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Research Article

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Differential Effects of Metalloporphyrins on Messenger RNA Levels of δ-Aminolevulinate Synthase and Heme Oxygenase

Studies in Cultured Chick Embryo Liver Cells

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Abstract

The acute porphyrias in relapse are commonly treated with intravenous heme infusion to decrease the activity of δ-aminolevulinic acid synthase, normally the rate-controlling enzyme in heme biosynthesis. The biochemical effects of heme treatment are short-lived, probably due in part to heme-mediated induction of heme oxygenase, the rate-controlling enzyme for heme degradation. In this work, selected nonheme metalloporphyrins were screened for their ability to reduce δ-aminolevulinic acid synthase mRNA and induce heme oxygenase mRNA in chick embryo liver cell cultures. Of the metalloporphyrins tested, only zinc-mesoporphyrin reduced δ-aminolevulinic acid synthase mRNA without increasing heme oxygenase mRNA. The combination of zinc-mesoporphyrin and heme, at nanomolar concentrations, decreased δ-aminolevulinic acid synthase mRNA in a dose-dependent manner. The combination of zinc-mesoporphyrin (50 nM) and heme (200 nM) decreased the half-life of the mRNA for δ-aminolevulinic acid synthase from 5.2 to 2.5 h, while a similar decrease was produced by heme (10 μM) alone (2.2 h). The ability of zinc-mesoporphyrin to supplement the reduction of δ-aminolevulinic acid synthase mRNA by heme, in a process similar to that observed with heme alone, provides a rationale for further investigation of this compound for eventual use as a supplement to heme therapy of the acute porphyrias and perhaps other conditions in which heme may be of benefit. (*J. Clin. Invest.* 1994. 94:649–654.) Key words: δ-aminolevulinic acid synthetase • heme • heme oxygenase • metalloporphyrins • RNA, messenger

Introduction

Acute intermittent porphyria (AIP)[1] the most common form of the acute hepatic porphyrias (1–3), is due to an autosomal dominantly inherited defect in porphobilinogen (PBG) deaminase. During the acute phase of AIP, urinary excretions of δ-aminolevulinic acid (ALA) and PBG increase 20–200-fold, and excretions of uroporphyrin and coproporphyrin are also increased (1). Other acute hepatic porphyrias include hereditary coproporphyria and variegate porphyria (4). The biochemical feature common to all acute porphyrias in relapse is an uncontrolled induction of ALA synthase (E.C. 2.3.1.37), the first and normally rate-controlling enzyme in heme biosynthesis (1–3). The treatment of choice, for all but mild attacks of acute porphyria, is intravenous heme (5–8), first described 23 yr ago (8). Heme reduces hepatic ALA synthase levels in various experimental models of acute porphyria (9–12) and apparently has the same effect in patients suffering from acute porphyria (5–8). The mechanism(s) whereby heme exerts a repressive effect on hepatic ALA synthase may be manifold, including decreasing mRNA stability (13, 14) and decreasing import of the enzyme into mitochondria (15, 16).

Metalloporphyrins which inhibit heme oxygenase (HO) (E.C. 1.14.88.3) were first suggested as a possible treatment for acute porphyrias in studies with rats pretreated with allylisopropylacetamide, a potent experimental porphyrogen, and subsequently treated with tin-protoporphyrin (SnP) (17). (SnP has also been used to decrease bilirubin production in neonates and in experimental jaundice [18, 19].) Tin-mesoporphyrin (SnM) was later found to be a more potent inhibitor of HO activity than SnP and to be chemically more stable (20). Tin-diiododeuteroporphyrin has also been tested for its ability to inhibit HO and to produce photosensitivity (21).

Concerns regarding the clinical use of tin-porphyrins as inhibitors of HO include their propensity to produce phototoxicity and sensitivity (22, 23), their long biologic half-lives, their ability to cross the blood–brain barrier (24), and the possible toxicity of tin itself, if it were to be released from the porphyrin macrocycle (for review see reference 3). In addition, tin- or cobalt-metalloporphyrins that inhibit HO have been found to induce expression of the HO gene (25, 26).

