Prevention of neonatal hyperbilirubinemia by tin protoporphyrin IX, a potent competitive inhibitor of heme oxidation

[neonatal jaundice/heme oxidase (deacetylizing)/metalloporphyrins]

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ABSTRACT The effects of various metalloporphyrins on hepatic heme oxygenase (EC 1.14.99.3) activity were examined in order to identify compounds that could inhibit heme degradation to bile pigment and might therefore be utilized to suppress the development of hyperbilirubinemia in the newborn. Among nine metal-protoporphyrin IX chelates (i.e., metal-hemes) studied, Sn-heme, Mn-heme, and Zn-heme substantially diminished heme oxygenase activity in vivo in the rat. These metalloporphyrins act as competitive inhibitory substrates in the heme oxygenase reaction but are not themselves oxidatively degraded. Sn-heme was the most potent enzyme inhibitor ($K_i = 0.011 \mu M$) in liver, spleen, kidney, and skin. Sn-heme administered to newborn animals within the first 72 hr after birth blocked the postnatal increase in heme oxygenase activity that occurs in various tissues. Its effect on the enzyme levels was prompt and protracted. Sn-heme administration also entirely prevented the development of hyperbilirubinemia that normally occurs postnatally. The effect of the metalloporphyrin in lowering the increased concentrations of serum bilirubin in neonates was prompt (within 1 day) and persisted throughout the 42 days after birth. No deleterious effects of Sn-heme treatment of the newborn were observed. This demonstrates that a synthetic metalloporphyrin that is a potent competitive inhibitor of heme oxygenase can, when administered to the newborn, also prevent the hyperbilirubinemia that normally develops postnatally. The potential clinical implications of these findings are evident, and it is suggested that the pharmacological properties of Sn-heme and related synthetic metalloporphyrins merit further study.

Chemical blockade of the postnatal induction of heme oxygenase activity [heme, hydrogen-donor:oxygen oxidoreductase (amethene-oxidizing, hydroxylating), EC 1.14.99.3] and suppression of the hyperbilirubinemia that results from fetal erythrocyte lysis and exaggerated heme oxidation during this period has not been demonstrated in the whole animal. Total blockade of heme oxygenase induction by certain transition and related elements can, however, be achieved by concurrent administration of other metal ions (1, 2). Moreover, certain synthetic metalloporphyrins, as we earlier demonstrated (3), can act as competitive substrates for heme in the heme oxygenase reaction, though they do not themselves undergo oxidative degradation by the enzyme. These findings indicate that heme oxygenase activity can be regulated at the catalytic level as well as at the synthetic level. Appropriate competitive inhibition of heme oxidation by the enzyme might therefore be expected to suppress or prevent the hyperbilirubinemia that occurs in the neonatal period.

In this study we examined the ability of nine metalloporphyrins to alter heme oxygenase synthesis or function in liver, spleen, and other tissues of the rat. Of these compounds three markedly inhibited heme degradation by the enzyme; three substantially enhanced this process, presumably by inducing heme oxygenase; and three had intermediate effects on the enzyme. Two of these metal protoporphyrin IX complexes (Sn-heme and Mn-heme) were employed in whole animal studies to determine whether the ability to block heme oxygenase activity demonstrated in vitro could be shown to have physiological expression by also blocking the development of postnatal hyperbilirubinemia in the neonate during the 6-week period after birth.

We report here that a metalloporphyrin, Sn-heme, which in vitro acts as a potent competitive substrate for heme in the heme oxygenase reaction can also, when administered to newborn animals, entirely prevent the hyperbilirubinemia that develops in the postnatal period.

MATERIALS AND METHODS

Materials. Male (160–200 g) and 15-day-pregnant female Sprague–Dawley rats purchased from Holtzman (Madison, WI) were used. To the extent possible pregnancy was synchronized in the latter group of animals so that large numbers of newborn could be studied within the same postnatal time period; the study involved a total of 750 neonates divided approximately equally among the control and experimental groups described below. Metalloporphyrins were purchased from Porphyrin Products (Logan, UT) and all other chemicals were of the highest grade obtainable from either Fisher or Sigma.

