Interaction of Hemopexin with Sn-Protoporphyrin IX, an Inhibitor of Heme Oxygenase

ROLE FOR HEMOPEXIN IN HEPATIC UPTAKE OF Sn-PROTOPORPHYRIN IX AND INDUCTION OF mRNA FOR HEME OXYGENASE*

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Sn-protoporphyrin IX (SnPP), an inhibitor of heme oxygenase and a potential therapeutic agent for neonatal hyperbilirubinemia, is bound tightly by hemopexin. The apparent dissociation constant (Kd) at pH 7.4 is 0.25 ± 0.15 μM, but estimation of the Kd for the SnPP-hemopexin complex is hampered by the fact that at physiological pH SnPP exists as monomers and dimers, both of which are bound by hemopexin. SnPP is readily displaced from hemopexin by heme (Kd < 1 μM). The hemopexin-SnPP interaction, like that of heme-hemopexin, is dependent on the histidine residues of hemopexin. However, as expected from the differences in the coordination chemistries of tin and iron, the stability of the histidyl-metalloporphyrin complex is lower for SnPP-hemopexin than for mesoheme-hemopexin. Nevertheless, when SnPP binds to hemopexin, certain of the ligand-induced changes in the conformation of hemopexin which increase the affinity of the protein for its receptor are produced. Binding of SnPP produces the conformational change in hemopexin which protects the hinge region of hemopexin from proteolysis, but SnPP does not produce the characteristic increase in the ellipticity of hemopexin from 231 nm that heme does. Competition experiments confirmed that human serum albumin (apparent Kd = 4 ± 2 μM) has a significantly lower affinity for SnPP than does hemopexin. Appreciable amounts of SnPP (up to 35% in adults and 20% in neonates) would be bound by hemopexin in the circulation, and the remainder of SnPP would be associated with albumin due to the latter’s high concentration in serum. Essentially no non-protein-bound SnPP is present. Importantly, SnPP-hemopexin binds to the hemopexin receptor on mouse hepatoma cells with an affinity comparable to that of heme-hemopexin and treatment of the hepatoma cells with SnPP-hemopexin causes a rapid increase in the steady state level of heme oxygenase messenger RNA. These results show that hemopexin participates in the transport of SnPP to heme oxygenase and in its regulation by SnPP.

Hemopexin is the plasma glycoprotein which acts to convey heme† (iron-protoporphyrin IX) to hepatic heme oxygenase (1). In this receptor-mediated transport process (2–4), a 17.5-kDa heme-binding membrane protein (4) is thought to act in the intracellular transport of heme. Since tin-protoporphyrin IX (SnPP), a specific inhibitor of microsomal heme oxygenase (5), is being evaluated as a potential therapeutic agent for neonatal hyperbilirubinemia (6), it is important to define both the interaction of SnPP with metalloporphyrin-binding plasma proteins like hemopexin and the mechanism of the transport of SnPP to tissues. Human serum albumin (HSA) and myoglobin were shown to bind SnPP (7) and the liver, kidney, and spleen to be major sites of uptake of SnPP administered to rats (8, 9). Plasma clearance was dose-dependent with a t1/2 near 4 h for initial plasma levels of 7–15 μM, but more rapid (t1/2 near 15 min) for levels of 0.025–0.2 μM (9), suggesting that a saturable, receptor-mediated process may be involved. However, the mechanisms of transport of SnPP, and in particular the role of hemopexin in this transport, have not been elucidated. Since hemopexin is the major transport protein involved in liver uptake of heme and is present in neonates at near adult levels, characterization of the SnPP-hemopexin interaction and of the biological consequences of this interaction is required to develop and evaluate SnPP therapy.

The work reported here was undertaken to address some of these questions. The results demonstrate that hemopexin binds SnPP with sufficient affinity to participate in transporting SnPP to the liver. Moreover, the specific hemopexin receptor is involved in uptake of SnPP and intracellular routing of SnPP to heme oxygenase.

