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MECHANISM OF SYNERGISTIC INDUCTION OF HEPATIC HEME OXYGENASE BY GLUTETHIMIDE AND IRON: STUDIES IN CULTURED CHICK EMBRYO LIVER CELLS

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Summary: Heme oxygenase, the rate controlling enzyme for heme catabolism, is inducible by a variety of treatments, some of which induce by a heme-dependent mechanism and others by a heme-independent mechanism. This work shows that, in cultured chick embryo liver cells, synergistic induction of heme oxygenase by iron, added with the phenobarbital-like drug, glutethimide was heme-dependent. Addition of an inhibitor of heme biosynthesis abolished the synergistic induction of heme oxygenase providing evidence for the heme-dependent mechanism of induction. Glutethimide and iron appeared to induce at the transcriptional level since both heme oxygenase mRNA and protein levels correlate with changes in heme oxygenase activity.

Introduction: Heme oxygenase (E.C. 1.14.88.3) catalyzes the breakdown of heme to biliverdin IXα, carbon monoxide and iron and is the rate-controlling enzyme of the physiological pathway for heme degradation (1). Activity of heme oxygenase can be inhibited by certain non-iron containing metalloporphyrins (2,3) or induced by chemicals (4,5). In some (6,7), but not all (8) cells heme oxygenase can also be induced by heat shock as part of the so-called stress response. Recent results from our (4) and other (5) laboratories showed that there are at least two disparate mechanisms for induction of heme oxygenase by chemicals: one, produced by phenobarbital, glutethimide and similar drugs that is dependent upon heme, and a second, produced by metal ions, such as Co^{+2} or Cd^{+2}, that is independent of heme.

Effects of iron on heme metabolism are of particular importance not only because of the role of iron as an essential element and component of heme, but also because iron overload may be associated with exacerbations of acute (9) and chronic (10,11) porphyrias and with increases in activities of both heme oxygenase and ALA synthase (E.C.2.3.1.37) the rate-controlling enzyme of hepatic heme synthesis (12,13). In recently reported work from our laboratory, it was found that even low concentrations of FeNTA (≤ 5μM) were capable of producing remarkable synergism of drug-mediated increases of

Abbreviations: ALA- 5-aminolevulinic acid, BR- bilirubin, 4,6-DHA- 4,6-dioxoheptanoic acid, FeNTA-ferric nitrilotriacetate. SDS- sodium dodecyl sulfate, 1x SSC- 0.15 M sodium chloride, 0.015M sodium citrate (pH 7.0).

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heme oxygenase and ALA synthase and experimental uroporphyria (14). The work reported here was undertaken to elucidate the mechanism whereby FeNTA produces synergistic increases in heme oxygenase activities in cultured chick embryo liver cells treated with glutethimide.

Materials: Fertilized white leghorn eggs were from Hyline Farms (Mansfield, GA). BSA, dexamethasone, 4,6-DHA, DNase, glutethimide, heme, leupeptin, NADPH and tri-iodothyronine were from Sigma (St. Louis, MO). William's E medium, trypsin and penicillin/streptomycin were from Gibco (Grand Island, NY). RNAzol was from Cinna/Biotecx Laboratories (Friendswood, TX). Deferoxamine (Desferal mesylate) was from CIBA-Geigy (Edison, NJ). Alkaline phosphatase conjugated goat anti-rabbit IgG and nitrocellulose (0.45μm) were from Bio-Rad Laboratories (Richmond, CA). Phast gels, buffer strips and applicators were from Pharmacia (Piscataway, New Jersey). All other chemicals were of the highest purity commercially available.

