SELECTIVE INHIBITION OF HEME OXYGENASE, WITHOUT INHIBITION OF NITRIC OXIDE SYNTHASE OR SOLUBLE GUANYLYL CYCLASE, BY METALLOPORPHYRINS AT LOW CONCENTRATIONS

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ABSTRACT:
Studies on the physiological role of heme oxygenase (HO) require an inhibitor that will selectively inhibit HO activity without inhibiting the activity of either nitric oxide synthase (NOS) or soluble guanylyl cyclase (sGC). The objective of this study was to test a series of metalloporphyrins that have previously been shown to inhibit HO activity, for their ability to inhibit HO without inhibiting NOS or sGC activities. Measurement of activity of HO in rat brain microsomes and NOS in rat brain cytosol was made for samples incubated with metalloporphyrins (0.15–50 μM), including zinc protoporphyrin IX, zinc deuteroporphyrin IX, zinc protoporphyirn IX, chromium mesoporphyrin IX (CrMP), tin protoporphyrin IX, and zinc N-methylprotoporphyrin IX. CrMP and ZnBG were found to be the most selective inhibitors of HO activity (i.e., caused the greatest inhibition of HO activity, 89 and 80%, respectively, without inhibition of NOS activity). Based on these results, sGC activity in rat lung cytosol incubated with CrMP or ZnBG (0.15–15 μM) was measured. ZnBG did not affect basal sGC activity but did potentiate S-nitroso-N-acetylpenicillamine (SNAP)-induced sGC activity. CrMP did not affect either basal or SNAP-induced activity. It was concluded that of the five metalloporphyrins studied, CrMP, at a concentration of 5 μM, was a selective inhibitor of HO activity and was the most useful metalloporphyrin for the conditions tested. Thus, CrMP would appear to be a valuable chemical probe in elucidating the physiological role of HO.

It has been proposed that carbon monoxide (CO),1 which is formed endogenously during heme oxygenase (HO) catabolism of heme, plays a role in the regulation of cell function and communication (Marks et al., 1991; Verma et al., 1993). The biological role proposed for CO is similar to that of another endogenously formed gaseous molecule, namely, nitric oxide (NO), which is produced by NO synthase (NOS) from L-arginine (Moncada et al., 1991). Both gaseous molecules can mediate a physiologic effect, such as blood vessel relaxation, by activating soluble guanylyl cyclase (sGC). Inhibitors of enzymatic activity are useful chemical probes in establishing a physiological role for specific enzymes, such as the role of Nω-nitro-L-arginine methyl ester (L-NAME) has played in elucidating the biological roles of NOS (Moncada et al., 1991). Metalloporphyrins have been shown to inhibit HO, and their potency is affected by the metal cation associated with the porphyrin ring as well by different ring substituents (Vreman et al., 1993). Inhibition of HO activity has been demonstrated for each of the metalloporphyrins used in this study, specifically zinc protoporphyrin IX (ZnPP), tin protoporphyrin IX (SnPP), chromium mesoporphyrin IX (CrMP) (Vreman et al., 1993; Cook et al., 1995; Marks et al., 1997), zinc deuteroporphyrin IX (SnPP) (Chernick et al., 1989; Vallier et al., 1991; Vreman et al., 1992), and zinc N-methylprotoporphyrin IX (ZnMePP) (De Matteis et al., 1985). ZnPP has been exploited therapeutically to reduce hyperbilirubinemia in the neonate (Qato and Vincent, 1991; Meffert et al., 1994). More recently, metalloporphyrins have been used to test the hypothesis that CO has a physiological role. SnPP and ZnPP have been used to investigate a possible role for CO as a vasodilator (Zakhary et al., 1996). ZnPP also has been used to demonstrate an apparent role for CO in long-term potentiation (Zhao et al., 1993) and the inhibition of depolarization-induced glutamate release (Shinomura et al., 1994).

Several investigators have shown that metalloporphyrins are not only specific inhibitors of HO but also inhibit NOS and sGC (Luo and Vincent, 1994; Meffert et al., 1994; Grundemar and Ny, 1997). Based on these findings, the conclusions reached by investigators using metalloporphyrins to establish a physiological role for CO in biological systems, in which NOS and sGC are also active, have been criticized. In contrast, Zakhary et al. (1996) reported that SnPP was 10...
times more potent in inhibiting HO-2 than NOS or sGC and based on this finding have used SnPP to study CO-induced vasodilation.

