio at the 0.05 level was obtained, a Newman–Keuls test was used to determine the means that differed significantly from each other. For the results to be considered significant, the activity of the drug in the presence of NADPH had to be significantly different from that measured in control (−drug, +NADPH) and that measured in the presence of drug but absence of NADPH (+drug, −NADPH). For the *in ovo* enzymatic assays and P450 determinations, a randomized design one-way analysis of variance with a Newman–Keuls test was used to determine whether two means differed significantly ($P \leq 0.05$) from one another.

RESULTS AND DISCUSSION

Choice of doses of 4-ethyl DDC and TTMS and time of administration

The choice of the 4-ethyl DDC dose of 4 mg (0.014 mmol/egg) was based on previous studies carried out in chick embryos [41] in which it was demonstrated that 4 mg of 4-ethyl DDC administered *in ovo* produced maximal hepatic porphyrin accumulation in 17-day-old chick embryos. In several dose–response studies [15, 42], it was found that the potency of TTMS as a porphyrinogenic drug in chick embryo liver is comparable to that of 4-ethyl DDC, and for this reason the same dose of TTMS (4 mg, 0.014 mmol/egg) was selected for use in our studies.

The time point of 4 hr was chosen based on previous studies with 4-ethyl DDC and other dihydropyridines in the rat and chick embryo. In previous studies conducted on the rat, time periods of 2, 4, and 8 hr were selected for study [10]. The effects of the dihydropyridines were often pronounced after 2 hr and generally maximal after 4 hr. In the chick embryo, maximal destructive effects on P450 were noted within 4 hr [43].

Effect of in ovo *administration of 4-ethyl DDC and TTMS on EROD activity*

The EROD assay is a known catalytic marker for polycyclic aromatic hydrocarbon-inducible mammalian P450 1A1 and 1A2 activity [37, 44]. Recently, EROD activity has been reported to be associated with P450 isozymes isolated from the livers of pentachlorobiphenyl-, 3MC- and βNF-treated chickens [25, 26, 45], as well as the livers of βNF-, 3MC-, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-treated chick embryos [27, 46, 47]. Thus, the EROD assay is considered to be a marker for chick embryo P450 1A functional activity.

When microsomes were prepared from the livers of chick embryos, 24 hr following administration of βNF (2 mg/egg), a 120-fold increase in EROD activity was found when compared with the activity in hepatic microsomes from untreated chick embryos (Fig. 2). A similar induction of EROD activity by βNF has been reported previously in the rat [37] and the chick embryo [14]. When hepatic microsomes were prepared from βNF-induced chick embryos treated *in ovo* with 4-ethyl DDC and TTMS 4 hr prior to harvesting of liver tissue, a significant inhibition of EROD activity (Fig. 2) and decrease of P450 content (Table 1) was observed. Thus, 4-ethyl DDC caused EROD activity to fall to 29% of control and P450 content to fall to 33% of control, while TTMS caused EROD activity to fall to 5% of control and P450 to fall to 32% of control. Therefore, the conclusions reached in previous *in vitro* experiments concerning the chick embryo hepatic P450 1A isozyme [14] are in accord with the findings of the present *in ovo* experiment. In both *in vivo* and *in vitro* studies, rat hepatic P450 1A1 has been shown to be targeted for inactivation by porphyrinogenic compounds [8, 10, 11]. Thus, the *in ovo* and *in vitro* inactivation of P450 1A observed in chick embryo liver corresponds with results previously seen in the rat with P450 1A1.

Effect of in ovo *administration of 4-ethyl DDC and TTMS on ERND activity*

ERND has been used as a selective catalytic marker for the functional activity of P450 3A isozymes in the rat [38]. Using ERND and other functional markers, rat hepatic P450 3A1 and 3A2 have been shown to be targets for mechanism-based inactivation by both *in vivo* and *in vitro* administration of 4-ethyl DDC [4, 6, 8–11, 14, 48–50]. TTMS was also shown to cause *in vitro* mechanism-based inactivation of rat hepatic P450 3A-mediated ERND activity [14]. PB and DEX have been reported to

