CYTOCHROME CYP SOURCES OF N-ALKYLPROTOPORPHYRIN IX AFTER ADMINISTRATION OF PORPHYRINOCIC XENOBIOTICS TO RATS

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ABSTRACT:

Cytochrome P-450 (CYP) 3A2 and CYP2C11 are sources of 70 and 30%, respectively, of N-vinylprotoporphyrin IX (N-vinylPP) formation after administration of 3-[(arythio)ethyl]sydnone (TTMS) to rats. Female rats receiving TTMS were pretreated with dexamethasone, which induces CYP3A1 preferentially to CYP3A2. The resulting 12-fold increase in N-vinylPP formation showed that CYP3A1 was also a source of N-vinylPP. Phenobarbital (PB) pretreatment, which induces CYP2B1/2 and 3A1/2 in male rats, increased N-vinylPP formation after TTMS administration. Troleandomycin, a selective CYP3A inhibitor, was unable to decrease TTMS-mediated N-vinylPP formation in PB-treated male rats, indicating that CYP2B1/2 were sources of N-vinylPP. This conclusion was supported by demonstrating a 15-fold increase in TTMS-induced N-vinylPP formation in female rats after CYP2B1/2 induction with PB pretreatment. Allylisopropylacetamide (AIA) inactivates rat CYP2B1/2, 2C6, 2C7, 2C11, and 3A1/2. Troleandomycin was unable to decrease N-AIA protoporphyrin IX adduct (N-AIAAPP) formation, showing that CYP3A1/2 were not susceptible to AIA-mediated N-alkylation. N-AIAAPP formation in females was approximately 30% of that in males, and thus we attribute 30% of N-AIAAPP formation in males to the non-gender-specific isozymes (CYP2C6, 2C7, and/or 2B1/2), whereas approximately 70% originates from CYP2C11. PB treatment in female rats resulted in a 5-fold increase in N-AIAAPP formation, showing that CYP2B1/2 were also susceptible to N-alkylation mediated by AIA. 1-Aminobenzotriazole elicited formation of equivalent amounts of N-N-aryl bridged protoporphyrin IX in male and female rat liver, demonstrating that nonselective mechanism-based inactivation is accompanied by nonselective conversion of the CYP heme moieties to N’N-aryl bridged protoporphyrin IX.

Although the cytochrome CYP (CYP) family of drug-metabolizing enzymes maintains a wide catalytic diversity, individual CYP isoforms have demonstrated substrate selectivity, a property that has been attributed to structural differences within the active site. Studies on mechanism-based CYP inactivation, in which the substrate is catalytically activated, leading to the destruction of the CYP enzyme in a suicide-type manner, have helped elucidate the structure-function relationships of the CYP family (De Matteis et al., 1983). Porphyrogenic xenobiotics (Fig. 1, a–c) interfere with heme biosynthesis as a consequence of their mechanism-based inactivation of specific CYP isoforms (for a review, see Marks et al., 1988; Ortiz de Montellano and Correa, 1996). Mechanistically, these compounds can alkylate the CYP heme moiety at one of the four pyrrole nitrogens within the active site, producing a modified heme adduct referred to as N-alkylprotoporphyrin IX (N-alkylPP; Fig. 2). Depletion of free heme levels results from a combination of heme N-alkylation, and the potent inhibitory action of certain N-alkylPPs (i.e., N-ethylPP, and N-vinylPP) on ferrochelatase, the terminal enzyme in heme synthesis, as seen with the metabolism of two prototypical porphyrogenic agents, 3-[(arythio)ethyl]sydnone (TTMS; Fig. 1a) and 3,5-diethoxy carbonyl-1,4-dihydro-2,6-dimethyl-4-ethyl pyridine (4-ethylDDC, Fig. 1b; Tephly et al., 1979; Ortiz de Montellano et al., 1981a; Marks et al., 1985; McCluskey et al., 1986; Ortiz de Montellano and Grab, 1986; Sutherland et al., 1986). Alternatively, porphyria can be induced through continual heme depletion, where repeated mechanism-based inactivation dissociates the CYP modified heme moiety from an intact, and heme-reconstitutable, apoprotein, as seen with allylisopropylacetamide (AIA; Fig. 1c; Bornheim et al., 1987).