MATERIALS AND METHODS

Hemopexin was purified from rabbit serum as described (10), and the activity and purity (greater than 95%) of the isolated protein were determined from the characteristic absorbance and heme-binding properties of the molecule (1, 11, 12) as well as by SDS-polyacrylamide gel electrophoresis (13) using 4–20% acrylamide gradient gels. Rabbit hemopexin was used in this work since its interactions with heme and the hemopexin receptor are characterized more fully than those of other species of hemopexin. HSA was obtained from Kabi and SnPP, mesoporphyrin, and mesoheme from Porphyrin Products. Mesoporphyrin IX was labeled with 55Fe (Du Pont-New England Nuclear, Boston, MA) by refluxing in dimethylformamide (14) and freed of unincorporated iron and metal-free porphyrin by washing.

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†The abbreviations used are: heme, ferriprotoporphyrin IX; mesoheme, ferrimesoporphyrin IX; SnPP, Sn-protoporphyrin IX; DMEM, Dulbecco’s modified Earle’s medium; PBS, phosphate-buffered saline; 10 mM sodium phosphate, 0.15 mM NaCl, pH 7.4; HSA, human serum albumin; SDS, sodium dodecyl sulfate; DEP, diethyl pyrocarbonate; Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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with acid. Hemopexin was labeled with 125I (ICN, Irvine, CA) using IODO-BEADES (Pierce Chemical Co., Rockford, IL) according to the manufacturer's instructions, and unincorporated 125I was removed by passage over Sephadex G-25 (Sigma Chemical Co., St. Louis, MO) or by extensive dialysis. Iodinated hemopexin was routinely more than 95% precipitable by 10% final volume trichloroacetic acid, had no specific activity of 4000 to 5000 dpm/nmol, and retained full hemebinding and immunological activities.

Singly labeled metal-porphyrin-hemopexin complexes were prepared as described (1-3). Mesoheme (iron-mesoporphyrin IX) from Porphyrin Products (Logan, UT) was used in place of heme (ironprotoporphyrin IX) since it is more stable and more reactive than the heme-hemopexin complexes chemically and biologically equivalent to heme-hemopexin (1-4). Concentrations were measured spectrophotometrically using published extinction coefficients (mM^-1 cm^-1) of 1.7 x 10^4 at 394 nm for mesoheme in dimethyl sulfoxide (15), 1.1 x 10^4 at 280 nm for apohemopexin (16), of 1.3 x 10^4 at 405 nm for mesoheme-hemopexin (17), and of 3.6 x 10^4 at 280 nm for HSA (18). To minimize dimerization, solutions of SnPP (Porphyrin Products) in dimethyl sulfoxide were prepared fresh each day, and their concentrations were determined using an extinction at 406 nm of 1.6 x 10^4 in 0.5% pyridine containing 1 drop of NHLOH/100 ml.

Protein concentrations were obtained using a Cary 219 spectrophotometer, fluorescence spectra with a Perkin-Elmer 650-40 fluorometer, and circular dichroism spectra with a Jasco 500C spectropolarimeter. Fluorescence binding titrations were carried out in 10 mM sodium phosphate containing 0.1 M NaCl, pH 7.4, or in 0.1 M sodium borate, pH 9.2, by adding portions of concentrated hemopexin solutions to a known amount of SnPP, usually 0.5-1.5 µM. The excitation was at 410 nm, and the emission was recorded from 500 to 650 nm for each addition. Titrations of binding using absorbance change upon adding aliquots of porphyrin to protein were conducted by the small change in absorbance of the SnPP upon binding and the steady changing degree of dimerization as the SnPP concentration increased.

Complexes of hemopexin with SnPP or with mesoheme were prepared by mixing 1 or 2 eq of tetrapyrrole with 1 eq of protein. Unbound tetrapyrrole was removed by passage over DEAE-cellulose (DE-52, Whatman, Clifton, NJ) or by extensive dialysis by 600,000 Mx. Modification reactions using diethyl pyrocarbonate (DEP) were carried out for 1 h on ice by adding the indicated amount of DEP to apohemopexin (at a concentration of 4.6 µM) in 0.1 M sodium phosphate, pH 6.3. The number of DEP-modified histidine residues was calculated using a millimolar difference extinction coefficient of 3.2 X 10^5 at 280 nm for HSA (18). To minimize dimerization, solutions of DEP-modified histidine residues were allowed to equilibrate at the indicated temperatures before measurement.

