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Research Article

The synthetic heme analogue Sn-protoporphyrin is a potent competitive inhibitor of heme oxygenase, the rate-limiting enzyme in heme degradation to bile pigment, and can entirely suppress hyperbilirubinemia in neonatal animals and significantly reduce plasma bilirubin levels in a variety of circumstances in experimental animals and man. To further explore the mechanism by which this metalloporphyrin reduces bilirubin levels in vivo, we have examined its effects on bilirubin production in bile duct-cannulated rats, in which bilirubin derived from heme catabolism is known to be rapidly excreted in bile. The administration of Sn-protoporphyrin (10-50 mumol/kg body weight) was followed by prompt (within approximately 1 h) and sustained (up to at least 18 h) decreases in bilirubin output, to levels 25-30 percent below the levels of bilirubin output in control bile fistula animals. The metalloporphyrin had no effect on bile flow or the biliary output of bile acids. Infusions of heme, which is taken up primarily in hepatocytes, or of heat-damaged erythrocytes, which are taken up in reticuloendothelial cells, resulted in marked increases in bilirubin output in bile in control animals; these increases were completely prevented or substantially diminished by Sn-protoporphyrin administration. By contrast, the metalloporphyrin did not alter the high levels of bilirubin in plasma and bile that were achieved in separate experiments by the constant (16 h) infusion of unconjugated bilirubin […]

Studies on the Mechanism of Sn-Protoporphyrin Suppression of Hyperbilirubinemia

Inhibition of Heme Oxidation and Bilirubin Production

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Abstract

The synthetic heme analogue Sn-protoporphyrin is a potent competitive inhibitor of heme oxygenase, the rate-limiting enzyme in heme degradation to bile pigment, and can entirely suppress hyperbilirubinemia in neonatal animals and significantly reduce plasma bilirubin levels in a variety of circumstances in experimental animals and man. To further explore the mechanism by which this metalloporphyrin reduces bilirubin levels in vivo, we have examined its effects on bilirubin production in bile duct-cannulated rats, in which bilirubin derived from heme catabolism is known to be rapidly excreted in bile. The administration of Sn-protoporphyrin (10–50 μmol/kg body weight) was followed by prompt (within ~1 h) and sustained (up to at least 18 h) decreases in bilirubin output, to levels 25–30 percent below the levels of bilirubin output in control bile fistula animals. The metalloporphyrin had no effect on bile flow or the biliary output of bile acids. Infusions of heme, which is taken up primarily in hepatocytes, or of heat-damaged erythrocytes, which are taken up in reticuloendothelial cells, resulted in marked increases in bilirubin output in bile in control animals; these increases were completely prevented or substantially diminished by Sn-protoporphyrin administration. By contrast, the metalloporphyrin did not alter the high levels of bilirubin in plasma and bile that were achieved in separate experiments by the constant (16 h) infusion of unconjugated bilirubin to bile duct-cannulated rats. Thus, Sn-protoporphyrin exerts no major effects on the metabolic disposition of preformed bilirubin. Heme oxygenase activities were markedly decreased in microsomal preparations from liver, spleen, and kidneys in these experiments, to a degree comparable to the decreases we have observed in the intact rat. We also demonstrated that a substantial proportion (19–35%) of a dose of Sn-protoporphyrin is promptly excreted in bile and that the time course of biliary excretion of this compound more closely reflects plasma concentrations of the metalloporphyrin, which decline rapidly, rather than concentrations in liver, which are considerably more persistent.

These results indicate that Sn-protoporphyrin substantially reduces the in vivo production of bilirubin from the degradation of endogenous as well as exogenous heme in the rat. Moreover, this inhibitory effect of the synthetic metalloporphyrin on bilirubin production occurs in both hepatocytes and reticuloendothelial cells, which are the major tissue sites for bilirubin formation. In other studies, we have established that heme oxygenase blockade by Sn-protoporphyrin leads to a marked and rapid excretion of heme into bile presumably because the synthetic metalloporphyrin blocks heme from binding to the catalytic site of heme oxygenase, thereby preventing its metabolism to bile pigment and making it available for excretion via the biliary system into the gut. These studies strongly suggest that Sn-protoporphyrin diminishes hyperbilirubinemia in animals and man by inhibiting the production of the bile pigment in vivo, and that its principal mode of action involves a potent and sustained competitive inhibition of heme oxygenase.

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Introduction

Sn-protoporphyrin is a synthetic heme analogue that potently inhibits the activity of heme oxygenase (1, 2), the rate-limiting enzyme for the degradation of heme to bile pigment (3). This inhibition is competitive in nature, and has been demonstrated in a number of human as well as animal tissues including liver, spleen, and kidney when the metalloporphyrin is administered in vivo, or when it is added to microsomal preparations or to purified heme oxygenase in vitro (1, 2, 4–7). We have shown that administration of Sn-protoporphyrin shortly after birth prevents the development of neonatal hyperbilirubinemia in the rat (1, 2, 8), and this effect has been confirmed by others in the rhesus neonate (9). The compound also decreases plasma bilirubin levels in adult mice with congenital forms of severe hemolytic anemia (10), in the post-natal suckling rat with hyperbilirubinemia resulting from the administration of heme or the heme precursor δ-aminolevulinic acid (11), in the bile duct-ligated rat (12), and in patients with sustained jaundice due to primary biliary cirrhosis (12). It also evokes a marked increase in the excretion of heme in the bile of bile duct-cannulated rats (11). Using a fluorometric method for the measurement of Sn-protoporphyrin in adult rats (13), we have found that the metalloporphyrin is rapidly cleared from plasma, and persists in tissues where it can inhibit heme oxygenase activity promptly and for prolonged periods of time (14).

