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IRREVERSIBLE BINDING OF HEME TO MICROSOMAL PROTEIN DURING INACTIVATION OF CYTOCHROME P450 BY 4-ALKYL ANALOGUES OF 3,5-DIETHOXYCARBONYL-1,4-DIHYDRO-2,4,6-TRIMETHYLPYRIDINE

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Abstract—The porphyrinogenicity of 4-alkyl analogues of 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC) is related to the process of mechanism-based destruction of cytochrome P450 (P450) heme, accompanied by conversion of heme to *N*-alkylprotoporphyrins (*N*-alkylPPs). Certain DDC analogues (4-isopropyl, 4-isobutyl, 4-hexyl) are weakly porphyrinogenic in comparison to the potent porphyrinogen, 4-ethyl DDC. We have examined the abilities of these DDC analogues to promote irreversible binding of radiolabeled heme to protein in rat liver microsomal preparations. The goals of this study were to determine whether DDC analogues with different porphyrinogenicities differ in the extents to which they cause heme adduct formation, and whether P450 isozymes differ in their capacities to catalyze heme covalent binding. Incubation of microsomes with NADPH alone promoted heme covalent binding, while loss of spectral P450 heme was minimal or absent. In microsomal incubations containing NADPH, the 4-ethyl, 4-isopropyl, and 4-isobutyl analogues caused heme covalent binding to extents which paralleled their P450 destructive activities. In contrast, 4-hexyl DDC caused less heme covalent binding as a function of P450 loss than the other analogues in microsomes from untreated and β-naphthoflavone (βNF)-treated rats. Thus, the weakly porphyrinogenic DDC analogues do not cause greater heme covalent binding than 4-ethyl DDC. Weak porphyrinogenicity, therefore, cannot be explained by diversion of the heme moiety of P450 from conversion to *N*-alkylPPs towards utilization for formation of heme-derived protein adducts. Treatment of rats with P450 inducing agents altered the degree to which DDC analogues caused heme covalent binding. The greatest heme adduct formation occurred in microsomes from untreated and dexamethasone (DEX)-treated rats, whereas treatment with phenobarbital and especially βNF reduced heme covalent binding as a function of P450 loss. Thus, these microsomal studies suggest that constitutive P450 isozymes and members of the DEX-inducible P450IIIA subfamily appear to catalyze heme covalent binding, while βNF-inducible forms such as P450IA1 (P450c) seem to be relatively inactive in this regard.

In recent years, it has been established that a variety of structurally diverse xenobiotics and endogenous peroxides cause mechanism-based inactivation of liver microsomal cytochrome P450 (P450†) (EC 1.14.14.1), resulting in the covalent binding of the prosthetic heme to protein [1–7, reviewed in Ref. 8]. The most extensively studied compounds in this regard have been carbon tetrachloride [1, 9] and allylisopropylacetamide (AIA) [1, 2, 5]. Although a diverse array of chemical structures promote the formation of such heme-derived protein adducts, a unifying feature appears to be their metabolism by P450 to radical intermediates [8]. The formation of a covalent link between heme and the P450 apoprotein could conceivably result from an attack of the radical on either the prosthetic heme or amino acids located in the active site. The production of heme-derived protein adducts can be reproduced in simpler model systems which employ myoglobin as the hemoprotein and $BrCCl_3$ [10] or H_2O_2 [11] as destructive species. In the case of H_2O_2, the chromophore of the protein-bound prosthetic group remains similar to that of heme itself [11]. Irreversible binding of heme to the P450 apoprotein may act as a physiologically important marker to enhance protein degradation [6, 8].

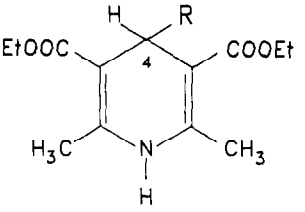

Fig. 1. Structure of dihydropyridine analogues.
(a) R = —CH_3, DDC;
(b) R = —CH_2CH_3, 4-ethyl DDC;
(c) R = —$CH(CH_3)_2$, 4-isopropyl DDC;
(d) R = —$CH_2CH(CH_3)_2$, 4-isobutyl DDC; and
(e) R = —$(CH_2)_5CH_3$, 4-hexyl DDC.

