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EFFECT OF HEME ON ALLYLISOPROPYLACETAMIDE-INDUCED CHANGES IN HEME AND DRUG METABOLISM IN THE RHESUS MONKEY (*MACACA MULATTA*)*

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Abstract—In rhesus monkeys, in which porphyria was induced by the administration of allylisopropylacetamide (AIA), hepatic δ-aminolevulinic acid synthase (ALA-S) was increased. Cytochrome P-450 and associated monooxygenase activities and microsomal heme oxygenase activity were decreased in these animals. Administration of heme for 4 days concurrently with AIA prevented the induction of hepatic ALA-S but produced further decreases in cytochrome P-450 and monooxygenase activities. The decrease in heme oxygenase activity elicited by AIA alone was partially reversed. Administration of heme alone caused an impairment of hepatic drug metabolism but had no significant effect on heme metabolism. The porphyric monkeys showed elevation of porphyrin levels in blood and urine. When heme was administered concurrently with AIA, blood porphyrin levels were further elevated, while the urinary excretion of porphyrins was lower than that following treatment of monkeys with AIA. Following the administration of heme alone, blood and urinary porphyrin levels were minimally affected. These results suggest that repeated heme administration in the primate may adversely affect drug metabolism by the liver.

Genetic diseases of the class known as inducible porphyrias are caused by partial blocks in the heme biosynthetic pathway, ultimately resulting in a negative feedback induction of δ-aminolevulinic acid synthase (ALA-S), which is the major rate-limiting enzyme in heme biosynthesis. Dependent upon the defect in the heme biosynthetic pathway, excessive porphyrin precursors may accumulate in the liver, serum, and urine of these patients. Clinically, these patients may experience a wide variety of symptoms, the major manifestation being disturbances of the autonomic nervous system. The underlying mechanisms causing the symptomatology have not been clarified. It has, however, been shown that heme administration can effect remission of acute attacks of acute intermittent porphyria which at times are life-threatening [1, 2]. In acute intermittent porphyria, derangement of heme metabolism has been shown to cause decreases in drug metabolism [3].

A variety of drugs can interfere with heme metabolism and produce porphyria both in humans and in experimental animals. One such agent which induces the biochemical manifestations of experimental porphyria in several species is allylisopropylacetamide (AIA). Treatment of rats with AIA produces a rapid loss of hepatic heme, particularly the heme of phenobarbital-inducible cytochrome P-450, and a concomitant increase in the activity of ALA-S [4]. This induction in ALA-S activity is attributed to an interference with the negative feedback control of ALA-S by heme, and it is prevented by heme administration [5–9].

The present study was undertaken to evaluate the effect of intravenously administered heme, given on several days, on key enzymes of heme and drug metabolism in monkeys treated with AIA. Rhesus monkeys were used in the present studies, since they are used for preclinical drug testing and as models of human responses to drugs and other environmental chemicals. Heme given in a dose schedule used in the treatment of acute attacks of inducible porphyria in humans, was administered to untreated and AIA-treated (porphyric) monkeys. Heme metabolism was evaluated by measuring the activities of ALA-S and microsomal heme oxygenase and the total porphyrin concentrations in liver, blood and urine. Drug metabolism was monitored by measuring cytochrome P-450 content and the activities of the P-450 dependent monooxygenases, benzo[*a*]pyrene hydroxylase and ethylmorphine *N*-demethylase.

MATERIALS AND METHODS

Experimental protocol. Two adult female monkeys, weighing 2.5 and 7.3 kg, were injected on four consecutive days with AIA, 300 $mg \cdot kg^{-1} \cdot day^{-1}$ subcutaneously, and/or with heme, 4 mg/kg intravenously as a bolus. Heme will be used throughout this paper for iron protoporphyrin IX, irrespective of the redox state. Heme, purchased from the Sigma

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Chemical Co., St. Louis, MD, was dissolved in 0.25 M Na_2CO_3 solution adjusted to pH 8 with 0.1 N HCl, diluted with 0.15 M NaCl to a concentration of 2 mg/ml, and passed through a 0.22 μm Swinnex filter. The millipore filtration resulted in a less than 10% loss of heme. This loss in heme concentration was not considered when reporting the amount of heme administered to the monkeys. AIA, a gift from Hoffmann-La Roche Inc., Nutley, NJ, was dissolved in 0.15 M NaCl.

