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Effects of induction of heme oxygenase by cobalt and tin on the *in vivo* degradation of myoglobin

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The oxidation of heme (Fe-protoporphyrin IX) to the open tetrapyrrole, biliverdin, is catalyzed by the microsomal heme oxygenase system [1, 2]. Biliverdin is reduced subsequently to bilirubin in the presence of the cytosolic enzyme biliverdin reductase [3]. The activity of heme oxygenase in various organs is inducible by a host of agents, including heavy and transitional metal ions. Certain metal ions, namely cobalt (Co^{2+}) and tin (Sn^{2+}), are among the most potent inducers of the enzyme activity [4].

The mechanism for the oxidation of the heme molecule by heme oxygenase, when in the free form, is well-established. However, there is a paucity of information concerning the metabolic fate of hemoproteins, in general, and that of myoglobin, in particular. It follows that, with the exception of an early isolated report [5], no attempt has been made to investigate the degradation *in vivo* of myoglobin under control or conditions of induced heme oxygenase activity. Moreover, although it has been shown that *in vitro* the heme moiety of myoglobin can be non-enzymatically degraded in the presence of ascorbic acid and O_2 to the intermediate product verdoheme, to our knowledge no attempt has been made to date to study the degradation of the heme moiety of myoglobin, when bound to apoprotein, by microsomal heme oxygenase. The present study investigates the possible involvement of heme oxygenase in the degradation *in vivo* and *in vitro* of the heme moiety of myoglobin.

Materials and methods

Materials. δ-Amino-[4-^{14}C]levulinic acid (50 mCi/mmole) was a product of Amersham, Arlington Heights, IL. All reagents were purchased from the Sigma Chemical Co., St. Louis, MO. Myoglobin was isolated from the skeletal muscles of rats treated with δ-amino-[4-^{14}C]levulinic acid (100 μCi/100 g body wt, i.p. 24 hr) employing the procedure of Yamazaki *et al.* [6], as modified by Wittenberg and Wittenberg [7]. The preparation was homogeneous when analyzed by polyacrylamide gel electrophoresis [8]. Various preparations of [^{14}C-heme]myoglobin exhibited radioactivity of 2000–3000 dpm/nmole heme. Biliverdin reductase was purified from rat liver as described before [3], and the specific activity in the various preparations ranged between 2500 and 3000 nmoles bilirubin/mg protein/min.

Animals and treatment. Fed male Sprague–Dawley rats (180–200 g) were used. The animals were treated (s.c.) with $CoCl_2 \cdot 6H_2O$ (250 μmoles/kg body wt) or $SnCl_2 \cdot 2H_2O$ (125 μmoles/kg body wt). The control animals received physiological saline. [^{14}C-heme]Myoglobin (60 nmoles/kg body wt) was administered intravenously 24 hr following metal ion treatment to bile duct-cannulated rats [9]. Animals were maintained hydrated throughout the study. Bile samples were collected at the time intervals indicated in Fig. 1. For *in vitro* experiments, the animals were killed after 24 hr of treatment, and the organs were perfused *in situ* with saline. The procedures used for the preparation of the subcellular fractions and the assessment of heme oxygenase activity were similar to those described previously [10].

Estimation of degradation of heme moiety of myoglobin. A reaction mixture (1.5 ml) containing [^{14}C-heme]myoglobin (25 μM, 3000 dpm/nmole), NADPH-generating system (0.8 mM NADP, 0.85 mM glucose-6-phosphate, 3 units of glucose-6-phosphate dehydrogenase and 2 mM $MgCl_2$), potassium phosphate buffer (0.09 M, pH 7.4), human serum albumin (2 mg/ml), purified biliverdin

