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3,5,5-TRIMETHYLHEXANOYLFERROCENE INDUCTION OF HEME OXYGENASE ACTIVITY IN NORMAL HEPATOCYTES

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Abstract—Recent work showed that the combination of 50 μM glutethimide plus 50 μM ferric nitrilotriacetate (FeNTA) synergistically induces heme oxygenase (HO) activity in cultured chick embryo liver cells (Cable *et al.*, *Biochem Biophys Res Commun* **168**: 176–181, 1990). This synergistic induction is due to increased heme synthesis, which then acts to increase HO gene transcription. The aim of the current studies was to characterize the effects on hepatic heme metabolism of (3,5,5-trimethylhexanoyl)ferrocene (TMH-ferrocene), which causes hepatic iron-loading in rats. Unlike FeNTA, TMH-ferrocene alone maximally induced HO activity at 5–10 μM TMH-ferrocene. At higher concentrations, HO activities declined, as did total cellular protein synthesis. Induction of HO was maximal after a 12-hr exposure to TMH-ferrocene, similar to induction by glutethimide plus FeNTA. The effect of TMH-ferrocene on HO could not be ascribed to greater cellular uptake of iron, since cell-associated iron levels were higher after FeNTA than after TMH-ferrocene treatment. TMH-ferrocene (up to 20 μM) did not induce δ-aminolevulinic acid synthase activity. Uroporphyrin accumulation in cells treated with TMH-ferrocene was minimal, but the combination of TMH-ferrocene and glutethimide caused a synergistic increase in uroporphyrin accumulation, similar to treatment with glutethimide plus FeNTA. 4,6-Dioxoheptanoic acid, an inhibitor of heme synthesis, blocked the induction of HO caused by glutethimide and FeNTA, but did not decrease the induction of HO by TMH-ferrocene. TMH-ferrocene-mediated induction of HO does not appear to be due to lipid peroxidation, since malondialdehyde formation was greater for ferrocene (a structural analog of TMH-ferrocene that does not induce HO) than for TMH-ferrocene. Furthermore, the anti-oxidant, butylated hydroxyanisole, which prevented lipid peroxidation, decreased HO induced by glutethimide plus FeNTA, but butylated hydroxyanisole did not affect HO induced by TMH-ferrocene. We conclude that, unlike the combination of glutethimide plus FeNTA, TMH-ferrocene induces HO activity by a mechanism that is independent of cellular heme synthesis.

Key words: heme oxygenase; induction; TMH-ferrocene; primary hepatocytes; chick embryo; glutethimide

Primary cultures of chick embryo liver cells have long been used to study the mechanisms of regulation of heme biosynthesis and degradation [1, 2]. Using this experimental model, we have shown previously that the combination of glutethimide plus FeNTA† synergistically induces HO (EC 1.14.88.3) [1] and that this synergistic induction depends on intracellular heme biosynthesis [3]. We recently demonstrated a role of HO in modulating ALA synthase (EC 2.3.1.37) via changes in the regulatory heme pool [4]. In an effort to further characterize the synergistic induction of HO by the combination of glutethimide plus FeNTA, we looked for ways to optimize iron uptake into cultured chick embryo liver cells.

Longueville and Crichton [5] have developed an animal model of iron overload using TMH-ferrocene. They observed a conspicuous hepatocellular siderosis after only 14 days of oral administration of this compound to rats. Recently these observations have been extended [6–9], and the emerging picture is that TMH-ferrocene causes in rats a rapid and intense iron-loading that has many of the characteristics of human primary hemochromatosis. In rats, Düllmann *et al.* [8] observed hepatic iron levels of 5.15 mg/g wet wt after 4 weeks of feeding 0.5% (w/w) TMH-ferrocene and 28.00 mg/g wet wt after 43 weeks. Our working hypothesis was that TMH-ferrocene should cause a more rapid and intense iron-loading of chick embryo liver cells than FeNTA, and when given in combination with glutethimide, TMH-ferrocene should also cause a synergistic induction of HO.

The major finding of the current study was that TMH-ferrocene *alone* produced nearly maximal induction of HO activity. In this paper, we describe conditions for the TMH-ferrocene-mediated induction of HO activity in cultured chick embryo liver cells and investigate the mechanism by which TMH-ferrocene causes this induction.

MATERIALS AND METHODS

Materials. TMH-ferrocene was provided by Dr.

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† Abbreviations: FeNTA, ferric nitrilotriacetate; HO, heme oxygenase; ALA, δ-aminolevulinic acid; TMH-ferrocene, (3,5,5-trimethylhexanoyl)ferrocene; and TCA, trichloroacetic acid.

