PROOF THAT Sn-PROTOPORPHYRIN INHIBITS THE ENZYMATIC CATABOLISM OF HEME IN VIVO

Suppression of ^{14}CO Generation from Radiolabeled Endogenous and Exogenous Heme Sources

By STEPHEN A. LANDAW, SHIGERU SASSA,* GEORGE S. DRUMMOND,* AND ATTALLAH KAPPAS*

From the Veterans Administration Medical Center, Syracuse, New York 13120; and
*The Rockefeller University Hospital, New York 10021

An extensive series of studies by Kappas and Drummond (see review in reference 1) has demonstrated that the synthetic heme analogue, tin(Sn)-protoporphyrin (SnPP) acts as a potent competitive inhibitor of heme oxygenase (2–4), and can suppress hyperbilirubinemia in experimentally induced and naturally occurring forms of jaundice in animals and man (2, 3, 5, 6). SnPP also significantly diminishes the output of biliary bilirubin derived from endogenous and exogenous heme sources (7), does not affect the metabolic disposition of preformed bilirubin (7), and elicits a marked biliary excretion of untransformed heme in bile duct–cannulated animals (8).

In the process of heme degradation catalyzed by the enzyme heme oxygenase, one mole of carbon dioxide (CO) is generated for each mole of bile pigment produced (9, 10). Thus the findings reported above would support the expectation that SnPP would diminish the generation of CO from the catabolism of various heme moieties in vivo. We have examined this question by studying the generation of ^{14}CO from radiolabeled heme sources in the intact animal. The use of specifically labeled heme is crucial in answering this question since CO may be generated from sources other than heme (11–14). The results of these experiments prove conclusively that SnPP markedly inhibits the enzymatic oxidation in vivo of heme from both endogenous and exogenous sources.

Materials and Methods

Animals used were 300–400 g Sprague-Dawley rats obtained from Taconic Farms, Germantown, NY. SnPP was provided by Porphyrin Products, Logan, UT. The labeled precursor δ-aminolevulinic acid (5-[^{14}C]ALA) (Sp act, 49 mCi/mM) was obtained from New England Nuclear, Boston, MA. Labeled [^{14}C]hemin (Sp act, 3.0×10^4 dpm/mg) was prepared in rats by methods previously described (9). Before injection into animals, 1.0 mg of the crystallized hemin was dissolved in 1 ml of alkalinized rat plasma, and injected intravenously within 15 min of preparation. SnPP was dissolved in a small amount of 0.2 N NaOH, a 3.5-fold greater volume of 0.9% NaCl was added, and pH was adjusted to
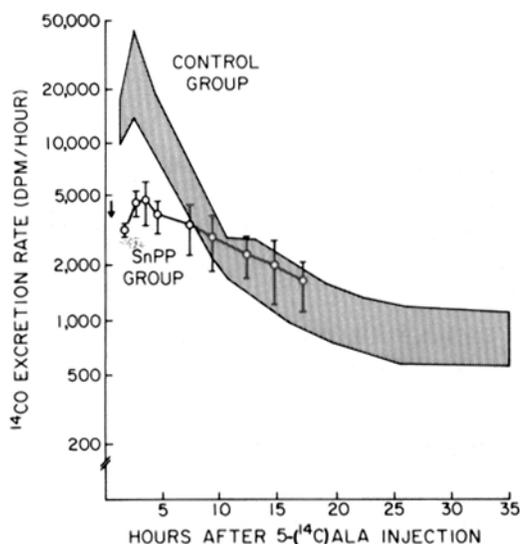
FIGURE 1.   Semilogarithmic plot of the excretion rate of ^{14}CO (dpm/h, ordinate) vs. time after injection of 5-[^{14}C]ALA (h, abscissa). To facilitate comparisons within this figure and with the results shown in Fig. 2, all ^{14}CO excretion data for control (saline-injected) animals were normalized such that total ^{14}CO excretion in the period 0–12 h after ALA injection was set at 100,000 dpm. For experimental animals, data were normalized for a dose of ALA that yielded 100,000 dpm in the first 12 h for the control animal run on the same date. The shaded area denotes the total range of normalized values for four control animals injected with saline 1 h before 5-[^{14}C]ALA. Results from four animals injected with SnPP (50 μmol/kg body weight) 1 h before injection of 5-[^{14}C]ALA are shown (*open circles*, mean ± SD). Note that the inhibition of ^{14}CO excretion produced by SnPP was rapid, and the effect appeared to last ~8–9 h.