The ideal metalloporphyrin (MePn) for eventual clinical use in the treatment of porphyria should reduce ALA synthase activities and mRNA levels without causing photosensitivity or other toxicity and without exerting other unwanted effects, such as induction of HO mRNA and protein levels. Zinc-containing MePns are of interest in this regard since they inhibit HO (27–30), cause little or no phototoxicity (30, 31), and do not cross the blood–brain barrier (24). In addition, zinc is an essential trace metal, and normal human erythrocytes (and perhaps other cells as well) contain zinc protoporphyrin (32, 33).

Primary chick embryo liver cell cultures are a simple, reproducible system for studying hepatic porphyrin metabolism and its regulation (9, 12, 15, 30, 34–37). The kinetics of the conversion of ALA to heme in human liver resembles that of chicks

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1. *Abbreviations used in this paper:* AIP, acute intermittent porphyria; ALA, δ-aminolevulinate; CoP, cobalt protoporphyrin IX; CrM, chromium mesoporphyrin IX; HO, heme oxygenase; MePn, metalloporphyrin; MnP, manganese-protoporphyrin IX; NiM, nickel mesoporphyrin IX; PBG, porphobilinogen; poly-T, poly-thymidylate; SnM, tin-mesoporphyrin IX; SnP, tin-protoporphyrin IX; ZnM, zinc-mesoporphyrin IX.

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more closely than that of rats (38, 39). Heme–albumin solutions have been effective in cultured cells and humans at reducing ALA synthase activity (9, 12, 30, 36) and excretions of ALA and PBG (1–3, 5–8, 40). We have used the chick embryo liver culture system to test various MePn–albumin complexes for effects on heme metabolism (30, 41).

An important recent finding was that zinc-mesoporphyrin (ZnM) (50 nM) significantly reduced the high ALA synthase activities in cultured chick embryo liver cells treated with glutethimide and iron (30) or glutethimide and 4,6-dioxoheptanoic acid, an inhibitor of heme synthesis (41). Furthermore, low concentrations of ZnM enhanced the effectiveness of nanomolar concentrations of heme to reduce ALA synthase activities in a dose-dependent manner, showing for the first time that the combination of an MePn inhibitory for HO and heme was capable of additively repressing ALA synthase activity, in addition to prolonging the heme-dependent reduction of ALA synthase activity (23, 42).

We show in this work that several MePns that inhibit HO decrease ALA synthase mRNA levels, in chick embryo liver cells pretreated with glutethimide and iron, a pretreatment that markedly increases ALA synthase activity and mRNA levels (30, 36) as well as activity and mRNA of HO (37). In these cultures, ZnM effectively reduced ALA synthase mRNA and, in other cultures not treated with glutethimide and iron, it did not increase levels of mRNA of HO. In contrast, SnM and heme, although equally as effective as ZnM at reducing ALA synthase mRNA, induced HO mRNA levels when tested in a similar fashion. The time course of HO mRNA induction was quite rapid for both heme and other MePns (SnM and cobalt-protoporphyrin IX [CoP]), and, following SnM or CoP treatment, high levels of HO mRNA were maintained for at least as long as 18 h. Furthermore, combinations of ZnM and heme reduced levels of ALA synthase mRNA in a dose-dependent manner at concentrations up to 200 nM[2] with the effective combinations reducing the half-life of ALA synthase mRNA.