In this study, the objective was to test five metalloporphyrins that have been shown previously to inhibit HO activity, for their ability to selectively inhibit HO relative to NOS and sGC activities. For each metalloporphyrin, a concentration was determined that inhibited HO activity, without inhibiting NOS activity. The two most selective inhibitors, CrMP and ZnBG, were further studied to determine whether they affected basal or S-nitroso-N-acetylpenicillamine (SNAP)-induced sGC activity. It was found that CrMP, at a concentration of 5 μM, was a selective inhibitor of HO activity and appeared to be the most useful HO inhibitor based on the studies conducted.

Materials and Methods

Drugs and Solutions. EDTA disodium salt, hemin, ethanolamine, BSA, HEPES, L-arginine, leupeptin, Amberlite IRP-69, L-NNAME, heparin, cGMP, NADPH, GTP, and SNAP were obtained from Sigma Chemical Co. (St Louis, MO). Tris-HCl, benzamidine HCl, and 3-isobutyl-1-methylxanthine were purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). CrMP, ZnBG, ZnPP, SnPP, and ZnMePP were purchased from Porphyrin Products, Inc. (Logan, UT). Neutral alumina was obtained from EM Science (Gibbstown, NJ). All other chemicals were at least reagent grade and were obtained from BDH Inc. (Toronto, Ontario, Canada). Stock solutions of methemalbumin (1.5 mM hemin and 0.15 mM BSA) and of each of the five metalloporphyrins (1.0 mM) were prepared as described previously (Vreman et al., 1993). Briefly, hemin or metalloporphyrin was dissolved in 0.5 ml of 10% (v/v) ethanolamine. BSA dissolved in 2 ml of deionized water was added to the hemin solution only. The volume was made up to 7 ml and slowly adjusted to pH 7.4 with 1 M HCl and vigorous stirring. The final volume for each stock solution was adjusted to 10 ml with deionized water. The metalloporphyrin vehicle was prepared as described above without the addition of any metalloporphyrin. The methemalbumin and metalloporphyrin stock solutions were prepared with the laboratory lights turned off and were stored at −20°C for up to 1 month.

Preparation of Subcellular Fractions of Rat Brain and Lung. Adult male Sprague-Dawley rats (300–350 g) were obtained from Charles River Canada, Inc. (Montreal, Quebec, Canada). Rats were given ad libitum access to Ralston Purina Laboratory Chow (5001; Ren’s Feed and Supplies, Ltd., Oakville, Ontario, Canada) and water. All animals were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care and the experimental protocol was approved by the Queen’s University Animal Care Committee. For measuring HO and NOS activity, each rat was sacrificed by decapitation and its brain was excised and weighed. For measuring sGC activity, the rat was injected i.p. with heparin (3 mg/kg b.wt.) and then 45 min later anesthetized with 93 mg/kg b.wt. sodium pentobarbital (MTC Pharma- decapitation and its brain was excised and weighed. For measuring sGC activity, the rat was injected i.p. with heparin (3 mg/kg b.wt.) and then 45 min later anesthetized with 93 mg/kg b.wt. sodium pentobarbital (MTC Pharma-
determine which experimental groups were statistically different ($p < 0.05$), a post hoc Newman-Keuls test was conducted to determine which experimental groups were statistically different ($p < 0.05$).

**Data Analysis.** The activity of HO, NOS, and sGC following incubation with each concentration of metalloporphyrin was expressed as a percentage of total activity, measured in the absence of metalloporphyrin. The data are presented as group means ± S.D. of four tissue preparations from different animals, unless otherwise stated. Parametric statistical analysis of the data was conducted by repeated-measures, one-way ANOVA. For a statistically significant F statistic ($p < 0.05$), a post hoc Newman-Keuls test was conducted to determine which experimental groups were statistically different ($p < 0.05$).

