Dual control mechanism for heme oxygenase: Tin(IV)-protoporphyrin potently inhibits enzyme activity while markedly increasing content of enzyme protein in liver

(heme oxidation/enzyme induction/enzyme inhibition/immunoquantitation)

MOHINDER K. SARDANA AND ATTALLAH KAPPAS

The Rockefeller University Hospital, 1230 York Avenue, New York, NY 10021

Communicated by Maclyn McCarty, November 12, 1986 (received for review October 1, 1986)

ABSTRACT Tin(IV)-protoporphyrin (Sn-protoporphyrin) potently inhibits heme degradation to bile pigments in vitro and in vivo, a property that confers upon this synthetic compound the ability to suppress a variety of experimentally induced and naturally occurring forms of jaundice in animals and humans. Utilizing rat liver heme oxygenase purified to homogeneity together with appropriate immunooquantitation techniques, we have demonstrated that Sn-protoporphyrin possesses the additional property of potently inducing the synthesis of heme oxygenase protein in liver cells while, concurrently, completely inhibiting the activity of the newly formed enzyme. Substitution of tin for the central iron atom of heme thus leads to the formation of a synthetic heme analogue that regulates heme oxygenase by a dual mechanism, which involves competitive inhibition of the enzyme for the natural substrate heme and simultaneous enhancement of new enzyme synthesis. Cobaltic(III)-protoporphyrin (Co-protoporphyrin) also inhibits heme oxygenase activity in vitro, but unlike Sn-protoporphyrin it greatly enhances the activity of the enzyme in the whole animal. Co-protoporphyrin also acts as an in vivo inhibitor of heme oxygenase; however, its inducing effect on heme oxygenase synthesis is so pronounced as to prevail in vivo over its inhibitory effect on the enzyme. These studies show that certain synthetic heme analogues possess the ability to simultaneously inhibit as well as induce the enzyme heme oxygenase in liver. The net balance between these two actions, as reflected in the rate of heme oxidation activity in the whole animal, appears to be influenced by the nature of the central metal atom of the synthetic metalloporphyrin.

The synthetic heme analogue tin(IV)-protoporphyrin (Sn-protoporphyrin) is an extremely potent inhibitor, in vitro and in vivo, of the enzyme heme oxygenase (heme-hydrogen-donor:oxygen oxidoreductase (α-methene-oxidizing, hydroxylating), EC 1.14.99.3) (1–4). This property of the metalloporphyrin has been successfully used to inhibit heme degradation to bilirubin in vivo and to suppress various experimentally induced and naturally occurring forms of jaundice in animals and humans (5–10). The potential use of Sn-protoporphyrin for ameliorating severe postnatal jaundice in human newborns has been of special interest since diminishing the production of the neurotoxic heme metabolite, bilirubin, may prove to be a more logical approach to the therapy of severe hyperbilirubinemia in the newborn than attempting to dispose of bilirubin after the bile pigment has already been formed (5).

In continued studies of the manner in which Sn-protoporphyrin regulates heme oxygenase, we have purified the rat liver enzyme to homogeneity and used purified immunooquantitative techniques to determine the content of heme oxygenase protein as well as the level of enzyme activity in the liver of metalloporphyrin-treated animals.

We report here that Sn-protoporphyrin regulates heme oxygenase by a dual mechanism—potently inhibiting the enzyme at the catalytic site by acting as a competitive substrate for heme while enhancing the synthesis of new enzyme protein. Inhibition of heme oxygenase by Sn-protoporphyrin is so pronounced that, despite the marked increase in synthesis of new enzyme protein, suppression of heme oxidation is the prevailing biological action of the metalloporphyrin that is expressed in the whole animal. This newly defined property of Sn-protoporphyrin probably extends to other metal–porphyrin complexes, as well as to certain inorganic metals that may be chelated with protoporphyrin in vivo. The ability of synthetic heme analogues to simultaneously inhibit heme oxygenase activity and to induce new enzyme synthesis provides useful new insights into the control mechanisms that determine the rate of heme degradation to bile pigments in vivo.