Peter Nielsen, Universitätskrankenhaus Eppendorf, Hamburg, Germany, as an orange powder. Butylated hydroxyanisole, bovine serum albumin, dexamethasone, 4,6-dioxoheptanoic acid, DMSO, DNase, ferric chloride, glutamine, glutethimide, insulin, penicillin/streptomycin, sodium nitrilotriacetate, TCA, 2-thiobarbituric acid, 3,5,3′-triiodo-L-thyronine, and trypsin were from the Sigma Chemical Co. (St. Louis, MO). Porphyrin standards and hemin chloride were from Porphyrin Products (Logan, UT). Ferrocene and 4-(2-pyridylazo)resorcinol were from the Aldrich Chemical Co. (Milwaukee, WI). [^{14}C]-Leucine (333 Ci/mol; 0.1 μCi/μL) was from Dupont NEN (Wilmington, DE). Williams' E medium was from Gibco (Grand Island, NY). All chemicals were of the highest purity available, and water purified with a MilliQ system (Millipore, Milford, MA) was used throughout.

Liver cell cultures. Fertilized eggs of Barred Rock chickens were obtained from a local supplier (Carousel Farm, Holliston, MA). The eggs were maintained at 37° in a humidified Petersime Incubator, model 4 (Gettysburg, OH). Livers were excised from 16- to 17-day-old chick embryos, immediately placed in calcium-free Hanks' solution, minced, washed, treated with 5% trypsin (w/v; final trypsin concentration in Hanks' solution), and incubated at 37° for 20 min. DNase (1 mg/mL) was added for the last 10 min of incubation. The cell suspension was washed in fresh cold Hanks' solution and centrifuged at 250 *g* for 90 sec. The pellet was resuspended and washed twice in sterile buffer (0.13 M ammonium chloride, 0.017 M Tris, 0.01 M potassium carbonate, pH 7.5). The cells were finally suspended in Williams' E medium, supplemented with glutamine (200 mM), dexamethasone (0.3 μg/mL), tri-iodothyronine (1 μg/mL), and insulin (1 μg/mL). Portions (6 mL) of the suspension were dispensed into sterile 6-cm diameter plastic culture dishes, and the dishes were incubated at 37° in an atmosphere of room air supplemented with 5% CO_2. Eighteen hours later, the medium was changed to one free of insulin (5 mL/dish), and the indicated treatments were added. Cells were harvested 18 hr later (or as indicated), and sonicated, as described previously [4].

Glutethimide, 4,6-dioxoheptanoic acid and the components of FeNTA (ferric chloride and sodium nitrilotriacetate) were all freshly prepared on the day of use. Glutethimide was dissolved in DMSO (10.8 mg glutethimide/mL DMSO). 4,6-Dioxoheptanoic acid was prepared in water (15.8 mg/mL) and sterilized through a 0.22 μm filter before use. FeNTA was prepared by combining 1 mL of a solution of 4.05 mg ferric chloride/mL water with 1 mL of a solution of 14.2 mg sodium nitrilotriacetate/mL water and adding 3 mL water. Heme was administered as heme-albumin (about 1.66 mol heme/1 mol albumin) which was prepared by adding 100 μL of 10 mM heme (6.5 mg hemin chloride/mL DMSO) to 3.9 mL of a solution of bovine serum albumin (11.1 mg albumin/mL of 40 mM Tris–HCl, pH 7.4).

Assays. HO activity was assayed as described previously [3]. Incorporation of [^{14}C]-leucine into protein was measured by reducing the volume of the medium on the 6-cm plates to 1.5 mL, adding 0.2 μCi of labeled leucine, and incubating for 60 min at 37°. The medium was removed from the plates, which were then washed three times with phosphate-buffered saline. The cells were harvested in 2 mL of 5% TCA (w/v), sonicated, and centrifuged at 200 *g* for 10 min. The supernatant was removed and the pellet was washed three more times in 5% TCA. The final pellet was dissolved in 1 mL of 0.2 N NaOH/0.1% sodium dodecyl sulfate (w/v); 0.5 mL of this solution was added to 15 mL Opti-Fluor scintillation mixture and counted for 1 min on a Minaxi B liquid scintillation counter (Packard, Downers Grove, IL). Cell-associated iron was measured by washing the cells twice with 40 mM Tris buffer (pH 7.6), harvesting the cells in 700 μL of buffer, and sonicating the cells. A portion of this sonicate (500 μL) was combined with 500 μL of a 1:1 mixture of concentrated sulfuric and nitric acids in a Kjeldahl tube and refluxed for 3 min. After cooling, 3.7 mL of concentrated ammonium hydroxide was added to the Kjeldahl tube, followed by 50 μL of 10% hydroxylamine (w/v). After 15 min, 250 μL of a 0.1% solution (w/v) of 4-(2-pyridylazo)resorcinol was added. After 1 hr of color formation, the absorbance at 500 nm was determined for the samples, blanks and appropriate standards. ALA synthase was assayed according to a modi-

