

Brief Definitive Report

BILE PIGMENT FORMATION BY SKIN HEME OXYGENASE: STUDIES ON THE RESPONSE OF THE ENZYME TO HEME COMPOUNDS AND TISSUE INJURY*

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Bilirubin is formed from heme (iron-protoporphyrin-IX) in a two step enzymatic process. Microsomal heme oxygenase is the rate-limiting enzyme which oxidizes the δ -methylene carbon bridge of heme to form carbon monoxide with release of the central iron atom and the resultant formation of the linear tetrapyrrole biliverdin. Bilirubin is subsequently formed by the action of the cytosol enzyme, biliverdin reductase (1).

Microsomal heme oxygenase is inducible by certain endogenous compounds including heme (2-5), as well as a number of heavy metals and transition elements (5-11). Metal inducible heme oxygenase activity is observed in a number of tissues and organs such as kidney (3), and liver in whole animals (5), and cell preparations including cultured avian hepatocytes (11), human skin fibroblasts, and intraperitoneal macrophages (4). In all these cases it has been demonstrated through utilization of inhibitors of protein and nucleic acid synthesis that the increased activity of heme oxygenase represents a *de novo* induction phenomenon of enzyme protein rather than an enzyme stimulation effect. The latter possibility has been further ruled out by observing that metals are inhibitory to the activity of heme oxygenase *in vitro* (5).

There has been thus far no direct evidence for heme oxygenase activity in intact skin, its response to various stimuli, or its developmental pattern, although the commonly observed changes in color of bruised skin (from the deep blue color of methemoglobin to the yellow color of bilirubin) imply the presence of heme oxygenase in skin. Accordingly, the present study was undertaken to investigate these and related phenomena.

Materials and Methods

Heme (hematin) and myoglobin (equine heart) were purchased from Sigma Chemical Co., St. Louis, Mo. Cobalt-heme (cobalt-protoporphyrin-IX) and protoporphyrin-IX were purchased from Porphyrin Products, Inc., Logan, Utah. Solutions of heme, cobalt-heme, and protoporphyrin-IX were prepared immediately before use as described earlier (5).

Male Sprague-Dawley rats weighing 150-180 g were used. The animals were treated as indicated in the appropriate experiments. Rats were starved for 15 h before killing. The develop-

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mental patterns of heme oxidation in skin and the liver were determined on pooled tissue from animals at different ages.

24 h after treatment, the rats were stunned, and hair was shaved off the skin at the injection site and at a distant area. A patch of skin approximately 3 cm² in area was then removed from each site. Skin 9,000 g fraction was prepared for enzyme assays by the method of Bickers et al. (12) and routinely used as the enzyme source. Liver and skin microsomal fractions were prepared as described elsewhere (5). The protein contents of skin and liver enzyme fractions were adjusted to 4-6 mg/ml of which 3 ml or 2 ml, respectively, were used in a 6 ml assay medium. Biliverdin reductase was added to the assay medium when measuring heme oxidation activity in liver or skin microsomal fractions. This addition was not made when heme oxidation activity of the skin 9,000 g fraction was assayed.

Enzyme Assay. Heme oxidation activities of skin and liver were studied as described earlier (5).

All reported data are the means of at least three experiments. The data were analyzed using the Student's *t* test and a value of *P* < 0.05 was considered to denote significance.

Results

The 9,000 g supernatant fraction of skin, and skin microsomal fraction supplemented with biliverdin reductase, were able to form bilirubin from heme during incubation with a reduced nicotinamide adenine dinucleotide phosphate-generating system. The heme oxidation activities of skin microsomal fractions were two- to threefold higher than those of 9,000 g supernatant fractions, indicating the microsomal nature of the enzyme. Skin heme oxygenase activity exhibited other similarities to the liver heme oxygenase system including kinetic parameters and the cofactor requirements.

Skin heme oxygenase responds to heme in the same manner as does the enzyme of other tissues such as the liver. As depicted in Fig. 1, two daily subcutaneous (s.c.) injections of heme caused a threefold increase in heme oxidation activity at the site of injection, relative both to an area distant to the site of injection, and to skin at the site of injection from rats receiving saline. By contrast, this much heme administered intraperitoneally increased only slightly skin heme oxygenase activity. Thus the elevation of enzyme activity in skin after s.c. injection of heme is largely a local rather than a systemic phenomenon.

Cobalt was the first metal discovered to possess potent heme oxygenase-inducing ability (6). Cobalt-heme is also an active inducer of the enzyme in liver (5). However, the tetrapyrrole nucleus, protoporphyrin-IX, lacking a central metal ion, is not an inducer of the hepatic enzyme. Accordingly, the effects of a single s.c. injection of cobalt, cobalt-heme, or protoporphyrin-IX on heme oxidation activity of skin and liver were compared. As shown in Table I, skin heme oxygenase activity was also significantly increased (at the injection site) by cobalt-heme or cobalt, and the enzyme activity was not altered by protoporphyrin-IX. Elevation of skin heme oxygenase by these nonphysiological substances was also a localized action since the enzyme activity from skin at the injection site was significantly increased relative to the enzyme activity of skin from the same animal at a distant area. In vitro cobalt at low concentrations (up to 150 μM) did not alter heme oxidation activity, and at high concentrations (150-500 μM) cobalt was inhibitory to enzyme activity (data not shown).