Each experimental period lasted for 5 days, during which 24-hr urine samples were collected on days 3–5. Blood was drawn on day 1, prior to the administration of AIA or heme, and on day 5, i.e. 24 hr after the last injection. Liver biopsies were performed on day 5, with the monkeys placed under ketamine and N_2O anesthesia. On day 5 of the last experimental period, the monkeys were killed by intravenous injection of ketamine and air, and the livers were removed and frozen until assayed. Since previous studies [10] have shown that serum hemopexin levels return to pretreatment values within 2 weeks after a series of heme injections, we assumed that homeostasis of hepatic heme metabolism had occurred and used the same monkey for the next experiment. Sequential studies were carried out in the same monkey at 2-week intervals with the drugs being administered in the following order: heme, AIA, and heme plus AIA.

Sample preparation. Urine samples were collected in containers wrapped in aluminum foil and stored at −20° until they were assayed. Blood samples were drawn in heparinized vacutainers, and hematocrits were determined. The blood samples were quick frozen in liquid nitrogen to lyse the cells. Liver biopsy samples, weighing approximately 3 g each, were placed immediately in 1.15% KCl and rinsed, blotted, weighed, and homogenized in 1.15% KCl solution. Each milliliter of homogenate contained the equivalent of 250 mg of liver, wet weight. Aliquots of this homogenate were used for assaying ALA-S activity and total porphyrin contents. The remaining homogenate was centrifuged at 9000 *g* for 20 min, and an aliquot of supernatant fraction was used for the benzo[*a*]pyrene hydroxylase assay. The remaining supernatant fraction was centrifuged at 105,000 g for 1 hr. The resulting supernatant fraction was used as a source of biliverdin reductase. The microsomal pellet was suspended in 0.1 M KH_2PO_4–Na_2HPO_4 buffer, pH 7.4, such that each milliliter contained microsomes equivalent to 250 mg of liver, wet weight.

Assays. All enzyme assays were performed on liver samples collected on day 5 of each experimental period. ALA-S activity in liver homogenate was determined by the method of Sassa *et al.* [11]. The porphyrin contents of blood and liver were assayed fluorometrically by the method of Granick *et al.* [12] using coproporphyrin as a standard. Cytochrome P-450 was assayed by the method of Omura and Sato [13]. Benzo[*a*]pyrene hydroxylase activity was determined as described previously [14]. Ethylmorphine *N*-demethylase activity of liver microsomes was determined as described by Alvares and Mannering [15]. Addition of heme, in concentrations of up to 20 μM, to microsomal suspensions or to the incubation mixtures used to assay monooxygenase activities, did not affect the enzyme activities. Microsomal heme oxygenase activity was assayed as described previously [11]. Urinary porphyrin levels were measured by the method of Schwartz *et al* [16]. The urinary values were corrected for creatinine excretion, as determined by the method of Bonsnes and Taussky [17].

RESULTS

Although the body weight of the two test animals (2.5 kg for monkey #1 and 7.3 kg for monkey #2) differed greatly, the control values for most of the hepatic variables tested were very similar for both monkeys (Table 1). The greatest difference seen between the control values of the two monkeys was in liver porphyrin content, the initial liver porphyrin levels in the younger monkey being 70% of that of the older monkey.