The purpose of the present study was to examine the effect of Sn-protoporphyrin on bilirubin production in vivo, as assessed by measuring bilirubin output in the bile duct-cannulated (bile-fistula) animal. Bilirubin formed from endogenous heme catabolism is normally quite rapidly and completely excreted in bile. Thus, a change in total biliary output of bilirubin in the absence of alterations in plasma bilirubin levels or bile flow represents good evidence of an altered rate of bilirubin production. In addition, we have examined the ability of Sn-protoporphyrin to influence the rate of conversion of exogenous heme to bilirubin in hepatocytes and reticuloendothelial cells utilizing both infused heme and infused heat-damaged erythrocytes in appropriate experiments. Finally, we

have examined the effect of Sn-protoporphyrin on plasma and biliary bilirubin levels during constant infusions of exogenous bilirubin to determine whether the metalloporphyrin significantly alters the metabolic disposition of the preformed bile pigment.

The results of these studies support the idea that Sn-protoporphyrin suppresses plasma bilirubin levels primarily by diminishing, in a potent and sustained manner, the rate of oxidation of endogenous as well as exogenous heme to bile pigment; that the metalloporphyrin can inhibit the conversion in vivo of heme to bilirubin in both hepatocytes and in reticuloendothelial cells; and that Sn-protoporphyrin does not affect in a major way the metabolic disposition of preformed bilirubin itself.

Methods

Male Sprague-Dawley rats (Taconic Farms, Germantown, NY) weighing 260–360 g were fasted overnight and anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight [bw],[1] Abbott Laboratories, North Chicago, IL). This anesthetic was used because, unlike ether and a number of other agents, it does not interfere with bile flow (15) or alter heme oxygenase activity (16). The bile duct of each rat was ligated distally 1 cm from the hepatic ducts and cannulated with PE 10 polyethylene tubing (Becton-Dickinson & Co., Parsipanny, NJ). The bile duct cannula was secured with two silk sutures, the distal end brought out through a stab wound in the abdomen, and the abdomen was then closed. The jugular vein was cannulated with PE 50 tubing. The animals were placed in restraining cages and the jugular vein catheter was infused throughout the experiment with a sterile solution containing equal amounts of 0.45% (wt/vol) NaCl and 5% glucose at a constant rate of 1.1 ml/h using an infusion pump (Harvard Apparatus Co., Millis, MA). A heat lamp was used during and after surgery to maintain body temperature, which was monitored periodically with a rectal thermometer. The animals were allowed to recover from surgery and bile flow was permitted to stabilize for ~4 h before each experiment (including the appropriate control period) was begun. Up to three animals (one to two treated and one control) were studied at one time.

$Sn(4^+)$-protoporphyrin IX HCl (Porphyrin Products, Logan, UT) was dissolved in a small amount of 0.2 N NaOH, a 3.5-fold greater volume of 0.9% NaCl was added, and the pH was then adjusted to 8.0 by dropwise addition of 0.5 N HCl, (1). For experiments involving heme administration, heme (Fe-protoporphyrin IX HCl; Sigma Chemical Co., St. Louis, MO) was dissolved in 0.1 ml 0.05 N NaOH, and 1.9 ml of cold rat serum was added dropwise with stirring (17). Solutions of unconjugated bilirubin (Sigma Chemical Co.) were prepared on the day of each experiment. 20 mg was dissolved in a small volume of 0.1 N NaOH; a fivefold volume of cold rat serum was added dropwise with stirring, followed by a 3.8-fold greater volume of a mixture of equal volumes of 0.9% NaCl and 10% dextrose; the pH was adjusted to 8.0 with the dropwise addition of 0.2 N HCl, so that the final solution contained 20 mg of bilirubin in 40 ml. Bilirubin solutions were kept in the dark during the infusion by wrapping the syringe and tubing with aluminum foil. The purity of unconjugated bilirubin was assessed by thin-layer chromatography (silica gel G, E. Merck & Co., Darmstadt, Federal Republic of Germany), using 1% glacial acidic acid in chloroform to develop the plates and light absorption spectroscopy (18). Thin-layer chromatography showed three different bands, whose R_f values and absorption spectra (after elution from the plates with chloroform) indicated that the material consisted of 85% bilirubin IXα, 11% bilirubin XIIIα, and 4% bilirubin IIIα (18). Other chemicals used were reagent grade and were obtained from Sigma Chemical Co. or Fisher Scientific, Pittsburgh, PA.

For preparation of heat-damaged erythrocytes, blood was obtained by translumbar puncture of the inferior vena cava (19) of rats that had been fasted overnight and then anesthetized with ether. Erythrocytes were sedimented by centrifugation at 1,100 *g* for 10 min, washed twice with 0.9% NaCl, resuspended to the original volume in 0.9% NaCl, and heated at 49.5°C in a temperature-controlled water bath for 40–60 min as described by Harris et al. (20) and Jandl et al. (21). For most experiments, the cells were heated for 40 min; longer periods of heating were likely to produce a variable degree of hemolysis. An aliquot of each heated erythrocytes preparation was centrifuged, and the degree of hemolysis determined by measuring hemoglobin concentration by the cyanomethemoglobin method (22), in the resuspended erythrocytes and in the supernatant after centrifugation of the resuspended cells.

All experiments were carried out in subdued light. Bile was collected serially for 30–60-min periods in tared plastic tubes with captive plugs, weighed to determine the amount collected, and stored in the dark at 4°C. Blood samples were obtained from the tail, anticoagulated with heparin, and the plasma stored at 4°C in the dark for bilirubin measurement. Bilirubin was determined by the fluorometric method of Roth (23) within 24 h. Total bile salt concentrations in bile were measured by the enzymatic method of Talalay (24); these determinations were kindly made by Professor Robert H. Palmer, Columbia University College of Physicians and Surgeons, New York. At the end of each 24-h experiment, the rats were exsanguinated by decapitation, the carcasses perfused with cold 0.9% NaCl, and microsomes were prepared from liver, kidney, and spleen for measurement of heme oxygenase activity as described previously (1). Microsomal protein was measured by the method of Lowry et al. (25). Concentrations of Sn-protoporphyrin in bile, plasma, and tissues were measured fluorometrically, as previously described (13). Statistical analysis was by *t* test, and *P* values < 0.05 were considered significant.