Methods: Chick embryo liver cell cultures were prepared from the livers of 16-18 day old chick embryos as described previously (4), except that newborn calf serum and serX-Tend were omitted. Chemicals were prepared and added as previously described (4). Appropriate solvent controls showed no effects on heme oxygenase or ALA synthase activity. Cultures were harvested as described at the indicated times and heme oxygenase was assayed as described (4), except that the reaction was allowed to proceed for 10 minutes and was terminated by the addition of 1.0 μmole p-hydroxymercuric benzoate. Bilirubin formed was quantified by absorbance spectroscopy (470-540 nm, $\Delta\varepsilon$ = 66 mM^{-1} cm^{-1}) with use of a suitable blank. Total cellular RNA was prepared (15) and northern blots done as described (16) except that a synthetic oligonucleotide probe identical to a portion of chick heme oxygenase cDNA cloned in our laboratory was used, and the blots were washed in 0.1X SSC, without SDS, at room temperature. The amount of heme oxygenase protein present was assessed with the use of western blots using the following procedure: Cultured cells were homogenized in 0.1 M potassium phosphate buffer (pH 7.5), 20% glycerol (v/v), 1mM EDTA containing leupeptin (40 μg/ml) as a protease inhibitor. Samples for western blotting were diluted 2:1 into a concentrated (3x) Laemmli (17) sample buffer. The samples were separated by electrophoresis on a PhastSystem, Pharmacia (Piscataway, New Jersey) according to manufacturers instructions on 8-25 gradient gels. The samples were transfered to nitrocellulose using the following procedure: Nitrocellulose, gels, and 3 sheets of blotting paper were soaked in transfer buffer (25 mM Tris-HCl, 5% methanol, 192 mM glycine, pH 9.2) for 10 minutes then assembled in a petri dish containing transfer buffer in the following order: Phast gel (with the plastic backing facing the bottom of the petri dish), nitrocellulose, blotting paper, and a heavy weight. The transfer was complete after incubation for 2 hours at 70°C. The blot was blocked overnight with PBS containing 1% BSA and then incubated with the specified antibodies. The primary antibody was IgG from rabbit immunized with purified heme oxygenase (18), and the secondary antibody was goat anti-rabbit IgG conjugated to alkaline phosphatase. Details of the antibody preparation and cloning of cDNA for chick heme oxygenase will be presented elsewhere (in preparation).

Results and Discussion: Heme oxygenase can be induced through two different mechanisms. The induction of heme oxygenase by FeNTA alone (Figure 1) is small, <2-fold (Table 1). This induction is non-heme mediated since 4,6-DHA, which inhibits heme biosynthesis at the level of porphobilinogen synthase (19), did not reduce heme oxygenase activity. In contrast, the induction of heme oxygenase by glutethimide was 3-fold (Table 1) and has previously been shown to be heme-mediated (4).

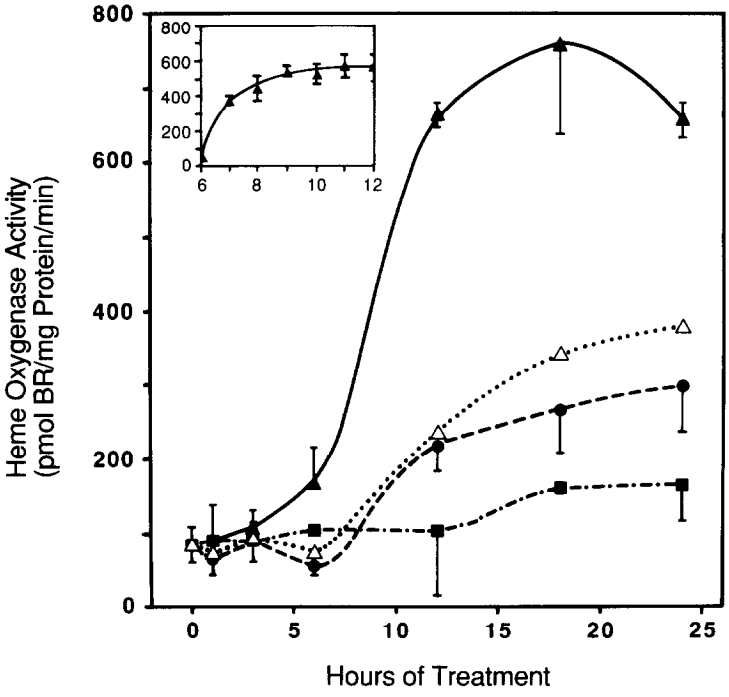

Figure 1: Time course of induction of hepatic heme oxygenase by glutethimide and ferric nitrilotriacetate. Chick embryo liver cell cultures were treated with 50 μM glutethimide (●), 50 μM FeNTA (■) or 50 μM glutethimide and 50 μM FeNTA together (▲). The expected additive values (Δ) were obtained by summing the inductive effects of glutethimide alone and iron alone. The inset shows a detailed time course for the combination of glutethimide and iron between 6 and 12 hours. The activities for the combination treatment are significantly greater ($p<0.02$) than the additive values at 12, 18, 24 hours. (The errors for the expected additive values that were used to determine the p-values were the sums of the errors for the glutethimide alone and FeNTA alone data points.) Points represent the means and standard deviations of three determinations. When no error bars are shown the standard deviation falls within the size of the symbol.