Several 4-alkyl analogues of 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC) (Fig. 1,

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† Abbreviations: P450, cytochrome P450; AIA, allylisopropylacetamide; DDC, 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine; *N*-alkylPP, *N*-alkyl protoporphyrin IX; DEX, dexamethasone; ALA, δ-aminolevulinic acid; PB, phenobarbital; and βNF, β-naphthoflavone.

compound a) cause mechanism-based inactivation of P450 via heme destruction [12–14]. The destructive species appears to be the alkyl radical derived from the 4-position of the dihydropyridine which is liberated during oxidative metabolism by P450 [12, 15]. In most cases, the result is the production of an *N*-alkylprotoporphyrin IX (*N*-alkylPP) which inhibits ferrochelatase (EC 4.99.1.1) and causes porphyrin accumulation [reviewed in Ref. 16]. Recent studies with a prototype dihydropyridine, 4-ethyl DDC (Fig. 1, compound b), have demonstrated that destruction of P450 heme by this compound is also accompanied by covalent binding of the prosthetic heme to P450 apoprotein [2, 6].

Certain isozymes of P450 are targets for inactivation by 4-ethyl DDC and related DDC analogues. In general, these compounds inactivate rat liver P450 forms h (IIC11*), k (IIC6), c (IA1), and P450p-related forms (IIIA), and loss of catalytic activity can be accompanied by loss/alteration of the corresponding apoprotein *in vivo* [6, 18–20]. It has been suggested by Correia *et al.* [6] that dexamethasone (DEX)-inducible forms of the P450IIIA subfamily may be particularly active in directing formation of heme-derived protein adducts, and that such protein modification may serve as a signal to enhance proteolysis.

In the present study, we have examined heme adduct formation by four DDC analogues (Fig. 1) which differ markedly in their porphyrinogenicities [13, 21]. The 4-ethyl analogue is extremely porphyrinogenic because of its ability to convert P450 heme to a ferrochelatase-inhibitory *N*-alkylPP [13, 21, 22]. The other three analogues (Fig. 1, compounds c–e) are much less porphyrinogenic [13, 14, 21]. The 4-isopropyl analogue (Fig. 1, compound c) destroys P450 heme [12, 13]; however, the heme appears to be degraded to products other than an *N*-alkylPP [12]. The 4-isobutyl (Fig. 1, compound d) and 4-hexyl (Fig. 1, compound e) analogues also destroy P450 heme resulting in the formation of *N*-alkylPPs which lack or possess little ferrochelatase-inhibitory activity respectively [14, 21, 22].

In principle, compounds which promote only the formation of heme-derived protein adducts should display reduced porphyrinogenicity for the following reasons: (a) lack of *N*-alkylPP formation and, hence, no ferrochelatase inhibition; and (b) the covalently modified P450 apoprotein would not be amenable to heme reconstitution and, hence, there would be minimal drain of the regulatory "free heme" pool. Thus, we were interested in determining whether DDC analogues with relatively low porphyrinogenicities tend to promote greater heme covalent binding than the highly porphyrinogenic 4-ethyl DDC. Of particular interest is the 4-isopropyl analogue, for which no fate of the destroyed P450 heme has yet been described.

The specific aims of the present work were: (a) to determine whether DDC analogues with different porphyrinogenicities differ with respect to their abilities to promote heme covalent binding; and (b) to determine whether certain P450 isozymes differ in their capacities to catalyze heme covalent binding.

* P450 designations according to the nomenclature system of Nebert *et al.* [17].

MATERIALS AND METHODS

Source of chemicals. DDC analogues were synthesized by the method of Loev and Snader [23] as described previously [12, 14]. AIA was obtained as a gift from Hoffmann-LaRoche Ltd. (Vaudreil, Quebec, Canada). Chemicals were purchased from the following sources: δ-amino[4-^{14}C]levulinic acid hydrochloride (ALA) (sp. act. 57.8 mCi/mmol, radiochemical purity 97%) and Aquasol-2®, Dupont Canada Inc. (Mississauga, Ontario, Canada); sodium phenobarbital (PB), BDH Inc. (Toronto, Ontario, Canada); β-naphthoflavone (βNF), Aldrich Chemical Co. (Milwaukee, WI, U.S.A.); DEX and NADPH, Sigma Chemical Co. (St. Louis, MO, U.S.A.); Soluene-350, Canberra-Packard Canada Ltd. (Mississauga, Ontario, Canada).