Methods

Materials. All tissue culture dishes were from Corning Glass Inc. (Corning, NY). Chloroform, dimethylformamide, and isopropanol were from Fisher (Pittsburgh, PA). Dexamethasone was from Gensia Pharmaceuticals (Irvine, CA). pd(N)$_6$ and Oligo dT$_{(12-18)}$ were from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Cobalt (Co^{+3})-protoporphyrin IX·Cl, chromium (Cr^{+3})-mesoporphyrin IX·Cl, ferric (Fe^{+3})-protoporphyrin IX·Cl (Hemin), manganese (Mn^{+3})-protoporphyrin IX·Cl, nickel (Ni^{+2})-mesoporphyrin IX, protoporphyrin IX, tin (Sn^{+4})-mesoporphyrin IX·2Cl, and zinc (Zn^{+2})-mesoporphyrin IX were from Porphyrin Products (Logan, UT). All MePns were tested for purity at Porphyrin Products by thin layer chromatography and all were > 97% pure. In addition, the absorption spectra in pyridine were identical to those observed with the corresponding methyl esters (reference 43 and J. Bommer, personal communication). The MePns were stored in a desiccator at −20°C, and, when used, the crystals were dry and free flowing. The plasmid pALX (44) was a generous gift from J. D. Engel (Northwestern University, Evanston, IL). AMV-Reverse Transcriptase, dTTP, Klenow DNA polymerase, and RNAsin were from Promega Corp. (Madison, WI). Actinomycin D, ammonium chloride, diethylpyrocarbonate, DMSO, formaldehyde (37%), formamide, D-(+)-glucose (tissue culture grade), glutethimide, penicillin/streptomycin, polyadenylic acid, salmon sperm DNA, sodium chloride, sodium citrate, sodium dodecyl sulfate, sodium hydroxide, 3,4,3′ triiodo-L-thyronine, trizma base, and trypsin were from Sigma Chemical Co. (St. Louis, MO). Fertilized Barred Rock chicken eggs were from Carousel Farms (Hopkinton, MA). Williams' E medium was from GIBCO BRL (Grand Island, NY). Ultraspec RNA was from Biotexc (Houston, TX). Nitrocellulose (0.45 μm) was from Schleicher and Schuell, Inc. (Keene, NH). All ^{32}P radionucleotides were from New England Nuclear (Boston, MA).

Methods. Cultures of primary chick embryo liver cells were prepared and chemicals were administered to the cell cultures as described previously (30, 36). All MePns were administered as BSA complexes. They were prepared by adding 100 μl of 10 mM MePn (dissolved in DMSO) to 3.9 ml of a solution of BSA, 11.1 mg/ml, dissolved in 40 mM Tris–HCl (pH 7.4). The final solutions had a molar ratio of 1.66 mol MePn/1 mol BSA. The MePn–BSA solutions were prepared under subdued light and immediately added to the cultures. The cultures were incubated in the absence of light.

Total RNA was obtained by harvesting the cells from one 60-mm culture dish directly into 500 μl of Ultraspec RNA and isolating the RNA by following the manufacturer's directions. RNA concentrations were estimated by the absorbance at 260 nm (1 AU = 40 μg/ml RNA).

Dot-blots were performed on total RNA with a probe that had previously been shown to specifically hybridize with the mRNA of interest under similar hybridization and washing conditions (36, 37, 45, 46). 20 μg of RNA in a final volume of 40 μl was treated with 24 μl of 20 × SSC and 16 μl of 37% formaldehyde and incubated at 65°C for 15 min. The samples were diluted to 400 μl with 10 × SSC and 10 μg of RNA was loaded per dot and duplicate nitrocellulose filters were prepared. The blots were crosslinked by exposure to 2.4 μJ of UV radiation using a UV-Stratalinker (Stratagene Inc., La Jolla, CA) and prehybridized at 42°C in 15 ml of a buffer containing 50% formamide, 5 × SSC, 100 μg/ml salmon sperm DNA, 5 × Denhardt's, and 1% SDS. After 1–2 h, the volume of the solution was reduced to 5–7 ml, and a boiled radioactive probe was added to 3.0×10^7 counts per blot. RNA concentrations and loading were normalized for total poly-A mRNA by hybridizing duplicate blots to a poly-thymidylate (poly-T) probe (45, 47). After hybridization for 18 h at 42°C, the blots were washed with 0.1 × SSC at room temperature for 30 min with two buffer changes and either exposed to x-ray film at −80°C or quantitated on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The amount of specific mRNA was normalized to total poly-A mRNA.

All experiments used at least triplicate samples for each treatment condition studied and statistical analyses were done with JMP 2.0 (SAS Institute, Cary, NC) using ANOVA, Tukey-Kramer test for multiple comparisons, or MANOVA as appropriate. P values < 0.05 were considered significant.

Results

MePns (10 μM) were screened for their ability to reduce ALA synthase mRNA in cell cultures that had been pretreated for 14 h with glutethimide and iron (Fig. 1 *A*, abscissa). Previously, this treatment had been shown to induce ALA synthase mRNA to high levels (48). Addition of heme, SnM, or ZnM for 4 h was equally effective at reducing ALA synthase mRNA levels, whereas CoP, nickel mesoporphyrin (NiM), and chromium mesoporphyrin (CrM) were less effective, and MnP did not reduce ALA synthase mRNA. These same MePns (10 μM) were tested for their ability to induce HO mRNA, in the absence of glutethimide and iron, after treatment for 5 h (Fig. 1 *A*, ordinate), a time previously shown to be associated with maximal HO mRNA induction by heme (45). Heme and MnP were the most effective inducers, while potency decreased in the order: CrM > CoP ≈ SnM > ZnM ≈ NiM ≈ No MePn (ANOVA, $P < 0.01$).

