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EFFECT OF DESFERRIOXAMINE AND CHRONIC IRON DEFICIENCY ON HEME METABOLISM

COMPARISON WITH THE PORPHYROGENS 2-ALLYL-2-ISOPROPYL-ACETAMIDE AND 3,5-DIETHOXYCARBONYL-1,4-DIHYDROCOLLIDINE

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Abstract—Desferrioxamine (DF), an iron chelator, inhibits mitochondrial heme synthase (heme-S) activity and stimulates the activity of microsomal heme oxygenase (MHO) in the liver of rats. However, the inhibitory effect on heme-S is not accompanied by an increased activity of δ-aminolevulinic acid synthase (ALA-S). Consequently, no prolonged accumulation of porphyrins is found in the liver. The proposed mechanism of the induction of ALA-S activity by the porphyrogen, 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC), has been thought to involve first a decrease in heme-S activity followed by an increase in ALA-S activity. DF and DDC both inhibit heme-S, but ALA-S was not induced by DF as it was by DDC. Therefore, the concept of derepression of ALA-S following decreased heme production cannot in itself explain the observed results. However, they do indicate a relationship between iron and heme metabolism which is above that of a simple reduction in substrate availability for heme formation by DF and do implicate a role for iron in the regulation of ALA-S activity by heme.

The clinical use of desferrioxamine (DF) has found wide acceptance in the treatment of iron excess [1–6]. Prolonged DF therapy to prevent iron accumulation and progression of hepatic fibrosis in certain chronic anemias has been performed successfully [7]. Yet it is not known which hepatic iron source in humans is chelated [8] and whether changes in iron metabolism, such as those produced by DF treatment, have an effect on the synthesis and/or degradation of hepatic heme* and hemeproteins.

The iron chelated by DF in the normal rat is derived from parenchymal cells and is excreted in the bile [9, 10]. Recently, Hershko *et al.* [11, 12] found evidence for another pathway of iron chelation by DF using normal and hyper-transfused rats. This involved chelation of extracellular iron with subsequent excretion in the urine. Whether DF has a direct effect on hepatic enzyme systems which utilize iron has not been investigated.

A study from this laboratory in starved rats [13] showed a reduction in the iron content of liver ferritin to 60 per cent of control values from 4 to 16 hr after DF administration. This reduction in liver ferritin iron was much greater, i.e. to 5 per cent of control values, in animals kept on a low-iron diet for 10 days before receiving DF. The total liver porphyrin content transiently increased 3-fold at 4 hr after DF administration and was further increased when the iron intake had been restricted. The level of the major hemeprotein of the liver, cytochrome P-450, was unaffected by DF or dietary iron restriction alone, but decreased between 2 and 8 hr after DF treatment in the iron-deficient rats. It was postulated that under these conditions, DF inhibits heme synthesis by making iron unavailable for insertion into protoporphyrin at the heme synthase, heme-S (ferrochelatase EC 4.99.1.1), step. In another preliminary study employing the isolated perfused rat liver [14], an increase in bile bilirubin content caused by DF was interpreted to represent increased liver heme catabolism.

The purpose of the present study, the results of which have been presented in abstract form [15], was to extend previous work by investigating the hepatic activities of key enzymes in heme metabolism: two enzymes of the heme biosynthetic pathway, heme-S and δ-aminolevulinic acid synthase, ALA-S [succinyl-CoA: glycine *c*-succinyl transferase (decarboxylating) EC 2.3.1.37], and the first enzyme of heme degradation, MHO [heme, hydrogen donor: oxygen oxidoreductase (α-methene-oxidizing, hydroxylating) EC 1.14.99.3], under conditions of acute and chronic iron deficiency. We expected to answer the following questions: (1) does acute iron withdrawal by DF exert its effect on heme metabolism principally by removing the iron substrate of heme-S and/or by changes in heme turnover influencing MHO activity, (2) does chronic iron deficiency, like DF injection, cause a similar effect on the activities of heme-S, MHO and ALA-S, and (3) do changes occur in response to porphyrogens in chronic and acute iron deficiency? Thus, the effects of two porphyrogens, 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) and 2-allyl-2-isopropylacetamide (AIA), in the presence of acute iron withdrawal were compared with the effects of the two drugs in chronic iron deficiency. Both of these compounds affect heme metabolism concomitantly with a decrease in cytochrome P-450 [16–18]. One site of DDC action is considered to

* Heme is defined as iron protoporphyrin IX.

be inhibition of heme-S activity (19–21). AIA administration exerts its effect through increased ALA-S activity by a mechanism not yet shown to be associated with concurrent inhibition of another enzyme involved in heme synthesis. instead, AIA is considered to act via negative feedback inhibition by changing a hepatic regulatory heme pool [22].