Results

Effect of Sn-protoporphyrin on the biliary output of bilirubin derived from endogenous heme catabolism. The output of bilirubin in bile in eight rats treated with Sn-protoporphyrin, 10 μmol/kg i.v., and in eight controls treated with saline, is shown in Fig. 1; the data shown are the mean values±SE of all experiments conducted. The dose of 10 μmol/kg of Sn-protoporphyrin was chosen because this amount of the metalloporphyrin was shown previously in intact adult male rats to

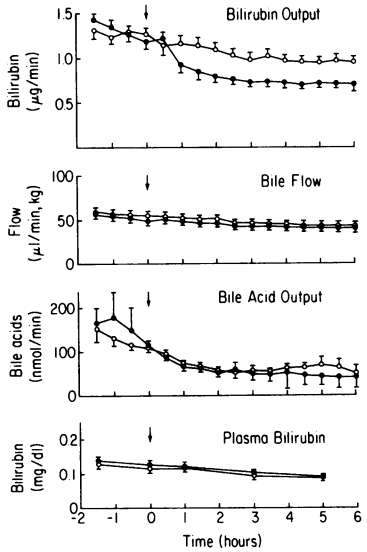

Figure 1. Effect of Sn-protoporphyrin on bilirubin output in bile, and on bile flow, bile acid output, and plasma bilirubin concentrations in bile duct-cannulated rats. Bile was collected from male rats (260–300 g bw) at 30-min intervals before and after treatment with Sn-protoporphyrin or saline (arrow). Blood was sampled at 1.5 h and again immediately before treatment, and at 1–2-h intervals after treatment. Values shown are mean±SE for a control group of eight rats administered saline (open circles) and a group of eight rats given Sn-protoporphyrin, 10 μmol/kg bw i.v. (closed circles); biliary bile acids were measured in three treated and three control rats. See text for details.

1. *Abbreviation used in this paper:* bw, body weight.

result in a prompt (within 1 h) and persistent inhibition of heme oxygenase activity in liver, spleen, and kidney (14); this dose is also the minimum amount of the metalloporphyrin which has consistently proved effective in completely suppressing post-natal hyperbilirubinemia in the newborn rat (2). As described in Methods, there was a period of ~4 h after insertion of the bile cannula and before the control period of bile collection was begun which allowed the animals to recover from anesthesia and which permitted bile flow and bilirubin output to stabilize. As shown in Fig. 1, there was a gradual decline in bilirubin output, as well as bile flow, in both groups before the metalloporphyrin (or saline) was injected, as expected after interruption of the enterohepatic cirulation of bile salts. In the group of animals treated with Sn-protoporphyrin, there was in addition a prompt drop in the output of bilirubin in bile occurring by ~1 h after administration of the metalloporphyrin; after this drop, bilirubin output remained at a diminished level, compared with the control group for the succeeding 6 h of observation. In unreported studies, we have observed this decline in biliary bilirubin output after Sn-protoporphyrin treatment to persist for at least 18 h. The metalloporphyrin-induced fall in bilirubin output in bile was not associated with changes in the rate of bile flow or in the output of bile salts (Fig. 1), which are major determinants of hepatic bile formation and biliary excretion of organic anions such as bilirubin (26, 27). Moreover, plasma bilirubin levels measured before treatment and 1, 3, and 5 h after treatment with either Sn-protoporphyrin or saline showed no significant changes after treatment with the metalloporphyrin in the bile fistula animals, using a sensitive fluorometric method (23) capable of quantitating the low levels of bile pigment normally found in the plasma of rats (1) (Fig. 1). These findings indicate that Sn-protoporphyrin can decrease biliary bilirubin output derived from endogenous heme catabolism without impairing bile flow and bile salt excretion or by causing bilirubin to accumulate in plasma.

The effects of larger amounts of Sn-protoporphyrin in bile duct-cannulated animals were also examined. In these experiments, doses of 20 or 50 μmol/kg bw of the metalloporphyrin were administered to a total of seven animals subcutaneously; the same group of untreated animals (total number, four) served as controls for each of the treated groups. The subcutaneous route of metalloporphyrin administration was employed to avoid the administration of large volumes of solvent by vein, and because such doses given by this route have been used extensively in previous studies in neonatal as well as adult rats (1, 14). In such experiments we have shown that absorption of the metalloporphyrin after subcutaneous administration is rapid and complete (>98% [14]), and that the tissue distribution patterns of the metalloporphyrin after administration by the intravenous and subcutaneous routes are comparable (14). Decreases in bilirubin output in bile after administration of the 20- and 50-μmol/kg doses of the metalloporphyrin are shown in Table I, and are compared with the effects of the 10-μmol/kg dose given intravenously. The decreases in bilirubin output with the larger doses were comparable to those produced by the 10 μmol/kg dose. These results indicate that the maximal decrease in bilirubin production by Sn-protoporphyrin administered in single doses in this animal model appears to be ~25–30% of the total output of biliary bile pigment.

Heme oxygenase activities in liver, kidney, and spleen measured when the bile fistula animals were killed at the end of each experiment are shown in Table II. There was no clear evidence that greater inhibition of heme oxygenase activity was achieved by increasing the dose in the range from 10 to 50 μmol/kg in these animals, even though there were dose-related increases in tissue levels of Sn-protoporphyrin (not shown). The tissue levels of Sn-protoporphyrin and the degrees of inhibition of heme oxygenase in these animals were comparable to those previously reported in normal adult rats treated with the same doses of Sn-protoporphyrin (14).