7.5 by dropwise addition of 0.5 N HCl (6). The compound was injected subcutaneously in a dose of 50 μmol/kg body weight at various time periods before or after injection of the labeled materials. Equal volumes of saline were injected by the same route for the control animals.

The degradation of labeled heme was followed by quantitation of the excretion of ^{14}C-labeled carbon monoxide (^{14}CO), using previously described methods (9). Immediately after injection of labeled material, rats were placed in flow-through metabolic chambers provided with food, water, and bedding. Labeled ^{14}CO was then collected continuously over the next 24–30 h, and expressed as dpm of ^{14}CO excreted over various time intervals. Student's *t* test was used to determine the statistical significance of results.

Results

Effect of SnPP on ^{14}*CO Generation after 5-[^{14}C]ALA Administration.*   In the rat, 5-[^{14}C]ALA is incorporated almost exclusively into nonerythropoietic hemes (15, 16), labeling bilirubin and CO to very high activity within minutes of injection (17, 18). The peak of ^{14}CO excretion occurs early, about 2–3 h after injection of 5-[^{14}C]ALA, and is followed by a rapid exponential decline (Fig. 1), with 3–4% of the injected dose appearing as ^{14}CO within the first 12 h after injection (Table I).

When 50 μmol/kg body weight of SnPP was injected 1 h before injection of 5-[^{14}C]ALA, there was a prompt reduction in ^{14}CO excretion, lasting ~8–9 h (Fig. 1). The peak of ^{14}CO excretion at 2–3 h was virtually abolished, and total ^{14}CO excretion in the first 12 h was reduced by ~60% ($p < 0.005$, Table I). Thereafter, ^{14}CO excretion was identical to the control animals (Table I and Fig. 1).

In separate experiments, SnPP or saline was injected subcutaneously into four rats 11 h after 5-[^{14}C]ALA; ^{14}CO excretion was monitored for the entire time period 0–35 h after 5-[^{14}C]ALA injection. When compared with controls, ^{14}CO excretion was reduced 48% for the time period for 12–24 h after injection of 5-[^{14}C]ALA corresponding to 1–13 h after injection of SnPP (Fig. 2). ^{14}CO excretion rates were similar to controls before injection of SnPP (0–10 h after

TABLE I

Effect of SnPP on Cumulative Excretion of ^{14}CO after Hemin or ALA ^{14}CO Excretion
(Percent of Injected Dose)

Compound injected	0–12 h			12–24 h		
	Control group	SnPP group	Percent change	Control group	SnPP group	Percent change
5-[^{14}C]ALA	3.10	0.90	−71	0.34	0.41	+20
	3.92	1.37	−65	0.57	0.68	+19
	3.35	1.45	−57	0.64	0.63	−2
	2.79	1.36	−51	0.64	0.56	−12
Mean	3.29	1.27	−61*	0.55	0.57	+6
[^{14}C]Hemin	4.63	1.92	−59	0.99	0.62	−37
	6.56	2.23	−66	1.03	0.74	−28
	20.04	13.59	−32	3.42	2.41	−30
	21.70	16.30	−25	9.21	5.63	−39
Mean	13.23	8.51	−46‡	3.66	2.35	−34§