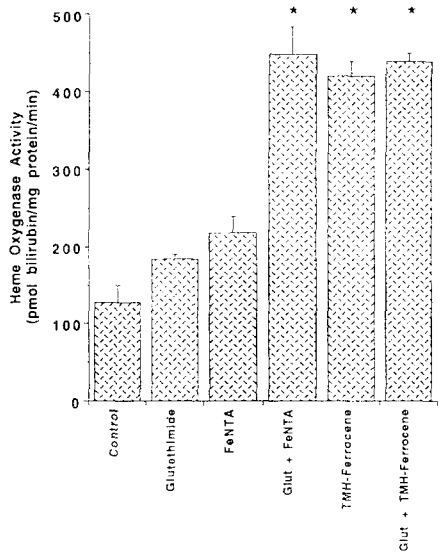

Fig. 1. Comparison of heme oxygenase activity in cells treated with glutethimide, FeNTA, glutethimide plus FeNTA, TMH-ferrocene, or glutethimide plus TMH-ferrocene. Chick embryo liver cell cultures were treated with DMSO (vehicle control; 5 μL/6-cm plate), glutethimide (50 μM), FeNTA (50 μM), TMH-ferrocene (5 μM), or the indicated combinations for 18 hr prior to harvest. The cells were harvested, sonicated and assayed for HO activity, as described in Materials and Methods. Data represent means ± SEM, N = 3. Cells treated with glutethimide (Glut) plus FeNTA, TMH-ferrocene, or glutethimide plus TMH-ferrocene had significantly increased HO activity compared with the other treatments (*$P < 0.01$), but they did not differ significantly from each other.

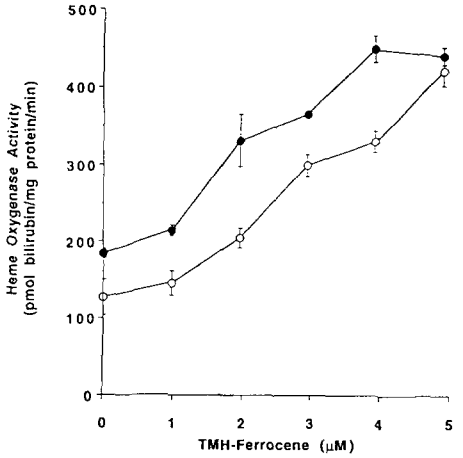

Fig. 2. Effects of increasing concentrations of TMH-ferrocene on heme oxygenase activity in the presence or absence of glutethimide. Chick embryo liver cell cultures were treated with the indicated concentrations of TMH-ferrocene (○), or the combination of glutethimide (50 μM) plus TMH-ferrocene (●) for 18 hr prior to harvest. The cells were harvested, sonicated and assayed for HO activity, as described in Materials and Methods. Data represent means ± SEM, N = 3. Cells treated with the combination of glutethimide (50 μM) and FeNTA (50 μM) were used as a positive control, and had HO activities of 448 ± 36 pmol bilirubin/mg protein/min.

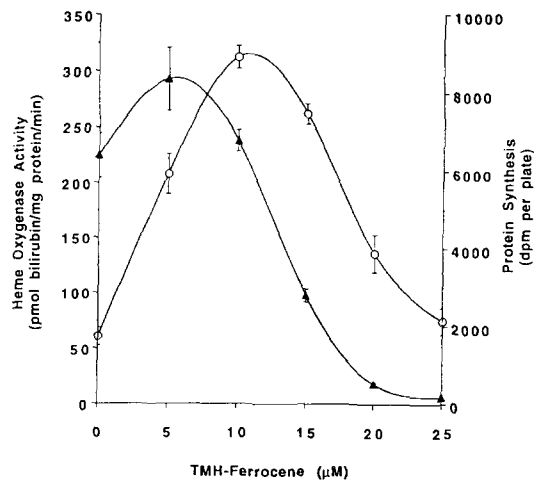

Fig. 3. Effects of increasing concentrations of TMH-ferrocene on heme oxygenase activity and protein synthesis. Chick embryo liver cell cultures were treated with the indicated concentrations of TMH-ferrocene for 18 hr prior to harvest. The cells were harvested, sonicated and assayed for HO activity (○) and protein synthesis (▲) measured as incorporation of [^{14}C]-leucine into TCA-precipitable material, as described in Materials and Methods. Data represent means ± SEM, N = 3. Where no error bars are shown, the standard errors fell within the size of the symbols. The total amount of protein per plate was not decreased by treatment with TMH-ferrocene, except for the highest concentration of TMH-ferrocene (25 μM) which decreased protein values by 17% versus control plates.

fication [4] of published methods [10, 11]. Porphyrin accumulation was measured by the method of Grandchamp *et al.* [12]. Malondialdehyde formation was measured by a modification [13] of the method of Buege and Aust [14]. Protein concentrations were determined using the bicinchoninic acid method, using bovine serum albumin as the standard [15].