Effect of Sn-protoporphyrin on bilirubin output in bile after infusions of exogenous heme. To determine the effects of Sn-protoporphyrin on bilirubin formation from exogenous heme (hematin; heme hydroxide), which is known to be degraded principally in hepatocytes (28–30), studies were carried out after acute infusions of heme in bile duct-cannulated animals. As shown in Fig. 2, administration of a bolus of heme (4 mg/kg bw or 6.1 μmol/kg bw i.v.), as described by Snyder and Schmid (17), was followed by a prompt increase in bilirubin output in bile in a group of four control rats; the peak increases in bilirubin output observed were ~3 h in duration. The administration of Sn-protoporphyrin (10 μmol/kg bw i.v.) immediately before the injection of heme to five other bile duct-cannulated rats entirely prevented this increase in bilirubin output in bile in four out of five animals. The mean values are shown in Fig. 2. In fact, in only one of the five individual rats treated with Sn-protoporphyrin was there a discernible peak in bilirubin output after heme administration.

Table I. Effect of Three Different Doses of Sn-Protoporphyrin on Biliary Bilirubin Output in Bile Duct-cannulated Rats at Two Time Intervals After Treatment

Sn-protoporphyrin dose	Number of animals	Sn-protoporphyrin-induced decreases in bilirubin output			
		2–4 h	2–4 h	4–6 h	4–6 h
μmol/kg		*μg/min*	*%*	*μg/min*	*%*
10	8	0.36±0.05	28.0±7.95	0.34±0.06	24.6±2.63
20	3	0.37±0.17	24.3±5.54	0.44±0.15	30.4±5.75
50	4	0.41±0.04	32.0±1.38	0.43±0.04	30.2±1.56

Bile flow was allowed to stabilize for ~4 h after insertion of bile duct cannulae into adult male rats, and bile was then collected for 30-min periods for 2 h before and 6 h after treatment with Sn-protoporphyrin or saline. Values shown are mean±SE of the decreases in bilirubin output 2–4 h and 4–6 h after treatment (expressed as microgram per minute and percent of the pretreatment period) and are corrected for the mean decreases observed in the control groups. The results were not significantly different for the three groups (*t* test).

Table II. Heme Oxygenase Activities in Liver, Kidney, and Spleen of Bile Duct-cannulated Rats After Treatment with Saline or Sn-Protoporphyrin

Treatment	Number of animals	Heme oxygenase activity		
		Liver	Kidney	Spleen
		nmol bilirubin/mg protein · h	*nmol bilirubin/mg protein · h*	*nmol bilirubin/mg protein · h*
Control	8	2.35±0.12	0.84±0.06	5.61±0.38
Sn-protoporphyrin, 10 μmol/kg	8	0.72±0.12 (−69%)	0.17±0.05 (−80%)	2.50±0.39 (−56%)
Control	4	1.64±0.27	0.97±0.04	7.11±1.09
Sn-protoporphyrin, 20 μmol/kg	3	0.39±0.002 (−76%)	0.12±0.05 (−88%)	2.22±1.06 (−69%)
Sn-protoporphyrin, 50 μmol/kg	4	0.46±0.03 (−72%)	0.36±0.06 (−63%)	2.83±0.52 (−60%)

Heme oxygenase was measured when the animals were sacrificed 24 h after insertion of bile duct cannulae and 18 h after treatment with Sn-protoporphyrin. As described in Methods, food was withheld for 16 h before surgery and until the time of killing, and an intravenous infusion of a mixture of equal volumes of 5% glucose and 0.45% NaCl was provided at a rate of 1.1 ml/h after the bile cannulae were inserted. Values are mean±SE. Percentage values shown in parentheses represent the difference between the mean values of control and Sn-protoporphyrin-treated groups at each dose level; there was one control group for the two groups treated with the two higher doses of Sn-protoporphyrin.

We calculated the increase in bilirubin output after heme administration in these experiments based on the areas under the peak observed in the bilirubin excretion vs. time curves for each rat during the 3-h period after heme administration. These calculations (not shown) showed that in the four control bile duct-cannulated rats given heme alone, ~14±3.9% of the injected dose of heme (this dose amounted to 2.2±0.05 μmol heme administered per animal) could be accounted for as increased bilirubin excretion in bile. As noted, this increase was entirely prevented in four of the five heme-treated rats that were also given Sn-protoporphyrin. The prevention by Sn-protoporphyrin of the expected increase in bilirubin output after heme administration in these rats indicates a major suppressive effect of Sn-protoporphyrin on bilirubin production in liver parenchymal cells (28–30).

It was also of interest that there was a slight increase in bile flow after the heme injections, which, although not statistically significant, was observed in all animals studied; this effect has been reported by others (31). Sn-protoporphyrin did not prevent this small increase in bile flow (not shown). Decreases in heme oxygenase activity in the bile duct-cannulated rats given heme as well as Sn-protoporphyrin were substantial (Table III), and were comparable to the decreases seen in bile fistula rats treated with Sn-protoporphyrin alone (Table II), which indicated that heme administration, despite its ability to induce heme oxygenase (11, 32), did not alter the inhibitory effect of Sn-protoporphyrin on this enzyme activity. Sn-protoporphyrin has also been shown to block the increases in heme oxygenase produced by metals known to potently induce this enzyme, such as inorganic cobalt, cadmium, platinum, nickel, or antimony, or metalloporphyrins such as Fe- or Co-protoporphyrin (33). Thus, Sn-protoporphyrin can inhibit heme oxygenase activity induced by foreign chemicals as well as by the natural substrate of the enzyme itself.

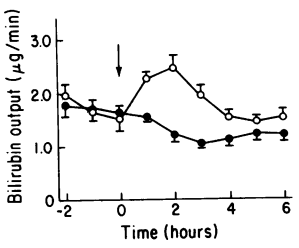

Figure 2. Effect of Sn-protoporphyrin (10 μmol/kg bw i.v.) on biliary output of bilirubin in bile duct-cannulated rats (330–360 g bw) treated with heme. Heme (6.1 μmol/kg bw i.v.) was administered as a bolus (arrow) to a total of nine rats; five of the rats received Sn-protoporphyrin (closed circles) also as a bolus (arrow) and the four others saline (open circles), 5 min before the infusion of heme. Values are mean±SE for each group. See text for details.

