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In vitro hepatic, renal, and pulmonary N-dealkylation of amiodarone

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Amiodarone, an iodinated benzofuran derivative, is used clinically for treating arrhythmias refractory to other drug therapies. However, its use is often associated with serious adverse effects, including life-threatening pulmonary fibrosis and hepatitis [1]. In light of recent evidence indicating a role for the N-dealkylated amiodarone metabolite, desethylamiodarone, in amiodarone-induced toxicities [2, 3], it is of interest to characterize the biotransformation of amiodarone to desethylamiodarone.

Experiments performed with inhibitors and inducers of cytochrome P450 have confirmed the role of the polysubstrate monooxygenase system in the conversion of amiodarone to desethylamiodarone [4–6]. In examining the effects of inducing agents on *in vitro* desethylamiodarone formation, phenobarbital treatment was found to enhance greatly rat hepatic microsomal activity, and to result in measurable activity in lung and kidney, whereas none was found in lung or kidney microsomes from control animals [6]. Larrey and colleagues also reported that amiodarone can form an inactive complex with cytochrome P450, and that formation of this complex is increased greatly by prior treatment with dexamethasone [7], which induces products of the cytochrome P450III gene family, principally the isozyme referred to as P450PCNa [8] or P450p [9]. However, the effect of dexamethasone treatment on amiodarone biotransformation has not been explored.

The aim of the present study was to investigate the role of steroid-inducible cytochrome(s) P450 on the biotransformation of amiodarone to desethylamiodarone, by examining the effect of dexamethasone treatment on hepatic and extrahepatic *in vitro* desethylamiodarone formation. These activities also were compared to those found in tissues from animals treated with the polycyclic aromatic inducing agent, β-naphthoflavone. To confirm the inducing effects of dexamethasone and β-naphthoflavone, we employed the isozyme-selective marker enzyme assays. erythromycin *N*-demethylase and 7-ethoxyresorufin *O*-deethylase.

Methods

Animals and treatments. Male Sprague–Dawley rats (Charles River Canada, Inc., St. Constant, Quebec, Canada) weighing 250–300 g, were maintained on a 12-hr light/12-hr dark cycle and fed laboratory chow and water *ad lib.* They were treated with dexamethasone (50 mg/kg) or β-naphthoflavone (80 mg/kg) i.p. in corn oil (2.0 mL/kg). Controls for the above treatments received corn oil (2.0 mL/kg). Treatments were given once daily for three successive days, and rats were killed by cervical dislocation 48 hr after the final dose.

Chemicals. Chemicals were obtained from suppliers as follows: β-naphthoflavone, dexamethasone, erythromycin, and chemically reduced NADPH from the Sigma Chemical Co., St. Louis, MO, U.S.A.; resorufin and ethoxyresorufin from Molecular Probes Inc., Junction City, OR, U.S.A.; and amiodarone hydrochloride and desethylamiodarone hydrochloride from Ayerst Pharmaceuticals, Montréal, Quebec, Canada. All other chemicals were of reagent grade and were obtained from common commercial suppliers.

Preparation of microsomes. Kidneys, lungs, and livers were perfused *in situ* with ice-cold 1.15% KCl. Minced tissues were homogenized in 4 vol. of 1.15% KCl–0.1 M potassium phosphate buffer (pH 7.4) using a Potter–Elvehjem glass–teflon tissue homogenizer. The homogenate was centrifuged at 10,000 *g* for 20 min at 4°. The 10,000 *g* supernatant fraction was centrifuged at 104,000 *g* for 60 min at 4°. The microsomal pellets were resuspended in 0.25 M sucrose–0.1 M potassium phosphate buffer. Aliquots of resuspended pellet were frozen in liquid nitrogen and stored at −70°. Protein concentration was assessed by the method of Lowry *et al.* [10].