Effect of AIA and heme on hepatic enzyme activities and porphyrin content. The administration of

Table 1. Effects of AIA and/or heme administration on hepatic heme and drug metabolism*

Assay	Monkey	Control	AIA	AIA + heme	Heme
ALA synthase (nmoles ALA formed · $g^{-1} \cdot hr^{-1}$)	#1	5.89	10.12	5.46	6.72
	#2	5.06	16.01	5.04	7.56
Porphyrin content (pmoles/mg protein)	#1	2.04	5.05	0.96	0.55
	#2	1.40	4.37	1.13	0.38
Cytochrome P-450 (nmoles/mg protein)	#1	1.30	0.26	0.20	0.72
	#2	1.26	0.45	0.18	0.60
Benzo[*a*]pyrene hydroxylase [nmoles OHBP formed · (mg protein)$^{-1} \cdot hr^{-1}$]	#1	20.27	3.26	0.77	9.53
	#2	23.70	6.39	0.46	9.79
Ethylmorphine *N*-demethylase [nmoles HCHO formed · (mg protein)$^{-1} \cdot hr^{-1}$]	#1	502.4	291.2	199.0	317.4
	#2	505.0	360.0	168.3	346.4
Microsomal heme oxygenase [nmoles bilibrubin formed · (mg protein)$^{-1} \cdot hr^{-1}$]	#1	3.00	0.31	1.96	2.43
	#2	2.76	0.94	1.66	2.99

* Two female rhesus monkeys weighing 2.5 kg (#1) and 7.3 kg (#2) were used in this study. Each animal served as its own control and was administered 300 mg · $kg^{-1} \cdot day^{-1}$ AIA subcutaneously and/or 4 mg · $kg^{-1} \cdot day^{-1}$ heme intravenously for 4 days. Liver biopsy samples were taken 24 hr after the last injection. Each value is the mean of duplicate determinations.

AIA for 4 days caused a significant alteration in all of the variables examined (Table 1). The ALA-S activity increased 2- to 3-fold. A concomitant rise in hepatic porphyrin content was observed. The cytochrome P-450 contents decreased to 20 and 36% of control values. Likewise, the activities of the two cytochrome P-450-dependent monooxygenases decreased after AIA treatment, with benzo[*a*]pyrene hydroxylase activity being affected to a greater degree than ethylmorphine *N*-demethylase activity. The hydroxylase activity decreased to 16 and 27% of control values, whereas *N*-demethylase activity decreased to 58 and 71% of control values. The activity of liver microsomal heme oxygenase activity was also decreased, i.e. to 10 and 34% of control values. Liver samples from both monkeys treated with AIA had a greenish tint, which can be attributed to adduct formation of heme and AIA metabolite [18, 19].

When heme was administered concurrently with AIA, hepatic ALA-S levels returned to control levels (Table 1). The mean hepatic porphyrin content was decreased in the two monkeys studied. Although heme administration prevented the AIA-induced increase in ALA-S activity and porphyrin accumulation, it did not prevent the decrease in drug-metabolizing activities. Cytochrome P-450 content and the activities of the two monooxygenases fell to even lower levels than those observed after treatment of the monkeys with AIA. The cytochrome P-450 contents were 15 and 14%, ethylmorphine *N*-demethylase activities were 40 and 33%, and benzo[*a*]pyrene hydroxylase activities were 4 and 2% of the respective control values in liver biopsy samples of monkeys #1 and #2. The heme oxygenase activities were reduced to a lesser extent than after AIA treatment, the reduction being 65 and 60% of the control values.

The administration of heme alone daily for 4 days to the monkeys appeared to affect the drug-metabolizing activities to a much greater extent than the activities of ALA-S and heme oxygenase. The ALA-S activity was increased slightly and microsomal heme oxygenase activity was 81 and 108% of the control values in monkeys #1 and #2 respectively. Cytochrome P-450 concentrations in liver microsomes decreased to 55 and 48% of control values. Benzo[*a*]pyrene hydroxylase activities fell to 47 and 41% of control values, and ethylmorphine *N*-demethylase activities fell to 63 and 69% of control values in monkeys #1 and #2 respectively. As with AIA treatment, the hydroxylase activity appeared to be affected to a greater extent than the demethlyase activity.