Minimal deviation hepatoma cells (mouse Hepa cells) from mouse solid tumor line BW 7756 were grown in Dulbecco's modified Earle's medium (DMEM) containing 2% fetal calf serum and 90 µg/ml gentamycin. For all studies, the Hepa cells were maintained in the log phase of growth. This cell line has been shown to synthesize hemopexin and to possess all the cellular components needed for specific, hemopexin-mediated cell uptake of heme.6 Binding of 125I-labeled protein was measured by adding 1-ml aliquots of Hepsbuffered DMEM, pH 7.4, containing the protein to 1.0-1.5 x 10^6 cells in one well of a 6-well tissue culture dish. At the indicated time, the medium was aspirated from the well, and the cells were washed with 3 x 2 ml of cold 10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl. Then NaOH (0.1 M, 2 ml) was added to dissolve the cells, and the suspension was removed and counted in a Beckman 7300C spectrophotometer. At least three wells were used for each determination. Uptake of 125I-labeled hemopexin was measured in a similar manner except that 0.23 ml of 50% acetic acid was added to neutralize the dissolved cells before 15 ml of liquid scintillation fluid (Betablend, Westchem Scientific, San Diego, CA). The cell-associated 125I was measured in a Beckman 9800 scintillation counter. Specific binding or uptake is defined as the difference in binding or uptake in the presence and absence of excess unlabeled mesoeheme-hemopexin added to the cells before the labeled complex. Additional details are given in the legends to the figures and tables. Cell counts were made on at least two wells before each experiment by releasing the cells with trypsin and counting the cells in a hemocytometer. Protein was determined on aliquots of the neutralized cell extracts using the Pierce BCA protein assay system according to the manufacturer's directions with bovine serum albumin as a standard.

Measurement of cellular heme oxygenase mRNA applied Northern blot analyses using the plasmid pHRO1 containing a full-length cDNA for rat heme oxygenase (23) kindly provided by Dr. S. Shibahara, Friedrich-Miescher Institute, Basel. Plasmid DNA was labeled to a specific activity of 1 x 10^10 cpm/µg using a nick translation kit (Bethesda Research Laboratories) and [α-35S]dCTP (5000 Ci/ mmol) (Du Pont-New England Nuclear). For isolation of total cellular RNA, Hepa cells were grown as described above. Subconfluent cells (1-3 x 10^5 cells in 150-cm2 culture flasks) were rinsed three times with 10 ml of prewarmed, serum-free DMEM buffer with 10 mM Hepes, pH 7.2. The cells were subsequently incubated with 10 ml of the same medium containing various concentrations of SnPP or SnPP-hemopexin for 4 h. Cytoplasmic RNA was isolated from Hepa cells according to the procedure of Greenberg and Ziff (21). The RNA was fractionated on a denaturing formaldehyde/agarose (1%) gel (22) and transferred to Zeta-Probe (Bio-Rad) nylon membrane according to the manufacturer's recommendations. Vacuum baked filters were incubated for 18 h at 45°C with 10 ml of hybridization buffer: 3 X SSPE (1 X SSPE = 0.15 M NaCl, 3 mM EDTA, 10 mM sodium phosphate, pH 7.4), 40% (v/v) deionized formamide, 7% (w/v) S DS, and 200 µg/ml denatured salmon sperm DNA. The filters were subsequently incubated with fresh hybridization buffer containing labeled DNA probe (1 x 10^8 cpm/ml of solution) for 36 h at 45°C. After hybridization, the filters were washed with solutions of 2 X SSC/0.1% SDS (1 X SSC = 0.15 M NaCl, 15 mM sodium citrate, pH 7.0) at room temperature for 30 min and 0.1 X SSC/0.1% SDS at 55°C for 60 min. The blots were exposed to x-ray film (Kodak X-Omat RP) with an intensifying screen at ~70°C for 36 h. Quantitation of the developed autoradiographs was carried out using densitometry.

RESULTS AND DISCUSSION

The interaction between hemopexin and SnPP is evident by the changes in both the absorbance and fluorescence spectra of SnPP in the presence of hemopexin. Hemopexin binds SnPP to form a complex with an absorption maximum at 410.5 nm (Fig. 1), and the fluorescence of SnPP is quenched upon interaction with hemopexin (Fig. 2). Due to complexities introduced by dimerization of the ligand and the small spec-

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5 B. Burnham, Porphyrin Products, personal communication.
6 A. Smith and B. L. Ledford, manuscript submitted for publication.

