0031-3998/89/2604-0362$02.00/0
PEDIATRIC RESEARCH

Vol. 26, No. 4, 1989
Printed in U.S.A.

In Vitro Inhibition of Adult Rat Intestinal Heme Oxygenase by Metalloporphyrins[1]

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ABSTRACT. We determined the inhibitory effects of concentrations of tin- and zinc protoporphyrin (1–100 μM) and mesoporphyrin (0.1–10 μM) on the *in vitro* heme oxygenase (HO) (E.C.1.14.99.3) activity in liver, spleen, and intestine 13, 000 × *g* tissue supernatants from fasted adult male Wistar rats through measurement of carbon monoxide by gas chromatography. All four metalloporphyrins inhibited intestinal HO, under the light-limited conditions of these experiments. The zinc porphyrins showed a clear concentration dependency over the entire range, reducing activity to near zero levels at their highest concentrations. The tin porphyrins reduced HO activity to 26% of initial levels, but the inhibition was not clearly concentration dependent. Liver and spleen supernatants exhibited concentration dependent inhibition by all four metalloporphyrins. We also assessed the effect of light on HO activity measurements. HO determinations in the light (8 μW/cm²/nm) yielded higher HO activity than for reactions performed in the dark. The presence of light and SnPP appeared to stimulate the HO activity of intestinal preparations thus overcoming the observed inhibition. Light and SnPP also decreased to a lesser degree the inhibition for the spleen preparation, but not for the liver. We conclude that heme oxygenase activity measurements via CO determination need to be conducted in the absence of light, in particular when photosensitizers are present. Furthermore, it appears that intestinal HO behaves in a quantitatively different way from other tissues, under varying conditions of metalloporphyrin inhibition and light exposure. (*Pediatr Res* 26:362–365, 1989)

Abbreviations

HO, heme oxygenase
ZnPP, zinc protoporphyrin
ZnMP, zinc mesoporphyrin
SnPP, tin protoporphyrin
SnMP, tin mesoporphyrin

Jaundice, a problem common to the human neonate, results from increased bilirubin production and immaturity of the bilirubin-conjugating mechanism in the liver. HO, the rate-limiting enzyme (E.C.1.14.99.3) in the heme catabolic pathway, cleaves and oxidizes the α-methene bridge of the heme molecule, yielding CO, iron, and biliverdin in equimolar amounts (1). Biliverdin is rapidly converted to bilirubin by the enzyme biliverdin reductase.

SnPP and ZnPP, synthetic heme analogs (Fig. 1), have been shown to be effective in lowering serum bilirubin levels in a wide range of animal species (2–8), by competitively inhibiting heme oxygenase activity (7–10). Additionally, SnMP and ZnMP have recently been found to be equally effective at about 10-fold lower doses (11, 12).

Previous studies have shown that heme is excreted into the bile of SnPP-treated adult rats (13, 14). Inhibition of intestinal HO may be of clinical importance, because heme reaching the intestine can potentially be degraded by intestinal tissue HO (15–18) to CO and bilirubin, where the latter may enter the body via the enterohepatic circulation in the neonate (19). Previously we did not observe significant inhibition of intestinal HO *in vivo* or *in vitro* at SnPP concentrations (40 μM) which inhibited hepatic and splenic HO (18, 20–21). We could not explain this lack of inhibition, but considered the existence of an isoform of HO (22), specific to the intestine, which is relatively less inhibitable by SnPP or some mechanism (natural or experimental) by which access of SnPP to HO is limited or by which extraneous CO is generated. Although our gas chromatographic assay for HO activity measures the generation of CO (23), a nonlight-sensitive product of the HO reaction, we had observed the generation of extraneous CO from tissues treated with SnPP and light (24). Therefore, we studied the *in vitro* efficacy of SnPP and ZnPP, as well as their mesoderivatives, to inhibit adult rat intestinal HO in 13 000 × *g* tissue homogenate supernatants over a wide range of concentrations (0.1, 1, 10, 40, and 100 μM) and light exposure. Liver and spleen tissue preparations were used as controls to demonstrate the efficacy of the various metalloporphyrins to decrease HO activity in tissues known to be inhibitable.

MATERIALS AND METHODS

Animals. This protocol was approved by the research committee of the Stanford University Division of Laboratory Animal Medicine. Fasted (16 h) adult male Wistar rats (Simonsen Laboratories, Inc., Gilroy, CA) weighing 265–310 *g* were used for all experiments. The rats had unlimited access to water and were housed in a temperature-controlled room (25 ± 1°C) with a 12-h light cycle starting at 0700 h. All animals were killed by decapitation at the conclusion of each experiment.