In agreement with earlier results (14), the combination of glutethimide and FeNTA produced a marked synergistic induction of heme oxygenase activity, observed at iron concentrations as low as 0.5 μM (Figure 2, $p < 0.0005$ compared to glutethimide

Table 1: Heme oxygenase activity in chick embryo liver cell cultures after 18 hour treatments with 50 μM glutethimide, 50 μM FeNTA, and the combination of the two chemicals with of without 2 mM 4,6-DHA (Results are mean ± S.D., n=3). 4,6-DHA, an inhibitor of heme biosynthesis, abolished the synergistic induction of heme oxygenase by glutethimide and iron, indicating that ongoing heme synthesis is important for heme oxygenase induction

	Control	Glutethimide	FeNTA	Glutethimide and FeNTA	Glutethimide and FeNTA and 4,6 DHA
HO Activity (pmol BR/ mg protein/ minute)	112± 35	181± 17	149± 22	513± 15	202± 15

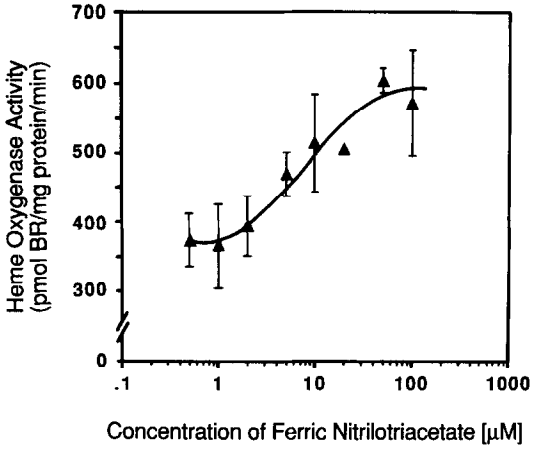

Figure 2: Dose response of the iron synergism for the induction of hepatic heme oxygenase by glutethimide. Chick embryo liver cell cultures were treated with 50 μM glutethimide and the indicated concentration of FeNTA for 18 hours. Heme oxygenase activity for untreated cells was 112 pmol bilirubin/mg protein/minute and the heme oxygenase activity for glutethimide alone was 181 pmol bilirubin/mg protein/minute. The points represent the means and standard deviations of three determinations. When no error bars are shown the standard deviation fell within the size of the symbol.

alone). Maximal synergism of induction of heme oxygenase occurred with FeNTA and glutethimide, both 50 μM. This potentiation of heme oxygenase induction by iron occurred rapidly, with maximal heme oxygenase activity by 9 h (Figure 1, Inset). This rapid rise suggests a heme-mediated mechanism since induction of heme oxygenase by metal ions in this system is not maximal until18-24 h (20, data not shown), whereas exogenous heme (10 μM) induces heme oxygenase maximally by 6-8 h (data not shown). Furthermore, exogenous heme (10 μM) did not further enhance the induction produced by glutethimide (50 μM) and FeNTA (50 μM) supporting the concept that the role of iron in synergism of heme oxygenase induction is mediated by heme. 4,6-DHA, an inhibitor of heme biosynthesis, abolished the synergistic effect of iron on drug mediated induction of heme oxygenase (Table 1), providing additional evidence that the synergism is a process mediated by heme. One possible reason for increased heme formation in the presence of added iron is that iron, combined with increased protoporphyrin accumulation stimulated by glutethimide treatment (4,14), to form heme (21).

Iron synergism of heme oxygenase induction was accompanied by increased levels of heme oxygenase protein (Figure 3A) and mRNA (Figure 3B), whereas inhibition of heme synthesis blunted these increases. These results suggest that the synergistic induction of heme oxygenase protein by iron and glutethimide depends upon an increase in the level of its message and thus the amount of heme oxygenase synthesized, rather than an increase in its half-life. This conclusion fits well with a heme-dependent mechanism, since heme in other systems has been found to increase levels of mRNA for heme oxygenase (6). Whether the increase in the mRNA level is due to increased transcription of nascent mRNA or stabilization of pre-existing mRNA is the subject of study currently ongoing in our laboratory.