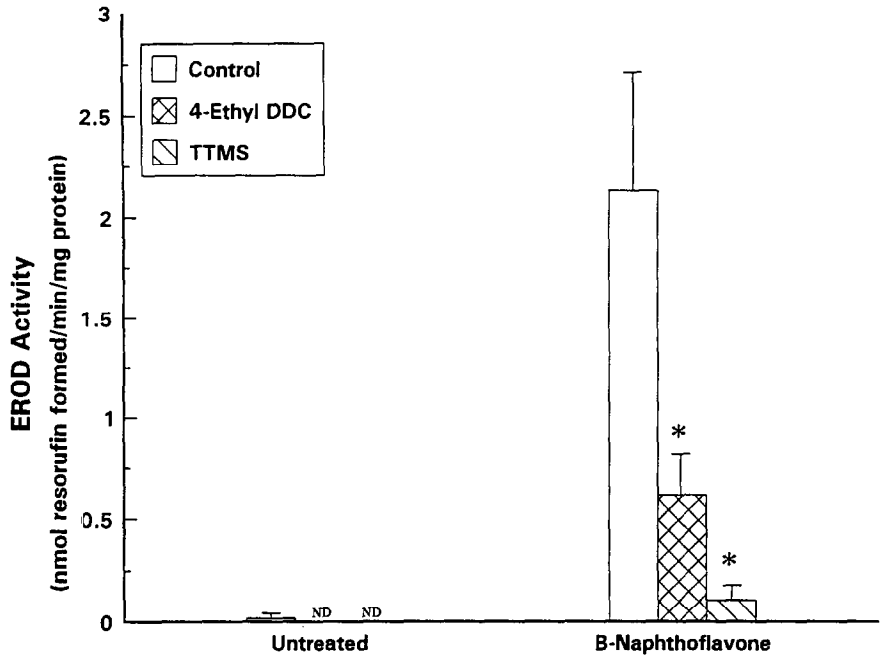

Fig. 2. Effect of *in ovo* administration of 4-ethyl DDC (4 mg/egg) and TTMS (4 mg/egg) on 7-ethoxyresorufin *O*-deethylase (EROD) activity in microsomes prepared from UT and βNF-treated 19-day-old chick embryo livers. Each bar represents the mean (± SD) of determinations from four experiments. The livers of 3–4 chick embryos were pooled to constitute one treatment group per experiment. Key: ND, not detectable; (*) significantly different ($P \leq 0.05$) from control within an induction group, as determined by a randomized design one-way analysis of variance and Newman–Keuls test.

Table 1. Effect of *in ovo* administration of 4-ethyl DDC and TTMS on total cytochrome P450 from hepatic microsomes prepared from UT, βNF-, DEX-, PB-, and GLUT-treated chick embryos

	Total microsomal P450 content (nmol/mg protein)		
Treatment	Control	4-Ethyl DDC	TTMS
UT	0.32 ± 0.08	0.25 ± 0.05	0.22 ± 0.04
βNF	0.71 ± 0.09	0.24 ± 0.04*	0.23 ± 0.04*
DEX	0.59 ± 0.11	0.42 ± 0.09*	0.37 ± 0.09*
PB	1.81 ± 0.26	0.76 ± 0.25*	0.33 ± 0.09*
GLUT	3.16 ± 0.61	1.13 ± 0.37*	0.56 ± 0.06*

All data are expressed as means ± SD of determinations from four experiments. The livers of 3–4 chick embryos were pooled to constitute one treatment group per experiment.

* Significantly lower ($P \leq 0.05$) from control in particular treatment group, as determined by a randomized design one-way analysis of variance and Newman–Keuls test.

elicit an induction of ERND activity in the livers of chick embryos and 1-day-old chicks [14, 29]. In addition, a monoclonal antibody selective for rat hepatic P450 3A isozymes has been demonstrated to cross-react with microsomal protein from PB-treated 18-day-old chick embryo livers, and both PB- and DEX-treated 1- and 36-day-old chick livers [29]. This evidence supports the existence of a chick embryo P450 isozyme that may be the equivalent of rat P450 3A.

In a previous study, the *in vitro* addition of 4-ethyl DDC and TTMS to hepatic microsomes from PB-treated chick embryos caused mechanism-based inactivation of ERND activity [14]. The objective of the current experiment was to determine whether a similar inactivation of chick embryo liver P450 3A occurs following the *in ovo* administration of 4-ethyl DDC and TTMS. Both PB and DEX caused a significant elevation of ERND when activity was expressed as nmol HCHO formed/min/mg protein (Fig. 3). However, as observed in other studies [14, 29], DEX caused a more specific induction of ERND activity than did PB when this enzymatic activity was expressed as a ratio of P450 content. When 4-ethyl DDC and TTMS were administered *in ovo*, 4 hr prior to harvesting of liver tissue, ERND activity measured in hepatic microsomes from PB- and DEX-treated chick embryos decreased significantly (Fig. 3). After TTMS administration, ERND activity was 33% of control and P450 was 64% of control in microsomes from DEX-treated chick embryos (Table 2); ERND activity was 22% of control and P450 was 18% of control in microsomes from PB-treated chick embryos (Table 2). Thus, TTMS was equally as effective in lowering ERND activity in hepatic microsomes from both PB- and