Statistical procedures. Data are presented throughout as means ± SEM. Statistical analyses were aided by the use of JMP software, version 2, run on a MacIntosh IIci computer. Preliminary evaluation of data indicated that they were normally distributed, and thus they were analyzed by ANOVA and multiple comparisons were made using the Tukey–Kramer test.

RESULTS

We have shown previously that the combination of glutethimide plus FeNTA causes a synergistic increase in HO activity in cultured chicken embryo liver cells [1, 3]. As shown in Fig. 1, TMH-ferrocene (5 μM) alone and the combination of TMH-ferrocene plus glutethimide (50 μM) increased HO activity to levels as high as that induced by the combination of glutethimide (50 μM) plus FeNTA (50 μM) in cells treated for 18 hr.

The results of a typical concentration–response study of TMH-ferrocene, (either alone or in combination with 50 μM glutethimide) on the induction of HO activity at 18 hr are shown in Fig. 2. In this experiment, the maximum concentration of TMH-ferrocene was 5 μM, and the HO activity was increased to the same level as was caused by the combination of glutethimide plus FeNTA, used as a positive control (see legend to Fig. 2). Treatment with the combination of glutethimide plus TMH-ferrocene resulted in higher levels of HO activity than treatment with TMH-ferrocene alone for all but the highest concentration (5 μM) of TMH-ferrocene tested. In other concentration–response studies, using higher concentrations of TMH-ferrocene, HO activity was maximal at concentrations of 5–10 μM TMH-ferrocene, but rapidly declined at higher concentrations. As shown in Fig. 3, the cause of this decline in HO activity was investigated by treating the cells for 18 hr with TMH-ferrocene (up to 25 μM), and measuring HO activity and protein synthesis, determined by the incorporation of [^{14}C]-leucine into TCA-precipitable material, as described in Materials and Methods. The protein synthesis results suggest that the observed decline in HO activity at higher concentrations of TMH-ferrocene (greater than 10 μM) was due to cellular toxicity of the TMH-ferrocene at these levels.

The time course of induction of HO activity by TMH-ferrocene and the combination of glutethimide plus FeNTA is shown in Fig. 4. Cells treated with glutethimide plus FeNTA had significantly increased HO activity at all times greater than 0 hr whereas cells treated with 5 μM TMH-ferrocene had significantly increased HO activity at 12 and 18 hr.

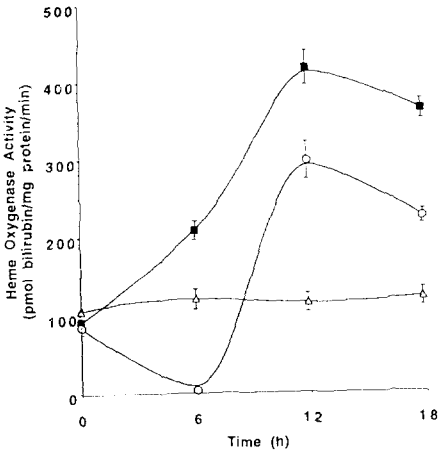

Fig. 4. Time course of induction of heme oxygenase activity by TMH-ferrocene and the combination of glutethimide plus FeNTA. Chick embryo liver cell cultures were treated with the vehicle (DMSO; 5 μL 6-cm plate; (△), 5 μM TMH-ferrocene (○) or the combination of 50 μM glutethimide plus 50 μM FeNTA (■) for the times indicated prior to harvest. The cells were harvested, sonicated and assayed for HO activity. Data represent means ± SEM, N = 3. Cells treated with glutethimide plus FeNTA had significantly increased HO activity at all times greater than 0 hr ($P < 0.01$), whereas cells treated with 5 μM TMH-ferrocene had significantly increased HO activity at 12 and 18 hr ($P < 0.01$).