FIG. 1. Absorbance spectra of SnPP-hemopexin and SnPP-HSA. The absorbance spectra of the equimolar complexes, 1.5 µM, of SnPP-rabbit hemopexin (solid line) and of SnPP-HSA (dashed line) were recorded in phosphate-buffered saline, pH 7.4, 5 min after mixing. For comparison, the spectrum of SnPP alone at the same concentration is also shown (dash-dot line). The Soret wavelength maxima were at 410-411 nm for the hemopexin complex, at 406-407 nm for the HSA complex, and at 406 nm for SnPP alone.
SnPP and hemopexin occurs at the unphysiological pH of 9.2 with approximately the same affinity (Fig. 3). This is not unexpected since the affinity of hemopexin for hormone decreases only at pH values above 9.2 (12). No explanation for the decreased quenching of the fluorescence of SnPP by hemopexin at this pH compared to that at pH 7.4 can be offered.

The association between SnPP and hemopexin, like that between heme and hemopexin (20, 25), requires intact histidine residues. As shown in Fig. 4, modification of histidine residues of hemopexin with DEP prevents formation of the SnPP-hemopexin complex as judged by the lack of change in both absorbance (panel A) or fluorescence spectra (panel B). However, as expected from the differences in coordination chemistry between heme and iron, the stability of the histidylmetalloporphyrin coordination complex is lower for SnPP-hemopexin than for heme-hemopexin. Several observations support this conclusion. First, SnPP does not protect the two heme-coordinating histidine residues of hemopexin from modification (data not shown) as heme does (20). Second, heme (Kd < 1 pm, Ref. 26) readily displaces SnPP from its complex with hemopexin (Fig. 5) confirming that the affinity of hemopexin for SnPP is lower than for mesoheme. Finally, the temperature at which the metalloporphyrin-hemopexin complex melts is significantly lower for SnPP-hemopexin than for mesoheme-hemopexin (Fig. 6).

Perhaps because of the differences in coordination, SnPP

![Fluorescence emission spectra of SnPP and SnPP-hemopexin.](image1)

![Titration of the SnPP-hemopexin interaction.](image2)

![Effect of modification of histidyl residues of hemopexin on its interaction with SnPP.](image3)

![Displacement of SnPP from hemopexin by heme.](image4)
pexin receptor was examined. Hemopexin has a higher affinity for SnPP-hemopexin than for HSA. Difference absorbance titrations at pH 7.4 yielded an apparent $K_d$ for SnPP-HSA of 4 ± 2 μM (not shown), providing evidence for tighter binding than the 10–30 μM value previously reported (7). The affinity of hemopexin for SnPP is sufficient to allow 1.5 μM hemopexin to bind a significant portion of SnPP (1.5 μM) when added to preformed HSA-SnPP mixtures containing HSA at concentrations ranging from 10 to 100 μM (Fig. 9, panels A–C). As expected, when complexed with heme, hemopexin does not affect the SnPP-HSA complex (Fig. 9, panel D). Conversely, hemopexin retains SnPP when HSA is added to preformed SnPP-hemopexin complexes under similar conditions (not shown).

Attempts to examine directly the extent of formation of the SnPP-hemopexin complex (maximum 410.5 nm) and the SnPP-HSA complex (maximum 412 nm) after adding SnPP to human serum were unsuccessful. The varying amounts of endogenous heme and high light scattering in the serum samples precluded quantitative evaluation of the two spectrally similar SnPP species using absorbance or fluorescence spectroscopy. In another series of experiments, SnPP con-

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Fig. 6. Thermal stability of SnPP-hemopexin and mesoheme-hemopexin. The thermal stability of the metalloporphyrin-hemopexin complexes was assessed by monitoring the absorbance spectra of the complexes in the Soret region after 10 min at the temperatures indicated. Shown are: mesoheme-hemopexin (filled circles) and SnPP-hemopexin (open circles). Each protein was present at 1.0 μM in 15 mM sodium phosphate, pH 7.4.