* $p < 0.005$.
‡ $p < 0.01$.
§ $p > 0.05$.
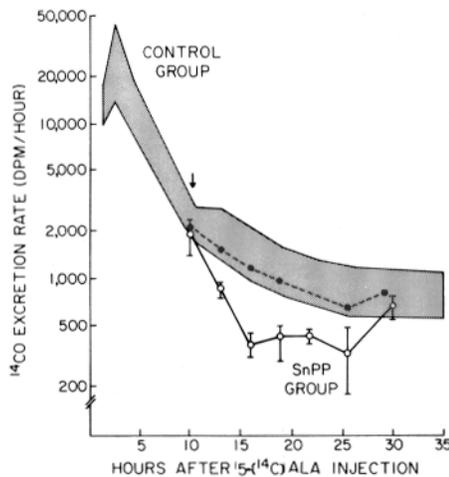

FIGURE 2. Semilogarithmic plot of excretion rate of ^{14}CO vs. time after injection of 5-[^{14}C]ALA details similar to those in Fig. 1. A single animal injected with saline 11 h after injection of labeled ALA is shown with the solid circles, while results from three animals injected with SnPP 11 h after injection of labeled ALA are shown as the open circles (mean ± SD). The shaded area represents the same values of the shaded area in Fig. 1. Data points in the experimental groups were normalized to 100,000 dpm during the control period (0–12 h after ALA). Inhibition of ^{14}CO excretion produced by SnPP began immediately, with the effect lasting about 14–20 h.

5-[^{14}C]ALA), and were again similar to controls by ~20 h after injections of SnPP.

Effect of SnPP on ^{14}CO Excretion after [^{14}C]Hemin Administration. [^{14}C]Hemin prepared in vivo from 2-[^{14}C]glycine (9) was injected intravenously in alkalinized plasma to eight rats, divided into four pairs. From each pair, one rat was injected with SnPP and the other with saline, to control for day-to-day variability in the preparation of solubilized hemin. As with 5-[^{14}C]ALA, excretion of labeled bilirubin and CO after [^{14}C]hemin is rapid (9), with peak excretion occurring 2–3 h after injection, 5–22% of the injected dose appearing as ^{14}CO within 12 h after injection (Table I).

When 50 μmol/kg body weight SnPP was injected 1 h before [^{14}C]hemin, there was a 46% reduction ($p < 0.01$, Table I) in ^{14}CO excretion over the ensuing 12 h. In the period 12–24 h after injection, there was a sustained and

consistent reduction in ^{14}CO excretion in SnPP-treated animals (mean decline, 34%), but this value did not reach statistical significance ($p > 0.05$, Table I).

Discussion

Drummond and Kappas proposed that (2) the mechanism of suppression of hyperbilirubinemia by SnPP involves a potent and sustained competitive inhibition of heme oxygenase by this synthetic metalloporphyrin. Such a mechanism would presuppose a concurrent diminution of production of bilirubin as well as of CO after treatment of animals with this synthetic heme analogue. Suppression of bilirubin production by SnPP has been confirmed (7). Milleville et al. (19, 20) and more recently Posselt et al. (21), using mature mouse and newborn rat models, respectively, but without using radiolabeled heme, have reported that SnPP reduces CO generation from endogenous heme and from heme derived from hematomas as well as exogenous sources, by ~18–30% and 35–56%, respectively. The variable time periods between injection of SnPP and its effects on CO production probably account for the differences in effects noted in these experiments.

The results of the present study, using highly specific techniques for quantitating ^{14}CO generation from the catabolism of radiolabeled heme moieties, conclusively establish that SnPP suppresses CO generation and does so at rates comparable to those by which biliary bile pigment output is diminished by SnPP (7). The experiments described here indicate that, when 5-[^{14}C]ALA is used as a heme precursor, the suppressive effect of SnPP on ^{14}CO excretion lasts about 8–20 h, whether the metalloporphyrin is given 1 h before (Fig. 1) or 11 h after (Fig. 2) the 5-[^{14}C]ALA. These observations thus suggest a duration of action in vivo of a single dose of SnPP in the range of about 12 h, despite the prolonged retention (in part in nonenzymatic tissue sites as previously suggested [22]) of the compound in the liver and other organs. Our findings also suggest that degradation of the heme moieties of both rapid and relatively slowly turning over hemeproteins in the liver is suppressed by SnPP.