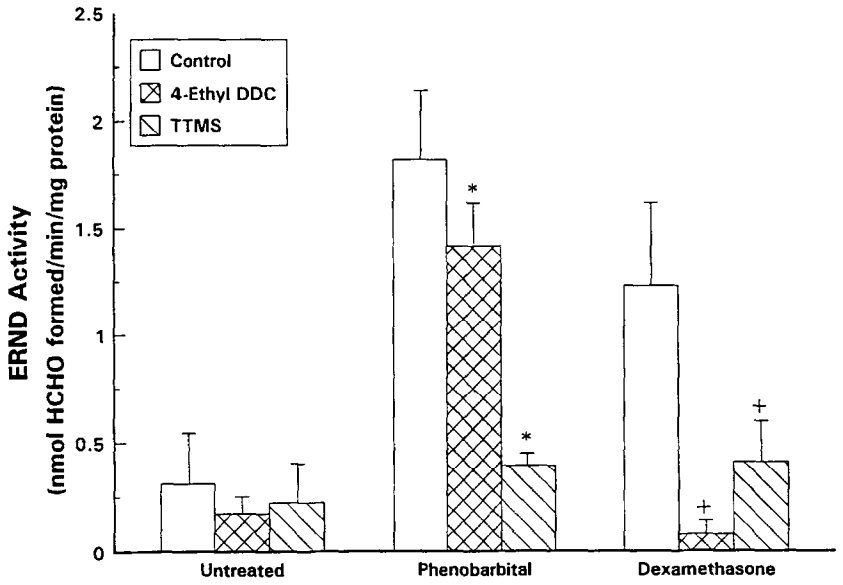

Fig. 3. Effect of *in ovo* administration of 4-ethyl DDC (4 mg/egg) and TTMS (4 mg/egg) on erythromycin *N*-demethylase (ERND) activity in microsomes prepared from UT, PB-, and DEX-treated 19-day-old chick embryo livers. Each bar represents the mean (± SD) of determinations from four experiments. The livers of 3–4 chick embryos were pooled to constitute one treatment group per experiment. Key: (*) significantly different ($P \leq 0.05$) from control in PB-treated chick embryos; and (+) significantly different ($P \leq 0.05$) from control in DEX-treated chick embryos, as determined by a randomized design one-way analysis of variance and Newman–Keuls test.

Table 2. Effect of *in ovo* administration of 4-ethyl DDC and TTMS on total cytochrome P450 content and enzymatic activity in hepatic microsomes prepared from DEX-, PB-, and GLUT-treated chick embryos

	Enzymatic activity and total P450 content (% of control)					
	Erythromycin *N*-demethylase activity		Benzphetamine *N*-demethylase activity		Total P450 content	
Treatment	4-Ethyl DDC	TTMS	4-Ethyl DDC	TTMS	4-Ethyl DDC	TTMS
DEX	6 ± 7	33 ± 15			71 ± 16	64 ± 16
PB	78 ± 11	22 ± 4	18 ± 4	18 ± 3	42 ± 14	18 ± 5
GLUT			42 ± 7	23 ± 6	36 ± 12	18 ± 2

All data (mean ± SD of four experiments) are expressed as a percent of control for a particular treatment group. The livers of 3–4 chick embryos were pooled to constitute one treatment group per experiment. Control values for total P450 content are given in Table 1, column 1; for erythromycin *N*-demethylase they are given in the open bars of Fig. 3, and for benzphetamine *N*-demethylase they are given in the open bars of Fig. 4.

DEX-treated chick embryos. However, the ability of 4-ethyl DDC to lower ERND activity was greater in DEX- than in PB-treated chick embryos. 4-Ethyl DDC caused ERND activity to fall to 6% of control and P450 to fall to 71% of control in microsomes from DEX-treated chick embryos (Table 2); ERND activity fell to 78% of control and P450 to 42% of control in microsomes prepared from PB-treated chick embryos (Table 2).