MATERIALS AND METHODS

Materials. Male Sprague–Dawley rats (180–200 g) purchased from Taconic Farms (Germantown, NY) were used. Metalloporphyrins were purchased from Porphyrin Products (Logan, UT). Rats were injected subcutaneously with a single dose of Sn-protoporphyrin and/or cobaltic(III)-protoporphyrin (Co-protoporphyrin) (50 μmol/kg of body weight). Solutions of the metalloporphyrins were prepared in subdued light by dissolving the compounds in 1 ml of 0.2 M NaOH, adjusting the pH to 7.4 with 1 M HCl, and diluting the solution to the final volume with 0.9% NaCl. These metalloporphyrin solutions were administered (0.2–0.4 ml/100 g of body weight) within 10 min of preparation. Control animals received an equivalent volume of 0.9% NaCl. All other chemicals used were of the highest grade obtainable from Sigma or Fisher. Radioiodinated protein A was purchased from New England Nuclear.

Tissue Preparation and Enzyme Assays. Livers were perfused in situ with ice-cold 0.9% NaCl and homogenized in 3 volumes of 0.1 M potassium phosphate, pH 7.4/0.25 M sucrose. The homogenates were centrifuged at 9000 × g for 20 min, and the resultant pellet was used to study δ-aminolevulinate (ALA)-synthase (EC 2.3.1.37) activity. The supernatant fraction was centrifuged at 105,000 × g for 1 hr; the microsomal pellet obtained was washed once and resuspended in 0.1 M potassium phosphate (pH 7.4) at a protein concentration of 15 mg/ml. The activity of heme oxygenase was determined in the microsomal fraction as previously described (11). Bilirubin produced in the heme oxygenase

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Abbreviations: Sn-protoporphyrin, tin(IV)-protoporphyrin; Co-protoporphyrin, cobaltic(III)-protoporphyrin; ALA-synthase, δ-aminolevulinate synthase.

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assay was calculated by using an extinction coefficient of 40 Mm⁻¹cm⁻¹ between 464 and 530 nm. The activity of ALA-synthase was determined in the mitochondrial fraction as previously described (12). Protein concentration was determined by the method of Lowry et al. (13) using bovine serum albumin as the standard.

**Metal Analysis.** All trace metal analyses were performed on a Zeeman/5000 atomic absorption spectrophotometer equipped with a HGA-500 graphite furnace (Perkin-Elmer) using Zeeman background correction for all graphite furnace determinations. The graphite furnace utilized the L'vov platform (14, 15) with appropriate wavelength and instrumental operating conditions as optimized in this laboratory for individual metal analyses. Cobalt and tin levels were measured in tissue digests at wavelengths of 242.5 and 286.4 nm, respectively.

**Purification of Heme Oxygenase and Preparation of Rabbit Antibody.** Rat liver heme oxygenase was purified from microsomes of rats treated with CoCl₂ (250 μmol/kg of body weight) for 16 hr to increase the specific content of the enzyme. Basically the procedure as described earlier (4) from this laboratory for purification of bovine spleen heme oxygenase was used. To achieve maximum purity of the liver enzyme, a final step of high pressure liquid chromatography on an anion exchange Mono Q column (Pharmacia) was introduced, and the enzyme was eluted by using a linear salt gradient from 0–0.12 M KCl in 20 mM Tris Cl, pH 7.7/10% (vol/vol) glycerol/0.1% Triton X-100. The enzyme eluted as a sharp peak, and the preparation was apparently homogeneous as judged from NaDodSO₄/polyacrylamide gel electrophoresis as shown in Fig. 1A. The molecular weight of rat liver heme oxygenase was 30,000, and the enzyme preparation was used to raise specific antibodies in rabbits as described earlier (16).