MATERIALS AND METHODS

Animals and dosages. Male Sprague–Dawley rats (Simonsen Laboratories, Gilroy, CA) were housed in plastic cages with stainless steel grid floors. Rats (250–400 g, at time of injection) were fed on Purina Lab Chow (Ralston Purina Co., St. Louis, MO) and were allowed tap water *ad lib.* Food, as in previous studies [14, 15] was withheld from the rats at 9.00 a.m. on day 1, and on day 2 either AIA (300 mg/kg, s.c., 20 mg AIA/ml of 0.15 M saline) or DDC (300 mg/kg,i.p., in 1.0 ml of warm corn oil) was injected at 5.00 p.m. Desferal mesylate (desferrioxamine mesylate, CIBA, NJ) was injected (100 mg/kg, i.m., in 1.0 ml saline) at 5.00 p.m. on both day 1 and day 2. Under light ether anesthesia, the rats were decapitated 16 hr after the last injection and the liver, or liver perfused with 50 ml of ice-cold 0.15 M saline through the portal vein, was quickly rinsed in cold water, blotted and placed in ice-cold 0.25 M sucrose.

Animals used in the study of chronic deficiency were housed in separate quarters (as were appropriate control animals), and kept strictly free of detergent and insecticides. Weanling animals (50–60 g) were fed for 30 days on McCall's low iron test diet (Teklad Test Diets, Madison, WI; iron content 1.4 to 2.8 mg/kg) as described previously [13]. The rats received tap water to drink and were fasted for 48 hr before death. In some experiments, weanling rats were divided into two groups. The body weights of the animals fed Purina Chow were 175–245 g after 30 days, whereas those on the low iron diet weighed considerably less, ranging from 100 to 155 g. AIA and DDC were injected as described above, and control animals were injected with the appropriate vehicle. For the time course experiments, AIA or DDC was injected at 8.00 a.m. (the 10 hr time point rats were injected at 6.00 a.m. with AIA and at 5.00 a.m. with DDC), and rats were killed 0, 1, 2, 4, 10 and 24 hr later.

Liver ferritin content and liver mitochondrial ALA-S activity. These were determined as described previously [13] using saline perfused livers in most cases. Microsomes were prepared from the post-mitochondrial supernatant fraction (10,000 *g* for 10 min) in 0.25 M sucrose and 0.1 mM EDTA by centrifugation at 105,000 *g* for 1 hr. The microsomal pellet was rinsed, covered with 1.0 ml of 0.1 M sodium phosphate, pH 7.4, and 1 mM EDTA, frozen in liquid nitrogen and stored at −70°. Cytochromes P-450 and b_5 were determined by difference spectra [23, 24] after suspension of the pellet in 0.1 M phosphate (pH 7.4) and 1 mM EDTA. Protein for MHO and heme-S assays was determined by the Biuret method [25] and for the ALA-S assay by the method of Lowry *et al.* [26], using bovine serum albumin (fatty acid free, Sigma St. Louis, MO) as a standard.

MHO activity. MHO activity in liver and spleen was measured according to the method of Tenhunen *et al.* [27], as modified by Bissell *et al.* [28]. Formation of bilirubin was determined from the increase in absorbance at 468 nm recorded from 1 to 6 min after addition of NADPH to the sample cuvette (extinction coefficient 60 mM^{-1} cm^{-1}).

