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Pharmacological and Biological Effects of Tin-Protoporphyrin on Neonatal Hyperbilirubinemic Gunn Rats

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ABSTRACT. Our study was undertaken to examine the pharmacological and biological effects of tin-protoporphyrin, a competitive inhibitor of heme oxygenase, on 5- or 6-day-old homozygous (*j/j*) Gunn rats with hereditary unconjugated hyperbilirubinemia. When *j/j* neonates were injected subcutaneously with 20 μmol of tin-protoporphyrin/kg of body weight, hepatic heme oxygenase activity decreased to 30% of the initial level 2 h after administration and remained low during the next 46 h. However, the reduction of serum bilirubin was more rapid and transient, reaching the minimum value (40% of the initial level) at 1 h and increasing thereafter at a rate almost comparable to that in nontreated *j/j* rats. The mortality rate of *j/j* rats was strikingly reduced by the administration of 1 to 100 μmol of tin-protoporphyrin/kg; the most effective dose was 5 μmol/kg (8% compared with 80% in non-treated *j/j* rats). However, the protective effect of tin-protoporphyrin on bilirubin cerebellopathy (cerebellar hypoplasia) was less marked than expected. Possible implications of our results are discussed. (*Pediatr Res* 24: 209–912, 1988)

Abbreviations

SnPP, tin-protoporphyrin
***j/j*, homozygous to the jaundice gene**
***j/+*, heterozygous to the jaundice gene**
Ki, inhibition constant

Hyperbilirubinemia remains a significant problem in human neonates. Phototherapy and exchange transfusion are current therapies widely used for neonatal jaundice. However, there is an increasing number of surviving extremely low birth weight infants in whom these therapies may not be effective. Several drugs have been used to reduce the total serum bilirubin level in neonates (1, 2), but these carry significant risk (3).

Drummond and Kappas (4) reported the chemoprevention of neonatal jaundice by SnPP. This compound is a potent competitive inhibitor of microsomal heme oxygenase, a rate-limiting enzyme in the degradation of heme to bilirubin. SnPP ameliorates developing hyperbilirubinemia in newborn rats (4, 5) and rhesus monkeys (6) and reduces the plasma bilirubin level in adult mutant mice with hemolytic anemia (7, 8). *j/j* Gunn rats with an inherited deficiency of hepatic bilirubin:uridine diphosphate-glucuronyltransferase activity (9) are hyperbilirubinemic throughout life (10) and develop bilirubin-induced cerebellar hypoplasia (11–14). In our study we evaluated the pharmacological and biological effects of SnPP in this rat model of neonatal jaundice.

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MATERIALS AND METHODS

j/j and apparently normal *j/+* Gunn rats of the Sprague-Dawley strain, developed in our laboratory (14), were used in our study. The breeding conditions were as reported previously (14). The litter size was restricted to eight pups soon after delivery. SnPP was purchased from Porphyrin Products (Logan, UT). All other chemicals used were of the highest grade commercially available. SnPP was dissolved in a small volume of 0.1 N KOH, the pH was adjusted to 7.4 with 0.1 N HCl, and the solution was diluted to desired concentrations with 0.1 M phosphate buffer, pH 7.4. All solutions were prepared under dim light just before use. SnPP was administered subcutaneously to *j/j* and *j/+* rats at various doses up to 100 μmol/kg of body weight.

Treated and nontreated adult rats were anesthetized with chloroform and perfused in situ with ice-cold physiological saline. Livers and spleens were excised and homogenized in a Teflon-pestled glass homogenizer with 3 vol of 50 mM Tris-Cl buffer, pH 7.4, containing 0.25 M sucrose. The homogenate was centrifuged at 18,000 × *g* for 10 min. The supernatants from the livers and spleens of *j/+* rats, and from the spleens of *j/j* rats were used to assess the kinetic parameters of heme oxygenase. In the case of the *j/j* rat livers, the microsomal fraction was analyzed because of a very high concentration of bilirubin in the 18,000 × *g* supernatant. The microsomal pellet, obtained by centrifuging the supernatant at 105,000 × *g* for 60 min, was resuspended in 50 mM Tris-Cl buffer, pH 7.4, containing 0.25 M sucrose.