One explanation for this result is that PB induces a larger amount of P450 than does DEX (Table 1), and a greater variety of P450 isozymes than does induction with DEX. It is possible that many of these PB-induced P450 isozymes (other than P450 3A) have substantial binding affinity for 4-ethyl DDC, thus diverting 4-ethyl DDC from P450 3A metabolism. An alternate explanation for this difference is that PB induces a P450 isozyme that metabolizes 4-ethyl DDC to the corresponding pyridine without the loss of the 4-ethyl substituent, thereby reducing the amount of 4-ethyl DDC available for mechanism-based inactivation of P450

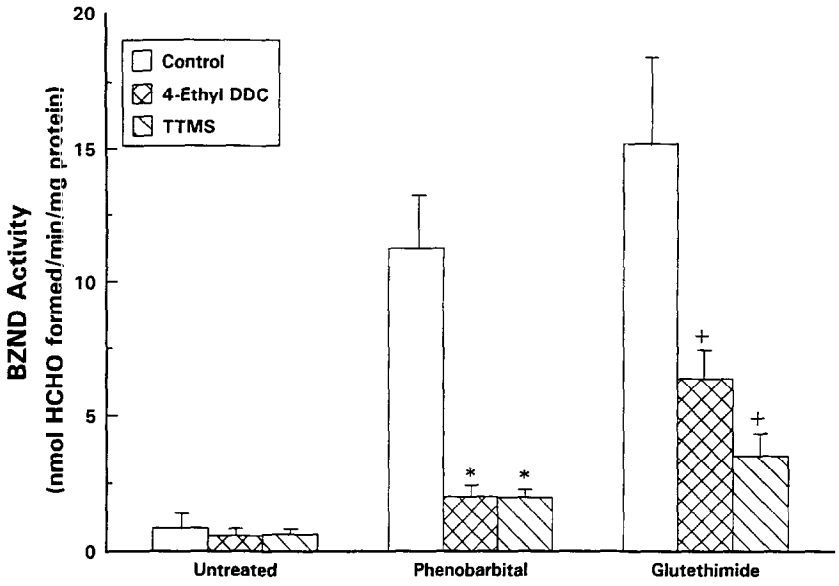

Fig. 4. Effect of *in ovo* administration of 4-ethyl DDC (4 mg/egg) and TTMS (4 mg/egg) on benzphetamine *N*-demethylase (BZND) activity in microsomes prepared from UT, PB-, and GLUT-treated 19-day-old chick embryo livers. Each bar represents the mean (± SD) of determinations from four experiments. The livers of 3–4 chick embryos were pooled to constitute one treatment group per experiment. Key: (*) significantly different ($P \leq 0.05$) from control in PB-treated chick embryos; and (+) significantly different ($P \leq 0.05$) from control in GLUT-treated chick embryos, as determined by a randomized design one-way analysis of variance and Newman–Keuls test.

3A. If either of these explanations is correct, it would follow that increasing the amount of 4-ethyl DDC should increase the inactivation of ERND activity. When 4-ethyl DDC was added *in ovo* at a higher concentration (15 mg/egg), ERND activity fell to 40% of control in PB-treated chick embryos (results not shown), as compared with a fall to 78% of control ERND activity produced by 4-ethyl DDC (4 mg/egg) in PB-treated chick embryos (Fig. 3). Thus, either of these explanations may be correct, and further work is required to decide between these two possibilities.

Therefore, the results obtained by *in ovo* administration of 4-ethyl DDC and TTMS correspond to results previously obtained from *in vitro* experiments, where the chick embryo P450 3A isozyme was found to be a target for mechanism-based inactivation [14]. The results with 4-ethyl DDC also correspond to *in vivo* and *in vitro* results observed in rat liver [4, 6, 8–11, 14, 48–50]. Thus, P450 3A isozymes are targeted for both *in vivo* and *in vitro* inactivation by 4-ethyl DDC in both the rat and chick embryo liver.

Effect of in ovo *administration of 4-ethyl DDC and TTMS on BZND activity*

In the rat, cytochrome P450 2B1 and 2B2 are the major PB-inducible isozymes, while P450 2H1 and 2H2 are the predominant isozymes expressed in the chick embryo liver following PB treatment. P450 2H1/2 isozymes have been isolated from acetone-treated chickens and were observed to possess BZND activity [45]. Polyclonal antibodies prepared to chicken P450 2H1/2 were capable of causing 80% immunoinhibition of BZND activity in microsomes obtained from GLUT-treated chickens [45]. Therefore, BZND activity is considered to be a marker for chick embryo P450 2H activity.

