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EXOGENOUS HEME RESTORES IN VIVO FUNCTIONAL CAPACITY OF HEPATIC CYTOCHROME P-450 DESTROYED BY ALLYLISOPROPYLACETAMIDE

Geoffrey C. Farrell and Rudi Schmid
Department of Medicine, School of Medicine

Kent L. Kunze and Paul R. Ortiz de Montellano
Department of Pharmaceutical Chemistry, School of Pharmacy;
and the Liver Center

University of California, San Francisco, California 94143

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SUMMARY: Administration of allylisopropylacetamide (AIA) produces a dose-related destruction of the heme moiety of the phenobarbital-induced subspecies of hepatic cytochrome P-450. This results in delayed plasma disappearance of the inactivating agent as determined after injection of [^{14}C]AIA. In phenobarbital-pretreated rats, infusion of heme reversed this AIA-mediated impairment of the plasma disappearance of [^{14}C]AIA. In the absence of phenobarbital pretreatment, cytochrome P-450 destruction by AIA was minimal and heme infusion failed to enhance plasma disappearance of [^{14}C]AIA. Since exogenously administered heme is incorporated into hepatic cytochrome P-450 in vivo, these observations suggest that the infused heme restored the functional capacity of the phenobarbital-induced mixed function oxidase system by substituting for the prosthetic heme moiety destroyed by AIA. Heme infusion is a potentially useful therapeutic modality for enhancing drug biotransformation after intoxication with compounds that inactivate cytochrome P-450.

Microsomal cytochrome P-450 consists of a group of closely-related hemoproteins which constitute an integral part of the enzyme system catalyzing the mixed function oxidation of a wide variety of xenobiotics. There is conclusive evidence that heme used for assembly of holocytochrome P-450 is synthesized in the liver cell itself (1), but details of the mechanism and subcellular localization of this assembly process are unknown. It has been shown, however, that in liver homogenate prepared from rats under appropriate experimental conditions exogenously supplied heme can be utilized for reconstitution of functionally active cytochrome P-450 (2,3). Moreover, it has recently been demonstrated in intact rats that exogenously administered heme, taken up by the liver, is directly incorporated into the phenobarbital-inducible subspecies of cytochrome P-450 (4,5).

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Certain xenobiotics which are substrates of the mixed function oxidase system, such as allylisopropylacetamide (AIA) (6), secobarbital (7), fluoroxene (8), and ethinylestradiol (9), destroy the particular cytochrome P-450 subspecies involved in their metabolism. This destruction is highly selective in that other hepatic cytochrome P-450 subspecies, cytochrome b_5 and NADPH-cytochrome P-450 reductase are not affected (10-12). AIA, the most extensively studied of these compounds, produces a dose-dependent decrease of the phenobarbital-inducible cytochrome P-450 subspecies involved in its biotransformation (13-16). This destructive process requires metabolic activation of AIA by the mixed function oxidase system to a reactive intermediate (11, 12) which then binds covalently to the prosthetic heme group of the cytochrome resulting in formation of an equimolar adduct between AIA and cytochrome P-450 heme (17, 18). AIA appears to destroy only the heme moiety of cytochrome P-450, leaving the apoprotein intact (19).

The destruction of cytochrome P-450, which occurs during conversion of AIA to more polar metabolites (20-22), interferes with metabolism of the parent compound, and this is reflected by a delay in its plasma disappearance. Since exogenously administered heme is incorporated into hepatic cytochrome P-450 *in vivo* (4), it might be expected that heme would repair the AIA-mediated destruction of the cytochrome and thereby enhance AIA metabolism. We have investigated the effect of heme infusion on the plasma disappearance of AIA in rats, using a dose of the drug sufficiently large to produce a substantial reduction of the phenobarbital-inducible subspecies of cytochrome P-450. The rate of AIA plasma disappearance was compared before and during heme infusion, each rat serving as its own control.

Experimental Procedures

Materials: AIA and [^{14}C]AIA (specific activity 71.5 μCi/mg, and at least 99% pure by TLC) were gifts from Hoffmann-La Roche Inc., Nutley, NJ. Hemin was obtained from Sigma Chemical Co.