2. All stated concentrations represent the concentrations in the culture medium.

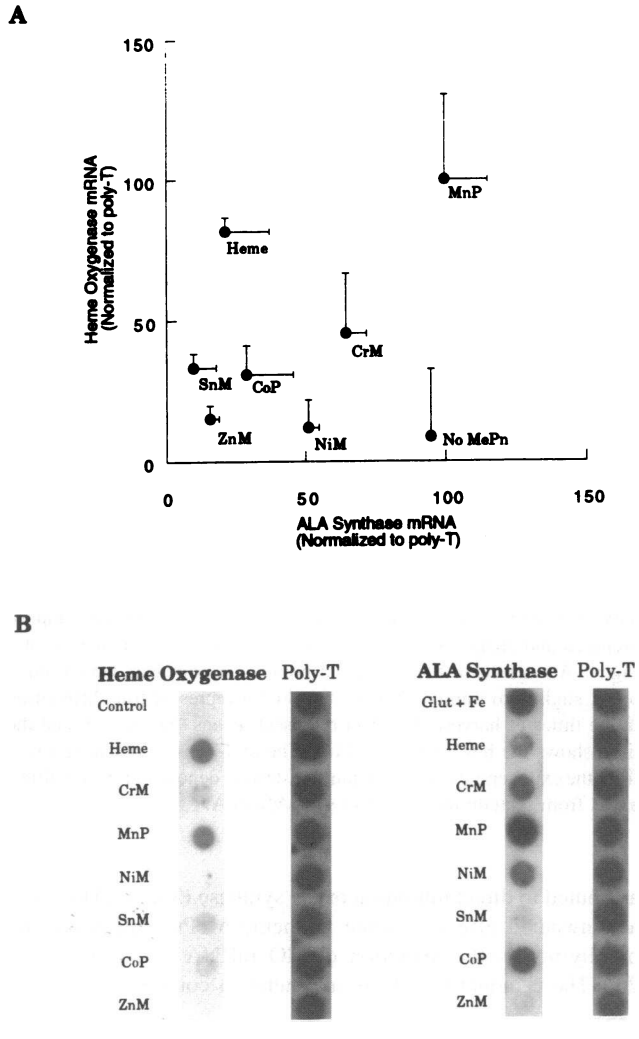

Figure 1. Effects of MePns on ALA synthase and HO mRNA. ALA synthase was induced in chick embryo liver cell cultures by treatment with 50 μM glutethimide and 50 μM FeNTA for 14 h before addition of 10 μM MePn. The cultures were exposed to the indicated MePn for an additional 4 h after which they were harvested and mRNA of ALA synthase was measured (abscissa). In separate experiments, mRNA for HO was measured after treatment of the cultures with 10 μM MePn alone for 5 h (ordinate). All cultures were harvested and samples prepared and analyzed as described in Methods. Quantitation of dot-blots was done using a PhosphorImager. The relative amounts of specific mRNAs present have been normalized by the amount of poly-T mRNA present in the sample, measured on a duplicate blot. The data represent means+SE, $n = 3$ (*A*), while the blot shows representative individual samples and the corresponding poly-T controls (*B*). There is no horizontal error bar for no MePn because, for this condition, the error fell within the size of the symbol.

The photograph (Fig. 1 *B*) shows individual samples and corresponding filters probed for poly-T content.

The time course of induction of HO mRNA demonstrated a rapid and transient increase in the presence of heme, while CoP and SnM produced a less rapid increase in HO mRNA with levels increasing for at least 9 h (Fig. 2). Subsequent experiments for longer times indicated that the induction of HO mRNA was the slowest for cobalt, with HO mRNA levels increasing steadily for at least 18 h (data not shown). Protoporphyrin IX had no effect on HO mRNA (data not shown). Cobalt was used to contrast the typical metal-dependent induction of HO, with the different heme-dependent induction of HO (49).