Treatment of Animals and Preparation of Microsomes. Male rats were injected at subcutaneous sites with the protoporphyrin IX chelates of manganese (Mn$^{2+}$), tin (Sn$^{2+}$), nickel (Ni$^{2+}$), zinc (Zn$^{2+}$), magnesium (Mg$^{2+}$), copper (Cu$^{2+}$), cadmium (Cd$^{2+}$), cobalt (Co$^{2+}$), or iron (Fe$^{2+}$) at various doses up to 125 $\mu$mol/kg body weight in order to determine the ability of these various heme complexes to induce or inhibit the activity of microsomal heme oxygenase in liver. The metalloporphyrins were taken up in a small volume of 0.2 M NaOH adjusted to pH 7.4 with 1 M HCl and made up to final volume with 0.9% NaCl. Control animals were injected with an equivalent volume of saline. The animals were given access to water but were starved for 16 hr after metalloporphyrin administration, prior to decapitation. Microsomes were prepared as described (1).

Neonates received subcutaneous injections of either Sn-heme or Mn-heme (100 $\mu$mol/kg body weight) prepared as described above but in a final volume of 0.1 ml. The regimen of administration was a single injection of the synthetic hemes at birth, and 12, 24, 48, and 72 hr later. The dose of metalloporphyrin employed was selected to fall within the range examined in the in vivo studies in the adult male animal (see above). The time period during which each compound was administered conforms with that in which, postnatally, heme oxygenase activity is known to be rapidly increasing (4). Control neonates received an equivalent volume of saline at each of these time periods.
points. Groups of neonates (6–30 animals per group) were then sacrificed at the times indicated in the figures and assays were performed for serum bilirubin and tissue enzymes as described below.

Enzyme Assays. Heme oxygenase was assayed as described (5), and bilirubin formation was calculated on the basis of an extinction coefficient of 40 mM⁻¹·cm⁻¹ and the difference of absorbance between 464 nm and 530 nm. The assay was carried out on the microsomal fractions isolated from liver, kidney, and spleen and on the 9000 x g fraction from skin. 3-Amino-levulinate synthase was measured as described (6). All assays were performed on an Aminco Chance DW2A spectrophotometer in the split beam mode.

Total bilirubin was estimated in serum fluorimetrically by the method of Roth (7); the variation in replicate samples of serum was <5% (7). Protein concentration was determined with crystalline bovine albumin as standard (8). The data were analyzed by the standard t test and the value of P < 0.05 was regarded as denoting significance.

RESULTS

Effect of Various Metal-Protoporphyrin IX Complexes on Heme Oxygenase and 3-Aminolevulinate Synthase Activities in Liver. Metalloporphyrins, administered at a dose of 50 μmol/kg body weight, were examined in adult male rats for their effects in vivo on the activities of heme oxygenase and 3-aminolevulinate synthase, the rate-limiting enzymes of heme catabolism and synthesis, respectively (Table 1). Hepatic heme oxygenase activity, measured 16 hr after metalloporphyrin administration, was lowered (50–60%) by Sn-heme, Mn-heme, and Zn-heme, increased (100–400%) by Cd-heme, Fe-heme, and Co-heme, and largely unaffected by the other compounds examined. This pattern of metalloporphyrin effects on heme oxygenase activity was maintained in liver up to the highest dose administered (125 μmol/kg body weight). Sn-heme, Mn-heme, and Zn-heme also significantly lowered renal heme oxygenase activity at all doses (50–125 μmol/kg body weight) studied (results not shown). Hepatic 3-aminolevulinate synthase activity was inhibited to various degrees by all metalloporphyrins (50 μmol/kg body weight) with the exception of Cu-heme and Sn-heme (Table 1); however, Sn-heme administered at a dose of 125 μmol/kg body weight inhibited the enzyme activity in vivo by 60% (results not shown).