Kappas et al. (8) have reported that the administration of SnPP leads to a marked increase in the biliary excretion of untransformed heme after the injection of both hemin and heat-damaged red blood cells, and that this excretion of heme quantitatively accounts for much of the concurrent decline seen in the output of biliary bilirubin. Similar results have recently been reported by Hintz et al. (23). The effect of SnPP on heme excretion after the administration of heat-damaged red blood cells, though significant, was less than that seen after injection of hemin, and was more variable in individual animals (7). This suggests that SnPP can inhibit to some extent extrahepatic heme degradation in vivo, as well as hepatic heme catabolism. The in vivo effects of SnPP in the liver and in extrahepatic tissues such as the spleen, however, are probably not equal, since the findings of Simionatto et al. (7), using heat-damaged erythrocytes, could be explained in part by the transport of unmetabolized, excess heme or hemoglobin from splenic to hepatic sites for degradation. Thus, significant inhibition of heme catabolism by SnPP may be primarily manifest on liver heme moieties or in circumstances when the organism is presented with a substantial excess of heme,

as in the immediate postnatal period in the newborn or when erythrocyte destruction is marked and sustained.

Summary

Sn-protoporphyrin (SnPP) suppresses generation of ^{14}CO from hepatic heme labeled with δ-aminolevulinic acid (5-[^{14}C]ALA) or from infused [^{14}C]hemin in rats. SnPP administered 1 h before administration of 5-[^{14}C]ALA virtually abolished the peak output of ^{14}CO occurring 2–3 h after injection of this heme precursor, and during the succeeding 12 h reduced ^{14}CO excretion by ~61% compared with controls. When [^{14}C]hemin was infused, SnPP diminished ^{14}CO excretion by ~50%. These findings, derived from experiments using radiolabeled endogenous and exogenous heme sources, establish conclusively that the synthetic metalloporphyrin SnPP inhibits the oxidative degradation of heme in the intact animal.

References

1. Kappas, A., and G. S. Drummond. 1986. Control of heme metabolism with synthetic metalloporphyrins. *J. Clin. Invest.* 77:335.
2. Drummond, G. S., and A. Kappas. 1981. Prevention of neonatal hyperbilirubinemia by Sn-protoporphyrin IX, a potent competitive inhibitor of heme oxidation. *Proc. Natl. Acad. Sci. USA.* 78: 6466.
3. Drummond, G. S., and A. Kappas. 1982. Chemoprevention of neonatal jaundice: potency of tin-protoporphyrin in an animal model. *Science (Wash. DC).* 217:1250.
4. Yoshinaga, T., S. Sassa, and A. Kappas. 1982. Purification and properties of bovine spleen heme oxygenase: amino acid composition and sites of action of inhibitors of heme oxidation. *J. Biol. Chem.* 257:7778.
5. Kappas, A., G. S. Drummond, C. S. Simionatto, and K. E. Anderson. 1984. Control of heme oxygenase and plasma levels of bilirubin by a synthetic heme analogue, tin-protoporphyrin. *Hepatology (Baltimore).* 4:336.
6. Anderson, K. E., C. S. Simionatto, G. S. Drummond, and A. Kappas. 1986. Pharmacokinetics of Sn-protoporphyrin and suppression of hyperbilirubinemia in humans. *Clin. Pharmacol. Ther.* 39:510.
7. Simionatto, C. S., K. E. Anderson, G. S. Drummond, and A. Kappas. 1985. Studies on the mechanism of Sn-protoporphyrin suppression of hyperbilirubinemia: inhibition of heme oxidation and bilirubin production. *J. Clin. Invest.* 75:513.
8. Kappas, A., C. S. Simionatto, G. S. Drummond, S. Sassa, and K. E. Anderson. 1985. The liver excretes large amounts of heme into bile when heme oxygenase is inhibited competitively by Sn-protoporphyrin. *Proc. Natl. Acad. Sci. USA.* 82:896.
9. Landaw, S. A., E. W. Callahan, and R. Schmid. 1970. Catabolism of heme *in vivo*: comparison of the simultaneous production of bilirubin and carbon monoxide. *J. Clin. Invest.* 49:914.
10. Berk, P. D., F. L. Rodkey, T. F. Blaschke, H. A. Collison, and J. G. Waggoner. 1974. Comparison of plasma bilirubin turnover and carbon monoxide production in man. *J. Lab Clin. Med.* 83:29.
11. Rodkey, F. L., H. A. Collison, and J. D. O'Neill. 1972. Carbon monoxide and methane production in rats, guinea pigs and germ-free rats. *J. Appl. Physiol.* 33:256.