Effect of Sn-protoporphyrin on bilirubin output in bile after infusion of heat-damaged erythrocytes. Heat-damaged rat erythrocytes (8.9×10^9 erythrocytes/kg bw [30], equivalent to 8.5 μmol heme/kg bw) were injected as a bolus intravenously into eight bile fistula rats. This treatment produced a prompt and marked increase in bilirubin output in bile, which lasted ~6 h (Fig. 3). In eight additional bile fistula animals this increase could be substantially prevented by Sn-protoporphyrin administration (10 μmol/kg bw i.v.), as shown by the mean values for both groups in Fig. 3. In two of the latter animals, the suppression of the bilirubin increase after the erythrocyte infusion was complete, while in seven other animals the increase in biliary bile pigment output was partially prevented. In five out of these eight animals, Sn-protoporphyrin was administered 1 h before, and in the other 3 animals only a few minutes before, infusion of the damaged erythrocytes; because we did not observe a different degree of inhibition of the increase in biliary bilirubin output with earlier administration of the metalloporphyrin, the results in all eight Sn-protoporphyrin-treated animals were combined as shown in Fig. 3. In these experiments, bile flow did not change after either the erythrocyte infusions or the Sn-protoporphyrin treatment (not shown).

Calculations of bilirubin output based on the areas under the bilirubin excretion vs. time curves indicated that 56.4±5.0% of the heme injected via the damaged erythrocytes preparations (the dose of heme amounted to 2.9±0.2 μmol heme/rat) could be accounted for as increased bilirubin in bile, whereas this increase was significantly smaller (33±7.5%) in the animals treated with Sn-protoporphyrin. Thus, the oxidation of ~23%

Table III. Effect of Sn-Protoporphyrin on Heme Oxygenase Activity in Liver, Kidney, and Spleen of Bile Duct-cannulated Rats After the Administration of Heme

Treatment	Number of animals	Heme oxygenase activity		
		Liver	Kidney	Spleen
		nmol bilirubin/mg protein · h	*nmol bilirubin/mg protein · h*	*nmol bilirubin/mg protein · h*
Heme	4	2.23±0.44	0.76±0.26	4.71±0.44
Heme plus Sn-protoporphyrin	4	0.71±0.20 (−68%)	0.09±0.04 (−88%)	1.69±0.53 (−64%)
P value		<0.02	<0.05	<0.01

The animals were treated intravenously with Sn-protoporphyrin (10 μmol/kg bw) or saline 5 min before the intravenous bolus of heme (6.1 μmol/kg bw). Bile cannulation and heme oxygenase measurement were carried out as described in Table II. Values are mean±SE for each group. Percentage values shown in parenthesis represent the difference between the mean values of control and Sn-protoporphyrin-treated groups. See text for details.

of that amount of injected heme which would ordinarily be metabolized to bilirubin (i.e., 56 vs. 33%) was prevented by Sn-protoporphyrin. The damaged erythrocytes preparations in these experiments contained a variable amount of free hemoglobin (average 12% of the total heme injected), but clearly this amount of free hemoglobin, which is taken up largely in hepatocytes (29, 30), could not account for the increases in bilirubin output produced by the damaged erythrocytes infusions. Moreover, the mean blockade in biliary bilirubin output produced by Sn-protoporphyrin after the erythrocytes infusions was considerably greater than could be accounted for by the fraction of free hemoglobin in the damaged erythrocytes preparations (12%). Since damaged erythrocytes are taken up in reticuloendothelial cells (30, 34), these experiments indicate that Sn-protoporphyrin can block, at least partially, the degradation of heme to bile pigment in reticuloendothelial tissues. This idea is substantiated by the inhibition by Sn-protoporphyrin of heme oxygenase activity in spleen, as well as liver and kidney, in bile duct-cannulated animals given damaged erythrocytes (Table IV); the degrees of enzyme inhibition in these experiments were comparable to those observed in other experiments in the bile duct-cannulated (Tables II and III) or in the intact rat (14).

Effect of Sn-protoporphyrin on bilirubin levels in plasma and bilirubin output in bile during the infusion of unconjugated bilirubin. The purpose of these experiments was to determine whether Sn-protoporphyrin altered the plasma levels or biliary excretion of exogenous bilirubin infused intravenously at a constant rate, as described by Hammaker and Schmid (35). In a group of five bile duct-cannulated rats, a mixture of 5% glucose and 0.45% NaCl was infused at a rate of 1.1 ml/h for 4 h, and was then followed by an infusion of unconjugated bilirubin at a rate of 9.5 μg/min (35) for a period of 16 h. As shown in Fig. 4, constant high concentrations of bilirubin in plasma and increased bilirubin output in bile were achieved during the bilirubin infusion. In the middle of the infusion period (8-h point), the rats were administered a single dose of Sn-protoporphyrin, 10 μmol/kg bw i.v. Administration of the metalloporphyrin produced no detectable changes in the constant bilirubin levels in plasma or bile that were achieved during the bilirubin infusion (Fig. 4), and there was no change in bile flow. The very large output of exogenous bilirubin in bile obscured the expected effect of Sn-protoporphyrin in decreasing by ~25–30% (Table I) the output of endogenous bilirubin. The plasma concentration of Sn-protoporphyrin measured fluorometrically (13) immediately after the bolus injection of the metalloporphyrin exceeded, on a molar basis, the plasma concentration of bilirubin by a factor of eight, and during the next 8 h decreased gradually; at the end of this period, the plasma concentration of the metalloporphyrin still exceeded the bilirubin concentration by a factor of three. Moreover, the uptake of Sn-protoporphyrin by the liver of these animals was not affected by the infusion of bilirubin, as excretion of Sn-protoporphyrin in bile began within 60 min after the administration of the metalloporphyrin, and concentrations of Sn-protoporphyrin in liver at the end of the experiment were similar to those of bile-duct cannulated rats not infused with bilirubin or of intact adult rats after treatment with the same dose of the metalloporphyrin (14). These results indicate that Sn-protoporphyrin at plasma concentrations greatly exceeding those of bilirubin did not alter significantly the steps involved in plasma transport and hepatic uptake of bilirubin, and that the conjugation and biliary excretion of the infused bilirubin in these animals was not affected by concomitant treatment with Sn-protoporphyrin. Minor changes in one or more of these parameters of bilirubin disposition might be detectable by more precise kinetic techniques of study, but the methods employed in these experiments are sufficiently sensitive to exclude major influences of Sn-protoporphyrin on the