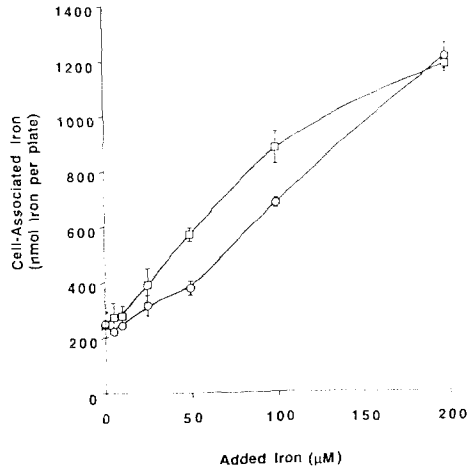

Fig. 5. Accumulation of iron in cells treated with TMH-ferrocene or FeNTA. Chick embryo liver cell cultures were treated with the indicated concentrations of TMH-ferrocene (○) or FeNTA (□) for 18 hr prior to harvest. The cells were harvested, sonicated and assayed for cell-associated iron, as described in Materials and Methods. Data represent means ± SEM, N = 3. Where no error bars are shown, the standard errors fell within the size of the symbols.

The 18 hr induction time was used in subsequent experiments.

To determine whether the TMH-ferrocene-mediated induction of HO activity was due to elevated intracellular iron levels, the accumulation of cell-associated iron was measured in cells treated with either TMH-ferrocene or FeNTA (Fig. 5). Both iron-containing compounds caused concentration-related increase in cell-associated iron levels, with FeNTA being somewhat more effective than TMH-ferrocene. These results suggest that the ability of TMH-ferrocene to induce HO activity is due to some cause independent of its ability to increase cell-associated iron in cultured chick embryo liver cells, since TMH-ferrocene was less effective than FeNTA in iron-loading the cells.

Cable *et al.* [4] have shown that the increase in HO activity caused by glutethimide plus FeNTA precedes an increase in ALA synthase activity by 4 hr. They concluded that the increase in HO activity caused a decrease in intracellular heme, and that decreased heme levels caused a subsequent induction of ALA synthase activity. To determine whether a similar process occurs with the TMH-ferrocene-mediated induction of HO activity, the ALA synthase activities in cells treated with TMH-ferrocene or glutethimide plus TMH-ferrocene were measured (Fig. 6). The combination of glutethimide plus TMH-ferrocene caused an initial increase in ALA synthase activity (up to 5 μM TMH-ferrocene) and then a subsequent decrease in ALA synthase activity, consistent with the decrease in protein synthesis

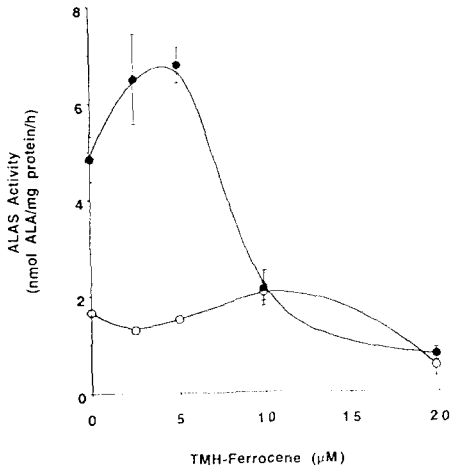

Fig. 6. Effects of increasing concentrations of TMH-ferrocene on ALA synthase activity in the presence or absence of glutethimide. Chick embryo liver cell cultures were treated with the indicated concentrations of TMH-ferrocene (○) or the combination of 50 μM glutethimide plus TMH-ferrocene (●) for 18 hr prior to harvest. The cells were harvested, sonicated and assayed for ALA synthase activity, as described in Materials and Methods. Data represent means ± SEM, N = 3.

previously observed at these higher concentrations of TMH-ferrocene (Fig. 3). Concentrations of less than 20 μM TMH-ferrocene alone caused little, if any, change in ALA synthase activity. These results suggest that TMH-ferrocene alone and the

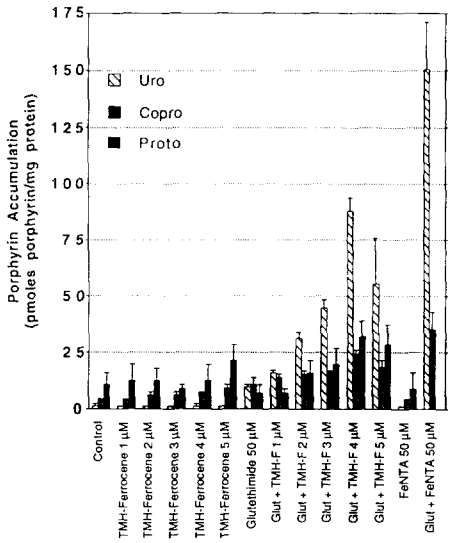

Fig. 7. Effects of TMH-ferrocene, glutethimide and FeNTA on porphyrin accumulation. Chick embryo liver cell cultures were treated with the indicated concentrations of TMH-ferrocene, glutethimide (50 μM) or FeNTA (50 μM) for 18 hr prior to harvest. The cells and medium were harvested together, sonicated and assayed for porphyrin content, as described in Materials and Methods. Data represent means ± SEM, N = 3. In the presence of glutethimide (Glut), TMH-ferrocene (2–5 μM) or FeNTA (50 μM) produced significant and synergistic increases in uroporphyrin accumulations ($P < 0.05$, compared with glutethimide or either iron compound alone). In the absence of glutethimide, TMH-ferrocene did not affect porphyrin accumulation ($P = 0.95$; ANOVA).