Recognizing that mechanism-based inactivation of CYP isoforms can play a critical role in experimental porphyria, the use of animal models to predict the potential human porphyrinogenicity of a drug is complicated by species differences in CYP isozyme expression. Drug-induced porphyria seen in animals may depend on N-alkylPP formation from a nonhuman isozyme, and these drugs may be unable to elicit porphyrinogenic effects in humans. Thus, to establish adequate animal test models for drug-induced porphyria, knowledge of human and animal CYP isozyme involvement in mechanism-based inactivation is required. Our laboratory has been interested in determining the in vivo CYP isozyme sources of N-alkylPP in rats after the administration of prototypical porphyrogenic agents (McNamee and Marks, 1996; Wong et al., 1998). Recognizing the limitations in the selectivity of CYP isozyme inhibitors, we have exploited gender differences.
in CYP isozyme expression in rats to determine the contribution of specific isozymes to N-alkylPP formation.

CYP3A2 has been shown to be a major source of N-vinylPP formation after administration of TTMS to male rats. However, the role of CYP3A1 in N-vinylPP formation was not determined. Dexamethasone (DEX) pretreatment in female rats has been shown to induce CYP3A1 with minimal effects on CYP3A2, in contrast to DEX pretreatment in males where both CYP3A1 and CYP3A2 are elevated (Cooper et al., 1993; Ghosal et al., 1996). Our first objective in this study was to determine whether CYP3A1 was a source of N-vinylPP formation by determining the effect of DEX pretreatment on TTMS-mediated N-vinylPP formation in female rats.

Previously, phenobarbital (PB) induction in male rats elevated N-vinylPP formation after TTMS administration (McNamee and Marks, 1996), but because the levels of both CYP3A and 2B1/2 are increased by this inducer (Waxman et al., 1985), the relative contributions of these two isozymes was uncertain. PB pretreatment in female rats, however, is more selective for CYP2B1/2 than CYP3A1/2 (Waxman et al., 1985). The second objective in this study was to determine the role of CYP2B1/2 in N-vinylPP formation after administration of TTMS to rats, by determining the effect of 1) troleandomycin (TAO), a selective CYP3A inhibitor, on N-vinylPP formation in PB-treated male rats and 2) PB pretreatment on N-vinylPP formation in female rats.

AIA (Fig. 1c) is another prototypical porphyrinogenic agent that elicits mechanism-based inactivation and experimental porphyria through continual heme depletion (De Matteis et al., 1980; Ortiz de Montellano and Mico, 1981). AIA inactivates rat CYP 2B1, 2C6, 2C7, 2C11, and 3A in vivo (Bornheim et al., 1987) and elicits in vitro NADPH-dependent mechanism-based inactivation of CYP2B1/2 (Ortiz de Montellano and Mico, 1981). Although it is a prerequisite for N-alkylation, mechanism-based inactivation does not always lead to N-alkylPP formation. For example, mechanism-based inactivation can involve CYP apoprotein alkylation or destruction of the heme moiety, rather than N-alkylPP formation. Whereas N-alkylPP formation from AIA has been previously documented, the CYP isozymes, which are important sources of N-alkylPP remain to be elucidated. Therefore, our third objective was to determine the CYP isozyme sources of AIA-protoporphyrin adduct (N-AIAPP, Fig. 2a) formation after AIA administration.

1-Aminobenzotriazole (ABT; Fig. 1d) is a mechanism-based CYP inactivator that elicits mechanism-based inactivation and experimental porphyria through continual heme depletion (De Matteis et al., 1980; Ortiz de Montellano and Mico, 1981). ABT inactivates rat CYP 2B1, 2C6, 2C7, 2C11, and 3A in vivo (Bornheim et al., 1987) and elicits in vitro NADPH-dependent mechanism-based inactivation of CYP2B1/2 (Ortiz de Montellano and Mico, 1981).
heme moiety with the formation of an N,N-aryl-bridged protoporphyrin IX (NNAPP; Fig. 2b; Ortiz de Montellano and Mathews, 1981). If N-alkylation is the primary mechanism by which ABT nonselectively inactivates CYP, NNAPP formation should be similar in male and female rats, despite the marked gender differences in CYP isozyme expression. Our final objective in this study was to investigate the hypothesis that ABT-mediated CYP inactivation proceeds through nonselective heme N-alkylation by comparing NNAPP formation in male and female rats after ABT administration.