Animals and treatment. Male Sprague–Dawley rats (175–200 g) were obtained from Charles River Canada Inc. (St.-Constant, Quebec, Canada). The rats were fed Purina Lab Chow and water *ad lib.* and were housed under controlled conditions (22°, 14-hr light/10-hr dark cycle). Rats received either no treatment or were injected intraperitoneally with PB (80 mg/kg in water daily for 4 days), βNF (40 mg/kg in corn oil daily for 3 days), or DEX (100 mg/kg in corn oil daily for 4 days). Twenty-four hours following the final inducer treatment, rats received a single intraperitoneal dose of [4-^{14}C]ALA (50 μCi/kg, 0.87 μmol/kg).

Preparation and storage of hepatic microsomes. Three hours after receiving [4-^{14}C]ALA, rats were killed by decapitation. Livers were perfused *in situ* with ice-cold (1.15%, w/v) KCl, removed, weighed, and homogenized in 4 vol. of cold phosphate-buffered KCl [1.15% (w/v) KCl, 10 mM K_2HPO_4, pH 7.4]. Microsomes were isolated by differential centrifugation, and the final microsomal pellets were frozen in liquid nitrogen and stored at −70°, as described previously [24]. Another study [6] revealed that after 3 hr of labeling with either 5 or 68.5 μCi doses of [4-^{14}C]ALA, approximately 85% of the microsomal radioactivity is attributable to P450 heme. The mean specific activity of our microsomal preparations was 2530 dpm/nmol P450 (≈1.1 nCi/nmol P450).

Destruction of microsomal P450 and heme. A typical incubation mixture (final volume, 4.5 mL) contained hepatic microsomes (3–4 mg protein/mL), a DDC analogue (1 mM) or AIA (10 mM), and NADPH (2 mM) in 0.1 M K_2HPO_4 buffer, pH 7.4, containing 1.5 mM EDTA. Control incubates, which excluded the DDC analogue or AIA, were always run in parallel. In some instances, control incubates which excluded both the DDC analogue or AIA and NADPH were run. After a 30-min incubation at 37°, reactions were terminated by cooling on ice, and aliquots were removed for determination of P450 (1 mL) and heme (0.5 mL) levels according to the spectrophotometric methods of Omura and Sato [25]. Protein was assayed by the method of Lowry *et al.* [26].

Table 1. Effects of NADPH on microsomal P450, heme, and heme covalent binding

Treatment group	P450 (nmol/mg protein)	Heme (nmol/mg protein)	Heme covalent binding (% initial radioactivity)
Untreated			
−NADPH	0.87 ± 0.15	1.78 ± 0.17	4.0 ± 0.2
+NADPH	0.81 ± 0.13*	1.76 ± 0.15	5.8 ± 0.4*
PB-treated			
−NADPH	1.62 ± 0.10	2.73 ± 0.32	3.3 ± 0.7
+NADPH	1.59 ± 0.10	2.62 ± 0.20	4.3 ± 0.9*
βNF-treated			
−NADPH	1.39 ± 0.03	2.28 ± 0.04	3.4 ± 1.4
+NADPH	1.31 ± 0.04	2.18 ± 0.10	4.0 ± 1.2*
DEX-treated			
−NADPH	1.66 ± 0.40	2.68 ± 0.63	5.4 ± 3.1
+NADPH	1.47 ± 0.37†	2.63 ± 0.63*	8.0 ± 2.8†

Three hours after receiving a single intraperitoneal dose of [4-^{14}C]ALA (50 μCi/kg) to radiolabel microsomal heme, rats were killed and hepatic microsomes prepared. Microsomes were incubated in 0.1 M K_2HPO_4 buffer, pH 7.4, containing 1.5 mM EDTA for 30 min at 37° in the absence or presence of 2 mM NADPH. The extent of irreversible binding of heme to microsomal protein is expressed as a percentage of the amount of radioactivity initially present in each incubation mixture. Data are expressed as means (±SD) of determinations from three rats. Mean absolute values for initial radioactivity were as follows (dpm): untreated, 12,960; PB-treated, 32,570; βNF-treated, 59,650; DEX-treated, 56,150.