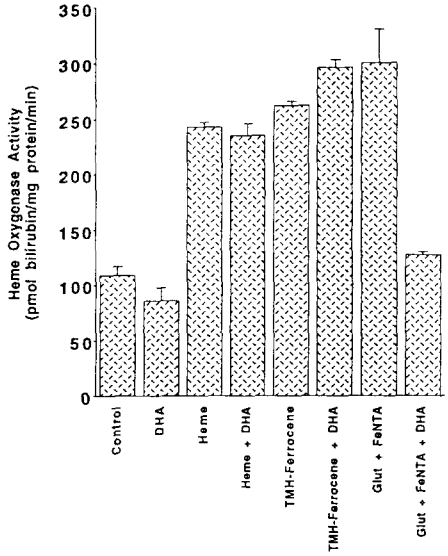

Fig. 8. Effects of 4,6-dioxoheptanoic acid on heme oxygenase activity induced by heme, TMH-ferrocene and the combination of glutethimide plus FeNTA. Chick embryo liver cell cultures were treated with heme (10 μM), TMH-ferrocene (5 μM) or the combination of glutethimide (50 μM) plus FeNTA (50 μM) in the presence or absence of 4,6-dioxoheptanoic acid (DHA; 1 mM) for 18 hr prior to harvest. The cells were harvested, sonicated and assayed for HO activity. Data represent means ± SEM, N = 3. Cells treated with heme (±DHA), TMH-ferrocene (±DHA) and glutethimide plus FeNTA had significantly increased HO activity compared with controls ($P < 0.05$), whereas cells treated with DHA or the combination of glutethimide plus FeNTA plus DHA did not.

combination of glutethimide plus FeNTA induce HO activity by different mechanisms.

The combination of glutethimide plus FeNTA has been shown previously to cause an impressive increase in porphyrin accumulation in cultured chick embryo liver cells [1]. To determine whether TMH-ferrocene caused a similar increase in porphyrin accumulation, cells were treated with increasing concentrations of TMH-ferrocene alone or in combination with glutethimide, and porphyrin accumulation was measured (Fig. 7). Cells treated with up to 5 μM TMH-ferrocene showed little, if any, change in the amount or pattern of accumulation of uroporphyrin, coproporphyrin or protoporphyrin. Cells treated with glutethimide plus TMH-ferrocene showed a concentration-related increase in porphyrin accumulation up to 4 μM TMH-ferrocene. Cells treated with the combination of glutethimide plus FeNTA showed the expected increase in both uroporphyrin and coproporphyrin. These results indicate that the pattern and amounts of porphyrin accumulation caused by TMH-ferrocene alone are substantially different from the porphyrin accumulation caused by glutethimide plus FeNTA.

To determine whether the induction of HO activity is a general property of ferrocene-type compounds, the inducing capability of up to 100 μM ferrocene (a structural analog of TMH-ferrocene) was measured. Cells treated with 1, 5, 10, 50, or 100 μM ferrocene showed no significant increase in HO activity compared with control cells, whereas cells treated with 5 μM TMH-ferrocene showed a significant increase (results not shown). These results suggest that a substituted cyclopentadiene ring structure is required to induce HO activity.

4,6-Dioxoheptanoic acid blocks heme biosynthesis because it is a potent inhibitor of ALA dehydrastase (EC 4.2.1.24) [16]. As shown in Fig. 8, 4,6-dioxoheptanoic acid had no effect on the induction of HO activity caused by either heme or TMH-ferrocene. By contrast, 4,6-dioxoheptanoic acid decreased the induction of HO activity caused by the combination of glutethimide plus FeNTA, in agreement with our earlier report [14]. These results are consistent with the hypothesis that induction of HO activity by glutethimide plus FeNTA requires intracellular heme synthesis, whereas the induction of HO by TMH-ferrocene does not.