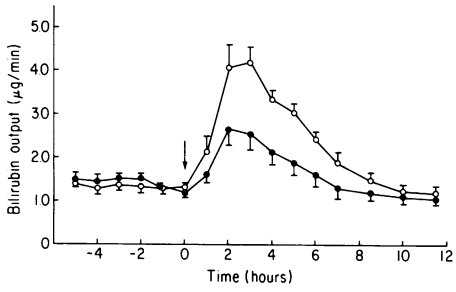

Figure 3. Effect of Sn-protoporphyrin (10 μmol/kg bw i.v.) on biliary bilirubin output in bile duct-cannulated rats (330–360 g bw) after administration of heat-damaged erythrocytes. Sn-protoporphyrin (closed circles) was administered as a bolus (arrow) to a group of eight rats, either 5 min (three rats) or 1 h (five rats) before the injection of damaged erythrocytes, and the combined results are shown. A control group of eight rats (open circles) was treated with saline. Values are mean±SE for each group. See text for details.

Table IV. Effect of Sn-Protoporphyrin on Heme Oxygenase Activity in Liver, Kidney, and Spleen of Bile Duct-cannulated Rats After the Administration of Heat-Damaged Erythrocytes

Treatment	Number of animals	Heme oxygenase activity		
		Liver	Kidney	Spleen
		nmol bilirubin/mg protein · h	*nmol bilirubin/mg protein · h*	*nmol bilirubin/mg protein · h*
Damaged erythrocytes	8	1.06±0.19	0.83±0.17	5.19±0.45
Damaged erythrocytes plus Sn-protoporphyrin	8	0.31±0.08 (−71%)	0.13±0.03 (−84%)	2.25±0.44 (−57%)
P value		<0.01	<0.01	<0.001

The animals were treated intravenously with Sn-protoporphyrin (10 μmol/kg bw) or saline 5 or 60 min before the intravenous bolus of heat-damaged erythrocytes (8.9×10^9 erythrocytes/kg bw). Bile duct cannulation and heme oxygenase measurement were carried out as described in Table II. Values are mean±SE for each group. Percentage values shown in parenthesis represent the difference between the mean values of control and Sn-protoporphyrin-treated groups. See text for details.

overall metabolic disposition of bilirubin in vivo. In other studies carried out with Prof. E. Breslow, Department of Biochemistry, Cornell University Medical College, New York, we have shown that extremely high concentrations of Sn-protoporphyrin do not influence the binding of bilirubin to its binding sites on purified human serum albumin (manuscript in preparation); nor is the glucuronidation of bilirubin affected by the metalloporphyrin.

Sn-protoporphyrin excretion in bile. The excretion of Sn-protoporphyrin in bile fistula rats after single doses of 10, 20, or 50 μmol/kg bw of the metalloporphyrin is shown in Fig. 5. There was a prompt, dose-related excretion of the metalloporphyrin in bile, with peak excretion occurring in the first 1–4 h after administration. The peak excretion of the metalloporphyrin was followed by a decline that was somewhat more gradual than the initial increase. Similar patterns of biliary excretion of Sn-protoporphyrin were observed in bile duct-cannulated animals infused with bilirubin, heme, and heat-damaged rat erythrocytes (not shown). We found previously that in the intact adult rat, the plasma half-life of Sn-protoporphyrin is short (~3.4 h after a dose of 10 μmol/kg bw i.v.), and that concentrations of the compound in the liver and a number of other tissues are persistent and show little or no decline within 24 h (14). In the present study, the levels of Sn-protoporphyrin in liver, as well as spleen and kidney, in bile duct-cannulated rats at the time of killing (at 18 h, results not shown) were similar to the levels seen in our previous study (14) in intact rats (at 4–24 h), which suggests that the processes of hepatic uptake and accumulation of the compound are similar in intact and in bile duct-cannulated rats even when animals are treated with exogenous bilirubin, heme, or heat-damaged erythrocytes. Therefore, although not directly proven in these experiments, it seems likely that the biliary excretion of Sn-protoporphyrin after a single dose reflects the more rapidly changing levels of the compound in plasma, and that the more persistent levels of the metalloporphyrin in liver represent one or more pools of the compound that are not immediately accessible for biliary excretion. Further studies of the determinants of hepatic accumulation and biliary excretion of Sn-protoporphyrin are in progress.

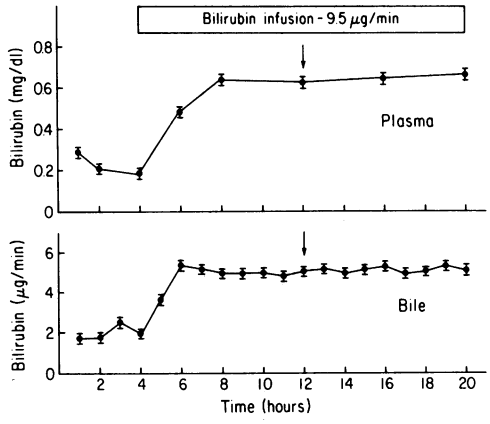

Figure 4. Effect of Sn-protoporphyrin (10 μmol/kg bw i.v.) on plasma bilirubin concentration and biliary bilirubin output in bile duct-cannulated rats during a constant intravenous infusion of unconjugated bilirubin. Bilirubin (9.5 μg/min) was infused into five male rats (330–360 g bw) for 16 h and the metalloporphyrin was administered as a bolus (arrow) at the midpoint (8 h) of the infusion period (arrow). Bile was collected at 1-h intervals and blood was sampled every 2–4 h. Values shown are mean±SE. See text for details.