Buffer, 0.1 M. KH_2PO_4, 13.61 *g* was dissolved in distilled water. The pH was titrated to 7.4 with 1.0 N KOH and the volume was adjusted to 1 l.

Metalloporphyrins. Stock solutions of 3.0 mM for the protoporphyrins, and 0.3 mM for the mesoderivatives, were prepared by dissolving SnPP (22.4 mg), ZnPP (18.7 mg), SnMP (2.25 mg) and ZnMP (1.89 mg) (Porphyrin Products Inc., Logan, UT) in 0.5 mL of 10% (v/v) ethanolamine (Sigma Chemical Co., St. Louis, MO). Human serum albumin (100 mg, fraction V, no. A1653, Sigma) was added, and the pH of all solutions was slowly

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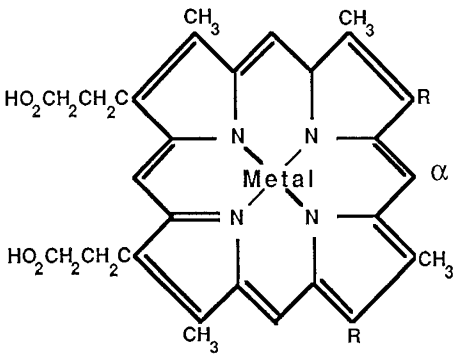
Supported in part by the Mead Johnson Nutritional Division, the Christopher Taylor Harrison Research Fund, and Grant HD14426 from the National Institutes of Health.

[1] Patent disclosure: Hendrik J. Vreman, Ph.D. and David K. Stevenson, M.D. have been issued U.S. Patent no. 4,831,024 for Method to Prevent Neonatal Jaundice, involving a screening procedure to detect elevated bilirubin production by CO detection technology combined with metalloporphyrin therapy.



Metals:

1. Fe-Heme
2. Sn-Tin Metalloporphyrin
3. Zn-Zinc Metalloporphyrin

R-Chemical Group:

1. Protoporphyrin, R=Vinyl (CH_2=CH-)
2. Mesoporphyrin, R=Ethyl (CH_3-CH_2-)

Fig. 1. Chemical structure of the metalloporphyrins studied.

titrated with a manual titrator (Gilmont, Cole-Parmer Instrument Co., Chicago, IL) under stirring to 7.4 with 1.0 N HCl. The final volume was then adjusted to 10 mL. Solutions were kept in the dark at 4°C and used within 2 wk of preparation. Inhibitor working solutions were prepared by appropriately diluting the stock solutions with buffer so that 2 μL added to 60-μL reaction mixture yielded the desired final concentration of 0.1 to 100 μM.

Methemalbumin, 150/11.2 μM. Hemin (13.1 mg, Sigma) was dissolved in 2.5 mL of 0.4 M Na_3PO_4. Distilled water (6.5 mL) and 100 mg BSA (fraction V, no. A4503, Sigma) were added. The pH of the solution was slowly titrated to 7.4 with 1.0 N HCl under stirring. The volume of this stock solution (2/0.15 mM) was adjusted to 10 mL with distilled H_2O. The stock solution was stored at 4°C in the dark for up to 1 mo. The working solution was obtained by diluting (0.75 to 10.0 mL) the stock solution with buffer.

NADPH, 4.5 mM. Four mg NADPH, tetrasodium salt (8.5% H_2O), type 1 (Sigma) was dissolved in 1.0 mL buffer. This solution was prepared fresh daily.

Tissue collection and preparation. Tissue supernatants were prepared for assay as follows. Organs were removed immediately from the decapitated animal and washed with ice-cold buffer. Liver and spleen were homogenized (20 s) with a Biohomogenizer (Biospec Products, Inc., Bartlesville, OK) in buffer (4 and 9 mL/g, respectively). The intestine was cut at the pyloric sphincter and at the anus. The entire length was flushed with 40 mL of ice-cold buffer to remove all intestinal contents. The tissue was homogenized with buffer (4 mL/g). Homogenates were centrifuged at 13 000 × *g* for 15 min at 4°C. The supernatants were analyzed for HO activity.