Experimental Protocol: Male Sprague-Dawley rats (214 ± 9 g) were pretreated for 5 days with sodium phenobarbital (80 mg/kg intraperitoneally, daily), except for control animals, and were fasted overnight prior to experiments.

Under ether anesthesia, polyethylene cannulas were inserted into the left jugular vein and carotid artery. The animals were placed in restraining cages, maintained at normal body temperature, and kept adequately hydrated by continuous infusion of 0.87% aqueous NaCl (1 ml/h). Heme was dissolved in a small volume of 0.1N NaOH, rapidly neutralized with 0.1 N HCl, and mixed with 2 ml of rat serum and sufficient 0.1 M potassium phosphate buffer, pH 7.4, to yield a final heme concentration of 1 mg/ml. Before injection, this heme solution was passed through a 0.45 μm millipore filter to remove aggregations and, if required, appropriately diluted with 0.87% aqueous NaCl.

An aliquot of [^{14}C]AIA (45-70 x 10^3 dpm), dissolved in 0.87% aqueous NaCl and mixed with unlabeled AIA, was injected as an intravenous bolus in a dose of 100 mg/kg body weight. Eighty minutes after administration of the drug, an intravenous bolus of 500 μg heme was injected, followed by infusion of heme at a constant rate of 100 μg/h. Blood samples (150 μl) were collected through the carotid cannula at 10 min intervals from 20 to 170 min after administration of the drug.

Determination of Plasma AIA Disappearance: After separation of the blood cells, plasma, buffered at pH 7.4 with 0.1 M potassium phosphate buffer, was extracted twice with 3 volumes diethylether and the radioactivity of the combined ether extracts was determined in a liquid scintillation spectrometer. Efficiency of extraction for authentic [^{14}C]AIA in this system is 95%. Only minor amounts of AIA metabolites appear in the ether extract. Hence, in plasma samples containing approximately equal proportions of labeled AIA and its metabolites, at least 90% of the ether-extractable radioactivity consisted of unchanged AIA as determined by TLC in the following systems: (a) benzene/acetone (1:1, v/v) (b) chloroform/acetone (10:1, v/v) (22). The small amount of AIA metabolites partitioned into the ether phase migrated as a single radioactive band exhibiting R_F values corresponding to those of AIA-diol (4,5-dihydroxy-2-isopropylpentenamide) (21). For computation, the concentration of AIA in plasma samples was taken as the radioactivity extracted by diethylether and was expressed as a percentage of the injected dose per ml. The plasma fractional disappearance rate of AIA (k) was determined by the least squares method.

Determination of Hepatic Cytochromes P-450 and b_5 : AIA, 100 mg/kg in 0.87% aqueous NaCl, was administered intravenously to groups of weight-matched rats to determine the extent of destruction of hepatic microsomal hemoproteins in phenobarbital-pretreated or untreated animals; controls were injected with 0.87% aqueous NaCl. The rats were killed 15 min or 3 h after AIA injection, the liver perfused with ice-cold 0.87% aqueous NaCl and microsomes prepared from 25% w/v homogenate in 0.1 M phosphate buffer, pH 7.4 (2). Hepatic content of cytochromes P-450 and b_5 was determined spectrophotometrically by the methods of Raj and Estabrook (23) and Omura and Sato (24) and is expressed per mg microsomal protein. Protein was measured with the Folin reagent (25).

Statistical Methods: The Student's t test, paired or unpaired where appropriate, was used for statistical comparison of the results.