**NaDodSO₄/Polyacrylamide Gel Electrophoresis/Immuno-blotting.** Microsomal preparations were subjected to NaDodSO₄/polyacrylamide slab gel electrophoresis essentially as described by Laemmli (17), with 5 and 10% acrylamide in the stacking gel and separating gel, respectively. Proteins were then transferred from gel to nitrocellulose sheets (Millipore) in the cold at 0.9 A constant current in 25 mM Tris Cl, 192 mM glycine/20% (vol/vol) methanol as described by Towbin et al. (18). Transfer was complete within 90 min as monitored by silver staining of the polyacrylamide gels (19). Parallel gels not transferred to nitrocellulose were stained with Coomassie blue R-250 to visualize the protein bands. The nitrocellulose sheets with transferred protein samples were blocked by incubation in 10 mM sodium-phosphate/0.15 M NaCl, pH 7.4 (PBS)/0.1% Tween 20 buffer containing 5% (wt/vol) bovine serum albumin for 2 hr at room temperature. The sheets were washed twice with PBS, and the filters were then incubated overnight with appropriately diluted antiserum in PBS/0.1% Tween 20 with gentle shaking. The sheets were washed thrice with PBS/0.1% Tween 20/2.5% (wt/vol) bovine serum albumin and then incubated with 125I-labeled protein A. After 3 hr at room temperature, washing was again carried out as described earlier. The sheets were dried, and radioactive bands were localized by autoradiography on Kodak XRP-5 film with enhancing screens. The intensity of bands was quantitated by using an LKB laser densitometer and by directly measuring the radioactivity in the bands in a Packard gamma counter.

**RESULTS**

**Characterization of Heme Oxygenase Antiserum.** Antibodies directed against heme oxygenase were elicited in rabbits as described above. Double-immunodiffusion analysis of antiserum is shown in Fig. 1B. Precipitation lines resulted from reaction between purified heme oxygenase protein (well 1), a 1:10 dilution of heme oxygenase (well 4), and immune serum (center well). The anti-heme oxygenase antibody used in this study was specific for rat liver heme oxygenase enzyme and did not crossreact with other components of the enzyme sequence—i.e., liver NADPH-cytochrome P-450 reductase (wells 2 and 5) or liver biliverdin reductase (wells 3 and 6).

**Effects In Vivo of Sn- and Co-Protoporphyrin Administration on Heme Metabolism in Liver.** The synthetic heme analogues, Sn-protoporphyrin and Co-protoporphyrin, have been reported to be potent in vitro inhibitors of heme oxygenase activity in rat liver and in a variety of other tissues and species including human spleen (1–4). Paradoxically, Co-protoporphyrin acts in vivo as a powerful inducer of heme oxygenase (20, 21), whereas the enzyme-inhibiting action of Sn-protoporphyrin is manifest both in vivo and in vitro (1, 4, 6, 9, 10). The effects of these two metalloporphyrins, administered at a dose of 50 μmol/kg of body weight, on the activity of heme oxygenase (the rate-limiting enzyme of heme degradation) were examined in adult male rats as shown in Table 1. The enzyme activity in response to Co-protoporphyrin treatment increased (700%) as compared to control levels, while the enzyme activity measured 16 hr after Sn-protoporphyrin administration was markedly diminished (90%). The in vivo activity of hepatic ALA-synthase was inhibited by both metalloporphyrins to various degrees, ranging from 45% in the case of Sn-protoporphyrin to 75% with Co-protoporphyrin. The simultaneous treatment of rats with Sn- and Co-protoporphyrin resulted in the blockade of the Co-protoporphyrin-mediated increase in heme oxygenase activity (Table 1).

In contrast to the potent in vivo inhibitory effect of heme oxygenase activity displayed by Sn-protoporphyrin, immunoquantitation of heme oxygenase protein after Sn-protoporphyrin treatment showed a marked increase (15-fold) in the hepatic content of enzyme protein (Table 1 and Fig. 2B). Heme oxygenase protein was also increased in Co-protoporphyrin-treated animals as expected, but the induction of the enzyme protein was ~2- to 4-fold greater than the increase in the enzyme activity produced by the metalloporphyrin. Simultaneous administration of Sn-protoporphyrin and Co-protoporphyrin did not abolish the increase in heme oxygenase protein elicited by the individual compounds, but it did reverse, almost to normal, the increase in heme oxygenase activity.
Table 1. Effect of Sn- and Co-protoporphyrin administration on heme oxygenase activity, heme oxygenase content, and ALA-synthase activity in liver