Effect of AIA and heme on blood and urinary porphyrin contents. Blood porphyrin levels were determined 24 hr after the last injection of AIA and/or heme. Control values were obtained by drawing blood prior to initiation of each treatment. In data not shown, blood porphyrin levels returned to baseline levels before the start of each experimental period. Treatment with AIA produced a 50 and 47% increase in blood porphyrins (Table 2). Administration of AIA and heme increased blood porphyrin contents even further, to approximately 2-fold of control values. Administration of heme alone produced minimal changes in the porphyrin concentrations.

Changes in urinary porphyrin concentrations elicited by the administration of AIA and/or heme are shown in Table 2. Monkeys receiving AIA showed a 3- to 4-fold increase in urinary porphyrin excretion over the 3-day experimental period. Concomitant administration of heme with AIA partially reversed the marked urinary excretion of heme precursors observed when the monkeys were treated with AIA. Administration of heme alone caused an approximately 50% mean increase in urinary porphyrin excretion. The data demonstrate that the minimal effects observed in heme metabolism in livers of monkeys treated with heme are reflected in minimal effects on porphyrin excretion. However, heme did partially reverse the marked increase in porphyrin excretion elicited by AIA.

DISCUSSION

The present study was undertaken to evaluate the effect which repeated intravenous heme injections may exert on hepatic heme metabolism in a primate model of the inducible porphyrias. Attention was focused on monitoring changes occurring in the liver since this is the major site of porphyrin and porphyrin precursor overproduction in these diseases. In a previous study with human subjects, a single dose of heme resulted in a decrease in the biliary secretion of coproporphyrin [20]. Since liver biopsy sampling may not be justified in porphyric patients, the present studies were carried out in rhesus monkeys. Results of studies in rodents may, because of

Table 2. Changes in blood and urinary porphyrin levels during the course of treatment with AIA and/or heme*

Assay	Monkey	Control	AIA	AIA + heme	Heme
Blood porphyrin content	#1	0.18	0.27	0.39	0.15
[nmoles·(ml RBC)$^{-1}$]	#2	0.19	0.28	0.68	0.10
Urinary porphyrin excretion	#1	128	384	261	202
(nmoles/24 hr)	#2	134	488	383	211

* Monkeys were treated with AIA and/or heme as described in the legend to Table 1. Blood samples were drawn immediately before each treatment period and 24 hr following the last injection. Each blood porphyrin content value is the mean of duplicate determinations, except the control value which is the mean of duplicate determinations of four blood samples drawn prior to each 2-week experimental period. Urine data are corrected using the 24-hr creatinine excretion in urine. Each value is the mean of three 24-hr urine samples, except the controls which are the mean of nine 24-hr urine samples.

known species differences in drug metabolism, not be applicable to primates.

We chose to analyze hepatic heme and drug metabolism after 4 days of heme, 4 mg/kg, treatment because of the following observations: (a) large fluctuations in hepatic ALA-S activity are observed up to 3 days after a single injection of heme to rats [5, 21]; (b) levels of hemopexin, the major heme binding serum protein which is produced in the liver and the synthesis of which responds rapidly to the level of circulating heme, shows no fluctuations after the third day of daily heme administration in rhesus monkeys [10]; (c) heme is being administered to humans intravenously at a dosage of 4 mg/kg for several days in treatment of acute attacks of inducible porphyrias [1, 2]; and (d) in a study in humans, we failed to observe an increase in the biliary excretion of bilirubin up to 8 hr following a single bolus infusion of heme [20].