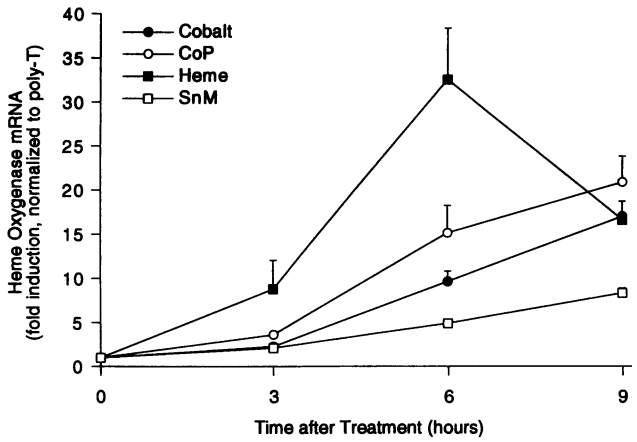

Figure 2. Temporal effects of cobalt and selected MePns on HO mRNA. Chick embryo liver cell cultures were treated with 10 μM heme, CoP, SnM, or 75 μM cobalt chloride for the indicated times. Cultures were harvested and samples prepared and analyzed for HO and poly-T mRNAs as described in Methods. The relative amounts of HO mRNA have been normalized as described in the legend to Fig. 1. The data represent means+SE, $n = 3$; when no error bars are shown the error fell within the size of the symbol.

In other experiments, addition of ZnM to cultures treated with heme did not affect the heme-dependent induction of HO mRNA. In the presence of 10 μM heme, a concentration that induced HO mRNA 10-fold after 5 h in culture, 10 μM ZnM had no effect on the level of HO mRNA. Even in the presence of 1 μM heme, a concentration that induced HO mRNA three- to fourfold after 5 h, 10 μM ZnM did not further increase HO mRNA.

Combinations of low concentrations of ZnM and heme decreased ALA synthase mRNA in a dose-dependent fashion with respect to both heme and ZnM (Fig. 3). Minimal ALA synthase mRNA levels were obtained in the presence of 200 nM heme alone, or 100 nM heme and > 50 nM ZnM (Tukey-Kramer, $P < 0.05$).

ZnM (50 nM) and heme (200 nM), either alone or in combination, reduced the half-life of ALA synthase mRNA to the same extent as that produced by 10 μM heme (Fig. 4, Table I) (13, 14). In contrast, SnM (50 nM) had no effect on the half-life of ALA synthase mRNA (Table I).

Discussion

The striking overproduction of ALA and PBG in the acute porphyrias led to the suggestion that overexpression of hepatic ALA synthase was the fundamental underlying defect in all these related disorders (50). Indeed, elevated ALA synthase activity plays a key role in the pathogenesis of these disorders, as evidenced by increased urinary excretions of ALA and PBG, while reversion of ALA synthase activity to normal is associated with an asymptomatic state. Discovery of the feedback repression of heme on ALA synthase, and the development of the regulatory heme pool theory (51, 52) provided a sound theoretical basis for the introduction of intravenous heme as a therapy

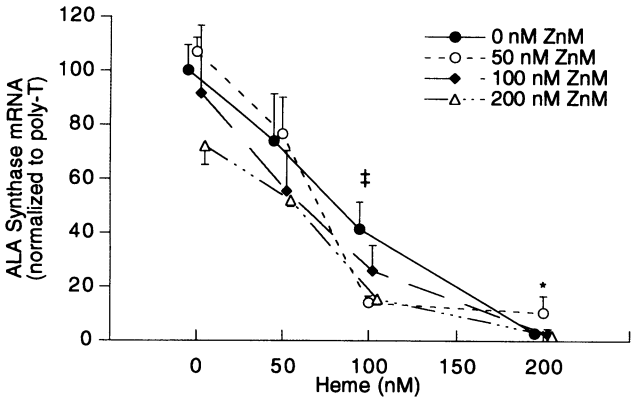

Figure 3. Effects of increasing concentrations of heme and ZnM to reduce ALA synthase mRNA. To increase ALA synthase mRNA levels, chick embryo liver cell cultures were treated with 50 μM glutethimide and 50 μM FeNTA for 14 h before addition of MePn. The cultures were exposed to the indicated concentrations of heme and/or ZnM for an additional 4 h, after which they were harvested and samples prepared and analyzed as described in Methods and in the legend to Fig. 1. To improve clarity, the individual points and their error bars have been offset slightly; however, the actual concentrations of heme used were always 0, 50, 100, or 200 nM. For all concentrations of heme < 200 nM, addition of 200 nM ZnM significantly decreased levels of the mRNA of ALA synthase, compared with 0 nM ZnM. At a heme concentration of 100 nM, all concentrations of ZnM tested (50, 100, and 200 nM) produced significant decreases compared with 0 nM. At the heme concentration of 200 nM, no additional repressive effect of ZnM could be detected since levels of mRNA of ALA synthase were so markedly decreased by heme alone.