Mitchondrial heme-S. This activity was determined

Table 1. Effects of AIA, DDC and/or desferal on ALA-S, heme-S and MHO enzyme activity and total prophyrin in the rat liver*

	ALA-S	Heme-S (nmoles hr^{-1} mg $protein^{-1}$)	MHO+	Total porphyrin (μg porphyrins/g liver)
Control	0.59 ± 0.12 (8)	14.11 ± 1.65 (12)	0.37 ± 0.06 (12)‡	1.77 ± 0.14 (6)
AIA	2.14 ± 0.12§ (4)	11.9 ± 0.88 (8)	0.20 ± 0.03‖ (8)¶	8.17 ± 2.35**(4)
DDC	5.97 ± 0.95§ (10)	3.46 ± 0.35§ (8)	0.82 ± 0.28 (7)	47.89 ± 16.63§ (4)
Desferrioxamine	0.78 ± 0.08 (12)	7.09 ± 0.83** (12)	0.70 ± 0.05**(12)	1.83 ± 0.08 (4)
AIA + desferrioxamine	2.26 ± 0.30§ (6)	7.06 ± 0.70** (6)	0.15** (6)++	ND‡‡
DDC + desferrioxamine	2.02 ± 0.29§ (6)	1.33 ± 0.11§ (6)	1.12 ± 0.10§ (6)	ND

* Rats weighing 250–400 g were fasted for 2 days before being killed. Sixteen hr before death, each rat received AIA (300 mg/kg, s.c.) or DDC (300 mg/kg, i.p.) and/or desferrioxamine (100 mg/rat, i.m.). Desferrioxamine was administered twice, 40 hr and 16 hr before death. Results are expressed as means ± S.E.M. with numbers of experiments in parentheses.

+ The lowest activity that could be measured for MHO with the assay used in this study was 0.15 nmole hr^{-1} mg $protein^{-1}$. For the purpose of statistical analysis, activities too low to be measured were given the value of 0.15.

‡ Two out of twelve were less than 0.15 nmole hr^{-1} mg protein $^{-1}$.

§ $P < 0.001$.

‖ $P < 0.05$.

¶ Four out of eight were less than 0.15 nmole hr^{-1} mg $protein^{-1}$.

** $P < 0.01$.

++ All six values were less than 0.15 nmole hr^{-1} mg protein $^{-1}$.

‡‡ ND = not determined.

using a method described by Porra [29]; total liver porphyrin was measured according to Abbritti and De Matteis [30].

RESULTS

Effects of AIA, DDC and/or DF on ALA-S, heme-S and MHO activities and total porphyrin in the rat liver. A comparison of the effect of the porphyrogens with DF (Table 1) showed the expected increase in ALA-S activity after AIA or DDC (17), whereas the iron chelator, DF, had no effect. Heme-S activity was not decreased significantly after AIA treatment. The inhibitory effect of DDC and DF was additive. The enzyme activity was decreased to such an extent that only 10 per cent of the control values was found when DDC and DF were administered together. The 45 per cent decrease in MHO activity by AIA was decreased further by DF to levels below 0.15 nmole mg^{-1} hr^{-1}. On the contrary, the activity of MHO was doubled by DF and further increased when DDC was given together with DF. The mean MHO activity after DDC was elevated, but because of the wide individual variation was not significantly different from controls ($P > 0.05$). In the spleen (Table 2) the activity was reduced after AIA and especially after a combination of AIA and DF. Contrary to the liver enzyme, neither treatment with DF nor treatment with DF in combination with DDC led to an increase in MHO activity.

DF did not affect the hepatic porphyrin content (Table 1), which rose in response to AIA and especially after DDC administration.

Table 2. Effects of AIA, DDC and/or desferal on MHO enzyme activity in the rat liver and spleen*

	MHO† (nmoles hr^{-1} mg protein $^{-1}$) Liver	Spleen
Control	0.37 ± 0.06 (12)‡	2.61 ± 0.42 (6)
AIA	0.20 ± 0.03 (8)§	1.82 ± 0.21 (4)
DDC	0.82 ± 0.28 (7)	ND‖
Desferrioxamine	0.70 ± 0.05¶ (12)	2.45 ± 0.17 (12)
AIA + desferrioxamine	0.15¶ (6)**	1.01 ± 0.14¶ (6)
DDC + desferrioxamine	1.12 ± 0.10†† (6)	2.35 ± 0.32 (6)

* Rats weighing 250–400 g were fasted for 2 days before being killed. Sixteen hr before death, each rat received AIA (300 mg/kg, s.c.) or DDC (300 mg/kg, i.p.) and/or desferrioxamine (100 mg/rat, i.m.). Desferrioxamine was administered twice, 40 hr and 16 hr before death. Results are expressed as means ± S.E.M. with numbers of experiments in parentheses.