<table>
<thead>
<tr>
<th>Metalloporphyrin</th>
<th>Heme oxygenase, nmol bilirubin/mg protein per hr</th>
<th>3-Aminolevulinate synthase, nmol aminolevulinic acid/mg protein per hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.47 ± 0.12</td>
<td>0.178 ± 0.01</td>
</tr>
<tr>
<td>Manganese</td>
<td>1.05 ± 0.16*</td>
<td>0.068 ± 0.01*</td>
</tr>
<tr>
<td>Zinc</td>
<td>1.24 ± 0.14*</td>
<td>0.098 ± 0.01*</td>
</tr>
<tr>
<td>Tin</td>
<td>1.08 ± 0.13*</td>
<td>0.177 ± 0.04</td>
</tr>
<tr>
<td>Copper</td>
<td>2.82 ± 0.10</td>
<td>0.198 ± 0.01</td>
</tr>
<tr>
<td>Nickel</td>
<td>3.70 ± 0.46</td>
<td>0.110 ± 0.01</td>
</tr>
<tr>
<td>Magnesium</td>
<td>3.65 ± 0.68</td>
<td>0.043 ± 0.01*</td>
</tr>
<tr>
<td>Cadmium</td>
<td>5.26 ± 0.50*</td>
<td>0.025 ± 0.01</td>
</tr>
<tr>
<td>Iron</td>
<td>6.50 ± 0.59*</td>
<td>0.040 ± 0.01</td>
</tr>
<tr>
<td>Cobalt</td>
<td>10.92 ± 0.79*</td>
<td>0.108 ± 0.01</td>
</tr>
</tbody>
</table>

Metalloporphyrins were administered subcutaneously in a single dose (50 μmol/kg body weight) to the animals, which were killed 16 hr later. Three to six animals were used for each determination and the assays were performed in duplicate. Data are presented as mean ± SEM.

* P < 0.05, compared with control.

Competitive Inhibition of Heme Oxygenase Activity In Vitro by Metalloporphyrins. The apparent K<sub>i</sub> for heme in the splenic (Fig. 1) and hepatic heme oxygenase systems was determined to be 12.5 μM, which is within the range reported previously for microsomal preparations (3, 9). The addition of Sn-heme (0.1 μM) to splenic heme oxygenase incubation mixtures resulted in a marked competitive inhibition of enzyme activity, with a >10-fold increase [12.5 μM to 136 μM (Fig. 1)] in the K<sub>i</sub> for heme. In separate experiments with both spleen and liver heme oxygenase preparations, the K<sub>i</sub> (inhibitor constant) for Sn-heme was determined to be 0.011 μM. Zn-heme produced an increase in the apparent K<sub>i</sub> for heme to 71 μM (spleen microsomes) and its K<sub>i</sub> was determined to be 0.212 μM. Mn-heme, as previously noted (10), interfered somewhat with the spectrophotometric determination of the product of the heme oxygenase assay; Mn-heme, however, appeared to be a potent inhibitor of both spleen and liver heme oxygenase activity, with a K<sub>i</sub> that was intermediate between the K<sub>i</sub> of Sn-heme and Zn-heme.

On the basis of these findings (Table 1, Fig. 1) Sn-heme and Mn-heme were selected for in vivo studies in neonatal rats to determine if they were capable of blocking the development of hyperbilirubinemia in the postnatal period. Treatment began immediately after birth, as indicated in Materials and Methods, and the study continued throughout the subsequent 6-week period.

Effects of Sn-Heme and Mn-Heme Administration on Heme Oxygenase Activity in the Neonate. The effects of Sn-heme and Mn-heme administration on hepatic heme oxygenase levels in neonates during the 6-week study period are shown in Fig. 2. Hepatic heme oxygenase activity rose rapidly after birth as expected (4), reaching levels 4-fold above those characteristic of adult liver before gradually declining to normal adult levels.