HO assay. The HO activity of tissue supernatants was determined with the gas chromatographic assay which measures the amount of CO produced from heme in the presence of NADPH, as described in detail elsewhere (23). Twenty μL supernatant, 20 μL methemalbumin solution (50 μM final concentration), and either 20 μL of NADPH (for total reaction) or buffer (for blank reaction) were pipetted into duplicate septum-sealed amber vials (12 × 32 mm, 2 mL, Alltech Associates, Inc., San Jose, CA). For inhibitor testing reactions, small volumes (2 μL) of SnPP and ZnPP working solutions were added to yield 1.0, 10, 40, and 100 μM concentrations. The meso derivatives, SnMP and ZnMP, were aliquoted to give 0.10, 1.0, 4.0, and 10 μM concentrations. After temperature equilibration in a 37°C waterbath for 5 min, the vial headspace was purged with CO-free air. The reaction was continued for 15 min at 37°C. Enzyme activity was terminated by quick-freezing the vial in a dry ice-acetone bath (−78°C). The CO produced in the vial head space was then quantified by gas chromatography using CO in N_2 as a standard as described previously. The entire assay was carried out in the dark.

We assessed the effect of light on HO activity determinations by performing the HO assay according to the procedure outlined above, except that the reactions were carried out in clear glass vials, which were placed in a water bath with a clear plexiglass bottom. One cool white fluorescent tube (T20T12CW, Philips, Inc., Salem, MA), emitting radiation primarily between 450–700 nm, was placed below the water bath so that the vial bottoms were exposed to a radiance of 8 μW/cm²/nm as measured with a Bili Meter (Olympic Medical, Seattle, WA). The vials were exposed to the light only during the 15-min reaction period.

Protein determination. The protein concentration of supernatant was determined by the method of Lowry *et al.* (25), using BSA as the standard

Statistics. All data are expressed as mean ± SD. For intergroup comparisons (Table 1), the null hypothesis that there was no difference between the groups of a given tissue preparation at any concentration was tested by using one-way analyses of variance. Statistical difference between each concentration and the control was tested by using the Bonferroni multiple comparions *t* test. Student's *t* test for independent and paired samples was used for statistical analysis of the results in Table 2. Differences with $p < 0.05$ were considered to be significantly different.

RESULTS

The results of *in vitro* addition of various concentrations of metalloporphyrins on the activity of heme oxygenase in the intestine, liver, and spleen 13 000 × *g* supernatants are summarized in Table 1. The null hypothesis that there was no difference between the groups of a given tissue preparation at any concentration was rejected with a $p < 10^{-6}$ for all tissues. The data show that 1 μM ZnPP significantly ($p < 0.001$) inhibited (to 47%) adult rat intestinal HO activity from 0.19 ± 0.04 to 0.09 ± 0.04 nmol CO/h/mg protein. Furthermore, increased ZnPP concentrations progressively inhibited HO activity to completeness. A similar pattern was observed for ZnMP, but the inhibition was not complete at 10 μM. Both SnPP and SnMP significantly inhibited intestinal HO activity at all of the concentrations tested, but this inhibition was not clearly concentration dependent over the range examined. All of the metalloporphyrins significantly inhibited hepatic and splenic HO activity, even at the lowest concentration evaluated. The degree of inhibition tended to increase with increasing concentration.

Table 2 compares the results of HO activity measurements performed in the dark *versus* the light on intestine, liver, and spleen 13 000 × *g* supernatants. HO determinations in the light (8 μW/cm²/nm) in the absence of added metalloporphyrin (+ buffer only) yielded higher values for intestine (122%), liver (123%), and spleen (153%) than for reactions performed in the dark. When SnPP was added and determinations were performed under dark conditions, significant inhibition of all preparations by 40 μM SnPP was observed. Liver and spleen were nearly completely inhibited and intestine was inhibited by nearly 50%. Light (8 μW/cm²/nm) appeared to stimulate the HO activity of the intestinal preparation exposed to SnPP, completely overcoming the inhibition observed in the dark. A similar effect, although

Table 1. *Adult rat tissue intestinal heme oxygenase activity in 13 000 × g supernatant compared to that of liver and spleen and inhibition by various metalloporphyrins in vitro; only intestinal preparation was studied at 40 μM concentration*