† The lowest activity that could be measured for MHO with the assay used in this study was 0.15 nmole hr^{-1} mg protein $^{-1}$. For the purpose of statistical analysis, activities too low to be measured were given the value of 0.15.

‡ Two out of twelve were less than 0.15 nmole hr^{-1} mg protein $^{-1}$.

§ Four out of eight were less than 0.15 nmole hr^{-1} mg protein $^{-1}$.

‖ ND = not determined.

¶ $P < 0.01$.

** All six values were less than 0.15 nmole hr^{-1} mg $protein^{-1}$.

†† $P < 0.001$.

Table 3. Effect of a single dose of AIA or DDC on ALA-S, heme-S and MHO enzyme activity of normal and iron-deficient rat livers*

	ALA-S	Heme-S (nmoles hr^{-1} mg $protein^{-1}$)	MHO†
Control	0.34 ± 0.02 (6)	6.99 ± 0.78 (12)	0.48 ± 0.06 (12)
AIA	2.36 ± 0.16‡ (6)	4.32 ± 0.68§ (6)	0.22 ± 0.05‖ (6)¶
DDC	1.04 ± 0.16‖ (6)	0.73 ± 0.14‖ (6)	0.39 ± 0.05 (6)
Iron-deficient	0.26 ± 0.02 (12)	4.43 ± 0.58§ (12)	0.44 ± 0.05 (12)
Iron-deficient + AIA	4.72 ± 0.66‡ (6)	2.37 ± 0.31‡ (6)	0.33 ± 0.04 (6)
Iron-deficient + DDC	4.84 ± 1.30‡ (6)	0.84 ± 0.14‡ (6)	0.93 ± 0.20‖ (6)

* Control (175–245 g) and iron-deficient (100–155 g) rats of the same age were fasted for 2 days before being killed. The drugs were administered as described in the legend to Table 1 and under Materials and Methods.

† The lowest activity that could be measured for MHO with the assay used in this study was 0.15 nmole hr^{-1} mg $protein^{-1}$. For the purpose of statistical analysis, activities too low to be measured were given the value of 0.15.

‡ $P < 0.001$.

§ $P < 0.05$.

‖ $P < 0.01$.

¶ Four out of six were less than 0.15 nmole hr^{-1} mg $protein^{-1}$.

Effects of AIA and DDC on liver enzymes in iron-deficient rats. Heme-S was the only enzyme activity which was decreased in iron deficiency *per se* (Table 3). Chronic iron deficiency, similar to the effect of acute iron withdrawal by DF, potentiated the effect of these porphyrogens on heme-S and ALA-S activities up to 4-fold. In iron-deficient rats, only DDC caused a significant rise in MHO activity.

Temporal effects of AIA and DDC on hepatic enzymes in iron deficiency. AIA treatment caused the ALA-S activity to increase during the 24-hr period (Fig. 1A), while that of heme-S rose within 1 hr after administration of the drug and then gradually decreased to less than preinjection values at 24 hr.

In response to DDC treatment, ALA-S activity was optimal around 10 hr after administration and had not returned to preinjection values at 24 hr. Heme-S activity was greatly reduced within 1 hr and remained low throughout the experiment.

MHO activity was inhibited within 1 hr after treatment with AIA, and this low activity level was maintained for the duration of the experiment (Fig. 1C). To determine whether this effect was due to a soluble inhibitor which was formed after treatment with AIA, equal amounts of post-mitochondrial supernatant fluid of a liver from an AIA-treated rat (16 hr after AIA) and that of a control rat were mixed. the resulting MHO activity was equal to half the sum (0.47 nmole $mg^{-1} hr^{-1}$) of activities from the AIA-treated rat (0.29 nmoles mg^{-1} hr^{-1}) and the control (0.79 nmole mg^{-1} hr^{-1}). Thus, the presence of an inhibitor is unlikely. DDC stimulated MHO activity, reaching a maximum at 10 hr after its administration.