The control values of cytochrome P-450 and ethylmorphine *N*-demethylase in the liver of the rhesus monkeys observed in this study were similar to the values reported by Litterst *et al.* [22] for female rhesus monkeys. In the present study, treatment of the monkeys with AIA produced changes in heme and drug metabolism which paralleled changes observed by others using the rat. For example, following AIA treatment of the monkeys, ALA-S activity was increased, while microsomal cytochrome P-450 content was decreased. Concomitant with this decrease in the hemeprotein, the activities of both benzo[*a*]pyrene hydroxylase and ethylmorphine *N*-demethylase were reduced markedly below control values. Benzo[*a*]pyrene hydroxylase activity was decreased to a greater extent than *N*-demethylase activity. These changes in rates of drug metabolism in primates may be of clinical significance. In a previous study [3], the mean antipyrine half-life in the plasma of a group of ten patients with hereditary hepatic porphyria (including eight with acute intermittent porphyria) was significantly greater than that observed in twenty normal subjects and in seven asymptomatic gene carriers of acute intermittent porphyria. In contrast to the data obtained with antipyrine, the porphyric patients did not show any significant difference in the mean plasma elimination rate of phenylbutazone, when compared to normal subjects. Both drugs are metabolized by the cytochrome P-450 system and have been used to assess cytochrome P-450 function *in vivo* in humans. In AIA-treated experimental animals, decreases in rates of drug metabolism are related to suicide inactivation of cytochrome P-450 by AIA. This mechanism, however, would not account for the decrease in antipyrine metabolism observed in humans having the genetic disease. In the present investigation, microsomal heme oxygenase was decreased after AIA administration, a finding similar to that reported for AIA-treated rats by Rothwell *et al.* [23], Maines and Kappas [24], and by Bissell and Hammaker [9]. In the monkeys studied, AIA treatment increased total blood porphyrin levels and increased the urinary excretion of heme precursors, findings similar to those reported for rodents [25].

Heme administered concurrently with AIA blocked the induction of ALA-S and caused a decrease in porphyrin content in the liver, when compared to the values obtained in monkeys administered AIA. These findings are similar to those seen in rodents [25]. The combined effect of AIA and heme on the drug-metabolizing enzyme system was profound. Cytochrome P-450 levels fell to 15% of control levels and the activity of benzo[*a*]pyrene hydroxylase decreased to 3% of the control activity. The additive effect of heme administration on the AIA-mediated reduction in drug metabolism in the rhesus monkeys is a novel finding. Previous short-term experiments have shown that, in phenobarbital-treated rats, heme administration partially restored the reduction of cytochrome P-450 and associated enzymic activities [26, 27]. In one study [28], heme administered intraperitoneally to rats at dosages higher than those used in the present studies did not reverse the decrease in cytochrome P-450 content induced by AIA. The decrease in microsomal heme oxygenase activity following AIA treatment was partially reversed by the concomitant administration of heme, a finding similar to that observed for the rat by Bissell and Hammaker [9].

Some of the results obtained after the administration of heme *per se* were unexpected. The activity of hepatic heme oxygenase in the monkeys did not increase. Since heme oxygenase converts heme to biliverdin, which in turn is converted in the cytosol to bilirubin, this finding is of interest in context with the observation of a lack of biliary excretion of bilirubin up to 8 hr after a single injection of heme to humans [20]. These observations suggest that, in the primate, administration of heme need not increase heme oxygenase activity and associated bilirubin formation. Although in the present studies, the heme degradative enzymic activity was not altered significantly, a definite decrease in cytochrome P-450 was observed. Similar findings have been reported using rats after repeated heme injections [8, 28, 29]. These data suggest that in primates, such as the rhesus monkeys, heme administered at a dose of $4\ mg \cdot kg^{-1} \cdot day^{-1}$ for several days leads to the destruction of cytochrome P-450 heme by a pathway independent of heme oxygenase, as has been suggested previously to occur in rats [30].

Whether the changes observed in the present studies using rhesus monkeys occur in patients with porphyria, who are treated with heme, is not known at the present time. The most significant effect of repeated heme administration in the porphyric monkeys was on drug metabolism. Since heme administration is currently being used in the treatment of acute intermittent porphyria, it is important to further investigate the optimal mode of administration and schedule of heme dosage on heme and drug metabolism.

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