![Fig. 1. Effect of Sn-heme (0.1 μM) on the oxidation of Fe-heme in vitro by microsomal splenic heme oxygenase prepared from the spleens of adult male animals. The enzyme assay was performed in duplicate.](image-url)
between days 21 and 42. In Sn-heme-treated neonates, the metalloporphyrin completely prevented the normal marked increase (4) in hepatic heme oxygenase activity after birth (days 2–10); indeed, in these neonates heme oxygenase activity declined immediately (within 1 day) after administration of the compound to levels below those of normal adults (day 7 level), where they remained for a considerable time before returning to normal levels by day 42. Mn-heme administration resulted in effects on the pattern of postnatal development of hepatic heme oxygenase intermediate between those of control and Sn-heme-treated animals. Responses of heme oxygenase activity in skin and kidney of control and Mn-heme- and Sn-heme-treated neonates paralleled those found in liver; Sn-heme administration profoundly lowered the enzyme activity in these tissues, whereas Mn-heme treatment produced intermediate effects (results not shown).

Postnatal development of splenic heme oxygenase (Fig. 3) differed from that in the other tissues examined (Fig. 2); enzyme activity, which, as we have previously reported (4), is low at birth, increased rapidly in the postnatal period (days 2–4), then more gradually, until adult levels were finally reached at days 14–21 in control neonates concurrent with an increase in spleen size. Mn-heme administration produced a more rapid increase in splenic heme oxygenase activity during the first 4 days postnatally, plateauing at adult levels thereafter. In contrast, Sn-heme administration entirely prevented the increase in splenic heme oxygenase activity observed in control neonates, with the enzyme activity remaining at the day 1 level throughout the first postpartum week; enzyme activity then declined between days 7 and 14, before returning towards normal adult levels, which were not attained until 42 days after birth.

**Effect of Sn-Heme and Mn-Heme Administration on Total Bilirubin Levels in the Neonate.** Sn-heme was selected for study because this metalloporphyrin displayed a potent ability to inhibit heme oxygenase activity in vivo and in vitro in all tissues examined. Mn-heme was studied because its inability to inhibit heme oxygenase in vivo in the spleen (Fig. 3), despite its in vitro effects on the enzyme, permitted an assessment in the whole animal of the role of splenic heme oxygenase activity in the development of neonatal hyperbilirubinemia. The effects of Sn-heme and Mn-heme treatment on serum bilirubin levels of neonates during the first 6 weeks after birth are shown in Fig. 4. The total bilirubin concentration in the serum of untreated neonates was increased at birth as expected for this species and increased to a maximum at day 3. The serum bilirubin at this time was 4-fold higher than that on day 21; the latter value is within the range typical of the normal adult animal. Sn-heme administration resulted in an immediate (within 1 day) and significant lowering of serum bilirubin levels in treated animals, and the decline continued throughout the period when the serum concentration of the bile pigment in control neonates was increasing or remaining above normal adult levels (Fig. 4). In Sn-heme-treated animals near-normal levels of serum bilirubin were quickly reached (day 3) and were maintained throughout the 42 days of study. The bilirubin concentration in the serum of control neonates did not match the level in Sn-heme-treated animals until the second postnatal week. It is of interest that this developmental pattern of serum bilirubin changes corresponds closely to the developmental pattern that hepatic heme oxygenase undergoes in untreated neonates (Fig. 2; ref. 4).

The bilirubin levels in Mn-heme-treated neonates paralleled those in the control neonates even though this metalloporphyrin significantly inhibited heme oxygenase activity in vitro in liver, kidney, spleen, and skin. The fact that Mn-heme administration could not block in vivo the postnatal development of spleen heme oxygenase activity (Fig. 3) may account for the lack of effect of this metalloporphyrin on serum bilirubin levels postnatally. This finding also indicates that blockade of heme oxygenase activity in spleen as well as in other tissues is probably essential to the prevention of hyperbilirubinemia postnatally.