Heme oxygenase activity was measured by the method of Tenhunen *et al.* (15). The reaction mixture (2.1 ml) contained 18,000 × *g* supernatant (1.2 to 2.6 mg of protein); 41.7 μM methemalbumin (16); 270 μM $NADPH_2$; and 90 mM potassium phosphate buffer, pH 7.4, bubbled with O_2 just before use. To measure enzymatic activity in the *j/j* rat liver, the 18,000 × *g* supernatant was replaced by a microsomal suspension (0.15 to 0.47 mg of protein), and the 105,000 × *g*/60 min supernatant (2.3 mg of protein) from the *j/+* rat liver was included as a source of biliverdin reductase. The reaction mixture was incubated at 37° C for 10 min. Bilirubin formed during the incubation

was extracted by the method of Katoh *et al.* (17)—a 1.8-ml aliquot of the reaction mixture was transferred into 5.25 ml of an ice-cold chloroform-methanol solution (2:5, vol/vol), followed by the addition of 3 ml of cold distilled water. After shaking the mixture vigorously for 20 s, the organic phase was separated by centrifugation at 1000 × *g* for 10 min at room temperature.

Bilirubin extracted was calculated from the difference between absorbances at 452 and 520 nm by using a molecular extinction coefficient at 452 nm of 62,600 (17). All procedures were carried out under dim light. The amount of bilirubin formed was linear with respect to incubation time or protein concentration under the conditions used. The specific activity was expressed as nmol of bilirubin formed/mg of protein/10 min. Protein was estimated by the method of Lowry *et al.* (18) using crystalline bovine serum albumin as a standard. The total serum bilirubin concentration was determined according to the method of Malloy and Evelyn (19). Group mean comparisons were made by the *t* test or alternatively a modified *t* test (the Cochran and Cox method) when group variances were unequal.

RESULTS

Figure 1 shows representative double reciprocal plots of splenic heme oxygenase activity *versus* methemalbumin (substrate) with and without SnPP in adult *j*/+ rats. The two lines converged to almost the same point on the ordinate, which is consistent with previous reports that SnPP is a competitive inhibitor with respect to the substrate (4, 5). Apparent Km values for methemalbumin were calculated to be 33.4 and 7.3 μM in the presence and absence of the inhibitor, respectively. Table 1 summarizes kinetic parameters obtained from four separate experiments with enzyme preparations from the spleen and liver of *j*/+ and *j*/*j* adult rats. The competitive nature of SnPP inhibition with regard to the substrate was common in all cases. Although some variations were observed in Km values for the substrate among four enzyme sources, they were not greatly different from each other. This was also true of apparent inhibition constants (*Ki*) for SnPP.

As shown in Figure 2, hepatic heme oxygenase activity in *j*/*j* neonates decreased rapidly after administration of SnPP to a minimum value, 2 h later. Enzyme activity increased gradually thereafter to about two-fifths of the initial level at 48 h. The response of serum bilirubin to SnPP is demonstrated in Figure 3. Reduction in the concentration of serum bilirubin was rapid and transient. One h after SnPP treatment the bilirubin level decreased about 60% but increased steadily thereafter at a rate comparable to that in nontreated *j*/*j* rats.

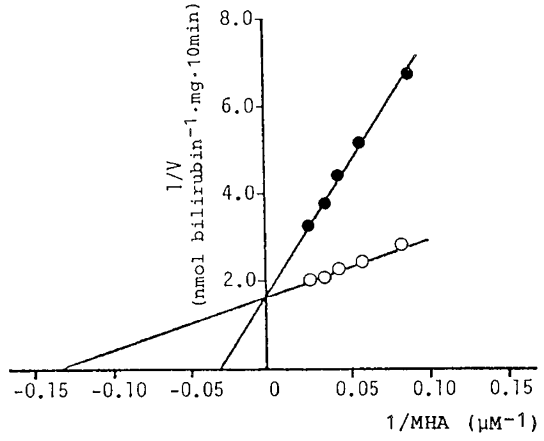

Fig. 1. Double reciplocal plots of heme oxygenase activity (*V*) *versus* methemalbumin (*MHA*) in the presence (●) and absence (○) of 0.2 μM tin-protoporphyrin. The microsomal fraction prepared from the spleen of adult heterozygous Gunn rats was used as an enzyme source. Each *point* represents the average of triplicate determinations.