,† Significantly different from −NADPH ($P \leq 0.05$ and †$P \leq 0.01$), based on Student's *t*-test for paired observations. In these experiments, a paired design was used in which microsomal samples derived from three separate rats were each subjected to −NADPH and +NADPH treatments. Thus, a significant difference is determined by the presence of a consistent trend among treatment pairs, rather than the mean ±SD for the groups. This approach partially eliminates extraneous sources of variation, thus increasing the statistical power of Student's *t*-test.

Irreversible binding of radiolabeled heme to microsomal protein. The remaining 3-mL aliquot of each incubate was used to determine the extent of formation of heme-derived protein adducts by the method of Davies *et al.* [2]. Microsomal protein was precipitated with 10 vol. of ethyl acetate : acetic acid (4:1, v/v) and allowed to stand for 20 hr at −20°. The pellets were washed twice with the ethyl acetate/acetic acid solution. Two washes were shown to be sufficient to reduce the counts in the supernatants to background (data not shown). After additional washes in ethyl acetate/acetic acid and ethyl acetate, the microsomal pellets were solubilized by incubating at 50° for 1.5 hr in the presence of Soluene-350 (1.5 mL). After addition of Aquasol-2® (15 mL) and neutralization with glacial acetic acid (0.1 mL), radioactivity was measured in a Searle Mark III 6880 liquid scintillation counter utilizing the external standard channels ratio method of quench compensation. Counting efficiencies ranged from 87 to 94%. The amount of radiolabeled heme irreversibly (covalently) bound to microsomal protein is expressed as a percentage of the total radiolabel initially present in each incubate.

Statistical analysis. For comparison of two treatment groups derived from the same animals, significant differences were identified using a paired-difference *t*-test ($P \leq 0.05$ or 0.01). For comparison of more than two treatment groups derived from the same animals, a repeated measures design one-way analysis of variance was used. For comparison of more than two independent treatment groups, a randomized design one-way analysis of variance was used. In the latter two cases, significantly different groups were identified using a Newman–Keuls test ($P \leq 0.05$ or 0.01).

RESULTS

Increased formation of heme-derived protein adducts in the presence of NADPH. Before examining the abilities of DDC analogues to promote heme covalent binding in rat liver microsomes, we were interested in determining the extent to which this process occurs in control incubates. It is well known that incubation of rat liver microsomes in the presence of NADPH results in lipid peroxidation and a concomitant loss of P450 heme [27, 28]. These effects can generally be prevented by the inclusion of EDTA in the reaction mixture [24, 27]. In a previous study [19], we demonstrated that in the presence of EDTA, NADPH causes lipid peroxidation and P450 heme loss only in microsomes from DEX-treated rats. As shown in Table 1, the present results confirm this finding; in addition, we observed NADPH-dependent loss of spectral P450 in microsomes from untreated rats. However, in all four microsomal systems, NADPH increased the extent of heme covalent binding above the background level observed following incubation in the absence of NADPH. The effects of NADPH were greatest in microsomes from untreated and DEX-treated rats. Because of this NADPH-dependent heme covalent binding, we have

Table 2. Effects of DDC analogues and AIA on microsomal P450, heme, and heme covalent binding