**Results and Discussion.**

The metalloporphyrins used in this study have been shown previously to inhibit HO activity (De Matteis et al., 1985; Chernick et al., 1989; Vreman et al., 1992, 1993). The use of metalloporphyrins in investigating a physiological role for HO has been criticized because some metalloporphyrins have been shown also to inhibit NOS and sGC activity (Lou and Vincent, 1994; Meffert et al., 1994; Grundemar and Ny, 1997). In contrast, Zakhary et al. (1996), in a study demonstrating HO-derived CO as a vasodilator, reported a dose of SnPP that appeared to selectively inhibit HO relative to NOS. Thus, the potential exists for finding a metalloporphyrin and/or a metalloporphyrin concentration that would inhibit HO activity without inhibiting NOS or sGC activity. In the present study, five metalloporphyrins were investigated to determine whether there is a metalloporphyrin and/or a metalloporphyrin concentration that can be used as a selective inhibitor of HO relative to NOS and sGC.

HO activity in rat brain microsomes and NOS activity in rat brain cytosol were 3.2 ± 0.3 nmol of CO formed/mg protein/h ($N = 6$) and 5.0 ± 1.1 nmol of L-citrulline formed/mg protein/h ($N = 4$), respectively. All five metalloporphyrins (CrMP, ZnBG, SnPP, ZnPP, and ZnMePP) inhibited both HO and NOS activity (Fig. 1). However, there was a concentration for each metalloporphyrin at and below which only HO activity was inhibited. The metalloporphyrin vehicle did not affect HO or NOS activity (data not shown).

In comparing the metalloporphyrins as selective inhibitors of HO, the first step was to determine a maximum selective inhibitory (MSI) concentration for each metalloporphyrin, at or below which there is inhibition of HO, with no effect on NOS or sGC activity (Table 1). The next step was to compare the percentage of inhibition of HO activity at the MSI concentration for each metalloporphyrin. Also, the percentage of inhibition of HO activity at concentrations 3- and 10-fold lower than the MSI concentration was compared (Table 1). This latter comparison was made because in an experiment, a concentration lower than the MSI concentration, at which the metalloporphyrin becomes nonselective, would normally be used. Based on these criteria, the inhibition of HO activity was lowest and/or declined most rapidly with metalloporphyrin concentration for ZnMePP, ZnPP, and SnPP (Table 1). Thus, it was concluded that CrMP and ZnBG at concentrations at and below 5 μM were selective inhibitors of HO activity relative to NOS activity in rat brain.

It is interesting to compare the above data (Fig. 1) with that of Meffert et al. (1994). These workers concluded that CrMP and ZnPP, but not tin mesoporphyrin or ZnBG, inhibited NOS in the rat hippocampus. Based on these findings, it was emphasized that some metalloporphyrins are nonselective and would therefore not be useful in biological studies involving CO. The concentration of metalloporphyrin used in these studies ranged from 10 to 100 μM, whereas in the present study metalloporphyrin concentrations from 0.15 to 50 μM were used. The results of Meffert et al. (1994), showing that CrMP and ZnPP inhibited NOS at concentrations of 10 μM and higher are in agreement with the results of the present study. In contrast, it was found in this study that ZnBG inhibits NOS at concentrations >5 μM. Tin mesoporphyrin was not tested in the present study. The message that emerges in comparing the data is that to achieve selectivity of metalloporphyrin-induced inhibition of HO versus NOS, the concentration used is critically important and must be kept <5 μM.

To characterize further the selectivity of CrMP and ZnBG as inhibitors of HO, the effect of the two metalloporphyrins on basal and SNAP-induced sGC activity was determined for metalloporphyrin concentrations similar to those used for the study of HO and NOS activity. The basal and SNAP-induced sGC activities in the rat lung cytosol were 121 ± 56 (N = 6) and 2059 ± 821 (N = 3) pmol of cGMP formed/mg protein/h, respectively. Thus, the addition of 100 μM SNAP produced an ~17-fold increase in sGC activity from basal level. Neither CrMP nor ZnBG had any effect on basal sGC activity (Fig. 2) at the concentrations tested. However, ZnBG elevated SNAP-induced sGC activity, which is consistent with reports from investigators who found that certain metalloporphyrins, such as cobalt protoporphyrin, enhance NO-induced sGC activity (Dierks et al., 1997). CrMP had no effect on SNAP-induced sGC activity for the concentration range tested, a range that included the MSI concentration of 5 μM, at and below which CrMP selectively inhibit HO activity, with no effect on NOS activity. Thus, CrMP was found to be the most...
selective and useful inhibitor of HO activity compared with NOS and sGC activities in rat brain and lung.