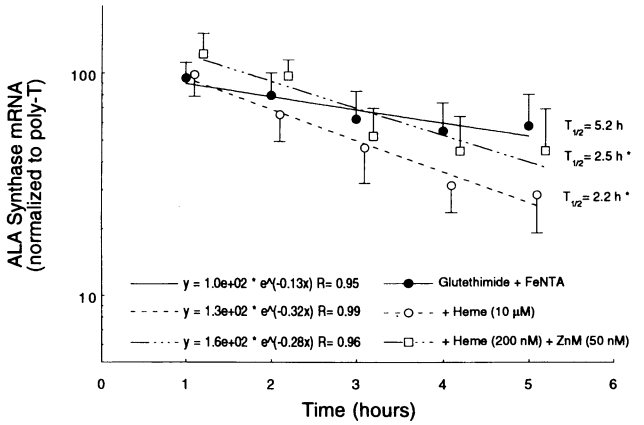

Figure 4. Effects of MePns on the half-life of ALA synthase mRNA. To increase ALA synthase mRNA levels, chick embryo liver cell cultures were treated with 50 μM glutethimide and 50 μM FeNTA for 14 h before addition of MePn. Then, 10 μM heme or the combination of 200 nM heme and 50 nM ZnM was added to the cultures for an hour, followed by addition of actinomycin D (0.4 μM). The cultures were harvested hourly for 5 h after addition of actinomycin D and samples prepared and analyzed as described in Methods and in the legend of Fig. 1. At each hour, the individual points and error bars have been offset slightly to improve clarity, but do not represent true differences in the times of harvest. The data represent means+SE, n = 3, and the lines shows the best exponential fits. The half-lives were calculated from the exponential decay constants. Asterisks denote significant differences from glutethimide + FeNTA, MANOVA.

for acute porphyria (8). Heme as a negative regulator of ALA synthase activity has not only been shown experimentally (30, 41, 53). Heme has also been used clinically for the prevention of attacks of acute porphyria (54) and suppression of attacks of acute porphyria, if given early during relapse (1–3, 5, 7, 55, 56). Although heme, 3–4 mg/kg per d, is effective in treating attacks of acute porphyria, many patients so treated develop unwanted side effects (57–62). Therefore, using lower doses of heme and prolonging its duration of action would be desirable therapeutic goals.

A nonheme MePn that inhibits HO, specifically SnP, was first tested in experimental and clinical hyperbilirubinemias (18, 63), especially neonatal jaundice. Later, SnP was shown to blunt the induction of ALA synthase in a rat model of acute porphyria (17). However, in human acute porphyria, at least at the doses given (1 μmol/kg per d for 3 d), there was little, if any, evidence of a therapeutic effect of SnP alone (23). In contrast, SnP, given with standard doses of heme (3 mg/kg per d) to patients with acute porphyrias prolonged the duration, although it did not increase the initial effect of, heme alone (23). Cutaneous, and perhaps CNS, toxicities were complications of SnP administration (23).

Recent results from our laboratory indicate that zinc porphyrins, especially ZnM, are as potent as SnP or SnM in decreasing activities of HO and ALA synthase. Indeed, for several MePns, their potencies to inhibit heme oxygenase were directly proportional to their abilities to decrease activities of ALA synthase in chick embryo liver cells in culture (30). Furthermore, low concentrations of ZnM and heme are additive or synergistic in decreasing activities of ALA synthase, an effect that cannot be attributed to direct inhibition of the synthase (30, 41). However, an unwanted effect of some nonheme MePns, including tin-porphyrins, is the induction of HO mRNA and protein (25, 26). These inductive effects may tend to counterbalance their