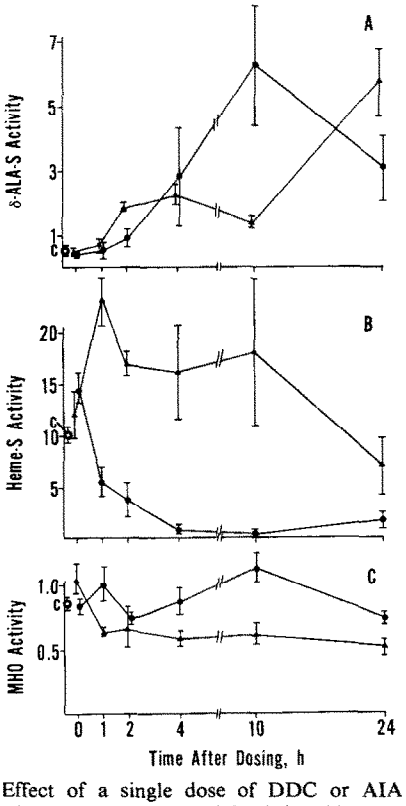

Fig. 1. Effect of a single dose of DDC or AIA on δ-aminolevulinic acid synthase activity (A) and heme synthase activity (B) of rat liver mitochondria and heme oxygenase activity (C) of rat liver microsomes. Each value is the mean ± S.E. for four to six experiments. The rats were iron-deficient and starved for 48 h before decapitation; 0, 1, 2, 4, 10 and 24 hr prior to death, the animals received DDC (300 mg/kg, i.p., in 1.0 ml warm corn oil) (●—●) or AIA (300 mg/kg, s.c., 20 mg AIA/ml of 0.15 M saline) (▲—▲). C = rats received no drugs. Panel A: δ-ALA-S = δ-aminolevulinic acid synthase activity in nmoles ALA hr^{-1} mg $protein^{-1}$. Panel B: heme-S = heme synthase activity in nmoles heme hr^{-1} mg $protein^{-1}$. Panel C: MHO = microsomal heme oxygenase activity in nmoles bilirubin hr^{-1} mg $protein^{-1}$.

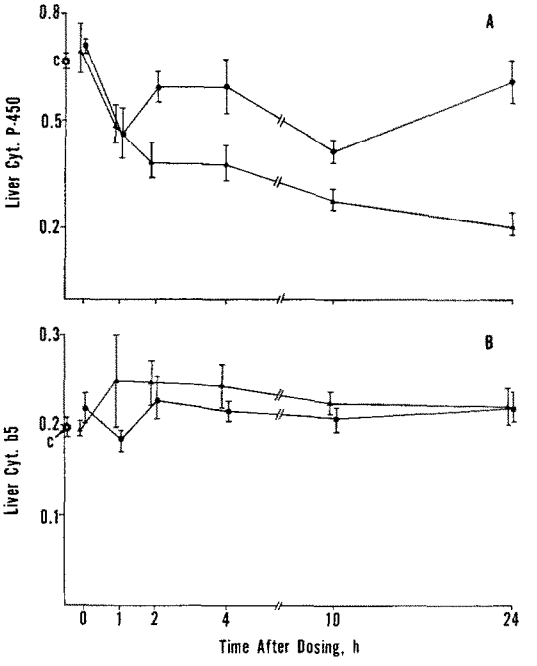

Fig. 2. Effect of a single dose of DDC or AIA on cytochrome P-450 (A) and cytochrome b_5 (B) levels of rat liver microsomes. The rats were treated as described in the legend to Fig. 1. Panel A: liver cytochrome P-450 in nmoles mg $protein^{-1}$. Panel B; liver cytochrome b_5 in nmoles mg $protein^{-1}$.