To our knowledge, no particular structural feature of metalloporphyrins has been identified that allows the prediction of the efficacy of the compounds to inhibit HO or NOS. For sGC, a mechanism to explain, at least in part, the interactions of metalloporphyrins with this enzyme has been proposed by Serfass and Burstyn (1998). These investigators postulate that a key requirement for sGC activation by metalloporphyrins is the absence of a bond between a proximal protein-histidine and the metal in the porphyrin. This proposed mechanism is based on the observation that activation of sGC by NO has been attributed to binding of NO to heme iron with concomitant breaking of a bond between a proximal protein-histidine and iron. In their study, the minimal activation of sGC by ZnPP is attributed to the likelihood that the bond between a proximal histidine and Zn atom is intact. However, the marked activation of sGC by SnPP is attributed to the absence of a bond between a proximal histidine and the Sn atom. Activation of sGC by SnPP, as demonstrated by Serfass and Burstyn (1998), provides further rationale for selecting CrMP, from among the metalloporphyrins tested, as the most selective metalloporphyrin to elucidate the physiological role of HO.

Fig. 1. Inhibition of HO and NOS activity by CrMP, SnPP, ZnBG, ZnMePP, and ZnPP.

Concentration-response curves for HO (\(\nabla\)) and NOS (\(\bullet\)) activity in rat brain microsomes and cytosol, respectively, were obtained in the presence of CrMP, SnPP, ZnBG, ZnMePP, and ZnPP. The data are presented as group means \(\pm\) S.D. \((N = 4)\). a, the MSI concentration at or below which there is selective inhibition of HO activity, with no effect on NOS activity; NOS activity for lower concentrations of metalloporphyrins were not different from the MSI concentration. Group means with different letters are statistically different from each other, \(p < .05\).
Luo and Vincent (1994) and Grundemar and Ny (1997) concluded that ZnPP, SnPP, and ZnBG cannot be used to establish a messenger role for CO. This comment is based on the fact that these metalloporphyrins inhibit sGC in addition to HO. However, in their study, the concentration of these metalloporphyrins ranged from 10 to 100 μM for sGC inhibition. This conclusion requires reconsideration in light of our data that demonstrates that concentrations of metalloporphyrins ranging from 10 to 100 μM can inhibit HO activity without inhibiting NOS and sGC activities. Thus, careful exploration of concentration-response relationships with a variety of metalloporphyrins potentially can lead to the identification of an appropriate selective inhibitor for the biological model being used. This conclusion is reinforced by the results of Zakhary et al. (1997), who used HO-2 knockout mice and SnPP to demonstrate that CO plays a role in nonadrenergic, noncholinergic (NANC) relaxation evoked by electrical field stimulation of mouse ileal segments. In wild-type mice, SnPP partially inhibited NANC relaxation. However, in mice where the gene for HO-2 had been deleted, SnPP did not affect NANC transmission.

There is considerable interest in the use of metalloporphyrins to inhibit HO in the treatment of juvenile jaundice (Qato and Maines, 1985; Valaes et al., 1998). CrMP and ZnBG appear to be promising candidates because of their high potency to inhibit HO as demonstrated in the present study and other studies (Vreman et al., 1998), good oral absorption (Vallier et al., 1991, 1993), resistance to metabolism by HO and inability to up-regulate HO-1 in cell culture (W.S. Zhang, P.R. Contag, D.K.S., and C.H. Contag, personal communication). Moreover, CrMP has the additional advantages of not distributing across the blood-brain barrier and being photochemically inactive. Although ZnBG is a photosensitizer, the potential low doses required for therapeutic use, due to its high potency as a HO inhibitor, may restrict its photoreactivity. For the above reasons, therapeutic and toxicological studies of CrMP and ZnBG are warranted.

In summary, of the five metalloporphyrins tested, CrMP and ZnBG inhibited HO to the greatest extent at and below the concentration for which there was no measurable inhibition of NOS activity. Furthermore, CrMP was found to have no effect on basal or SNAP-induced sGC activity, unlike ZnBG, which enhanced SNAP-induced sGC activity. Thus, in this study, CrMP, at or below 5 μM, was found to be the most selective inhibitor of HO relative to NOS and sGC, in rat brain and lung. In other studies with different biological models, it will be necessary to determine the concentration of CrMP or other metalloporphyrins that will selectively inhibit HO activity without inhibiting NOS and sGC activities.

**References**

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