**DISCUSSION**

Studies of the regulation of heme oxygenase by inorganic metals and by various metal-protoporphyrin IX chelates (i.e., metal-
hemes) have now revealed that within each of these groups of agents there are both inducers and inhibitors of the enzyme synthesis or of its catalytic function. Thus, many inorganic ions—i.e., Co, Ni, Cd, and Sn—are potent inducers of the enzyme in liver or kidney (2, 11–13), an action that can be partially or entirely blocked by the concurrent administration of certain other metal ions—i.e., Zn or Mn (1, 2). Similarly, metal-hemes, such as Fe-heme, Cd-heme, and Co-heme, also induce heme oxygenase synthesis (Table 1; refs. 14 and 15) whereas other metal-protoporphyrin IX chelates, such as Sn-heme, Mn-heme, and Zn-heme, significantly inhibit the enzyme activity (Table 1; refs. 3 and 16). The latter effect, as we showed earlier (3), reflects the activity of these synthetic metalloporphyrins (Fig. 1) to act as competitive substrates for the heme-binding site on heme oxygenase. Co-heme, now known not to be a substrate for heme oxygenase (17, 18), in contrast to earlier findings (3), is an interesting metalloporphyrin because it has the capacity to act both as a competitive substrate for heme in the heme oxygenase reaction and as a powerful inducer of the enzyme, producing marked as well as prolonged increases in its activity (Table 1; ref. 19). Functionally, the enzyme-inducing property of Co-heme predominates in vivo.

In undertaking this study of the comparative effects of various metal-heme chelates on heme oxygenase activity in vitro our intent was to identify compounds that might be potent inhibitors of this enzyme action and as such could be employed in experiments in vivo as competitive synthetic substrates for heme oxidation, thus possibly inhibiting the development of the hyperbilirubinemia characteristic of the newborn. Of the two metalloporphyrins chosen for study during the first 6 weeks of postpartum life, Sn-heme proved to be completely effective in blocking the normal development of increased bilirubin levels in the newborn animal (Fig. 4). This action is unambiguously attributable to the very potent ability of this metalloporphyrin to block heme oxygenase catalysis of heme in spleen (Fig. 3), as well as in other tissues such as liver (Fig. 2), kidney, and skin both in vitro and in vivo. The failure of Mn-heme treatment of neonates to suppress the increased levels of serum bilirubin in the 6 weeks after birth indicates that inhibition of spleen heme oxygenase activity in vivo is probably necessary for suppression of hyperbilirubinemia and that the demonstration of heme oxygenase inhibition in vitro by a specific metalloporphyrin does not guarantee that the compound in question will act comparably in vivo to block the enzyme activity in all tissues. Many biologic variables may influence the latter response—i.e., pharmacokinetic parameters, tissue distribution, subcellular localization of the synthetic metalloporphyrin, etc.—and, as the findings with Sn-heme demonstrate, whole animal studies are required to establish whether an inhibition of heme oxygenase activity identified in vitro will also be expressed in vivo by a comparable inhibition of the development of excessive hyperbilirubinemia.

Studies concerning the biological effects of tin in man are extremely meager; those in animals have focused on the role of the element as an essential micronutrient (20) and also as a possibly toxic material to which there may be excessive environmental exposure (21). Such toxicity can extend to man in certain circumstances, especially in relationship to exposure to organotin compounds such as triethyltin (22, 23). In this study Sn-heme had no apparent deleterious effects in the treated neonates in terms of morbidity, mortality, growth rate, organ size, or sucking and other behavior. Inorganic tin (Sn(II)) is known to impair 8-aminolevulinate dehydratase activity in vivo (24) and to potentiate induce renal heme oxygenase in vivo (2, 13). Sn(IV), on the other hand, does not display these biological activities (24), and it is the latter form (Sn(IV)) of the element that characterizes the valence state of the metal in Sn-heme.