Fig. 7. Circular dichroism spectra of SnPP-hemopexin. The circular dichroism spectra of hemopexin (open circles), equimolar SnPP-hemopexin (open squares), were recorded in phosphate-buffered saline, pH 7.4. For comparison, the spectrum of mesoheme-hemopexin (filled circles) is also shown. Hemopexin was present at 1.0 μM in each sample. The spectrum of the 2:1 SnPP-hemopexin complex (not shown) was the same as that of the 1:1 complex.

does not cause the characteristic increase in the ellipticity of hemopexin at 231 nm (Fig. 7) that heme does (11, 12). This is associated with one of the three types of conformational change which occur in hemopexin upon heme binding and influence its affinity for its receptor. SnPP does, however, produce the Type II change in conformation of hemopexin which confers the resistance to proteolysis of the interdomain hinge region (Fig. 8) that hemopexin complexes containing mesoheme (17) and iron-tetraphenylporphine sulfonate have.

To begin assessment of the physiological relevance of the SnPP-hemopexin interaction, first the affinity of hemopexin for the ligand was compared with that of the other major plasma metalloporphyrin binder, HSA; and second, the ability of the SnPP-hemopexin complex to interact with the hemopexin receptor was examined. Hemopexin has a higher affinity for SnPP than does HSA. Difference absorbance titrations at pH 7.4 yielded an apparent $K_d$ for SnPP-HSA of 4 ± 2 μM (not shown), providing evidence for tighter binding than the 10–30 μM value previously reported (7). The affinity of hemopexin for SnPP is sufficient to allow 1.5 μM hemopexin to bind a significant portion of SnPP (1.5 μM) when added to preformed HSA-SnPP mixtures containing HSA at concentrations ranging from 10 to 100 μM (Fig. 9, panels A–C). As expected, when complexed with heme, hemopexin does not affect the SnPP-HSA complex (Fig. 9, panel D). Conversely, hemopexin retains SnPP when HSA is added to preformed SnPP-hemopexin complexes under similar conditions (not shown).

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The amount of free SnPP and SnPP bound to hemopexin or to HSA in mixtures of the three components under the conditions shown were estimated using the dissociation constants measured here. These conditions reflect those used experimentally here and those encountered physiologically under therapeutic conditions. The concentrations of proteins are from Refs. 26 and 27, and the SnPP concentrations are from Refs. 9 and 25. The calculations are indicated concentrations for the interaction of heme-hemopexin to mouse Hepa cells was assessed by incubating the competing agent with the cells at the indicated concentrations for 15 min at 37 °C. The labeled heme-hemopexin complex (50 pM) was then added, and binding was assessed 15 min later. Data presented are the means of three experiments, triplicate wells per experiment, and the standard error of the mean was less than 15% for each. Specific binding which averaged 70% of the total was 0.25 pmol/mg of protein in this series of experiments. The ability of the agents shown at 2.5 μM concentration to inhibit the binding of mesoheme-125I-hemopexin to mouse Hepa cells was assessed by incubating the competing agent with the cells at the indicated concentrations for 15 min at 37 °C as described under “Materials and Methods.” The labeled heme-hemopexin complex (50 nM) was then added, and binding was assessed 15 min later. Data presented are the means of three experiments, triplicate wells per experiment, and the standard error of the mean was less than 15% for each. Specific binding which averaged 70% of the total was 0.25 pmol/mg of protein in this series of experiments.

**Table I**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Initial concentrations</th>
<th>Distribution of SnPP</th>
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**Table II**

**Competitive inhibition of the interaction of mesoheme-125I-hemopexin with mouse Hepa cells**

The amount of free SnPP and SnPP bound to hemopexin or to HSA in mixtures of the three components under the conditions shown were estimated using the dissociation constants measured here. These conditions reflect those used experimentally here and those encountered physiologically under therapeutic conditions. The concentrations of proteins are from Refs. 26 and 27, and the SnPP concentrations are from Refs. 9 and 25. The calculations are indicated concentrations for 15 min at 37 °C as described under “Materials and Methods.” The labeled heme-hemopexin complex (50 nM) was then added, and binding was assessed 15 min later. Data presented are the means of three experiments, triplicate wells per experiment, and the standard error of the mean was less than 15% for each. Specific binding which averaged 70% of the total was 0.25 pmol/mg of protein in this series of experiments.

