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# HEPATIC HEME METABOLISM: POSSIBLE ROLE OF BILIVERDIN IN THE REGULATION OF HEME OXYGENASE ACTIVITY

R. Krishnan Kutty and Mahin D. Maines*

Department of Pharmacology, University of Illinois
College of Medicine, Chicago, Illinois 60680

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Treatment of rats with biliverdin (48 h) resulted in an increase in microsomal heme oxygenase activity in the liver. This was accompanied by decreases in the microsomal heme and cytochrome P-450 contents. In these respects cellular responses elicited by biliverdin resembled those produced by hematin (48 h). When rats were treated with biliverdin for a short interval (3 h) an inhibition of the activities of heme oxygenase and biliverdin reductase, concomitant with an increase in microsomal heme and cytochrome P-450 contents, were observed. Hematin was ineffective in altering these parameters under similar conditions. Biliverdin, in a concentration-dependent manner, inhibited the activities of purified heme oxygenase and biliverdin reductase. The activity of purified rat liver heme oxygenase was refractory to bilirubin, whereas that of purified biliverdin reductase was severely inhibited. It is suggested that biliverdin regulates cellular heme degradation processes by occupying the heme binding site on heme oxygenase, thus hindering the access of the substrate to the catalytic site of the enzyme.

The microsomal heme oxygenase catalyzes the oxidation of heme (Fe-protoporphyrin IX, protoheme) to open tetrapyrrole, biliverdin (1,2). The activity of this enzyme is considered rate-limiting in heme degradation processes, and often an increase in heme oxygenase activity is accompanied by reductions in cytochrome P-450 and microsomal heme concentrations (3,4). The cytosolic enzyme -- biliverdin reductase -- reduces biliverdin to the bile pigment, bilirubin (5,6). Heme oxygenase has been characterized (7-10) and the regulatory action of a host of agents, including hematin, on the enzyme activity has been described (2-4,11,12). However, the effect of the product of the heme oxygenase activity on the oxygenase has not been investigated, although product regulation of activity is a rather common mechanism for the regulation of many enzymes. On the other hand, the activity of the purified rat liver

*To whom correspondence should be addressed.

Abbreviations: ALA, δ-aminolevulinate

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biliverdin reductase has been found subject to substrate inhibition (5). Moreover, the activity of the purified reductase was shown to be inhibited by those agents which induce heme oxygenase activity, such as hematin (5,6).

The aim of the present study was to investigate the possible regulatory effect, *in vivo*, of biliverdin on the activities of heme oxygenase, biliverdin reductase, and the heme metabolic processes in the liver.

## MATERIALS AND METHODS

Materials:

Sprague-Dawley male rats (180-220g) were purchased from Harlen Industries, Madison, WI. The animals were allowed access to food and water *ad libitum*. Hematin, biliverdin, bilirubin and other porphyrin derivatives were obtained from Porphyrin Products, Logan, UT. Other chemicals were the products of Sigma Chemical Company, St. Louis, MO. All injections were made between 8:00 and 8:30 a.m., unless otherwise indicated. Rats were injected subcutaneously in the loose skin of the neck with hematin or biliverdin solution. The solutions were prepared immediately before use by dissolving hematin or biliverdin in a small volume of 0.1N NaOH and adjusting the pH to 7.4. The control rats received saline. Animals were killed at time intervals indicated in the legend of appropriate tables and figures, and livers were perfused *in situ* with 0.9 percent NaCl. The procedure for the preparation of subcellular fractions have been described elsewhere (10).

Enzyme Purification: Heme oxygenase was purified from liver microsomes isolated from rats treated with $CoCl_2$ (250 μmol/kg, 24 h) as described before (10). The isolated enzyme preparation showed only minor contaminants when subjected to SDS-polyacrylamide gel electrophoresis, and exhibited the specific activity of 2000 - 3000 nmol bilirubin/h/mg protein. Biliverdin reductase was purified from rat liver cytosol using affinity chromatography on NADP-agarose as described before (5,6). The purified preparation displayed a specific activity of 3000 - 3500 nmol bilirubin/min/mg protein. NADPH-cytochrome *c* (P-450) reductase was purified from the liver microsomal fractions obtained from rats treated with phenobarbital (80 mg/kg/day, 2 times, sc) as described by Yasukochi and Masters (13). The enzyme preparations showed a specific activity of 30-50 μmol cytochrome *c* reduced/min/mg protein.