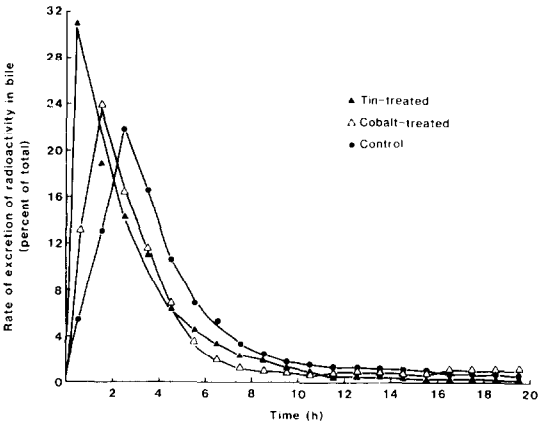

Fig. 1. Biliary excretion of radioactivity in control, cobalt chloride- and tin chloride-treated rats following the administration of [^{14}C-heme]myoglobin. The animals were treated (s.c.) with $CoCl_2 \cdot 6H_2O$ (250 μmoles/kg) or $SnCl_2 \cdot 2H_2O$ (125 μmole/kg). The control animals received physiological saline. [^{14}C-heme]Myoglobin (2400 dpm/nmole, 60 nmoles/kg) was administered intravenously 24 hr following metal ion treatment to bile duct-cannulated rats. Bile samples were collected at the indicated time intervals, and ^{14}C-activity was determined in aliquots as described in Materials and Methods. The data points shown are representative of three determinations.

reductase (5 μg protein/ml), and microsomal fraction (3–5 mg/ml) from the liver of Co^{2+}-treated rats or the kidney of Sn^{2+}-treated rats was incubated for 30 min at 37°. The reaction mixture was acidified with HCl, unlabeled bilirubin was added as the carrier, and the mixture was extracted with 3.0 ml of 2-butanone. The organic layer containing bilirubin, which separated following a brief centrifugation, was washed with water and dried under N_2. The residue was dissolved in chloroform and subjected to thin-layer chromatography on silica gel G plates using 2-butanone–*n*-hepatane–acetic acid (5:10:1, by vol.) as the solvent system. The radioactivity associated with the area corresponding to bilirubin was determined in samples using a Beckman LS 7500 counter. Automatic quench corrections were applied, and the counting efficiency approached 90%. Protein determinations were carried out using the method of Lowry *et al.* [11].

Results and discussion

The pattern of biliary excretion of end-products of [^{14}C-heme]myoglobin degradation and the effect of metal ion treatment on this process are shown in Fig. 1. As shown in the control animals, the presence of radioactivity was detected in the bile within 30 min following the administration of [^{14}C-heme]myoglobin. The analysis of the bile samples for bilirubin and ^{14}C-activity by the method of Ostrow *et al.* [12] revealed that the entirety of the ^{14}C-activity excreted in the bile was associated with bilirubin. In the control rats, the rate of the excretion of [^{14}C]bilirubin reached its maximum at 3 hr after the administration of myoglobin; the rate gradually decreased with time and was reduced markedly 8 hr after the administration of myoglobin. Figure 1 also shows the effect of the treatment of rats (24 hr) with Co^{2+} or Sn^{2+} on the degradation of

myoglobin *in vivo*. These metal ions are the most potent inducers of heme oxygenase activity in the liver and the kidney respectively [4]. As noted, Sn^{2+} treatment pronouncedly accelerated the rate of the excretion of [^{14}C]-bilirubin in the bile. The excretion rate attained its peak height within a mere 30 min after the injection of myoglobin, and nearly 80% of the ^{14}C-activity was excreted within the initial 4.5 hr. In Co^{2+}-treated rats, the rate of the excretion of [^{14}C]bilirubin was also enhanced, although not to the same extent as with Sn^{2+}. The maximum rate was observed 2 hr after [^{14}C-heme]myoglobin administration.

Although exposure to metal ions accelerated the rate of the excretion of [^{14}C]bilirubin in the bile, it did not noticeably affect the total amount of [^{14}C]bilirubin excreted in the course of the experiment. Regardless of the regimen of the treatment, the total amount of radioactivity excreted in the bile amounted to 67–74% of the total injected ^{14}C-activity. The fate of the remaining 26–33% of the injected myoglobin is not clear. The animals were killed at the termination of the experiments, and the activity of heme oxygenase in the kidneys and the liver was measured. As expected, the enzyme activities in both organs were increased markedly (Table 1). In the liver of Co^{2+}-treated rats, the microsomal heme oxygenase activity measured twelve times that of the control rats. However, in the Sn^{2+}-treated rats the liver enzyme activity was increased only by 4-fold. In Co^{2+}- and Sn^{2+}-treated rats, the kidney heme oxygenase activity measured five and twenty times that of the control respectively.