12. Westlake, D. W. S., J. M. Roxburgh, and G. Talbot. 1961. Microbial production of carbon monoxide from flavonoids. *Nature (Lond.)*. 189:510.
13. Wolff, D. G., and W. R. Bidlack. 1976. The formation of carbon monoxide during peroxidation of microsomal lipids. *Biochem. Biophys. Res. Commun.* 73:850.
14. Engel, R. R. 1977. Alternative sources of carbon monoxide. *In* Proceedings of the Fogarty International Symposium on Chemistry and Physiology of Bile Pigments. P. D. Berk, and N. I. Berlin, editors. Dept. of Health, Education and Welfare Publication NIH-77-110. 148–155.
15. Landaw, S. A. 1977. Kinetic aspects of endogenous carbon monoxide production. II: The erythroid component of the early labeled peak. *In* Proceedings of the Fogarty International Symposium on Chemistry and Physiology of Bile Pigments. P. D. Berk, and N. I. Berlin, editors. Dept. of Health, Education and Welfare Publication NIH-77-110. 134–147.
16. Robinson, S. H., M. Tsong, B. W. Brown, and R. Schmid. 1966. The sources of bile pigment in the rat: studies of the "early labeled" fraction. *J. Clin. Invest.* 45:1569.
17. Robinson, S. H., C. A. Owen, E. V. Flock, and R. Schmid. 1965. Bilirubin formation in liver from nonhemoglobin sources. Experiments with isolated, perfused rat liver. *Blood.* 26:823.
18. Ibrahim, G. W., S. Schwartz, and C. J. Watson. 1966. Early labeling of bilirubin from glycine and δ-aminolevulinic acid in bile fistula dogs, with special reference to stimulated versus suppressed erythropoiesis. *Metabolism.* 15:1129.
19. Milleville, G. S., M. D. Levitt, and R. R. Engel. 1983. Tin-protoporphyrin (TP) reduces endogenous carbon monoxide production (VCO). *Pediatr. Res.* 17:239A.
20. Milleville, G. S., M. D. Levitt, and R. R. Engel. 1985. Tin-protoporphyrin inhibits carbon monoxide production in adult mice. *Pediatr. Res.* 19:94.
21. Posselt, A. M., L. K. Kwong, H. J. Vreman, and D. K. Stevenson. 1986. Suppression of carbon monoxide excretion rate by tin-protoporphyrin. *Am. J. Dis. Child.* 140:147.
22. Anderson, K. E., C. S. Simionatto, G. S. Drummond, and A. Kappas. 1984. Tissue distribution and disposition of tin-protoporphyrin, a potent competitive inhibitor of heme oxygenase. *J. Pharmacol. Exp. Ther.* 228:327.
23. Hintz, S. R., L. K. Kwong, H. J. Vreman, and D. K. Stevenson. 1986. Carbon monoxide (CO) excretion as an accurate measure of suppressed endogenous heme catabolism in bile duct cannulated adult Wistar rats after tin-protoporphyrin (TP). *Pediatr. Res.* 20:350A.