Materials and Methods

Reagents and Chemicals. TTMS was obtained from Color Your Enzyme (Bath, Ontario, Canada). AIA was obtained as a gift from Hoffman-La Roche Ltd. (Vaudreuil, Quebec, Canada). Solvents (methanol, dichloromethane, and acetone) were purchased from VWR Canada (Mississauga, Ontario, Canada). Inhibitors (TAO) and inducers (DEX and PB) were purchased from Sigma Chemical Co. (St. Louis, MO.).

Treatment of Animals. Male Sprague-Dawley rats (200–250 g) were used for inhibitor studies with TAO and the erythromycin N-demethylase (ERND) assay (described below), and male and female rats (150–200 g) were used for the gender studies. All rats were obtained from Charles River Canada, Inc. (St. Constant, Quebec, Canada). All rats received Purina Laboratory Chow (5001) and water ad libitum and were housed under controlled conditions (22°C, 14:10 h light/dark cycle). Animals were allowed a 1-week environmental adjustment period.

Induction Studies. When appropriate, rats received either DEX or PB, dissolved in 0.5 ml vehicle, i.p. once daily for 4 days. The vehicle for DEX (100 mg/kg) was dimethyl sulfoxide, and the vehicle for PB (80 mg/kg) was deionized water. Control animals received vehicle (0.5 ml) once daily for 4 days.

Inhibitor Studies. The inhibitor TAO was coadministered with TTMS (111.3 mg/kg) or AIA (100 mg/kg) 24 h after the final dose of inducer, when appropriate. For experiments with TMS, 450 mg/kg TAO was used; for AIA, 150 mg/kg TAO was used.

Isolation, Purification, and Separation of N-AlkylIPP. Four hours after administration of porphyrinogenic compounds, in the presence or absence of inhibitor, rats were decapitated and their livers perfused in situ with 100 ml of ice-cold 1.15% potassium chloride solution. Livers were individually weighed and homogenized in ice-cold 5% concentrated H2SO4/methanol (v/v; 100 ml/liver) and stored in the dark at 4°C for 24 h. The resulting mixture of deionized water, and extracted with dichloromethane (230 ml) in a separatory funnel. After successive washes with 5% sodium bicarbonate (80 ml), the dichloromethane solution was dried over anhydrous sodium sulfate. After removal of the sodium sulfate by centrifugation and water ad libitum and were housed under controlled conditions (22°C, 14:10 h light/dark cycle). Animals were allowed a 1-week environmental adjustment period.

Elution of the extract (e 5 124,000 m2 1 cm2 1 at 432 nm; Ortiz de Montellano et al., 1981) may have been a problem for the indirect determination of NNAPP formation because the extinction coefficient for NNAPP is not available.

Inactivation of Microsomal CYP. Microsomal pellets were thawed and resuspended in 17 ml of 0.1 M K2HPO4 buffer (pH 7.4) containing 1.5 mM EDTA to yield a protein concentration of approximately 4 to 6 mg/ml. Two milliliters of the microsomal suspension and 2 ml of 2.0 mM NADPH (dissolved in buffer) were incubated with AIA (10 mM) for 30 min at 37°C in a shaking water bath. The reaction was stopped by cooling on ice, and samples were then centrifuged (106,000g) for 60 min at 4°C. Microsomal pellets were immediately frozen in liquid nitrogen.

Enzyme Assay. Microsomal pellets were resuspended in 0.1 M K2HPO4 buffer (pH 7.4) to yield a final protein content of 0.8–1.0 mg/ml and were assayed for ERND activity according to the method of Wrighton et al. (1985). Incubations were run at 37°C in the presence of 1.0 mM erythromycin and 1.2 mM NADPH for 10 min and then analyzed for formaldehyde content by the method of Nash et al. (1953). Microsomal protein was assayed by the method of Lowry et al. (1951).