Treatment group	P450 (nmol/mg protein)	Heme (nmol/mg protein)	Heme covalent binding (% initial radioactivity)
Untreated			
NADPH	1.05 ± 0.10	1.99 ± 0.20	5.4 ± 0.7
Ethyl + NADPH	0.72 ± 0.07*	1.57 ± 0.19*	22.4 ± 3.1*†‡
Isopropyl + NADPH	0.61 ± 0.10*†‡§	1.50 ± 0.14*‡	27.9 ± 3.6*†‡§
Isobutyl + NADPH	0.76 ± 0.07*	1.68 ± 0.19*	19.4 ± 1.8*†
Hexyl + NADPH	0.72 ± 0.07*	1.51 ± 0.17*‡	16.3 ± 1.1*
PB-treated			
NADPH	1.64 ± 0.24	2.62 ± 0.31	9.4 ± 9.2
Ethyl + NADPH	0.72 ± 0.07*	1.57 ± 0.19*	22.4 ± 3.1*†‡
Isopropyl + NADPH	0.82 ± 0.07*†‡§‖	1.61 ± 0.21*†‡§‖	28.7 ± 5.3*†‡§‖
Isobutyl + NADPH	1.15 ± 0.13*	1.96 ± 0.18*	21.7 ± 7.7*‖
Hexyl + NADPH	1.30 ± 0.21*	2.05 ± 0.32*	18.5 ± 9.4*
AIA + NADPH	1.19 ± 0.17*	1.86 ± 0.29*	15.4 ± 10.5*
βNF-treated			
NADPH	1.53 ± 0.21	2.48 ± 0.22	11.6 ± 9.1
Ethyl + NADPH	0.99 ± 0.03*	1.93 ± 0.07*	21.7 ± 7.8*†‡
Isopropyl + NADPH	0.90 ± 0.06*	1.94 ± 0.11*	22.7 ± 7.3*†‡
Isobutyl + NADPH	0.99 ± 0.03*	2.05 ± 0.08*	19.9 ± 7.5*†
Hexyl + NADPH	0.94 ± 0.09*	1.86 ± 0.10*	18.1 ± 8.8*
DEX-treated			
NADPH	1.48 ± 0.30	2.59 ± 0.52	7.8 ± 2.3
Ethyl + NADPH	0.63 ± 0.24*†	1.37 ± 0.28*†	33.7 ± 4.0*†
Isopropyl + NADPH	0.37 ± 0.13*†‡§	1.10 ± 0.25*†	44.7 ± 2.6*†‡§
Isobutyl + NADPH	0.57 ± 0.23*†	1.27 ± 0.35*†	35.4 ± 5.6*†
Hexyl + NADPH	1.12 ± 0.33*	1.89 ± 0.59*	20.8 ± 4.3*

Three hours after receiving a single intraperitoneal dose of [4-^{14}C]ALA (50 μCi/kg) to radiolabel microsomal heme, rats were killed and hepatic microsomes prepared. Microsomes were incubated in 0.1 M K_2HPO_4 buffer, pH 7.4, containing 1.5 mM EDTA and 2 mM NADPH for 30 min at 37° in the absence or presence of a DDC analogue (1 mM) or AIA (10 mM). The extent of irreversible binding of heme to microsomal protein is expressed as a percentage of the amount of radioactivity initially present in each incubation mixture. Data are expressed as means (±SD) of determinations from four rats. Mean absolute values for initial radioactivity were as follows (dpm): untreated, 41,200; PB-treated, 18,530; βNF-treated, 33,090; DEX-treated, 58,370.

*–‖ Statistical analysis: *significantly different from NADPH ($P \leq 0.01$); †significantly different from hexyl + NADPH ($P \leq 0.05$); ‡significantly different from isobutyl + NADPH ($P \leq 0.05$); §significantly different from ethyl + NADPH ($P \leq 0.05$);, and ‖significantly different from AIA + NADPH ($P \leq 0.05$), based on repeated measures design one-way analysis of variance and Newman–Keuls test.

used incubates containing NADPH as our control to examine the effects of DDC analogues.

Formation of heme-derived protein adducts: Effects of DDC analogues. Our objective was to determine whether DDC analogues differed in their capacities to promote heme covalent binding. The data derived from experiments designed to examine this idea are presented in Table 2.

In microsomes from untreated rats, all analogues caused loss of P450 and heme, with 4-isopropyl DDC being most active. While all analogues also promoted heme covalent binding, 4-hexyl DDC was less effective than the other compounds. For the three remaining analogues, the extent of heme covalent binding paralleled the trends observed for P450 and heme loss with the following rank order: isopropyl > ethyl > isobutyl.

The production of heme-derived protein adducts in microsomes from PB-treated rats by AIA has been studied previously [1, 2, 5]. We have used AIA as a positive control for our studies. In our hands, AIA caused 15.4% of the initial radiolabel to be bound irreversibly to microsomal protein, as compared to reported values of 15% [1] and 21.6% [2]. In microsomes from PB-treated rats, the isopropyl analogue promoted most heme covalent binding, followed by the ethyl and isobutyl analogues. 4-Hexyl was less effective than the isopropyl and ethyl analogues. Again, this rank order paralleled the trend observed for P450 loss.