Assays. O-Deethylation of 7-ethoxyresorufin was

Table 1. Monooxygenase activities in rat tissue microsomes

Tissue	Treatment	7-Ethoxyresorufin *O*-deethylase (pmol/min/mg protein)	Erythromycin *N*-demethylase (pmol/min/mg protein)	Amiodarone *N*-deethylase (nmol/hr/mg protein)
Liver	Control	10.1 ± 1.6 (4)	0.94 ± 0.30 (4)	20.7 ± 6.4 (4)
	β-Naphthoflavone	2740* (2)	0.76 ± 0.28 (4)	20.6 ± 4.8 (4)
	Dexamethasone	12.0 ± 1.7 (4)	4.59 ± 0.74* (4)	63.8 ± 15.9* (4)
Kidney	Control	0.51 ± 0.34 (4)	ND†	ND
	β-Naphthoflavone	554 ± 292* (4)	ND	Detected‡
	Dexamethasone	1.64 ± 1.14 (3)	ND	ND
Lung	Control	1.85 ± 0.45 (4)	ND	Detected
	β-Naphthoflavone	46.2 ± 30.7* (3)	ND	Detected
	Dexamethasone	3.09 ± 0.46 (3)	ND	Detected

Values are means ± SD, with one exception where the average of two values is given. The number of animals is shown in parentheses.

* Significant difference from control ($P < 0.05$) by one-way ANOVA and Newman–Keuls test.

† ND = not detected.

‡ Detected = desethylamiodarone was produced, but the amount formed was below the lower limit of quantitative sensitivity of the assay (1.5 nmol/mL incubate).

assayed by the fluorometric method of Burke *et al.* [11]. Erythromycin *N*-demethylase activity was determined by the generation of formaldehyde as described by Wrighton *et al.* [12].

For microsomal biotransformation of amiodarone to desethylamiodarone, 2.0-mL incubation mixtures contained approximately 5.0 mg microsomal protein, 2.0 mM NADPH, 1.0 mM EDTA, and 4.0 mM $MgCl_2$, in 0.2 M phosphate buffer (pH 7.4). Samples were incubated in a metabolic shaker bath at 37° for 1 hr. Reactions were initiated by the addition of amiodarone hydrochloride (0.5 μmol) dissolved in 10 μL of absolute ethanol. Reactions were stopped by the addition of 4.0 mL of HPLC mobile phase [acetonitrile: 5% (v/v) aqueous acetic acid, 80:20 (v/v) adjusted to pH 5.9 with ammonium hydroxide] to the incubation mixture. The contents were mixed and allowed to stand for 15 min before being centrifuged at 1350 *g* for 5 min. Aliquots (20 μL) of supernatant fraction were analyzed by HPLC according to a previously published method [13]. With this method, recovery of desethylamiodarone was 92.5 ± 5.8% (N = 5), and the lower limit of quantitative sensitivity was 1.5 nmol/mL incubate.

Results and discussion

Consistent with previous reports from other investigators [6, 14], we found appreciable amiodarone *N*-deethylase activity in rat liver microsomes from uninduced animals, but little or no activity in kidney or lung microsomes (Table 1). The parallel effects of dexamethasone treatment on amiodarone dealkylation and erythromycin *N*-demethylation (pronounced induction in hepatic microsomes and minimal effects in kidney and lung) indicate an important role for products of the steroid-inducible cytochrome P450III gene family in desethylamiodarone formation from amiodarone, and a deficiency of steroid-inducible cytochrome(s) P450 in kidney and lung. Interestingly, dexamethasone treatment also has been found to enhance greatly *in vivo* formation of an inactive cytochrome P450Fe(II)–amiodarone metabolite complex [7]. Thus, it appears that the same cytochrome P450 isozyme which forms an inactive complex with amiodarone, may also catalyse the dealkylation of the chemical.