The synthetic metalloporphyrins employed in this study do not bind molecular oxygen when complexed with heme oxygenase and are not degraded by the enzyme; their metabolic fate is therefore not known. However, if Sn-heme were degraded by some cellular mechanisms, thus releasing the Sn(IV) in vivo, our inability to detect, over a 6-week period, an induction effect on renal heme oxygenase [which is induced markedly by Sn(II) (2, 13)] would support previous observations (24) that Sn(IV) is not readily reduced during its absorption and systemic tissue distribution in the whole animal.

It should be noted that Sn-heme administration, with a total dose of 4 μmol of the metalloporphyrin given to each neonate, produced an immediate and substantial lowering of serum bilirubin (Fig. 4) and marked depressions of hepatic and splenic heme oxygenase activities (Figs. 2 and 3) for a prolonged period. The protracted nature of these effects in the whole animal, coupled with the in vitro kinetic data showing the profound inhibition of heme oxidation produced by the metalloporphyrin (Fig. 1), strongly suggests that much smaller amounts of Sn-heme than those used in this study would produce the same biological responses in neonates. Our preliminary findings indicate that single doses of Sn-heme in the range ≈5% of those employed in this study produce in vivo <60% inhibition of heme oxygenase activity in adult spleen and liver.

Hyperbilirubinemia in the newborn is common; the excess bile pigment is derived from exaggerated erythrocyte destruction, and its retention in plasma is due in part to an immaturity of the hepatic glucuronontransferase system; a variety of clinical events, such as prematurity, infection, hypoxia, acidosis, hypothermia, concurrent hemolytic disorders (i.e., glucose-6-phosphate dehydrogenase deficiency), etc., may result in concentrations of serum bilirubin greatly exceeding those considered "physiologic." Arbitrary levels of unconjugated serum bilirubin have been defined beyond which the risk of the severe and irreversible encephalopathy known as "kernicterus" is considered high; these levels extend, depending upon factors such as birth weight and clinical complications, as high as 20 mg/dl (see ref. 25 for review) vs. normal serum bilirubin levels of ≈1.0 mg/dl. Treatments may include phototherapy, which converts some fraction of serum bilirubin to metastable more hydrophilic geometric isomers that are excreted in bile (26), exchange transfusions, and the use of inducers of bilirubin glucuronontransferase such as phenobarbital (27, 28).

The decision to apply any of these therapies to neonates with excessive hyperbilirubinemia is usually based on the consideration that the bile pigment has reached a predetermined level which is thought, in the specific clinical situation, to be hazardous. However, as others have indicated (25, 29), it is unlikely that single levels of serum bilirubin can predict the occurrence of bilirubin-related brain damage, and neurotoxicity from this bile pigment may well represent a continuum of very subtle to quite overt neuropathological processes correlating in a general fashion with increasing serum levels of free bilirubin. Within this clinical context the availability of a chemical that can act as a potent competitive inhibitor of heme oxygenase could prove to have therapeutic value, because the biological action of such an agent on the enzyme would be immediate and bile pigment formation would be expected to be suppressed quickly.

Sn-heme, as shown in these studies, displays the appropriate biochemical properties of a model agent of this type. Suppression of the rate of heme breakdown by ferrous heme oxygenase inhibition would presumably lead to the sequestration of heme in the circulation or in various tissues. The consequences of this in the newborn are not known; in the adult, however, heme (as hematin) has been administered intravenously (doses
as well as the hereditary hepatic porphyrinas (32–34) as well as lead poisoning (35), and with the exception of a single individual (36) who had a transient renal complication after infusion of a massive amount of hematin (1000 mg within 30 min), such therapy has been essentially without detriment to the patient.

The pharmacokinetic characteristics, metabolic fate, and toxicity, if any, of small amounts of synthetic metalloporphyrins administered for short periods of time to humans are at present not known. We believe it is important to obtain such information, because the direct blockade of bile pigment formation at the catalytic site of heme oxygenase, exemplified in this study by the action of Sn-heme, defines a potentially important means for regulating this enzyme activity. A control mechanism of this type may be therapeutically useful in clinical situations in which excessive hyperbilirubinemia or exaggerated rates of heme oxidation occur.

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