In microsomes from βNF-treated rats, all analogues caused similar loss of P450 heme. However, 4-hexyl DDC was less effective than the other analogues at promoting heme covalent binding.

Treatment of rats with DEX revealed large differences between DDC analogues in their abilities to destroy P450. These differences were paralleled by differences in the extents to which these compounds promote heme covalent binding so that the following rank order was observed for both processes: isopropyl > isobutyl = ethyl > hexyl.

Formation of heme-derived protein adducts: Effects of P450 inducers. Our objective was to determine whether altering the microsomal P450 isoenzyme composition by treating rats with P450 inducing agents changes the abilities of DDC analogues to promote heme covalent binding. Because the absolute amount of P450 and the extent of P450 loss caused by DDC analogues differ in the various microsomal systems, we have expressed heme covalent binding as a percentage of the loss of spectral

P450 caused by each analogue in each system. These data are presented in Fig. 2.

In the case of 4-ethyl DDC, the greatest fractional heme covalent binding was observed in microsomes from untreated rats. Treatment with either PB or DEX reduced covalent binding as a function of P450 loss. Treatment with βNF reduced the degree of fractional heme covalent binding below that observed following PB or DEX treatment. Qualitatively similar patterns were observed with the isopropyl and isobutyl analogues. However, with these two analogues, heme covalent binding was not reduced significantly by DEX treatment. With 4-isopropyl DDC, PB treatment reduced heme covalent binding below levels seen in microsomes from untreated and DEX-treated rats.

Quite different results were obtained with 4-hexyl DDC. Compared to the other analogues, heme covalent binding was relatively low in microsomes from untreated rats and, again, treatment with βNF resulted in a further decrease. However, treatment with PB increased the extent of fractional heme covalent binding compared to that seen in microsomes from untreated rats. DEX treatment resulted in the greatest fractional heme covalent binding with this analogue.

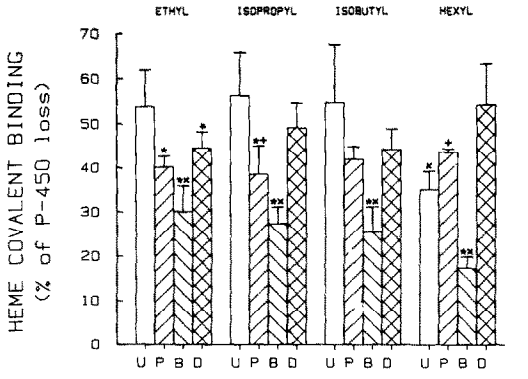

Fig. 2. Heme covalent binding caused by DDC analogues in hepatic microsomes from untreated (U), PB-treated (P), βNF-treated (B), and DEX-treated (D) rats. Heme covalent binding is expressed as a percentage of the spectrophotometrically-determined P450 loss, after first subtracting the amount of covalently bound radiolabel measured in parallel incubations run in the presence of NADPH but lacking a DDC analogue. Each bar represents the mean (±SD) of determinations from four rats. Key: (*) Significantly different from U ($P \leq 0.05$); (+) significantly different from D ($P \leq 0.05$); and (×) significantly different from P and D ($P \leq 0.05$), based on randomized design one-way analysis of variance and Newman–Keuls test.

DISCUSSION

Our initial studies demonstrated that the cofactor NADPH alone promoted significant irreversible binding of heme to microsomal protein (Table 1). Depending on the inducer treatment, between 4.0 and 8.0% of the radiolabel was bound to microsomes in the presence of NADPH. Previously reported values range from 1.5 to 6% for microsomes from PB-treated rats [1, 2, 5, 7] and from 3.5 to 10% for microsomes from DEX-treated rats [3, 6]. It seems likely that reactive oxygen species produced by the oxidase activity of P450 mediate the heme covalent binding in these incubations [8]. The results of the present work and our previous study [19] with microsomes from DEX-treated rats support a role for reactive oxygen species. Microsomes from DEX-treated rats are most susceptible to NADPH-dependent lipid peroxidation [19], P450 heme loss [19], and heme covalent binding (present study). It is also of interest that significant heme covalent binding occurred under conditions which produced no changes in spectrophotometrically-determined P450 or heme levels (Table 1). This indicates that at least a portion of the irreversibly-bound heme retains its ability to form a pyridine hemochromogen and a carbon monoxide adduct. Similar findings were demonstrated recently for the H_2O_2–myoglobin interaction [11]. Thus, the use of spectral assays to measure P450 heme loss may result in underestimation of the actual heme alteration caused by mechanism-based inactivators of P450.