Table I. Effects of MePns on the Half-Life of ALA Synthase mRNA

Treatment	Half-life	Exponential decay constants k±SE
	h	
Glutethimide + FeNTA	5.2	0.13±0.03
+ 10 μM heme	2.2	0.32±0.03*
+ ZnM (50 nM) + heme (200 nM)	2.5	0.28±0.06*
+ ZnM (50 nM)	1.8	0.38±0.06*
+ Heme (200 nM)	2.5	0.28±0.06*
+ SnM (50 nM)	5.4	0.13±0.08

To increase ALA synthase mRNA levels, chick embryo liver cell cultures were treated with 50 μM glutethimide and 50 μM FeNTA for 14 h before addition of MePn. The indicated MePns were then added to the cultures followed by addition of actinomycin D (0.4 μM) at 15 h after initial treatment. The cultures were harvested and samples prepared and analyzed as described in Methods and in the legend of Fig. 1. The data represent means±SE, n = 3. The exponential decay constants and the standard errors of the best fit lines are shown in the last column. An asterisk represents a significant increase in the decay constant versus glutethimide + FeNTA alone (Student's t test modified for testing regressions in two populations, $P < 0.05$). The half-lives are calculated from the equation: $t_{1/2} = \ln 2/k$.

inhibitory effects on HO activity. Depending upon the relative time courses of, and doses needed for, these opposing effects, the net outcome of administration of nonheme MePns may be to decrease or to increase activity of HO and may change over time. Indeed, induction of the mRNA and protein for HO by SnP may in part account for its modest effect on hyperbilirubinemia or acute porphyria.

Since in previous work all the MePns that were effective at inhibiting HO also increased protein or mRNA levels of the oxygenase (25, 26), one might expect that every nonheme MePn, inhibitory for HO activity, would also induce HO mRNA. While it is not unexpected that some MePns (e.g., MnP [Fig. 1]) have no effect on ALA synthase, the discovery that ZnM is as effective at reducing ALA synthase mRNA as heme or SnM, but without affecting HO mRNA (Fig. 1), is a novel and important finding in this work.

The data presented here, and in previous work (30), suggest that a combination of ZnM and heme may be a useful therapeutic regimen for the management of acute porphyria. The low doses (50 nM) at which ZnM exerts an effect on both ALA synthase mRNA and activity (Fig. 3, references 30, 41) are at least one order of magnitude lower than concentrations of MePns previously reported to be effective. Other data from Vreman et al. (27) have described ZnM as an ineffective catalyst of the photodegradation of tryptophan and other organic molecules in vitro. In vivo phototoxicity studies demonstrated a high mortality in rats given SnP at 11.7 μmol/kg or SnM at 30 μmol/kg, while ZnP or ZnM did not produce any mortality at 40 μmol/kg (31). Taken together, these data suggest that ZnM is at least as potent as, and has fewer side effects than, the tin-porphyrins currently in investigational human use.

The effects of heme and nonheme MePns on ALA synthase mRNA provide a biochemical explanation for the potency of ZnM and heme at reducing ALA synthase activity (Fig. 1, references 30, 41). ZnM (50 nM) and heme (200 nM), alone or in combination, were just as effective at reducing the stability of ALA synthase mRNA as 10 μM heme (Fig. 4, Table I) (13, 14). The quantitative relationship between an observed steady state level of ALA synthase mRNA and the half-life of the mRNA remains to be elucidated. The inability of SnM (50 nM) to change the half-life of ALA synthase mRNA reflects the inability of this concentration of SnM to affect steady state levels of ALA synthase (30). There may be other effects of nonheme MePns on ALA synthase, such as on the mitochondrial processing and import of the enzyme (15, 16). Additional studies exploring these possibilities are currently underway.

In summary, the rationale for using a combination of heme and a MePn inhibitory for HO is supported not only by the clinical findings of Dover et al. (23) but also by the work described in this paper. The ability of nonheme MePns to enhance the effectiveness of heme may have therapeutic potential for not only acute porphyrias but also other porphyrias and other disorders in which heme appears to have therapeutic potential (5, 64–67). The studies described here, in light of other recent results from our (30, 41) and other (27, 31) laboratories, provide a solid experimental basis for studies of low dose heme and ZnM for acute porphyria and perhaps other disorders as well.

Acknowledgments

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