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HO activity, (nmol of bilirubin)-mg⁻¹-hr⁻¹</th>
<th>HO content, μg/mg of microsomal protein</th>
<th>ALA-synthase, nmol ALA/g of liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>2.43 ± 0.16</td>
<td>0.45</td>
<td>16.1 ± 0.87</td>
</tr>
<tr>
<td>SnPP</td>
<td>0.19 ± 0.07</td>
<td>7.50</td>
<td>8.8 ± 0.36</td>
</tr>
<tr>
<td>CoPP</td>
<td>17.90 ± 2.46</td>
<td>10.11</td>
<td>4.1 ± 0.57</td>
</tr>
<tr>
<td>SnPP/CoPP</td>
<td>2.71 ± 0.31</td>
<td></td>
<td>2.2 ± 0.25</td>
</tr>
</tbody>
</table>

Rats were treated with an equimolar dose (50 μmol/kg of body weight) of Sn-protoporphyrin (SnPP), Co-protoporphyrin (CoPP), or both (SnPP/CoPP) at 0 hr. The enzymes heme oxygenase (HO) and ALA-synthase were assayed, and immunoquantitation analyses were performed 16 hr after metallocorphyrin administration as described in Materials and Methods. Data are expressed as the mean ± SEM for at least three animals.

activity produced by Co-protoporphyrin (Table 1 and Fig. 2B).

**Time-Dependent Dual Responses to Sn-Protoporphyrin In Vivo**. The effects of Sn-protoporphyrin on hepatic heme oxygenase activity in vivo and on immunooquantifiable heme oxygenase protein were examined at different time intervals after administration of the metallocorphyrin. A single dose of Sn-protoporphyrin (50 μmol/kg of body weight) caused a rapid and profound decrease in hepatic heme oxygenase activity (90%) within 30 min of metallocorphyrin administration (Fig. 3). This diminished level of enzyme activity persisted at all time intervals studied, and the enzyme remained markedly inhibited throughout the 24-hr study period. In contrast to the potent in vivo inhibitory action of Sn-protoporphyrin on heme oxygenase activity, there was an accompanying substantial increase in the immunooquantifiable enzyme protein, beginning between 4 and 8 hr. The maximum effect on induction of heme oxygenase protein was observed at 12 hr (20-fold); thereafter the increased level of the enzyme protein was maintained for at least 24 hr (Fig. 3).

**Time-Dependent Effects of Co-Protoporphyrin Administration on Heme Oxygenase**. The effects of Co-protoporphyrin on the activity as well as on the immunooquantifiable enzyme content of heme oxygenase in vivo were studied as shown in Fig. 4. Administration of Co-protoporphyrin produced a slight but transient decrease in the enzyme activity (35% within 30 min). This decrease in heme oxygenase activity reverted to normal by 4 hr, and at later time intervals the enzyme activity increased linearly, reaching a maximum (700% of control) at 16–24 hr. By comparison, the immunooquantifiable enzyme protein increased slowly up to 4 hr and was followed by a surge in heme oxygenase protein content (>20-fold) between 4 and 8 hr after Co-protoporphyrin administration. Enzyme protein content continued to increase and reached a maximum at 16–24 hr. The magnitude of increase for heme oxygenase protein was 2–4 times higher than that for the enzyme activity at all time intervals studied.

**Effects In Vivo of Sn- and Co-Protoporphyrin on Tin and Cobalt Content in Liver**. The effects of a single injection of either Sn- or Co-protoporphyrin at 50 μmol/kg of body weight on the content of the individual metals (presumed to reflect the content of the metallocorphyrins themselves since neither Co- nor Sn-protoporphyrin is enzymatically degraded in vivo) in rat liver were followed over time (Fig. 5). Metal determinations in liver digests were made as described in Materials and Methods. Administration of Sn-protoporphyrin produced a rapid increase in liver tin content that reached a maximum level (45 μg/g of liver) at 16 hr. There was no detectable tin in the livers of untreated control animals. The tin content of liver had increased more than 30-fold at 16 hr as compared to the level of 1.41 μg/g of liver, which was determined 30 min after the metallocorphyrin was administered, when heme oxygenase activity had declined by ~90%. The induction of heme oxygenase protein (4–8 hr after administration of Sn-protoporphyrin (Fig. 3) occurred when the content of tin in liver cells was 20–40 μg/g of liver. As compared to the large increase in liver Sn-protoporphyrin content necessary to initiate the enzyme induction response to administration of this metallocorphyrin, the absolute content of liver cobalt increased much more slowly when Co-protoporphyrin was administered at an equimolar dose (50 μmol/kg of body weight). The sharp increase in liver content of heme oxygenase protein elicited by Co-protoporphyrin (4–8 hr; Fig. 4) occurred when the liver content of cobalt ranged from 4 to 6 μg/g of liver and the
maximum increase in cobalt (6.5 μg/g of liver) was attained 16 hr after the metalloporphyrin treatment. The content of Sn-protoporphyrin in liver required to elicit an increase in heme oxygenase protein comparable to that produced by Co-protoporphyrin was therefore at least 7-fold greater than that of Co-protoporphyrin at equimolar doses of the metalloporphyrins, whereas marked heme oxygenase inhibition in liver was elicited within 30 min of administration of Sn-protoporphyrin, which is at a time when only very small amounts (<1.5 μg/g of liver) of the metalloporphyrin had localized in the liver.