Assay Procedures: The activity of δ-aminolevulinate (ALA) synthetase was measured by modifying the procedure of Marver *et al.* (14) as described before (15). Heme oxygenase activity in the microsomal fractions was measured as described earlier (10) using purified biliverdin reductase. The activity of the purified heme oxygenase preparations was determined in an assay system supplemented with NADPH-cytochrome *c* (P-450) reductase and biliverdin reductase, NADPH was used as the cofactor (10). Biliverdin reductase was assayed as described previously (5) at pH 8.7, NADPH was used as the cofactor. The cytochrome P-450 content of the liver microsomal fractions was determined by the method of Omura and Sato (16). Heme content was estimated by the pyridine hemochromogen method of Paul *et al.* (17). The protein concentration was measured by the method of Lowry *et al.* (18). All spectral studies were carried out using an Aminco DW2 spectrophotometer. The experimental results are expressed as the mean ± SD. The data were analyzed using Student's t-test; a P value of $\leq 0.05$ was considered significant.

## RESULTS

The comparative effects *in vivo* of hematin and biliverdin treatments on heme oxygenase activity in the liver are shown in Fig. 1. As shown, the

activity of heme oxygenase was increased by nearly 2-fold in rats treated with biliverdin for 48 h. As expected, in rats treated with hematin heme oxygenase activity also increased by nearly 4-fold. On the other hand, 3 h after biliverdin administration, the enzyme activity was significantly inhibited, whereas, in hematin-treated rats the activity remained unchanged at this time (data not shown). The comparative effects of hematin and biliverdin treatment on the microsomal cytochrome P-450 and heme are shown in Fig. 2a and 2b, respectively. As shown, biliverdin treatment (48 h) elicited effects similar to those of hematin (48 h) on the contents of cytochrome P-450 (Fig. 2a) and microsomal heme (Fig. 2b), i.e. significant decreases in these parameters were noted in the treated rats. Conversely, 3 h after biliverdin treatment significant increases in the contents of cytochrome P-450 (Fig. 2a) and microsomal

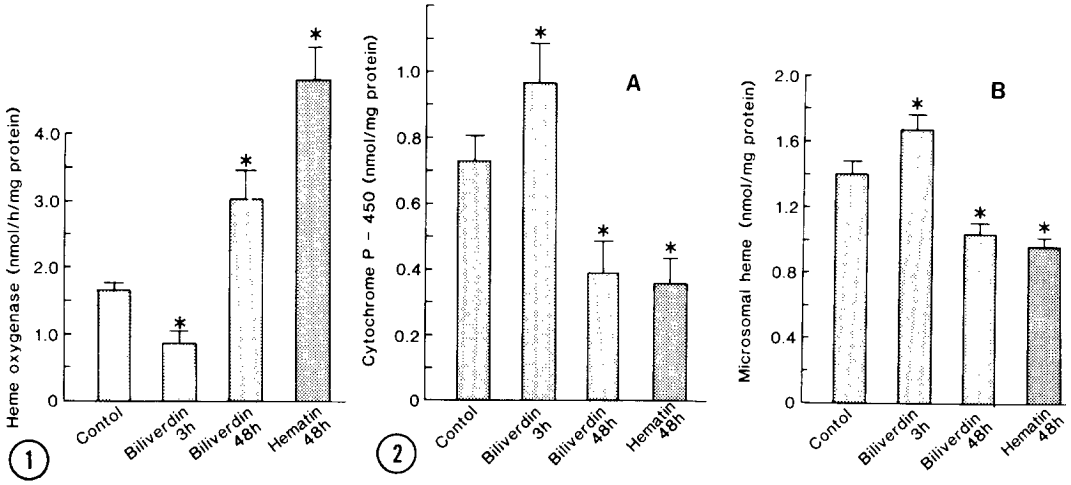


Fig. 1 Effect in vivo of biliverdin and hematin on heme oxygenase activity.
Rats were injected once with hematin or biliverdin at a dose of 40 μmol/kg and killed 3 h after the injections, or injected every 12 h, for a total of 4 injections, and are killed 12 h after the last injection. The subcellular fractions were isolated from livers and assayed for enzyme activities as described in Materials and Methods.
* $P \leq 0.05$