**Agent** | % specific binding inhibited
---|---
Mesoheme-hemopexin | 100
SnPP-hemopexin | 95
Mesoheme | 5
SnPP | 6

**Fig. 10. Induction of heme oxygenase mRNA in mouse Hepa cells by SnPP-hemopexin.** Hepa cells were incubated for 4 h in serum-free medium containing 0, 0.2, 1, and 2 μM SnPP (lanes 1–4, respectively) or 0, 5, and 10 μM SnPP-hemopexin (lanes 5–7, respectively). RNA isolation and Northern blot analysis were performed as described under “Materials and Methods.” The migrations of heme oxygenase (HO) mRNA and of the 28 S and 18 S ribosomal RNAs are indicated.

Depending on the relative amounts of hemopexin and albumin, appreciable portions of SnPP in the circulation are likely to be bound by hemopexin. For example, assuming 20 μM hemopexin, 500 μM albumin, and a total of 10 μM SnPP, 35% of the SnPP would be bound to hemopexin. Only negligible amounts of SnPP remain non-protein-bound.

Also important is the fact that SnPP-hemopexin is an effective competitive inhibitor of the interaction of hemopexin with its receptor on the plasma membranes of...
mouse Hepa cells (Table II). In fact, SnPP-hemopexin is nearly as effective as mesoheme-hemopexin in competitively inhibiting the binding of mesoheme-129-hemopexin to its receptor on these cells, suggesting that the affinities of these two complexes for the receptor are comparable. The dissociation constant of heme-hemopexin bound to the receptor on Hepa cells is approximately 20 nM. Thus, direct analysis of the binding of SnPP-hemopexin by the receptor, for example by a Scatchard-type analysis, is precluded by the dissociation of SnPP from hemopexin at the nanomolar concentrations of hemopexin required for such analyses.

The high affinity interaction of SnPP-hemopexin with the hemopexin receptor on mouse Hepa cells suggests that SnPP may be transported into these cells by the mechanism normally used to transport heme to heme oxygenase. The transport of heme into Hepa cells via the hemopexin receptor causes a rapid increase in the steady state level of heme oxygenase mRNA. Interestingly, incubation of Hepa cells with SnPP-hemopexin also induces the accumulation of heme oxygenase mRNA (Fig. 10). The 15-fold induction observed after 4 h with 10 μM SnPP-hemopexin is quantitatively similar to that observed with 10 μM heme-hemopexin. In contrast, free SnPP, even at concentrations up to 10 μM (data not shown), has little effect on the level of heme oxygenase mRNA in Hepa cells, suggesting that specific intracellular routing mechanisms are necessary for the observed mRNA induction. Recently, Sardana and Kappas (31) reported that hepatic heme oxygenase enzymic activity diminished to 10% of control levels within 30 min of subcutaneous injection of SnPP into rats. However, heme oxygenase immunoprecipi-
table protein in the liver actually increased after a 4-h lag period. Maximum induction of 17-fold in heme oxygenase protein occurred 12 h after SnPP injection. Our present data suggest that the SnPP-induced increase in hepatic heme oxygenase protein observed in vivo results from accumulation of heme oxygenase mRNA. Furthermore, since this induction requires SnPP-hemopexin complexes, the likelihood that hemopexin acts as a principle physiological transporter of SnPP in the circulation in humans and rats in vivo is strengthened.

In conclusion, the results presented here show that hemopexin binds SnPP sufficiently tightly to compete with albumin for SnPP in the circulation. The interaction between SnPP and hemopexin produces conformational changes in the protein which enable the SnPP-hemopexin complex to interact tightly with the hemopexin receptor. Thus, endocytosis of SnPP-hemopexin is likely to be a major route whereby SnPP reaches its site of action, heme oxygenase, as shown for hemopexin-mediated heme uptake by the liver (32). Furthermore, incubation of Hepa cells with SnPP-hemopexin, but not with SnPP alone, rapidly induces the mRNA for heme oxygenase in a dose-dependent manner, providing a mechanism whereby heme oxygenase protein levels are induced in vivo. Thus, binding of SnPP to hemopexin and conditions altering hemopexin metabolism are factors that must be considered in the development and application of SnPP therapy for neonatal hyperbilirubinemia.

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