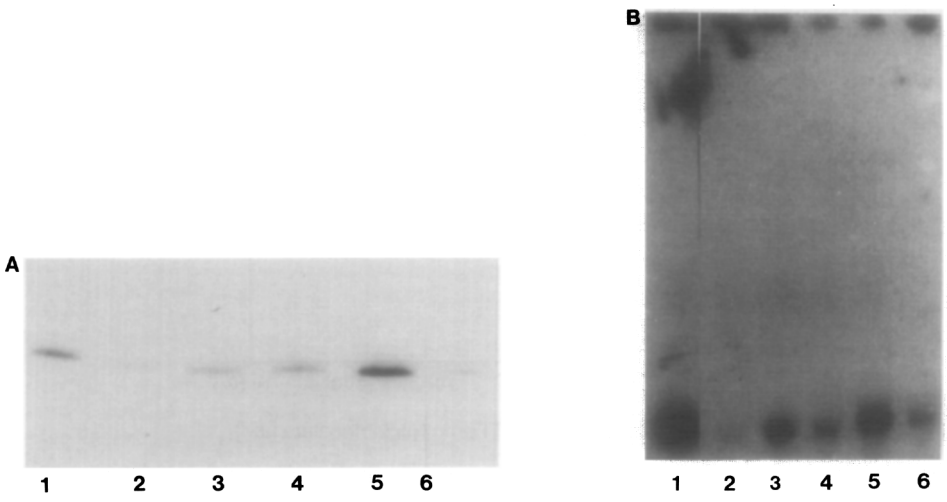

Figure 3: Western blots and northern blots showing the relative amounts of heme oxygenase protein and mRNA from chick embryo liver cell cultures treated to induce heme oxygenase with and without an inhibitor of heme synthesis. The western blot (A) lanes are 1 μg of the following: 1- purified avian hepatic heme oxygenase, 2-6 cultured chick embryo liver cells [2- control, 3- 50 μM glutethimide, 4- 50 μM FeNTA, 5- 50 μM glutethimide and 50 μM FeNTA, 6- 50 μM glutethimide and 50 μM FeNTA and 2 mM 4,6-DHA]. The northern blot (B) lanes are 1- 1 μg hepatic mRNA from Cd^{+2}treated chicken, 2-6 25μg of total RNA from cultured chick embryo liver cells [2- control, 3- 50 μM glutethimide, 4- 50 μM FeNTA, 5- 50 μM glutethimide and 50 μM FeNTA, 6- 50 μM glutethimide and 50 μM FeNTA and 2 mM 4,6-DHA]. (The material at the top of the northern blot shows the origin.) The figure shows the synergistic increase in the amount of heme oxygenase protein and mRNA in cells treated with glutethimide and iron the marked decrease when heme biosynthesis was inhibited by 4,6-DHA.

Our results provide a biochemical explanation for the previously described increased rate of hepatic heme breakdown in rats treated with iron (22) and may help to account for the known potentiation by iron of drug-mediated induction of ALA synthase (9,13) and for the propensity of iron to exacerbate or precipitate porphyrias (9-11). If synergistic induction of heme oxygenase were sufficient to deplete the regulatory heme pool (14,23), induction of ALA synthase and the severity of porphyria might be increased.

In summary, our results indicate that the iron synergism of heme oxygenase induction by glutethimide is associated with increased levels of heme oxygenase mRNA and protein, suggesting a transcriptional mechanism. That this synergistic induction of heme oxygenase mRNA and protein is mediated by heme is supported by several pieces of evidence: first, the induction is rapid and is analogous to the induction of heme oxygenase by heme alone; second, addition of heme does not further induce heme oxygenase; and third, the synergistic induction of heme oxygenase can be abolished with the addition of 4,6-DHA, a potent inhibitor of heme biosynthesis. To our knowledge, these studies are the first to characterize changes in levels of heme oxygenase mRNA and protein produced by chemicals or iron in a primary cell culture model that retains the same expression and inducibility of heme metabolic enzymes and hemoproteins as the intact animal (24,25).

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