As suggested previously [6, 8], the presence of small amounts of heme covalent binding in microsomes incubated in the absence of NADPH suggests that this may represent a process which occurs normally to a limited extent *in vivo*.

DDC analogues which differ markedly in their porphyrinogenicities were compared for their abilities to promote heme covalent binding. With the exception of 4-hexyl DDC, the remaining analogues demonstrated a relationship between P450 destruction and heme covalent binding (Table 2). Thus, when heme covalent binding was normalized for the amount of P450 destroyed in each microsomal system, similar results were obtained for the ethyl, isopropyl, and isobutyl analogues (Fig. 2). It therefore appears that during the inactivation of P450 by these three analogues the same fraction of altered P450 heme became irreversibly bound to microsomal protein. In contrast, in microsomes from untreated and βNF-treated rats, 4-hexyl DDC caused a smaller fraction of the heme which it destroyed to be covalently bound to protein (Fig. 2). Thus, we conclude that weakly porphyrinogenic DDC analogues (isopropyl, isobutyl, hexyl) are not in any cases more effective than 4-ethyl DDC at directing altered heme along the trajectory resulting in irreversible binding to protein. In particular, enhanced heme covalent binding cannot be invoked to explain the lack of formation of an *N*-alkylPP by 4-isopropyl DDC. The fate of a major fraction of the heme destroyed by this compound thus remains to be elucidated. Apparently, the major determinant of the porphyrinogenic activity of these DDC analogues relates to their ability or inability to produce *N*-alkylPPs of differing ferrochelatase-inhibitory potency [16].

It was suggested by Correia *et al.* [6] that different P450 isozymes may catalyze heme covalent binding to different extents. Our data indeed confirm this prediction. In general, we observed the greatest fractional heme covalent binding in microsomes from untreated and DEX-treated rats (Fig. 2). It appears

that constitutive isozymes destroyed by the ethyl, isopropyl, and isobutyl analogues are effective at directing heme covalent binding. The major P450 targets for 4-ethyl DDC in microsomes from untreated rats appear to be P450 h, k, and p-related forms [6, 18]. The fact that DEX treatment generally maintained high levels of fractional heme covalent binding supports a major role for members of the P450IIIA subfamily in this process. This was first suggested by Correia *et al.* [6] and indeed we now have additional evidence to support this idea. In microsomes from DEX-treated rats, various DDC analogues promote heme covalent binding (Table 2) and cause loss of catalytic activity of P450IIIA forms (erythromycin *N*-demethylase) [19] in the following rank order: isopropyl > isobutyl = ethyl > hexyl.

Treatment of rats with βNF reduced the formation of heme-derived protein adducts as a function of P450 loss. A major target for inactivation by DDC analogues in βNF-treated rats appears to be P450c [18–20]. This isozyme seems to be particularly poor at directing heme toward covalent linkage to protein. We have demonstrated recently that the P450c apoprotein is particularly susceptible to degradation/alteration concurrent with inactivation by DDC analogues *in vivo* [20]. Thus, it appears that the hypothesis of Correia *et al.* [6] that the covalent attachment of heme to P450 apoprotein acts to trigger proteolysis may not apply equally to all families of P450 proteins. Much work remains to determine the importance of heme covalent binding as a marker for enhanced proteolysis *in vivo*, the structure of the heme-derived protein adducts, and the role that P450 isozymes play in catalyzing their formation.

In summary, we found that the extent of heme covalent binding caused by DDC analogues did not reflect their porphyrinogenicities. P450 isozymes differed in their capacities to promote heme covalent binding: constitutive forms and members of the DEX-inducible P450IIIA subfamily were particularly active, while βNF-inducible P450c appeared to be particularly inactive in this regard.

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