Treatment		Heme oxygenase activity (nmol CO/h/mg protein)		
		Intestine ($n = 6$)	Liver ($n = 6$)	Spleen ($n = 6$)
Control	0 μM	0.19 ± 0.04	0.38 ± 0.10	1.29 ± 0.55
ZnPP	1 μM	0.09 ± 0.04*	0.16 ± 0.04*	0.78 ± 0.23†
	10 μM	0.07 ± 0.04‡	0.07 ± 0.03*	0.31 ± 0.13‡
	40 μM	0.02 ± 0.03‡		
	100 μM	<0.01‡	0.04 ± 0.02*	0.01 ± 0.11‡
ZnMP	0.1 μM	0.12 ± 0.04§	0.20 ± 0.05*	0.78 ± 0.56§
	1 μM	0.09 ± 0.04*	0.10 ± 0.15‡	0.17 ± 0.13‡
	4 μM	0.06 ± 0.03‡		
	10 μM	0.03 ± 0.02‡	0.08 ± 0.05‡	<0.01‡
SnPP	1 μM	0.09 ± 0.05*	0.12 ± 0.08‡	0.43 ± 0.36‡
	10 μM	0.08 ± 0.03‡	0.10 ± 0.04‡	0.08 ± 0.17‡
	40 μM	0.09 ± 0.06*		
	100 μM	0.09 ± 0.02*	0.09 ± 0.04‡	<0.01‡
SnMP	0.1 μM	0.10 ± 0.02§	0.09 ± 0.02‡	0.24 ± 0.33‡
	1 μM	0.10 ± 0.05*	0.06 ± 0.03‡	<0.01‡
	4 μM	0.05 ± 0.06‡		
	10 μM	0.06 ± 0.06‡	0.10 ± 0.10‡	<0.01‡

Statistical significance when compared to the control: Bonferroni t test: * $p < 0.001$; † $p < 0.01$; ‡ $p < 0.0005$; § $p < 0.005$.

Table 2. *Adult rat intestinal, hepatic, and splenic heme oxygenase activity in 13 000 × g supernatant, and inhibition by 40 μM SnPP determined in light (8 μW/cm2/nm) compared to determinations performed in dark*

Tissue	Heme oxygenase activity (nmol CO/h/mg protein)	
	Dark	Light
Intestine ($n = 6$)		
+ buffer	0.23 ± 0.05	0.28 ± 0.15
+ 40 μM SnPP	0.14 ± 0.06*	0.69 ± 0.31*
Liver ($n = 3$)		
+ buffer	0.34 ± 0.12	0.42 ± 0.1
+ 40 μM SnPP	0.04 ± 0.01	<0.01*
Spleen ($n = 3$)		
+ buffer	1.26 ± 0.39	1.93 ± 0.51
+ 40 μM SnPP	<0.01*	1.31 ± 0.16

Statistical significance when compared to the buffer control: Student's paired t test: * $p < 0.05$.

smaller in magnitude was observed for the spleen preparation. Light and SnPP did not appear to affect the inhibition of liver HO activity. The presence of light did not significantly affect the inhibition by 40 μM ZnPP which was observed in intestine, liver, or spleen preparations [intestine ($n = 6$) 0.23 ± 0.50 nmol CO/h/mg protein *versus* 0.11 ±0.06 ($p < 0.05$); liver ($n = 3$) 0.34 ± 0.12 *versus* 0.02 ± 0.01 ($p < 0.05$); spleen ($n = 3$) 1.26 ± 0.39 *versus* < 0.01 nmol CO/h/mg protein ($p < 0.05$)].

DISCUSSION

This is the first report to demonstrate that metalloporphyrins can significantly inhibit adult rat intestinal HO activity *in vitro*. Previous studies on our laboratory (18, 20, 21) could not demonstrate significant *in vitro* inhibition of intestinal HO in 13 000 × *g* supernatants of tissue homogenates from adult rats by concentrations of SnPP as high as 100 μM. This finding was a glaring exception for the intestine compared with other tissues, such as liver and spleen, which were dramatically inhibited under similar reaction conditions. Although no clear pattern of *in vitro* inhibition of intestinal HO could be demonstrated previously at 40 μM ZnPP, the standard deviation was large (36%), and the results of some individual experiments suggested that ZnPP was inhibiting intestinal HO. In fact, a higher dose of ZnPP (100 μM) appeared to inhibit intestinal HO (21). Considering our results with tin protoporphyrin (18, 20, 21), we questioned this finding and further queried whether conditions peculiar to intestinal tissue itself or to our experimental procedure could have influenced the results. Our findings suggest that both possibilities may have played a role and show that all four metalloporphyrins tested can significantly inhibit intestinal HO activity (as measured by CO generation) *in vitro*, at concentrations as low as 1 μM, under strictly light-limited conditions. The control tissues, liver and spleen were also significantly inhibited by the lowest concentrations (1 μM protoporphyrin and 0.1 μM mesoporphyrin) of the metalloporphyrins tested.