Table 1. *Apparent Km for methemalbumin and Ki for SnPP in heme oxygenase reactions in spleen and liver of adult heterozygous (j/+) and homozygous (j/j) Gunn rats**

	n	Km (μM)	Ki (nM)
j/+ spleen	3	6.1 ± 0.76	40.6 ± 10.8
j/*j* spleen	4	12.0 ± 3.0	45.0 ± 4.1
j/+ liver	3	13.8 ± 2.5	33.8 ± 2.4
j/*j* liver	4	14.0 ± 0.7	20.3 ± 4.5

* Results are expressed as the averages ± SD of the indicated number (*n*) of rats. Experimental details refer to "Materials and Methods." Km values for methemalbumin were determined from the double reciprocal plots of enzymic activity *versus* methemalbumin. Ki values for SnPP were calculated from the plots of SnPP concentration *versus* slope obtained from the double reciprocal plots in the presence and absence of SnPP.

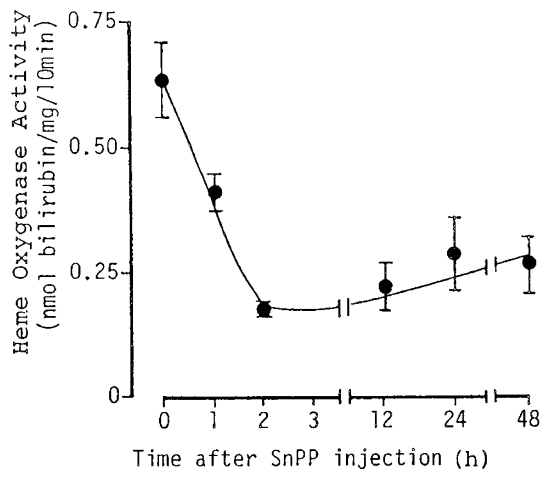

Fig. 2. Time course of hepatic heme oxygenase activity after subcutaneous administration of 20 μmol of SnPP/kg of body weight to 6-day-old homozygous Gunn rats. For experimental details refer to "Materials and Methods." Each *point* represents the average ± SD (*bar*) of two to five rats.

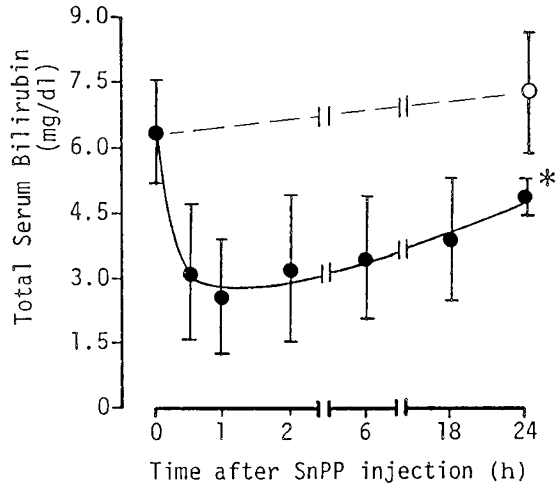

Fig. 3. Time course of the concentration of serum bilirubin after subcutaneous administration of 20 μmol of SnPP/kg of body weight to 6-day-old homozygous Gunn rats. Blood was collected from the tail vein of each rat according to the schedule. The *open circle* shows the average concentration of serum bilirubin in nontreated homozygous Gunn rats at 7 days of life ($n = 12$). Each *closed circle* represents the average ± SD (*bar*) of eight littermates. $^{*}p < 0.01$ compared with nontreated rats at 7 days of life.

Various doses of SnPP (0.5–100 μmol/kg of body weight) were tested to evaluate *in vivo* effects on the rate of mortality and cerebellar development in jaundiced neonates. As shown in Table 2 there were no differences between the morality rates of SnPP-treated and nontreated *j/+* rats. However, mortality was reduced in *j/j* rats injected with 1 to 100 μmol of SnPP/kg of body weight. The most effective dose was 5 μmol/kg of body weight. Although the cerebellar wet weight of any group of *j/+* rats treated with SnPP did not differ significantly from that of the nontreated control group, the cerebellar weights of the *j/j* rat groups given 1 and 5 μmol of drug/kg were significantly increased (by 51 and 45%, respectively) over the control value (Table 2).

DISCUSSION

The present kinetic study demonstrates that heme oxygenases prepared from liver and spleen have similar affinity for heme or SnPP between in the *j/+* and *j/j* Gunn rats. The apparent Km values for heme of hepatic and splenic enzymes were within the range reported previously for microsomal preparations from human (4) and rat (15) spleens, or rat liver (5). The Ki values for SnPP also were similar to values in previous reports (4, 5). Thus the heme oxygenase characteristics in liver and spleen were similar in nature independent of genotype.