**DISCUSSION**

These studies demonstrate the ability of the synthetic heme analogue Sn-protoporphyrin to regulate heme oxygenase by a dual mechanism. At the catalytic site of the enzyme, the compound acts as a potent competitive inhibitor of heme catabolism, an action manifest both in vitro and in vivo; Sn-protoporphyrin also displays the previously unsuspected property of markedly enhancing synthesis of the enzyme in liver as shown in this study by utilizing immunoquantitation of heme oxygenase protein. This latter action is presumably exerted at the genomic site where formation of new enzyme is initiated. The balance between enzyme inhibition and enzyme induction in vivo lies in favor of the former biological action of the compound, so that, despite the marked increase in liver content of heme oxygenase protein elicited by the metalloporphyrin, heme degradation is significantly inhibited by this metalloporphyrin in the whole animal. This accounts for the ability of Sn-protoporphyrin to suppress hyperbilirubinemia in a variety of experimentally induced and naturally occurring forms of jaundice in animals and humans.

The in vivo responses of heme oxygenase to Sn-protopor- phyrin and Co-protoporphyrin administration contrast significantly, despite the fact that the latter compound also acts in vitro as an inhibitor of heme oxygenase (1, 4). Co-protoporphyrin is a very potent inducer of the enzyme in the intact animal; this was demonstrated previously in experiments in which the metalloporphyrin, administered in a single dose, induced high levels of heme oxygenase activity in animals for periods of several weeks (20). The potent enzyme-inducing action of Co-protoporphyrin was manifest in the present study by a more pronounced enhancement of synthesis of heme oxygenase protein than that evoked by an equimolar dose of Sn-protoporphyrin. Moreover, far less accumulation of Co-protoporphyrin in liver was required to produce this increase in enzyme synthesis as compared with Sn-protoporphyrin. Nevertheless, there was a clear disparity between the Co-protoporphyrin-enhanced content of heme oxygenase protein in liver and Co-protoporphyrin-induced hepatic heme oxygenase activity; the ratio between the increase in content of enzyme protein and the increase in level of enzyme activity at all time points was approximately 2-4:1. This finding clearly suggests that the catalytic activity of some fraction of the newly synthesized enzyme protein was being concurrently inhibited by Co-protoporphyrin, which is in keeping with the known in vitro enzyme inhibitory action of this metalloporphyrin. Overall, enzyme activity (and consequently the rate of heme degradation) was greatly increased over normal levels in Co-protoporphyrin-treated animals. One consequence of this enhanced rate of heme catabolism is a marked depletion of cytochrome P-450 in liver cells of animals treated with this metalloporphyrin (20).

In contrast, at all time points studied, Sn-protoporphyrin acted as a potent inhibitor of heme oxygenase activity, and this inhibition extended both to preformed enzyme (since inhibition was nearly complete within 30 min) and to the 20-fold increase of newly formed heme oxygenase protein elicited by the metalloporphyrin. This finding attests to the profound capacity of Sn-protoporphyrin to inhibit heme catabolism in vivo.