Although the protocol employed for β-naphthoflavone treatments produced the expected induction of 7-ethoxyresorufin *O*-deethylase activity in all tissues examined (Table 1), production of desethylamiodarone was similar to control, indicating that the β-naphthoflavone-inducible cytochromes P450 do not play major roles in desethylamiodarone production. In kidney microsomes from β-naphthoflavone-treated rats, desethylamiodarone production was elevated to the point of being detected but not quantifiable (Table 1). 3-Methylcholanthrene, an agent with inducing properties similar to those of β-naphthoflavone, was found by Young and Mehendale [6] to increase slightly hepatic microsomal desethylamiodarone production relative to control. The difference between our results and those of Young and Mehendale may be due to a difference in efficacy between 3-methylcholanthrene and β-naphthoflavone in the induction of cytochrome P450 isozymes. The previously observed induction of amiodarone N-dealkylation following phenobarbital treatment [6] is not inconsistent with P450III being the principal cytochrome P450 family catalyzing this reaction, since the isozyme P450p is phenobarbital inducible [9].

In summary, the experiments performed have shown that steroid-inducible cytochrome(s) P450 plays a major role in amiodarone N-dealkylation in rat liver microsomes. Desethylamiodarone formation in kidney and lung was found to be minimal regardless of treatment with inducing agents. β-Naphthoflavone-inducible cytochrome P450 isozymes were found to play at best a minor role in kidney desethylamiodarone formation. Desethylamiodarone production in lung microsomes was detected in all three treatment groups, but was not quantifiable. This result is consistent with previous reports, indicating no significant plumonary biotransformation of amiodarone to desethylamiodarone.

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Comparison of Mongolian gerbil and rat hepatic microsomal monooxygenase activities: high coumarin 7-hydroxylase activity in the gerbil

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The Mongolian gerbil (*Meriones unguiculatus*) has been widely utilized as an animal model of unilateral hemispheric global ischaemia [1–3]. Although many pharmacological investigations have been undertaken using this model, studies of drug metabolism in the gerbil have not been well documented. Extensive studies on species differences in microsomal monooxygenase (MMO*) activities [4] do not include the gerbil amongst the species investigated. However, the pharmacokinetics, including limited metabolic studies, of coumarin (2*H*-1-benzopyran-2-one) in the gerbil have been reported [5]. Man is exposed to coumarin via its addition to toiletries and tobacco products [6]. Coumarin, in combination with cimetidine, is currently undergoing clinical trials for the treatment of various malignancies [7–9], and there have also been several human trials involving coumarin preparations for the treatment of lymphoedemas [10, 11]. A suitable animal model for man with respect to coumarin metabolism and toxicity has yet to be found [6].

It is well-recognized that the biotransformation of drugs, in particular by the cytochrome P450-dependent MMO system, can profoundly affect their pharmacological, and toxicological, activities. Hence, we have investigated various P450-dependent MMO activities [aniline 4-hydroxylase; benzphetamine *N*-demethylase; 7-ethoxycoumarin *O*-deethylase (7-ECOD); and, particularly, coumarin 7-hydroxylase (COH)] of gerbil liver microsomes, and compared these with those observed in the rat, a species for which extensive information on hepatic drug metabolism is available.

Materials and methods

All substrates, enzymes and cofactors were obtained from the Sigma Chemical Co. (Poole, U.K.) except for 7-ethoxycoumarin which was synthesized as described previously [12]. Other chemicals used were of AR grade. Adult male Wistar rats (115–140 g) and adult male Mongolian gerbils (60–70 g) were obtained from the University of Nottingham Medical School Animal Unit. They had access to standard laboratory diet and tap water *ad lib*.

Liver microsomes were prepared by the calcium aggregation technique as outlined previously [13]. Separate microsomal fractions were obtained for each animal. They were stored at −196° until required. Protein content was measured by the method of Lowry *et al.* [14]. Cytochrome P450 [15] and cytochrome b_5 [16] contents, and NADPH-cytochrome *c* reductase activity [16], were determined by the methods quoted. COH [17], 7-ECOD [12], aniline 4-hydroxylase [16] and benzphetamine *N*-demethylase [16] activities were assayed by standard methods. In addition, the glutathione content of liver homogenates was measured [18].

Statistical analysis was performed by means of an unpaired Student's *t*-test.

* Abbreviations used: COH, coumarin 7-hydroxylase; 7-ECOD, 7-ethoxycoumarin *O*-deethylase; 7-HC, 7-hydroxycoumarin; GSH, glutathione; MMO, microsomal monooxygenase; P450, cytochrome P450.