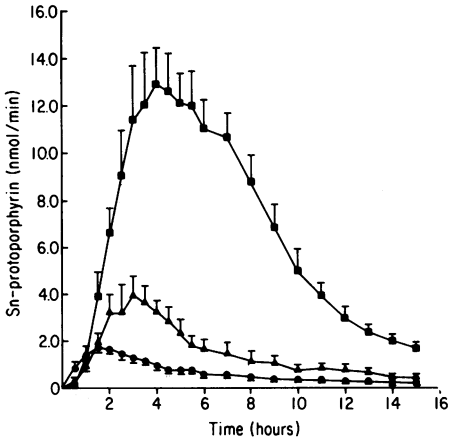

Figure 5. Excretion of Sn-protoporphyrin in bile in bile duct-cannulated rats. Three groups of rats were treated with single doses of 10 μmol/kg bw i.v. (eight rats, circles), 20 μmol/kg bw subcutaneously (three rats, triangles) or 50 μmol/kg bw subcutaneously (four rats, squares). Values shown are mean±SE for each group. See text for details.

The total recovery of Sn-protoporphyrin in bile, expressed as a percent of the dose in the bile duct-cannulated rats, was 18.9±2.3% after the 10 μmol/kg bw dose, 20.0±2.1 after the 20 μmol/kg bw dose, and 34.5±3.6% after the 50 μmol/kg bw dose. In previous studies in intact rats followed for 7 d, we found that ~15% of a dose of 20 or 50 μmol/kg bw Sn-protoporphyrin administered subcutaneously was recovered in the feces (14). It is possible, in the bile duct-cannulated rat, that a larger amount of the metalloporphyrin undergoes biliary excretion than in the intact rat, or that in the intact animal, intestinal absorption, or loss of Sn-protoporphyrin by an unknown mechanism during passage through the intestinal tract, occurs. These possibilities are being explored.

Discussion

The present studies demonstrate that Sn-protoporphyrin administered to bile fistula rats produces a prompt and sustained reduction in the output of endogenous bilirubin in bile. Reductions in bile pigment excretion were 25–30%, with doses of the metalloporphyrin ranging from 10 to 50 μmol/kg bw. This effect on endogenous bilirubin output was not accompanied by changes in the volume of bile flow or by changes in bile salt excretion, indicating that Sn-protoporphyrin did not alter bilirubin output by changing the hepatic formation or flow of bile. Moreover, Sn-protoporphyrin did not produce increases in the levels of bilirubin in plasma after administration of Sn-protoporphyrin. Because bilirubin produced from endogenous heme catabolism in vivo is rapidly excreted in bile, these findings in bile duct-cannulated rats thus indicate that Sn-protoporphyrin is capable of significantly reducing the production of this bile pigment from endogenous sources of heme for considerable periods of time.

The ability of Sn-protoporphyrin to inhibit bilirubin formation in both hepatocytes and in reticuloendothelial cells was also explored in bile duct-cannulated rats, using intravenous infusions of both heme and heat-damaged rat erythrocytes. Endogenous bilirubin formation normally takes place primarily from senescent erythrocytes taken up in the spleen, and in hepatocytes from the turnover of hepatic cytochromes, particularly cytochrome P-450 (36). The purpose of the experiments with exogenous heme sources was therefore to examine the effects of Sn-protoporphyrin on bilirubin formation from heme contained in hepatocytes as well as in the reticuloendothelial system, as assessed by the acute infusion of either exogenous heme alone, which is taken up primarily in hepatocytes (28–30), or damaged erythrocytes, which are taken up mostly in the spleen and other reticuloendothelial cell-containing tissues (20, 21).

The acute injection of heme to bile fistula rats produced a prompt increase in bilirubin output in bile (Fig. 2). This peak increase lasted ~3 h and accounted for ~14% of the injected dose of heme, a figure in general agreement with the report of Liem et al. (31) who found that ~20% of a similar dose of heme is transformed to bilirubin within 3 h in the isolated perfused rat liver (31). In our studies, the increase in bile pigment output in bile after heme injection was completely prevented in four out of five animals treated with Sn-protoporphyrin, and partially prevented by this treatment in the fifth animal, which indicates that Sn-protoporphyrin can markedly inhibit the degradation of heme in hepatocytes to bilirubin.

Administration of heat-damaged erythrocytes to control bile fistula rats also produced a prompt increase in bilirubin output in bile. The peak increase lasted ~6 h and accounted for ~56% of the hemoglobin administered in the damaged erythrocytes preparations. In two animals treated with Sn-protoporphyrin, this increase in biliary bilirubin was entirely prevented, and in six, the increase was partially prevented, such that the mean bilirubin output in bile after administration of the damaged erythrocytes accounted for an average of only ~33% of the hemoglobin administered. Thus, the oxidation of 23% (33 vs. 56%) of the administered heme in the damaged erythrocytes preparations, which would ordinarily be converted to bilirubin, was prevented by the administration of Sn-protoporphyrin. Although the peak increase in bilirubin output after administration of damaged erythrocytes was less often completely inhibited by Sn-protoporphyrin as compared with animals administered heme and Sn-protoporphyrin, the decreases in bilirubin output were greater when expressed as a percentage of the dose of administered heme or hemoglobin in the animals treated with damaged erythrocytes (23 vs. 14%). Thus, Sn-protoporphyrin can inhibit bilirubin formation from heme in reticuloendothelial tissues as well as in hepatocytes. The decreased biliary bilirubin output in all the experiments described above was confirmed by high performance liquid chromatography analysis of biliary bile pigments, which showed parallel and marked declines in both the mono- and diglucuronide forms of bilirubin in bile (37).