A possible connection between increased lipid peroxidation and induction of HO activity in rats has been suggested by Klimczak *et al.* [17]. To determine whether the TMH-ferrocene-mediated induction of HO was related to increased lipid peroxidation, chick embryo liver cells were treated with the combination of glutethimide plus FeNTA,

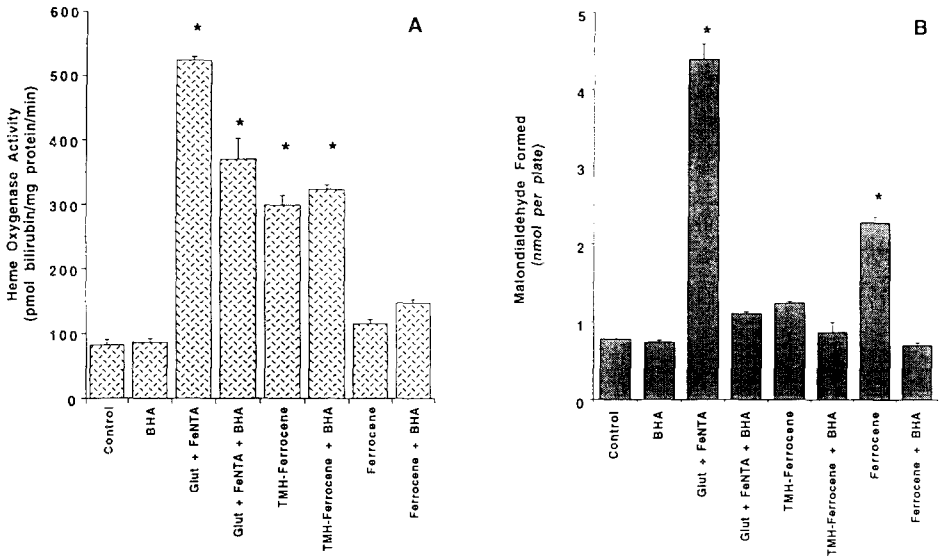

Fig. 9. Effects of butylated hydroxyanisole on heme oxygenase activity and malondialdehyde formation in cells treated with glutethimide plus FeNTA, TMH-ferrocene or ferrocene. Chick embryo liver cell cultures were treated with the combination of glutethimide (50 μM) plus FeNTA (50 μM), TMH-ferrocene (5 μM), or ferrocene (5 μM) in the presence or absence of butylated hydroxyanisole (67 μM) for 18 hr prior to harvest. The cells were harvested, sonicated and assayed for HO activity (A) and malondialdehyde formation (B), as described in Materials and Methods. Data represent means ± SEM, N = 3. Key: (*) significantly different from control, $P < 0.05$.

TMH-ferrocene or ferrocene, in the presence or absence of the anti-oxidant butylated hydroxyanisole, and HO activities and malondialdehyde accumulation, a measure of lipid peroxidation [13], were measured (Fig. 9). Butylated hydroxyanisole (at 67 μM) partially blocked the induction of HO activity caused by the combination of glutethimide plus FeNTA; however, butylated hydroxyanisole had no effect on HO activity induced by TMH-ferrocene (Fig. 9A). By contrast, Fig. 9B shows that malondialdehyde formation was only increased by the combination of glutethimide plus FeNTA and by ferrocene, but not by TMH-ferrocene. In all cases, treatment with butylated hydroxyanisole reduced malondialdehyde formation to nearly control levels. Since TMH-ferrocene does not increase malondialdehyde formation and since butylated hydroxyanisole does not decrease the TMH-ferrocene-mediated induction of HO activity, we conclude that induction of HO by TMH-ferrocene is not related to lipid peroxidation.

DISCUSSION

Since TMH-ferrocene has been shown to be highly effective at causing hepatic iron-loading in rats [5–9], this compound had the potential to iron-load cultured chick embryo liver cells more effectively than FeNTA. Our initial hypothesis, then, was that the combination of glutethimide plus TMH-ferrocene will synergistically induce HO activity in cultured chick embryo liver cells to the same levels produced by glutethimide plus FeNTA, but that this induction will occur more rapidly or will require less iron as TMH-ferrocene than FeNTA. Furthermore, this induction of HO was expected to be dependent on intracellular heme synthesis, and to result in a subsequent increase in ALA synthase activity, as previously shown for glutethimide plus FeNTA. In short, we anticipated that TMH-ferrocene would be more effective than FeNTA, but that it would affect HO and ALAS activities by the same mechanism.

The major finding of these studies was that TMH-ferrocene *alone* induced HO activity to levels nearly as high as our prototypic induction regimen (the combination of 50 μM glutethimide plus 50 μM FeNTA), as shown in Figs. 1, 2 (see legend to Fig. 2), 4, 8 and 9. However, the use of TMH-ferrocene for inducing HO in cultured chick embryo liver cells is limited because concentrations of TMH-ferrocene that maximally induced HO activity (5–10 μM depending on the experiment) also caused a decrease in protein synthesis (Fig. 3). We suspect that this TMH-ferrocene-induced decrease in protein synthesis is the reason for the variable levels of induction of HO activity observed between experiments.