Statistical Analysis. For the male-female rat gender studies, an unpaired Student’s t test was used to determine whether mean values of male and females were significantly different (P < .05). For the inhibitor studies with TAO, an unpaired Student’s t test was used to determine whether the mean data of inhibitor-treated rats was significantly different (P < .05) from the control animals receiving no inhibitor. For ERND activity, a randomized design one-way ANOVA with a Newman Keuls’ post hoc test was used to determine whether the means were significantly different.

Results and Discussion

Studies with TTMS. Effect of DEX induction on N-vinylIPP formation in female rats. Previously, CYP3A2 and CYP11 have been shown to be the major sources of TTMS-mediated N-vinylIPP formation in rats (McNamee and Marks, 1996; Wong et al., 1998); however, the role of CYP3A1 was not determined. Administration of DEX to female rats has been previously shown to induce CYP3A1, in contrast to the effect in males where both CYP3A1 and CYP3A2 are induced (Waxman et al., 1985; Cooper et al., 1993; Ghosal et al., 1996). Therefore, using DEX to induce primarily CYP3A1 in females would allow us to determine whether this isozyme was susceptible to TTMS-mediated N-vinylIPP formation. DEX induction (50 mg/kg for 4 days) produced a large (12-fold) increase in N-vinylIPP formation (Fig. 3a).

We conclude that the increased N-vinylIPP formation is due primarily to increased mechanism-based inactivation of CYP3A1 with concomitant N-vinylIPP formation and that TTMS elicits mechanism-based
induced animals, indicating that CYP3A was not an important source of N-alkylation. TAO was unable to significantly effect the AIA-porphyrin adduct formation if CYP3A was a major source of the N-alkylated heme adducts. The inability of CYP3A inhibition to decrease N-AIAPP formation indicates that this isozyme is not a source of N-alkylPP in PB-treated rats. Thus, Bornheim et al. (1987) demonstrated that AIA-mediated decreases in CYP3A activity, mediated by TAO, on the production of N-AIAPP, and the data are summarized in Fig. 5a. Administration of TAO resulted in a 2.4-fold increase in N-AIAPP formation. This observation was surprising because we anticipated a decrease in N-AIAPP formation if CYP3A was a major source of the N-alkylated heme adducts. The inability of CYP3A inhibition to decrease N-AIAPP formation indicates that this isozyme is not a source N-AIAPP. Other investigators have provided evidence suggesting that CYP3A is not a likely candidate for N-alkylPP formation. Thus, Bornheim et al. (1987) demonstrated that AIA-mediated decreases in CYP3A activity were not restored after the administration of heme and concluded that...

Studies with AIA. Effect of AIA on CYP3A activity. Previous studies have indicated that AIA inactivates CYP 2B1, 2C6, 2C7, 2C11, and 3A in vivo (Bornheim et al., 1987), although it was not determined whether this inactivation was NADPH-dependent and therefore likely to be mechanism-based. Other in vitro studies, however, have shown that the PB-inducible isozymes, namely CYP2B1/2, undergo NADPH-dependent mechanism-based inactivation (Ortiz de Montellano and Mico, 1981). Previously, CYP3A was shown to be susceptible to NADPH-dependent inactivation and the major source of N-vinylPP from TTMS (McNamee and Marks, 1996) in untreated and DEX-pretreated rats. Therefore, in the present study, we decided to determine whether AIA elicited similar effects on CYP3A. The effect of AIA on ERND activity, a selective catalytic marker for CYP3A isozymes (Wrighton et al., 1985), was determined in vitro, and the data are summarized in Fig. 4. ERND activity in microsomes receiving NADPH, regardless of the presence of AIA, was significantly lower than activity from microsomes where NADPH was absent. This finding is consistent with a previous report of Riddick et al. (1989), who observed NADPH-dependent decreases in total CYP content in microsomes isolated from DEX-treated rats, due to lipid peroxidation leading to destruction of CYP, despite the inclusion of 1.5 mM EDTA in the incubation medium. For this reason, the effects of mechanism-based inactivators were expressed with respect to the control incubation in the presence of NADPH alone. In the presence of NADPH, AIA significantly decreased ERND activity by 31%. However, in the absence of NADPH, AIA was unable to decrease ERND activity, showing that NADPH is required for CYP inactivation and that this process is likely to be mechanism-based. Thus, as observed with other porphyrinogenic agents such as TTMS and 4-ethylIDDC, AIA elicits mechanism-based inactivation of CYP3A.