As shown in several *in vivo* studies (4–8), the administration of SnPP to *j/j* neonates causes rapid decreases in hepatic heme oxygenase activity and serum bilirubin. However, the reduction of serum bilirubin occurred within 60 min after SnPP administration, whereas the decrease in hepatic heme oxygenase activity continued for 2 h with a trend similar to that reported by Anderson *et al.* (20). This is also recognized in the data reported previously (21–23). Furthermore, the recovery of serum bilirubin was much faster than that of enzymatic activity. It has been reported that the biological half-life of bilirubin in *j/j* Gunn rats is 33 to 62 h (24). Accordingly, even if heme oxygenase activity is completely blocked *in vivo* by SnPP, the serum bilirubin level would be expected to decrease more gradually than we observed.

There are several possible explanations about the observed phenomenon. SnPP may interfere with the bilirubin measurement. To assess this possibility, SnPP was mixed with the *j/j* rat serum at various concentrations (1–50 μM), and bilirubin assayed as described in "Materials and Methods." No interference was observed (data not shown). Alternatively there could be a preferential effect of SnPP in nonhepatic tissues such as spleen and kidney. However, Anderson *et al.* (20) reported that SnPP administered intravenously accumulates in liver, spleen, and kidney with similar time-course profiles up to at least 12 h, and 1-h levels of the inhibitor in these tissues are far larger than the Ki value. These authors also showed that heme oxygenase activities in liver, spleen, and kidney are inhibited in the same time-dependent manner by SnPP administered subcutaneously.

Another possibility is that there are heme degradation pathways not mediated by heme oxygenase (25) and that unconjugated bilirubin may be catabolized by mixed-function monooxygenases (26). We cannot exclude an effect of SnPP to stimulate these pathways and accelerate the reduction of serum bilirubin. SnPP could be acting as a photosensitizer to enhance bilirubin destruction (27). This appears unlikely because the present experiments, including the assay for bilirubin, were performed under dim light. Finally, SnPP may act to displace bilirubin from the albumin-bilirubin complex and cause pigment deposition in tissues with a concomitant decrease of serum levels. Recently Breslow *et al.* (28) reported that the albumin-bilirubin binding is little affected by SnPP. However, they conducted their experiments in an *in vitro* system and it is not clear if the same is also true *in vivo*, particularly in hyperbilirubinemic Gunn rats.

Keino *et al.* (14) showed that the cerebellar hypoplasia of *j/j* rats could be prevented by photoirradiation from postnatal day 5 to 10 and that this photo-effect was due to the reduction of serum bilirubin. Unlike phototherapy, the protective effect of SnPP (in a single dose of 20 μmol/kg) on the cerebellar hypoplasia was relatively limited (15% increase over the control; Table 2), although a significant response of serum bilirubin to SnPP was observed (Fig. 3). This contradiction may be explained by the long biological half-life of bilirubin in *j/j* rats (24). Surprisingly the mortality rate of *j/j* infants was most effectively reduced by the administration of SnPP at a dose of 5 μmol/kg of body weight (Table 2) and one-fifth of this dose also was effective in minimizing cerebellar hypoplasia (Table 2). The mechanism for the SnPP-induced decrease in mortality rate with only a limited effect on the cerebellar hypoplasia remains unclear.

Table 2. *In vivo effects of SnPP on mortality rate and cerebellar wet wt in j/+ and j/j Gunn rats**

Rat	SnPP (μmol/kg of body wt)	No. of rats used	Mortality rate (%)	Cerebellar wet wt (mg)
j/+	0	32	9	215.8 ± 14.6
	0.5	14	14	198.6 ± 5.5
	1	6	0	237.0 ± 7.5
	5	3	0	240.0 ± 3.5
	10	4	0	236.2 ± 7.6
	20	6	0	223.6 ± 5.9
	50	6	17	212.3 ± 7.4
	100	6	17	228.5 ± 8.3
j/j	0	70	80	49.9 ± 9.5
	0.5	26	73	53.4 ± 8.0
	1	10	20	75.1 ± 9.9†
	5	13	8	72.1 ± 10.7†
	10	12	25	68.2 ± 19.3
	20	10	40	57.3 ± 5.6
	50	10	30	55.5 ± 7.3
	100	10	30	50.3 ± 3.8

* Various doses of SnPP were injected subcutaneously to 5-day-old *j/+* and *j/j* Gunn rats and the effects of SnPP were examined at 30 days of life.

† $p < 0.01$ compared with nontreated *j/j* rats.

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