Fig. 2 Effect in vivo of biliverdin and hematin treatments on the microsomal concentrations of cytochrome P-450 and heme.
The livers of rats treated as described in the legend of Fig. 1 were used to prepare microsomal fractions.
a) The concentration of cytochrome P-450 was measured as described by Omura and Sato (16).
b) The heme content was measured using the pyridine hemochromogen method of Paul et al (17).
* $P \leq 0.05$

TABLE 1

Effect in vivo of Biliverdin and hematin on Biliverdin Reductase and δ-Aminolevulinic acid Synthetase Activities in rat liver

| Treatment | Duration (h) | Biliverdin reductase (nmol/h/mg) | ALA synthetase (pmol/h/mg) |
|---|---|---|---|
| Control | | 58.82 ± 0.60 | 73.37 ± 3.03 |
| Biliverdin | 3 | 47.41* ± 1.24 | 65.65 ± 7.11 |
| Biliverdin | 48 | 59.47 ± 6.59 | 103.22* ± 13.46 |
| Hematin | 3 | 57.63 ± 2.44 | 10.0* ± 1.01 |
| Hematin | 48 | 62.45 ± 0.68 | 57.12* ± 4.42 |

Rats were treated with biliverdin or hematin as descirbed in the legend of Fig. 1. The methods used for the preparation of subcellular fractions and assay procedures are described in the Materials and Methods section.
* $P \leq 0.05$

heme (Fig. 2b) were observed. Those parameters remained unchanged in 3 h after treating rats with hematin (data not shown).

Table 1 shows that biliverdin reductase activity in the liver was not altered in rats 48 h after treatment with biliverdin; whereas, 3 h after biliverdin treatment the enzyme activity was significantly decreased. Hematin treatment (3 h or 48 h) did not alter the reductase activity. Similarly, biliverdin and hematin exerted differential effects on the activity of ALA synthetase, the rate-limiting enzyme of the heme biosynthetic pathway. As shown, the enzyme activity was decreased following hematin treatments (3 and 48 h). However, the activity was significantly increased 48 h after biliverdin treatment and was unchanged at 3 h.

In vitro effects of biliverdin and bilirubin on purified preparations of liver heme oxygenase and biliverdin reductase are shown in Table 2. As shown, biliverdin reductase activity was inhibited by concentrations of biliverdin in excess of 10 μM. This observation is consistant with that reported earlier (5). Heme oxygenase activity was also noticably inhibited at biliverdin concentrations in excess of 10 μM. Biliverdin at concentrations of 10, 20 and 40

TABLE 2

*In vitro* Effect of Biliverdin and Bilirubin on Heme Oxygenase and Biliverdin Reductase Activities

| Compound | Concentration (μM) | Heme Oxygenase (nmol biliverdin/h/mg) | Biliverdin Reductase (nmol bilirubin/min/mg) |
|---|---|---|---|
| Biliverdin | 0 | 1,477 | 0 |
| | 2 | 1,482 | 2,715 |
| | 5 | 1,475 | 3,298 |
| | 10 | 1,240 | 2,974 |
| | 20 | 1,004 | 2,054 |
| | 40 | 665 | 983 |
| Bilirubin | 0 | 1,492 | 3,157 |
| | 0.5 | 1,430 | 2,201 |
| | 1.0 | 1,475 | 1,579 |
| | 5.0 | 1,459 | 789 |
| | 25.0 | 1,493 | 0 |