Results and Discussion

In phenobarbital-pretreated rats, cytochrome P-450 content of the liver fell to 48% of control values 3 h after intravenous injection of 100 mg/kg AIA (Table); the cytochrome b_5 level remained unaffected (Table). Approximately half of this reduction occurred during the initial 15 min (Table). In rats

TABLE: EFFECT OF AIA ON HEPATIC HEMOPROTEINS

HEMOPROTEIN	PHENOBARBITAL-PRETREATED RATS			UNTREATED RATS	
	Control	15 min after AIA	3 h after AIA	Control	3 h after AIA
Cytochrome P-450					
nmol/mg microsomal protein	2.96 ± 0.44	2.13 ± 0.32	1.41 ± 0.30	1.04 ± 0.13	0.82 ± 0.08
(n)	(7)	(7)	(5)	(5)	(5)
Cytochrome b_5					
nmol/mg microsomal protein	0.64 ± 0.20	0.63 ± 0.07	0.73 ± 0.08	0.52, 0.57	0.45, 0.43
(n)	(5)	(4)	(5)	(2)	(2)

Effects of a single intravenous injection of 100 mg/kg AIA on hepatic content of cytochromes P-450 and b_5 in groups of rats. Values represent mean ± SD; n indicates number of animals per group. In each group all differences from control for cytochrome P-450 are significant at $p < 0.005$.

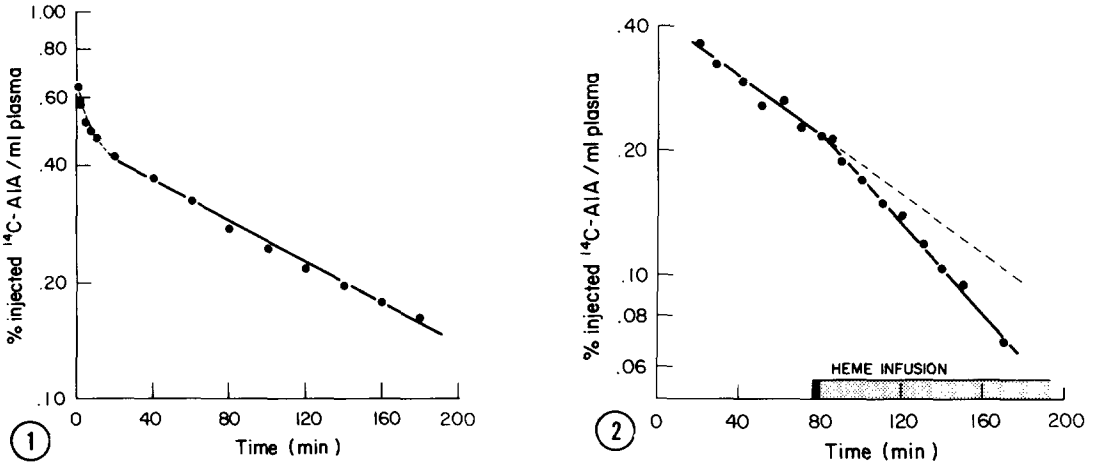

Figure 1. Plasma disappearance of [^{14}C]AIA after its intravenous administration in a single dose of 100 mg/kg body weight at time 0 to a phenobarbital-pretreated rat. The solid line is the computer-calculated line of best fit for [^{14}C]AIA plasma fractional disappearance between 20 and 180 min. It conforms to a single exponential (r^2=0.99) with a disappearance rate, k = -0.0065 min^{-1}. The dashed line represents the computer-calculated sum of this exponential and an earlier fast exponential, k = -0.170 min^{-1}. Results are representative of 3 individual experiments.

Figure 2. Plasma disappearance of [^{14}C]AIA in a phenobarbital-pretreated rat infused with heme beginning 80 min after drug administration. Solid lines are computer-calculated lines of best fit for [^{14}C]AIA plasma disappearance between 20 and 80 min, and between 85 and 170 min. The respective values for k are -0.0084 min^{-1} and -0.0126 min^{-1}. The dashed line is the extrapolation of the single exponential between 20 and 80 min. This study is representative of 6 individual experiments (see results).

that were not pretreated with phenobarbital, the reduction of hepatic cytochrome P-450 content after 3 h was much smaller (Table).