The presently observed accelerated rate of the biliary excretion of [^{14}C]bilirubin in the Sn^{2+}- and Co^{2+}-treated rats along with increased tissue heme oxygenase activity rather persuasively suggest that the enhanced rate of the degradation of myoglobin was a consequence of the elevated levels of heme degradative activity in the organs of the metal ion-treated rats. Since to date a heme oxidizing enzyme system other than heme oxygenase has not been identified, the present findings strongly suggest that heme oxygenase catalyzes the oxidative cleavage of the heme moiety of myoglobin. Moreover, considering the potent inducing action of Sn^{2+} on renal heme oxygenase activity, the finding that Sn^{2+} treatment was more effective in facilitating the rate of the conversion of myoglobin to bilirubin than Co^{2+} treatment may be interpreted as suggesting that the primary organ site of the degradation of the hemoprotein is the kidney.

In a series of *in vitro* experiments, the mechanism for the degradation of [^{14}C-heme]myoglobin by heme-oxygenase was explored by measuring the conversion of its heme moiety to bilirubin using the assay conditions described in the legend of Table 2. In the first series of experiments, the microsomal preparations obtained from the liver, kidney, spleen, heart and the muscles of control, Sn^{2+}- and Co^{2+}-treated rats were used as the heme oxygenase source, and [^{14}C-heme]myoglobin was utilized as the substrate. In the second series of experiments, the microsomal preparations from the kidneys of Sn^{2+}-treated rats and the liver of Co^{2+}-treated rats were used as the enzyme source; various derivatives of the [^{14}C-heme]myoglobin preparation (Table 2) were used as the substrate. The list of preparations included: oxymyoglobin, metmyoglobin, myoglobin denatured by treatment with 8 M urea, trypsin-treated myoglobin, heat-denatured myoglobin, or the isolated heme portion of myoglobin. The conversion of myoglobin to bilirubin was not detectable in the first series of experiments. On the other hand, as shown in Table 2, in the second series of experiments the formation of measurable amounts of [^{14}C]bilirubin in the presence of the kidney or the liver microsomal fractions of metal-exposed animals was detected when trypsin-digested, urea-treated, or heat-denatured myoglobin preparations were used as the substrate. As expected, a substantial degree of [^{14}C]-bilirubin formation was detected when the isolated heme moiety of myoglobin was used as the substrate. These results suggest that alterations in the structural configuration of myoglobin are prerequisite for its conversion to an active form of substrate, and that as reported before [13] the enzyme is incapable of catalyzing the oxidative cleavage of the porphyrin ring of native myoglobin.

Table 1. Induction of microsomal heme oxygenase activity of liver and kidney in rats following metal ion treatment

Treatment	Heme oxygenase Liver	Kidney (nmoles bilirubin formed/mg protein/hr)
Control	1.50 ± 0.18	0.93 ± 0.13
Co^{2+}	17.81 ± 1.86*	4.27 ± 0.59*
Sn^{2+}	6.63 ± 0.84*	18.02 ± 1.54*

The animals were treated (s.c) with $CoCl_2 \cdot 6H_2O$ (250 μmoles/kg) or $SnCl_2 \cdot 2H_2O$ (125 μmoles/kg). The control animals received physiological saline. The animals were killed at the end of 44 hr. The preparation of microsomal fraction from liver and kidney, and the determination of heme oxygenase activity were carried out as described before [10].

* $P < 0.05$.

Table 2. Oxidation of [^{14}C-heme]myoglobin derivatives by the liver microsomal fraction from cobalt chloride-treated rats and by the kidney microsomal fraction from tin chloride-treated rats

Substrate	Radioactivity in bilirubin (dpm) Enzyme source: Liver	Kidney
Oxymyoglobin	0	0
Metmyoglobin*	0	0
Myoglobin treated with 8 M urea†	160	120
Myoglobin treated with trypsin‡	790	870
Heat-treated myoglobin§	860	1100
Heme extracted from myoglobin	5160	6250

The conversion of the heme moiety of the indicated myoglobin derivatives to bilirubin was assessed in an assay system containing the liver microsomal fractions from Co^{2+}-treated rats or microsomal fractions from the kidney of Sn^{2+}-treated rats, an NADPH-generating system, and purified biliverdin reductase. The [^{14}C]bilirubin formed was estimated as described in Materials and Methods. The data represent the average of two determinations. Various myoglobin derivatives were prepared from [^{14}C-heme]-oxymyoglobin.

* Metmyoglobin was obtained by oxidizing oxymyoglobin with potassium ferricyanide followed by dialysis against water.