Effect of CYP3A inhibition on N-AIAPP formation. During mechanism-based CYP inactivation, one possible outcome is the N-alkylation of the isozyme’s heme moiety, followed by the dissociation of the AIA-porphyrin adduct from the apoprotein. Because AIA elicited mechanism-based inactivation of CYP3A, we decided to investigate the effect of CYP3A inhibition, mediated by TAO, on the production of N-AIAPP, and the data are summarized in Fig. 5a. Administration of TAO resulted in a 2.4-fold increase in N-AIAPP formation. This observation was surprising because we anticipated a decrease in N-AIAPP formation if CYP3A was a major source of the N-alkylated heme adducts. The inability of CYP3A inhibition to decrease N-AIAPP formation indicates that this isozyme is not a source N-AIAPP. Other investigators have provided evidence suggesting that CYP3A is not a likely candidate for N-alkylPP formation. Thus, Bornheim et al. (1987) demonstrated that AIA-mediated decreases in CYP3A activity were not restored after the administration of heme and concluded that...
this isozyme is inactivated through other destructive mechanisms without dissociation of modified heme-adducts from an intact apoprotein and without N-AIAPP formation. We propose that when TAO inactivates CYP3A, more AIA is available to react with other CYP isozymes that are susceptible to N-alkylation and can form N-AIAPP, resulting in the observed 2.4-fold increase in N-AIAPP formation. In the absence of TAO, a large percentage of AIA can interact with CYP3A without N-AIAPP formation, reducing the amount of AIA available to react with other isozymes that are susceptible to N-alkylation.

N-AIAPP formation in male and female rats. A marked gender difference in rat hepatic N-alkylPP formation has been demonstrated after administration of TTMS and 4-ethyl-DDC, which is attributable to the contributions of male-specific CYP2C11 (for both TTMS and 4-ethylDDC) and CYP3A2 (TTMS only) to N-alkylation. Figure 5b indicates that, as we previously observed with TTMS and 4-ethyl-DDC, male rats produced markedly more (3-fold) N-alkylPP than females after AIA administration, and because the TAO study ruled out CYP3A as a source of N-AIAPP, we attribute this difference to the contribution of CYP2C11 in males. Female rat N-AIAPP production, which was approximately 30% of that in males, must originate from the major CYP isozymes known to be constitutively expressed in females, namely CYP2B1/2, 2C6, and 2C12. Moreover, because the levels of CYP2C6 and 2B1 exist in similar levels in male and female rats (Waxman et al., 1985), the N-AIAPP production in females approximately represents the contributions of these isozymes in male rats. Therefore, in untreated male rats, approximately 30% of N-AIAPP formation originates from CYP2C11 in males, and because the levels of CYP2C6 and 2B1 exist in similar levels in male and female rats (Waxman et al., 1985), the N-AIAPP formation in females approximately represents the contributions of these isozymes in male rats. Therefore, in untreated male rats, approximately 30% of N-AIAPP formation originates from CYP2C11 in males, and because the levels of CYP2C6 and 2B1 exist in similar levels in male and female rats (Waxman et al., 1985), the N-AIAPP formation in females approximately represents the contributions of these isozymes in male rats.

Effect of PB induction on N-AIAPP formation in female rats. A previous study with AIA has suggested that PB-inducible isozymes, namely CYP2B1/2, are important targets for mechanism-based inactivation (Ortiz de Montellano and Mico, 1981), and if these isozymes are also major sources of N-alkylPP, then induction of CYP2B1/2 with PB should increase N-AIAPP formation. Thus, the effect of PB induction on N-AIAPP formation in female rats was determined and shown in Fig. 5c. PB induction (80 mg/kg for 4 days) resulted in a 5-fold increase in N-AIAPP formation. We conclude that the increased N-AIAPP formation is due to an increase in CYP2B1/2 levels, which has been reported to be 15-fold in female rats and that CYP2B1/2 are highly susceptible to AIA-mediated N-alkylation. This observation is consistent with previous studies indicating that PB-inducible isozymes were susceptible to AIA-mediated mechanism-based inactivation. Furthermore, our results are consistent with evidence demonstrating that AIA-mediated inactivation of CYP2B1/2 activity can be restored by heme administration, suggesting that the primary mechanism of inactivation is heme N-alkylation without destruction of the apoprotein.