There is considerable evidence that indicates that intracel- lular heme concentrations can be controlled at synthetic as well as at degradative levels. For example, it is believed that heme regulates its own synthesis by feedback repression of ALA-synthase, the rate-limiting enzyme in the heme biosynthetic pathway. Also at the catabolic level, heme has been implicated in the control of its own degradation in a positive feedback manner by acting to enhance heme oxygenase synthesis (22-24). Thus heme appears to be involved in two distinct feedback mechanisms that operate in opposite directions. Indeed, the ability of the porphyrinogenic drugs 2-alkyl-1,2-propylacetamide and 3,5-diethoxyacarbonyl-1,4-dihydrocollidine to deplete the “regulatory” heme pool has been suggested to lead to derepression of ALA-synthase and the accumulation of porphyrins (25, 26). In addition, administration of exogenous heme suppresses ALA-synthase and stimulates heme oxygenase synthesis (22-24). Since synthet- ic metalloporphyrins such as Sn- and Co-protoporphyrins are structural analogues of the naturally occurring metallopor-
phyrin, heme, these synthetic metalloporphyrins may also act at the same regulatory site(s) at which the natural ligand, heme, binds for induction of heme oxygenase and repression of ALA-synthase. In this respect it is of interest to note that both Sn- and Co-protoporphyrin bind to the heme receptor, which we have recently identified on the plasma membrane of murine erythroblasts (27).

The concurrent induction of an enzyme protein and inhibition of the catalytic activity of the induced enzyme by the same chemical agent is an unusual biochemical circumstance. Brown and Goldstein and their colleagues earlier described such a response with respect to the activity of 3-hydroxy-3-methylglutaryl-coenzyme A reductase in cultured human fibroblasts and Chinese hamster cells treated with compactin, a competitive inhibitor of the reductase (28, 29). In those cells, although the reductase activity was inhibited, the content of enzyme protein was increased greatly. The activity of the increased enzyme protein was masked by the presence of the inhibitor. The findings of the present study demonstrate that a comparable phenomenon can also be elicited by certain synthetic metalloporphyrins that regulate the enzyme heme oxygenase in liver.

In the present experiments, Sn-protoporphyrin, because of its uniquely potent capacity for binding to the catalytic site of heme oxygenase (1, 2, 3), almost completely blocked the activity of both preformed and newly synthesized enzyme, thus permitting its use as a pharmacological agent for suppressing heme catabolism in the whole animal. Inhibition of heme oxygenase activity was distinctly less effective with Co-protoporphyrin, in vitro, in contrast to that observed in vivo (1, 4); nevertheless, such an inhibitory action was probably expressed to some extent in the whole animal, as was suggested by the disparity between the substantial increase in the catalytic activity of heme oxygenase and the 2- to 4-fold greater increase in liver content of heme oxygenase protein induced by the metalloporphyrin. Thus it is clear that certain synthetic heme analogues possess the ability to inhibit the catalytic activity of heme oxygenase and to also induce new synthesis of the enzyme protein, that these properties may be differentially expressed in vitro and in vivo, and that heme oxygenase inhibition and induction can occur simultaneously in the whole animal. When the latter events occur, the prevailing biological response that becomes expressed—i.e., either inhibition or induction of heme oxidation activity—appears to be dependent on the nature of the central metal atom chelated by the porphyrin macrocycle.

The molecular mechanisms by which the content of heme oxygenase protein is increased in liver in response to certain synthetic metalloporphyrins have not yet been fully defined. However, induction of the enzyme protein can occur at transcriptional, translational, and posttranslational levels. Thus, several mechanisms may account for the regulation of this enzyme by synthetic heme analogues including increases in translatable mRNA, stabilization of mRNA, increases in translation of existing message, or stabilization of the final enzyme protein by decreased degradation. Direct analysis of the messages with cDNA probes would be important in answering such questions since regulation of heme oxidation by synthetic metalloporphyrins, as we have indicated earlier (9, 10), may prove to be of considerable pharmacological and clinical importance.

We thank Dr. Robert G. Lahita for his helpful discussions, Mr. David Markowitz for his excellent technical assistance, and Ms. J. A. Brighton and Mrs. Heidemarie Robinson for preparation of the manuscript. This research was supported by a grant from the Ogden Corporation and Public Health Service Grant ES-01055.