We were especially intrigued by the apparent difference between SnPP and ZnPP with respect to their capacity to generate CO from intestinal tissue in the presence of light. We would have expected similar capacities based on photophysical properties alone (26), but ZnPP appears to be much less photoreactive in physiologic environments (27). We knew that SnPP had been reported to be a strong photosensitizer (26–28). However, during the development phase of our HO assay (23), no evidence had been presented in the literature that CO could be a reaction product of SnPP-mediated photooxidation. Because the gas chromatographic method measures light-stable CO as the direct product of the HO reaction, we had initially not been particularly concerned about ambient light levels. The latter is, of course, a serious concern when light-sensitive bilirubin production is measured as a basis of HO activity determination by spectrophotometry techniques (1). The report by McDonagh and Palma (29) on SnPP-enhanced photooxidation of bilirubin *in vitro* is of particular relevance only to the spectrophotometric determination of HO activity. However, we recently discovered that SnPP, more readily than ZnPP, supports generation of CO from organic molecules exposed to cool white light *in vitro* (24). The apparent lack of inhibition of intestinal HO by SnPP can now be explained on the basis of the effect that light has on CO-based HO determinations in the presence of SnPP. This phenomenon is less prominent with ZnPP, which accounts most likely for ability of ZnPP to inhibit intestinal HO at light fluxes capable of overcoming inhibition by SnPP. It is not possible to estimate, in retrospect, the light quality and intensity encountered during the previous studies (18, 20, 21), but our results indicate that the previously observed lack of inhibition of intestinal HO with SnPP could be explained by the fact that HO activity determinations were performed under ambient light (a mixture of indirect summer daylight and cool white fluorescent light ranging in radiant flux from 0.5 to approximately 5 $\mu W/cm^2/nm$) in clear glass vials. Current activity measurements are performed in amber vials in the dark. The present results were obtained with metalloporphyrin solutions containing human serum albumin. The presence of albumin was determined to have no effect on the efficacy of metalloporphyrins as HO inhibitors, but it facilitates the preparation of homogenous solutions of metalloporphyrins at physiologic pH. Furthermore, our introduction of ethanolamine as a solvent has greatly facilitated the solubilization of metalloporphyrins, and addition of human serum albumin with the procedure of slow and gradual pH titration now yields clear solutions of pH 7.4–7.6.

The results of SnPP's interaction with intestinal HO *in vitro* are puzzling. Unlike liver and spleen preparations, the intestinal enzyme does not seem to be sensitive to increasing concentrations of SnPP and SnMP. Intestinal HO activity was only incom-

pletely inhibited by 100 μM SnPP or 10 μM SnMP to 42%, and 26% of control activity, respectively. Thus, our findings suggest that the intestinal enzyme seems to be of a different nature (or at least behaves differently under our current standard reaction conditions) than its hepatic and splenic analogues where generally more complete and concentration dependent inhibition of HO activity by SnPP and SnMP was observed. Additionally, these findings show the zinc compounds, ZnPP and ZnMP, to be more potent inhibitors of *in vitro* intestinal HO activity than the corresponding tin compounds. The reverse is usually true for other tissue preparations.

The validity of the CO-based determination HO activity assay could be questioned if the presently used blank would not adequately correct for non-HO generated CO. *In vivo* experiments have estimated that only 10–20% of the evolved CO is derived from nonheme degrading processes (30). Our *in vitro* blank values originate in part (<5%) from CO generated by the reaction vessel septum. The major portion, however, is derived from as yet unidentified dark reactions in the tissue preparation, at rates which are tissue-dependent (liver < spleen < intestine) (23). This non-HO-mediated CO production, determined by the blank reaction, is subtracted from the total CO production in the presence of NADPH to obtain the HO-mediated CO production rate. The blank rates (measured in the dark) for any of the tissues are not significantly affected by the presence of metalloporphyrins at any concentration. Only CO production due to the presence of NADPH (by definition HO activity) is decreased by the presence of metalloporphyrins. The HO-mediated CO generation in the dark becomes nondetectable for spleen and is reduced to approximately 20% of native activity for the liver at the highest metalloporphyrin concentrations. This is strong, though indirect evidence, that the CO-based HO activity assay measures true HO activity.

Light affects CO production by tissue preparations, even in the absence of added photosensitizers as can be seen in Table 2. The magnitude of this phenomemon is tissue dependent and is most likely due to the presence of endogenous photosensitizers such as riboflavin (31) which, like SnPP, also produce CO from organic molecules such as NADPH and proteins (Vreman HJ, Stevenson DK, unpublished data). This potential source of error is also prevented by performing the HO activity assay in the dark.

In conclusion, our results indicate that the central metal within the porphyrin ring and substituents on the ring appear to play a crucial role in the inhibitory potency of metalloporphyrins on HO activity, which may vary from tissue to tissue. Moreover, HO behaves differently from tissue to tissue under varying experimental conditions of inhibition and light exposure. Finally, light significantly affects the biochemical behavior of metalloporphyrins.

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