Heme oxygenase activity was measured in a reaction mixture (1.0 ml) containing purified enzyme (3 μg), NADPH-cytochrome c (P-450) reductase (20 μg), NADPH (1.0 mM), hematin (2 μM) and indicated concentration of biliverdin or bilirubin in 0.1 M potassium phosphate buffer, pH 7.4, at 37°. The reference incubation mixture did not contain NADPH (10). Enzyme activity was determined from rate of increase in absorbance at 680 nm due to the formation of biliverdin. The inhibitory effect on biliverdin on heme oxygenase activity was less pronounced at higher concentrations of hematin. Activity of biliverdin reductase was measured in a reaction mixture (1.0 ml) containing purified biliverdin reductase (0.2 μg), biliverdin (indicated concentrations) and NADPH (0.1 mM) in 0.1 M Tris-HCl buffer, pH 8.7, at 25°. Bilirubin formation was determined at 450 nm against a reference reaction mixture which did not contain NADPH (5). The effect of bilirubin on the reductase activity was measured using similar reaction mixtures except that they contained 5 μM biliverdin and the indicated concentrations of bilirubin. Enzyme preparations were preincubated for 5 min prior to the assay with indicated concentrations of biliverdin or bilirubin in buffer.

μM inhibited heme oxygenase activity by 16, 32 and 50 percent, respectively. The effect of bilirubin on heme oxygenase and biliverdin reductase activities was also investigated. As shown in this Table, bilirubin did not affect heme oxygenase activity, while that of biliverdin reductase was inhibited in a concentration-dependent manner.

## DISCUSSION

Biliverdin, the product of oxidation of heme, is shown to exert regulatory action *in vivo* on heme oxygenase activity. The response elicited by biliverdin treatment is that of initial inhibition followed by a rebound increase in enzyme activity. The latter effect is similar to that produced by the substrate hematin (Fig. 1). The initial inhibition of heme oxygenase activity

appears to reflect the direct inhibitory action of biliverdin on the enzyme. This suggestion is consistant with the finding that the activity of purified heme oxygenase was inhibited by biliverdin (Table 2). In turn, the mechanism of the inhibitory action may involve binding of biliverdin to, or near, the heme-binding site of the enzyme and preventing access of the substrate to heme oxygenase. Alternatively, the binding of biliverdin to heme oxygenase could render the enzyme more susceptible to degradation. The finding that heme oxygenase is invaribly purified associated with heme (8-10) may suggest the possible stabilizing action of the substrate on the enzyme protein integrity. Therefore, the possibility exists that heme oxygenase *in vivo* may be inactivated subsequent to accumulation of excess amounts of exogenously administered biliverdin in the proximity of the enzyme and the displacement of the bound substrate from the catalytic site of the oxygenase. It follows that the secondary increase in heme oxygenase activity may reflect the inhibition of heme oxygenase activity and the resulting increase in cellular heme level, which in turn would trigger *de novo* synthesis of the oxygenase.

An increase in heme oxygenase activity in the liver is frequently accompanied by a reciprocal decrease in the cellular heme and cytochrome P-450 levels. The present finding that the biliverdin-mediated inhibition of heme oxygenase activity (3 h) was accompanied by increases in cytochrome P-450 and microsomal heme contents is the first reporting of a reversal of the reciprocal relationship between the oxygenase activity and the cellular levels of heme and cytochrome P-450. The findings shown in Fig 2 and 3 that the increases in cytochrome P-450 and heme levels occurred in the absence of an alteration in the activity of ALA synthetase (Table 1) suggest the potential regulatory role of heme oxygenase in cellular cytochrome P-450 and heme homeostasis. Furthermore, the observed decreases in the liver microsomal cytochrome P-450 and heme contents 48 h after biliverdin treatment, in the face of an increased ALA synthetase activity, are consistent with the suggested role of heme oxygenase activity in the regulation of cellular heme and hemoprotein contents. It follows that the decrease observed in microsomal cytochrome P-450 and heme

levels in hematin-treated animals (Fig. 2) could, in part, reflect the elevation of heme oxygenase activity.

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