Effects of AIA and DDC on liver cytochrome P-450 and b_5 *in iron-deficient rats.* Cytochrome P-450 levels (Fig. 2A) in the iron-deficient rats were reduced significantly 1–2 hr after AIA or DDC, as had been observed after a single injection of DF [13]. The cytochrome P-450 levels in the rats treated with AIA continued to decline and were still only 30 per cent of controls 24 hr later. All microsome fractions after AIA administration to these iron-deficient rats were green in color. The intensity of the color was the same as seen previously with phenobarbital-pretreated animals [31]. The cytochrome P-450 levels after DDC also decreased, but had returned nearly to control values 24 hr later. On the other hand, cytochrome b_5 concentrations (Fig. 2B) were not affected significantly by treatment with AIA or DDC.

DISCUSSION

Several observations were apparent when the activities of the three enzymes of heme metabolism were compared among controls, iron-deficient rats and rats treated with DF. Heme-S activity was inhibited by DF as well as by DDC, but in contrast liver porphyrin

levels were not elevated 16 hr after DF, probably because the ALA-S activity was not increased. DDC and DF given in combination had an additive effect on Heme-S, suggesting a different mechanism of action for each of these agents. Hence, the effects of DF on heme metabolism appeared to be more complex than a simple removal of iron as substrate.

MHO activity increased after DF administration, but when given in combination with DDC the effect was additive, again implying the involvement of different mechanisms. On the other hand, AIA inhibited MHO activity, which was in agreement with a study by Rothwell *et al.* [32]. This inhibition was still maintained in the presence of DF, and preliminary experiments suggested that it was not due to a soluble inhibitor formed after treatment with AIA. The changes in MHO activities after DF, DDC or AIA were consistent with the observed bilirubin excretion patterns from endogenous and exogenous heme in a previous study using isolated perfused rat livers [14].

The lack of an effect of DF on ALA-S might depend on the extent of inhibition of heme-S necessary to cause a sufficient lowering of heme level with release of end-product repression sufficient to result eventually in ALA-S induction. The hepatic effect of DDC entails a decrease in heme-S activity, porphyrin accumulation and a lowering of heme production which causes derepression of ALA-S activity. We had expected to see a similar increase in ALA-S activity after DF treatment because of our previous observation of a transient hepatic porphyrin accumulation [13] and because of the decrease in heme-S activity. The lack of enhancement of ALA-S activity by DF may have several causes. The extent or duration of the inhibition of heme-S activity by DF may not have lowered heme production sufficiently for the end-product to derepress the activity of ALA-S. Alternatively, the iron–chelator complex, may, like heme, act as a repressor of ALA-S activity, or iron chelated by DF may play yet another role in the full expression of ALA-S. These mechanisms would explain why DF treatment did not enhance the stimulatory effect of AIA and DDC on ALA-S activity, which was seen in iron deficiency. The complexity of the drug effects do not allow further interpretation. The concepts of end-product repression by heme or by iron chelates cannot explain all the differences in the activities of heme-S and MHO observed in response to the various treatments with or without iron deficiency. Also, heme-S activity was decreased by AIA only after pretreatment with DF or in the iron-deficient animals. Thus, results of our studies suggest that the form and amount of iron in the liver exert an effect on heme metabolism above that of a simple reduction in substrate availability for heme formation. Whether the iron affecting the enzyme activities is ferritin bound [13], is the iron of iron-protoporphyrin as suggested by Maines and Kappas [33], or in some other form of hepatic cellular iron [34, 35] will require further investigations.

In chronic iron deficiency, as in normal animals [16,17], cytochrome P-450 levels were decreased within 1 hr after AIA or DDC. This decrease in iron deficiency is probably also due to a loss of pre-existing heme of cytochrome P-450. The effect of AIA on cytochrome P-450 was much greater than that observed previously after DF administration and was maintained for a much longer period of time [13]. These changes in iron deficiency may result in part from increased hepatic metabolism, as shown recently by Becking [36], which could produce alterations in AIA metabolism together with lipid peroxidation. The effects with time on heme metabolism of compounds such as AIA and DDC, which are metabolized by the cytochrome P-450 system, were different from normal [16, 18, 37], although in chronic iron deficiency cytochrome P-450 levels are maintained.

We have shown in this study that two injections of DF cause an inhibition of heme-S and a stimulation of MHO. This may reduce the available heme in the liver, affecting the turnover of heme proteins. Whether prolonged DF administration to humans affects the activity of these enzymes of heme metabolism, as well as enzymes which use heme for their prosthetic group, should be investigated.

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