The plasma disappearance of AIA was biphasic (Figure 1), consisting of an initial rapid phase with a $T_{1/2}$ of 2-4 min followed after 20 min by a much slower disappearance phase which exhibited first order kinetics for at least 3 h. The initial rapid phase of plasma disappearance has been ascribed to early metabolism of AIA by the hepatic cytochrome P-450 destined to be destroyed by the drug (22), but it undoubtedly also reflects drug distribution since a similar biphasic isotope disappearance pattern also was observed when a tracer dose of [^{14}C]AIA was administered 30 min after the injection of 100

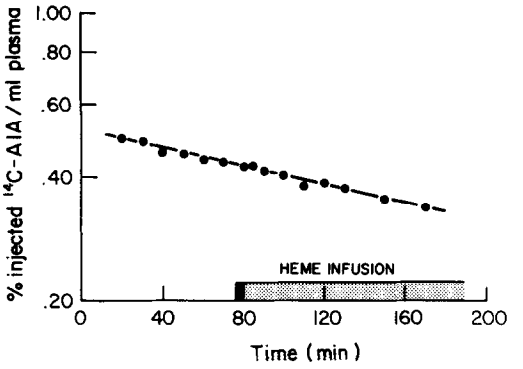

Figure 3. Plasma disappearance of [^{14}C]AIA in a rat that was not pretreated with phenobarbital. The plasma fractional disappearance rate of [^{14}C]AIA, k = -0.0026 min^{-1}, is not altered by heme infusion. Results are representative of 3 individual experiments.

mg/kg of unlabeled AIA. The slower monoexponential disappearance of AIA appears to reflect the rate at which the drug is metabolized in the liver by the residual cytochrome P-450 or by newly reconstituted cytochrome.

Infusion of heme in phenobarbital-pretreated rats during the monoexponential phase of AIA disappearance resulted in a prompt acceleration of the disappearance rate of AIA (Figure 2). During heme treatment, the AIA plasma fractional disappearance rate, k, of 6 rats changed abruptly from (mean $\pm$ SD) - 0.0088 $\pm$ 0.0025 min^{-1} to - 0.0124 $\pm$ 0.0052 min^{-1} ($p = 0.014$), resulting in a reduction of the $T_{1/2}$ from 79 to 56 min. These findings suggest that infused heme served to restore hepatic cytochrome P-450 that had been destroyed by AIA. This conclusion is strongly supported by the observation that effectiveness of heme was critically dependent on prior induction of cytochrome P-450 by phenobarbital pretreatment. In the absence of phenobarbital pretreatment, plasma disappearance of AIA was protracted (k = -0.0026 $\pm$ 0.0007 min^{-1}, $T_{1/2}$ = 267 min), destruction of hepatic cytochrome P-450 by AIA was considerably less (Table), and heme infusion was without effect (Figure 3). It is apparent, therefore, that functional reconstitution of cytochrome P-450 by exogenously administered heme was limited to the phenobarbital-inducible cytochrome subspecies that had been destroyed in the course of AIA metabolism. This selectiv-

ity of infused heme for the phenobarbital-inducible cytochrome P-450 subspecies eliminates the possibility that heme may have affected AIA disposition and metabolism by other mechanisms such as interference with protein binding of the drug, alteration of hepatic blood flow, or stimulation of AIA metabolism by alternate pathways. The rapidity with which heme infusion affects AIA metabolism and its selectivity for the phenobarbital-inducible cytochrome P-450 subspecies make it likely that cytochrome P-450 restoration occurs by incorporation of exogenous heme into apocytochrome that has survived intact the removal or displacement of the original AIA-damaged heme. It remains to be determined whether this is so, or, alternatively but less likely, whether apocytochrome for combination with the infused heme is newly synthesized in the liver.

Functional reconstitution of cytochrome P-450 by intravenous heme administration may be of therapeutic importance. We recently have suggested (4) that incorporation of exogenous heme into hemoproteins provides a plausible explanation for the successful treatment of acute attacks of hepatic porphyria with heme infusion (26, 27). It now is apparent that acute intrahepatic heme deficiency also may result from administration of drugs or toxins that destroy cytochrome P-450 heme (11, 12). In the event of overdosage, this would greatly prolong the rate of drug elimination and may thus result in extended pharmacologic effects. This is evident, for example, from recent European reports of suicidal use of the hypnotic diethylpentenamide (28-30), a congener of AIA. In such instances, heme infusion may be useful for accelerating drug biotransformation.

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