To explore the possibility that Sn-protoporphyrin might alter one or more steps in bilirubin metabolism and disposition other than bile pigment formation, studies were also carried out in bile fistula rats receiving a prolonged (16 h) and constant intravenous bilirubin infusion. This infusion produced marked and sustained increases in plasma bilirubin levels and in the biliary output of the bile pigment. The injection of a single intravenous dose of Sn-protoporphyrin (10 μmol/kg bw), which produced Sn-protoporphyrin concentrations in plasma which greatly exceeded on a molar basis plasma concentrations of bilirubin during the bilirubin infusion, did not alter the constant levels of bilirubin in plasma and bile during the exogenous bilirubin infusion. Thus, the observations that Sn-protoporphyrin, when given to bile duct-cannulated rats, decreases endogenous bilirubin output without causing bilirubin to accumulate in plasma, and produces no apparent alteration in bilirubin disposition in these animals during an infusion of exogenous bilirubin, provide further support for the idea that the metalloporphyrin acts in vivo primarily by inhibiting bile pigment formation rather than by affecting the disposition of preformed bilirubin.

Sn-protoporphyrin is rapidly excreted in bile as the present studies indicate (Fig. 5). In the intact adult rat, this synthetic metalloporphyrin is excreted both in urine and feces (14), and the amount in feces presumably originates from biliary excretion; whether any of the metalloporphyrin is excreted directly into the gut lumen by intestinal cells is not presently known. The time course of biliary excretion of Sn-protoporphyrin suggests that this process of excretion more closely reflects plasma concentrations, rather than hepatic concentrations, of the metalloporphyrin. Hepatic content of Sn-protoporphyrin is quite persistent after a single dose of the compound (14), and as shown in the present studies, biliary excretion decreases rapidly after the initial increase (Fig. 5). Studies on the nature of the fraction of Sn-protoporphyrin found in liver which is

apparently not immediately accessible to biliary excretion are presently in progress; presumably some portion of this Sn-protoporphyrin represents metalloporphyrin bound to various tissue proteins in addition to heme oxygenase, or is otherwise sequestered within the cell. There is a possibility, as we have noted earlier (14), that such tissue-bound Sn-protoporphyrin might in the tissue homogenization procedures involved in in vitro experiments redistribute to molecules of heme oxygenase, including newly synthesized enzyme molecues, to which the original dose of the metalloporphyrin had not become bound. This could lead to spurious estimates of the duration of heme oxygenase inhibition following Sn-protoporphyrin treatment; in addition, as we have clearly estabished in earlier studies with SKF525A, the level of heme oxygenase activity, even when markedly increased by chemical means, does not necessarily correlate with the in vivo rate of heme degradation, at least of cytochrome P-450 heme (38).

Functional determinants of the extent to which Sn-protoporphyrin inhibits heme degradation in vivo would be valuable in assessing the role of this compound as a competitive inhibitor substrate for heme oxygenase in the whole animal. We have reported earlier (11) that hepatic tryptophan pyrrolase rapidly (within ~30–60 min) becomes completely saturated with heme after Sn-protoporphyrin treatment, a process which subsides after several hours. This phenomenon suggests that after Sn-protoporphyrin administration some fraction of endogenous hepatic heme is preserved from degradation by heme oxygenase. We have also observed (11, 37) that Sn-protoporphyrin treatment evokes a marked increase in the excretion of heme into bile, a phenomenon which strongly supports the idea that the synthetic compound acts as a competitive substrate for heme oxygenase in vivo; through this action, Sn-protoporphyrin would inhibit heme degradation, thus making it available for excretion into the biliary system.

The production of carbon monoxide (CO) during heme catabolism in vivo is also a potentially useful determinant of the rate of heme oxidation in vivo. Cowan and colleagues (39) reported that the pulmonary excretion rate of CO in newborn rats was not affected by Sn-protoporphyrin. In contrast, Milleville and colleagues (40) reported that Sn-protoporphyrin markedly diminished endogenous CO production in treated adult mice, and that the compound also substantially diminished CO production from exogenous heme in these animals (declines in V_{CO} ranged from 30 to 57%). These decreases in CO production approximate the decreases in bilirubin production which have been produced by Sn-protoporphyrin in our own studies.

We have, in collaborative studies with Prof. S. A. Landaw, Veterans Administration Medical Center, Syracuse, NY (manuscript in preparation), examined CO production utilizing ^{14}C-labeled heme or heme labeled in vivo by the obligatory precursor, ALA-5-^{14}C, and quantitated the rate of expired ^{14}CO from these substrates in control and Sn-protoporphyrin-treated animals. The results of these studies indicate that the metalloporphyrin markedly decreased the rate of labeled CO expiration; in adult rats, this decrease ranged from 26 to 61% below control rates for exogenous heme and from 40 to 55% for endogenous heme.

The present studies on the effects of Sn-protoporphyrin on bilirubin output carried out in bile duct-cannulated rats, which are confirmed by observations on CO production reported by Milleville and colleagues (40, 41), as well as our own ongoing studies on the effects of Sn-protoporphyrin on CO generation from labeled endogenous and exogenous heme, indicate that this synthetic metalloporphyrin diminishes the rates of production of both bilirubin and CO in the whole animal. We have also noted previously (11) and described in detail in a recent study (37) that marked increases in biliary heme excretion rapidly occur after administration of this metalloporphyrin. These findings thus provide conclusive evidence that this synthetic heme analogue acts to suppress hyperbilirubinemia principally by binding to the catalytic site of heme oxygenase, thus inhibiting this rate-limiting enzyme of heme degradation, as we originally proposed (1). The subsequent marked excretion of heme into bile (11, 37) reflects this action of Sn-protoporphyrin on heme oxygenase and, by disposing of the unmetabolized substrate of the inhibited enzyme, enhances the ability of the synthetic metalloporphyrin to supress bilirubin production.

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