What is the mechanism by which TMH-ferrocene induces HO activity in chick embryo liver cells? Based on studies in rats, we initially postulated that TMH-ferrocene would cause increased iron-loading of chick embryo liver cells compared with FeNTA. However, as shown in Fig. 5, the levels of cell-associated iron caused by TMH-ferrocene were, if anything, lower than levels caused by FeNTA. Thus, we have disproven our initial hypothesis that, when

given in combination with glutethimide, TMH-ferrocene will surpass FeNTA at inducing HO activity because of an increased ability to iron-load the cells.

The possibility still remained that TMH-ferrocene induces HO by a mechanism similar to that of glutethimide plus FeNTA, that is, TMH-ferrocene is able to provide the cells with the same components as glutethimide plus FeNTA, namely a lipophilic drug and a source of iron. This possibility was supported by the similar time course of induction of HO activity for TMH-ferrocene and glutethimide plus FeNTA (Fig. 4), and by the similar accumulation of porphyrins, especially uroporphyrin, by cells treated with glutethimide plus TMH-ferrocene compared with cells treated with glutethimide plus FeNTA (Fig. 7; see Ref. 1 for a discussion of the underlying mechanism of porphyrin accumulation). However, several experimental observations, described below, appear to exclude the possibility that the same mechanism is responsible for induction of HO by TMH-ferrocene compared with glutethimide plus FeNTA.

Heme-dependent versus heme-independent induction of HO. 4,6-Dioxoheptanoic acid is an effective and specific inhibitor of heme biosynthesis [16]. We have shown that 4,6-dioxoheptanoic acid also effectively inhibits the induction of HO activity by the combination of glutethimide plus FeNTA ([3], and Fig. 8). We have interpreted these results to mean that intracellular heme biosynthesis is required for induction of HO activity by glutethimide plus FeNTA. Since the induction of HO activity by TMH-ferrocene was not decreased by the addition of 4,6-dioxoheptanoic acid (Fig. 8), it appears that the induction of HO activity by TMH-ferrocene is independent of intracellular heme biosynthesis. Thus, TMH-ferrocene seems to be more like cobalt and cadmium, whose ability to induce HO is also not affected by 4,6-dioxoheptanoic acid [18, 19]. TMH-ferrocene, however, is unlike cobalt and cadmium in that it takes the metals longer to maximally induce HO [about 24 hr for the metals (unpublished observations) versus the 12 hr required for TMH-ferrocene (Fig. 4)].

Induction of ALA synthase activity. We have shown previously that the combination of glutethimide plus FeNTA induces HO activity, that the increased HO activity reduces the intracellular heme levels, and that these lowered intracellular heme levels cause a delayed synergistic induction of ALA synthase [4]. In other words, the combination of glutethimide plus FeNTA first induces HO and then ALA synthase activity increases to high levels. By contrast, cultured chick embryo liver cells treated with TMH-ferrocene alone failed to show any increase in ALA synthase activity (Fig. 6) or any increase in uroporphyrin accumulation (Fig. 7), again suggesting that the mechanism by which TMH-ferrocene induces HO activity is different than the mechanism by which glutethimide plus FeNTA induce HO.

Oxidative stress. HO can be induced by heat-shock or oxidative stress in some experimental systems [20, 21]. The induction of HO activity by glutethimide plus FeNTA may involve oxidative stress in the liver cells, since treatment with glutethimide plus FeNTA increases both HO activity and malondialdehyde formation, and both of these parameters are decreased if butylated hydroxyanisole is added (Fig. 8). However, induction of malondialdehyde formation is not sufficient for induction of HO activity, since ferrocene (a structural analog of TMH-ferrocene) increased the former but not the latter (Fig. 9). We failed to obtain evidence that the induction of HO activity by TMH-ferrocene is mediated through oxidative stress, since malondialdehyde formation was increased only slightly and addition of butylated hydroxyanisole did not decrease HO activity, compared with TMH-ferrocene alone (Fig. 9).

We conclude that, unlike the combination of glutethimide plus FeNTA, TMH-ferrocene induces HO activity by a mechanism that is independent of cellular heme synthesis. The data suggest that induction of HO by TMH-ferrocene may occur by a unique mechanism, but confirmation of this would require further study, especially on the regulatory elements of the chick HO gene. In addition, we speculate that the mechanism of the heme-dependent induction of HO (by glutethimide plus FeNTA) involves an increase in oxidative stress in liver cells. This increase in oxidative stress may occur due to the pro-oxidant properties of endogenous cellular heme.

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