In our experiment, BZND activity was used to assay chick embryo hepatic microsomes prepared from 19-day-old UT, PB-, and GLUT-treated chick embryos for P450 2H activity. BZND activity from both PB- and GLUT-treated chick embryos was induced markedly compared with microsomes from UT chick embryos (Fig. 4). GLUT treatment induced BZND activity to the greatest extent, although PB treatment produced a more specific induction of BZND activity when expressed as a ratio of P450 content. *In ovo* administration of both 4-ethyl DDC and TTMS caused a significant depression of BZND activity from hepatic microsomes prepared from PB- and GLUT-treated chick embryos (Fig. 4). Thus, 4-ethyl DDC caused BZND activity to fall to 18% of control and P450 to fall to 42% of control in microsomes prepared from PB-treated chick embryos (Table 2), and BZND activity to fall to 42% of control and P450 to fall to 36% of control in microsomes prepared from GLUT-treated chick embryos (Table 2), while TTMS caused BZND activity and P450 to fall to 18% of control in microsomes prepared from PB-treated chick embryos (Table 2), whereas BZND activity fell to 23% of control and P450 fell to 18% of control in microsomes prepared from GLUT-treated chick embryos (Table 2). Thus, chick embryo liver P450 2H is targeted for inactivation by *in ovo* administration of 4-ethyl DDC and TTMS.

Table 3. Effect of *in vitro* administration of 4-ethyl DDC and TTMS on benzphetamine *N*-demethylase (BZND) activity in hepatic microsomes prepared from PB- and GLUT-treated chick embryos

		BZND activity (% of control)					
		4-Ethyl DDC			TTMS		
Treatment	NADPH	45 μM	150 μM	450 μM	45 μM	150 μM	450 μM
PB	−	87 ± 21	81 ± 8	60 ± 13	81 ± 16	73 ± 6	68 ± 7
	+	63 ± 5*	42 ± 2*	35 ± 4*	75 ± 9	58 ± 4*	48 ± 4*
GLUT	−	99 ± 4	96 ± 19	75 ± 9	101 ± 11	87 ± 8	68 ± 7
	+	90 ± 13	55 ± 12*	49 ± 7*	96 ± 11	71 ± 10*	59 ± 9*

All data (mean ± SD of four experiments) are expressed as a percent of control BZND activity for a particular treatment group. Control (−drug, +NADPH) values for all groups ranged between 5.22 and 6.53 nmol HCHO formed/min/mg protein.

* Significantly lower ($P \leq 0.05$) from control (−drug, +NADPH) and incubation of the respective drug in the absence of NADPH (+drug, −NADPH) as determined by a repeated measures design one-way analysis of variance and Newman–Keuls test.

Chick embryo P450 2H was also assayed for the *in vitro* effects of 4-ethyl DDC and TTMS on hepatic microsomes prepared from PB- and GLUT-treated chick embryos (Table 3). A 45 μM concentration of 4-ethyl DDC and TTMS did not elicit an *in vitro* mechanism-based inactivation of BZND activity in microsomes from GLUT-treated chick embryos, although BZND activity was inactivated by 45 μM 4-ethyl DDC in microsomes from PB-treated chick embryos. When the *in vitro* concentration of these inactivators was increased, mechanism-based inactivation of BZND activity was observed at 150 and 450 μM for microsomes prepared from both PB- and GLUT-treated chick embryos (Table 3). Thus, at higher concentrations, both 4-ethyl DDC and TTMS caused significant *in vitro* mechanism-based inactivation of BZND activity. The fact that the lowest concentration (45 μM) of 4-ethyl DDC and TTMS was unable to cause *in vitro* mechanism-based inactivation of BZND activity in microsomes from GLUT-treated chick embryos, in contrast to the observed inactivation of BZND activity in microsomes from PB-treated chick embryos by 4-ethyl DDC at 45 μM, might be explained by the relative induction of total P450 content by these inducers. Since GLUT induced a greater amount of total P450 than did PB, less 4-ethyl DDC and TTMS may have been available for P450 2H inactivation in microsomes from GLUT-treated chick embryos, due to non-specific binding and competition for the suicide substrate by other P450 isozymes.

In summary, the chick embryo hepatic P450 1A and 3A isozymes are targets for both *in ovo* and *in vitro* inactivation by the porphyrinogenic compounds 4-ethyl DDC and TTMS, reflecting a similar inactivation of rat hepatic P450 1A1 and 3A1/2 by 4-ethyl DDC. In addition, the chick embryo hepatic P450 2H isozyme(s) is also a target for both *in ovo* and *in vitro* inactivation by these compounds. The related P450 2B1/2 isozymes of rat liver are not targeted for mechanism-based inactivation by 4-ethyl DDC. Assuming that the inactivation of chick embryo hepatic P450 2H by 4-ethyl DDC and TTMS is accompanied by *N*-alkylPP formation, it is possible that mechanism-based inactivation of P450 2H in chick embryo liver, in contrast to rat liver P450 2B1/2, may be a factor in addition to low ferrochelatase activity [21, 22], which contributes to the greater sensitivity of the chick embryo than the rat to certain porphyrinogenic xenobiotics.

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