† A myoglobin preparation (1 mM) was treated with 8 M urea at 37° for 1 hr.

‡ Myoglobin (1 mM) was digested with trypsin (5 mg/ml) at 37° for 2 hr, and the digestion was stopped by the addition of a 10-fold excess of the trypsin inhibitor.

§ The myoglobin preparation was heated at 60° for 10 min.

‖ The myoglobin solution was acidified and extracted with 2-butanone, the organic layer was concentrated by evaporation under N_2, the residue was dissolved in a minimum amount of 0.1 N NaOH diluted with water, and the pH was adjusted to 7.4.

The possibility was explored that subsequent to the administration of myoglobin in circulation the hemoprotein undergoes biological transformations to render its heme moiety degradable by heme oxygenase. One mode of such a transformation could entail the introduction of a structural modification in the hemoprotein by serum factors, such as proteolytic enzymes, and the second could involve the transfer of the heme prosthetic moiety of myoglobin to serum albumin or other heme-binding proteins which would be degraded by tissue heme oxygenase subsequent to its cellular uptake. The heme–albumin complex is known to be an excellent substrate for heme oxygenase [1]. [^{14}C-heme]Myoglobin solutions (1 mM in physiological saline, pH 7.4) were preincubated with equal volumes of rat serum for various time intervals (0–2 hr) at 37°. Thereafter, the serum-treated myoglobin preparations were used as substrate for microsomal heme oxygenase employing the assay system and experimental procedures described in the legend for Table 2. No appreciable difference in the formation of [^{14}C]bilirubin was detected when the serum-treated and the untreated myoglobin preparations were used as substrates. Accordingly, it appears that serum factors do not convert myoglobin to a suitable substrate for heme oxygenase.

The findings reported in this communication suggest that, although myoglobin *in vivo* is a substrate for heme oxygenase, certain physicochemical changes *in vivo* in the structure of the hemoprotein are required prior to its conversion to a suitable substrate for the enzyme. Furthermore, these findings may be interpreted to suggest that such biotransformations of the hemoprotein take place at the cellular levels rather than at the plasma level. The latter suggestions are consistent with the suggested involvement of lysosomal enzymes in the initial process of the degradation of the globin moiety of hemoglobin [14]. Moreover, the present findings further suggest that the distinct possibility exists that the exposure of animals to metal ions not only promotes the induction of heme oxygenase activity but, in addition, stimulates the action of the biological processes which promote changes in the structural conformation of myoglobin to render the hemoprotein a suitable substrate for heme oxygenase.

On the basis of the present findings it would appear likely that, in the normal course of the degradation of musculature myoglobin, the hemoprotein is initially released into the circulation before it is transported to the ultimate cellular site(s) for degradation and bilirubin formation. This suggestion is consistent with the negligible levels of heme oxygenase activity detected in the musculature, and the appearance of myoglobin in the circulatory system and in the urine of patients suffering from certain skeletal and muscular disorders.

In summary the degradation of the heme moiety of myoglobin *in vivo* is a process which appears to involve the catalytic activity of heme oxygenase. This suggestion is based on the finding that [^{14}C]bilirubin was excreted in the bile of rats injected with [^{14}C-heme]myoglobin. Moreover, the treatment of rats with Sn^{2+} or Co^{2+}, potent inducers of heme oxygenase activity, enhanced the rate of the excretion of [^{14}C]bilirubin in the bile. Microsomal preparations obtained from the liver, kidney, spleen, heart and the muscles of control, Sn^{2+}- or Co^{2+}-treated rats failed to catalyze the formation of any detectable amount of [^{14}C] bilirubin from [^{14}C-heme]myoglobin. However, the formation of [^{14}C]bilirubin was detected when heme isolated from [^{14}C-heme]myoglobin or trypsin-, heat- or urea-treated [^{14}C-heme]myoglobin was used as substrate for the enzyme.

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The calcium antagonist nisoldipine stimulates the electrolyte transport of the isolated frog skin

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Calcium and sodium transport across epithelial cell membranes are thought to be linked by at least two mechanisms. First, a countertransport of sodium and calcium, energized by the sodium gradient, extrudes intracellular calcium. Second, sodium channels mediating passive influx of sodium are postulated to be modified by intracellular calcium [1]. The operation of such mechanisms, alongside Na/K-ATPase, should be able to regulate intracellular sodium