Studies with ABT. NNAPP adduct formation in male and female rats. The final objective of this study was to determine whether ABT elicited nonselective mechanism-based CYP inactivation through nonselective heme N-alkylation and the formation of NNAPP. ABT (100 mg/kg) was administered to male and female rats, and NNAPP was isolated (Fig. 6). There was no significant difference in NNAPP formation in male and female rats. Because NNAPP formation was similar in male and female rats despite the gender differences in rat CYP isozyme expression, we conclude that NNAPP formation is the
primary mechanism by which ABT elicits nonselective, mechanism-based inactivation.

Conclusions

In summary, the main male rat isozyme targeted for mechanism-based inactivation and N-alkylIPP formation by AIA is CYP2C11 (approximately 70%), with the remaining 30% originating from other important constitutively expressed isozymes, namely CYP2C6 and CYP2B1/2. The conclusion that CYP2C11 is susceptible to N-alkylation has been supported by our laboratory. CYP2C11 was a major source of N-ethylIPP formation (78%) after administration of 4-ethylDDC and also a source of N-vinylIPP formation from TTMS (32%); Wong et al., 1998). In contrast, whereas CYP3A is susceptible to mechanism-based inactivation by all three drugs, only TTMS elicits N-alkylation. Therefore, the unique mechanism by which TTMS interacts with CYP3A must facilitate N-vinylIPP formation, whereas interactions with 4-ethylDDC and AIA promote alternate pathways to inactivation, perhaps leading to destruction of the heme and/or apo-protein.

The consistently lower N-alkylIPP formation in female rats compared to males after the administration of TTMS, 4-ethylDDC, and AIA suggests that the female specific CYP2C11 is not as susceptible to N-alkylIPP formation as the male-specific CYP2C11. Therefore, there must be distinct structural properties of CYP2C11 that facilitate N-alkylIPP formation, whereas CYP2C12 is not susceptible to this form of mechanism-based inactivation despite the fact that the CYP2C family is closely related and has considerable immunological cross-reactivity (Bandiera et al., 1985). Furthermore, the observed rat gender differences in N-alkylIPP formation with all three compounds suggests that if rats are used for screening potentially porphyrinogenic drugs, studies in both male and female rats are required.

If hepatic CYP isozymes targeted for mechanism-based inactivation and N-alkylIPP formation by TTMS, 4-ethylDDC, and AIA in the rat are similarly targeted in human liver, it would be reasonable to deduce that these xenobiotics would also exhibit porphyrinogenic properties in humans. In previous studies, we found that 4-ethylDDC and TTMS caused mechanism-based inactivation of cDNA expressed human CYP1A1, 1A2, and 3A4, whereas only 4-ethylDDC was able to elicit mechanism-based inactivation of CYP2C9 (McNamee et al., 1997). Although CYP1A1 and CYP1A2 undergo TTMS-mediated mechanism-based inactivation in rat liver, induction studies with β-naphthoflavone showed that neither of these isozymes are quantitatively important sources of N-vinylIPP in the rat (McNamee and Marks, 1996). Considering this information and our previous finding that CYP3A2 is quantitatively the major source of N-alkylIPP in rats, the most likely source of N-vinylIPP formation in humans is CYP3A4. On the other hand, 4-ethylDDC causes mechanism-based inactivation and N-alkylIPP formation from rat CYP2C11, thus implicating a role for human CYP2C9 as a source of N-alkylIPP. Our future studies will therefore be directed to determining whether N-alkylIPPs are formed during the interaction of TTMS and 4-ethylDDC with human CYP3A4 and CYP2C9, respectively. AIA causes mechanism-based inactivation of several rat CYP isozymes, and it was therefore surprising to find that it did not inactivate any of the major human hepatic isozymes (McNamee et al., 1997). Our future studies will therefore be directed to determining whether AIA elicits mechanism-based inactivation of one or several of the minor hepatic human CYP isozymes and if it does, to determining whether N-alkylIPP formation accompanies this inactivation.