In another experiment the possibility that elevation of heme oxygenase might be a skin response elicited by certain pathophysiologic stimuli was also investigated. As shown in Fig. 2, skin heme oxygenase was increased at the treatment

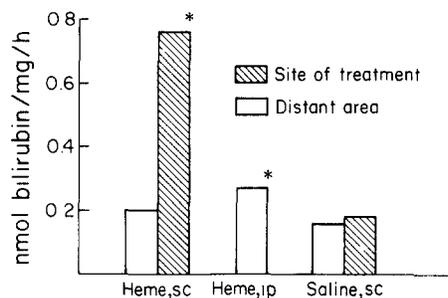


FIG. 1. Effect of heme on the bilirubin-forming capacity of rat skin. Heme ($40 \mu\text{mol/kg}$) or saline was administered s.c. or intraperitoneally to rats 24 and 48 h before killing. The $9,000 \text{ g}$ fractions were prepared from the specimens of skin from the site of injections and from a distant area from the site of injection of the same rats. Heme oxidation activity was analyzed as described in the Materials and Methods. Each reported value is the mean of at least three experiments. * $P < 0.05$ in respect to control.

TABLE I
Effect of Cobalt, Cobalt heme, and Protoporphyrin on the Bilirubin-Forming Capacity of Rat Skin and Liver

Treatment	Skin enzyme		Liver enzyme
	Injection site	Distant site	
	(nmol bilirubin/mg/h)		
Cobalt	0.54	0.20	13.5
Cobalt-heme	0.60	0.20	6.75
Protoporphyrin-IX	0.17	0.17	1.62
Saline	0.18	0.14	1.51

Cobalt-heme, protoporphyrin-IX ($40 \mu\text{mol/kg}$, each), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ($250 \mu\text{mol/kg}$), or saline were administered (s.c.) 24 h before sacrifice. Heme oxygenase activity was assayed in the $9,000 \text{ g}$ fraction of skin and in the hepatic microsomal fraction as described in the Materials Methods section.

site by stimuli such as severe bruising, injection of hemoglobin (blood), or myoglobin. The culminating response of the enzyme to injury was of considerable magnitude emphasizing the important role that heme oxygenase can play in the degradation and disposal of released intravascular heme subsequent to tissue injury. The increase of heme oxygenase by myoglobin is of interest and implies catabolism of myoglobin by this cutaneous enzyme. As noted, blood when injected s.c. also caused a localized elevation of heme oxygenase activity.

Table II shows the developmental patterns of skin and liver heme oxygenase activities. In both tissues, this activity immediately after birth as well as during the 1st wk of postnatal development was considerably higher than that of adult animals. After 2 wk postpartum the enzyme activity in both liver and skin approximated the adult values.

Discussion

This study demonstrates that microsomes from rat skin are capable of oxidatively degrading heme to bile pigment. Skin heme oxygenase activity is increased by the same compounds which have been demonstrated to induce this enzyme in the intact liver (5) and cultured avian hepatocytes (11). Moreover, all culture studies have shown that fibroblasts and macrophages, both of which are

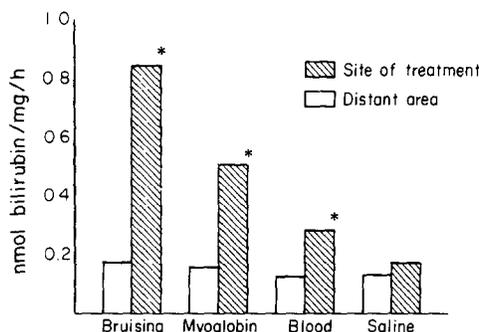


FIG. 2. Effect of bruising, myoglobin, and lysed blood on the bilirubin-forming capacity of rat skin. Rats were bruised by placing a fold of skin between a rubber stopper and a rubber mallet, and striking the mallet eight times. Rat blood (0.3 ml) was obtained by cardiac puncture, lysed by mixing with 0.3 ml sterile, distilled water, and injected s.c. into the same rat. Rats were anesthetized with ether during bruising or bleeding. Other rats received s.c. myoglobin (40 μ mol/kg) or saline. After 24 h, tissues were prepared for enzyme analysis as described in the Materials and Methods section. * $P < 0.05$ with respect to control values.

TABLE II
The Comparative Developmental Patterns of Skin and Hepatic Heme Oxygenase Activity in the Rat

Developmental stage	Skin	Liver
days	(nmol bilirubin/mg/h)	
0	0.33	1.7
4	0.27	4.3
7	0.21	5.1
14	0.16	2.8
21	0.15	2.1
Adult	0.15	1.8

Skin or liver were pooled from rats at the indicated ages. Tissues preparation and enzyme analysis are as in the Materials and Methods section.

present in skin, also possess heme (4) and metal (Co^{2+}) inducible heme oxygenase activity (2). Therefore, it can be inferred that the observed increased heme oxygenase activity in intact skin as a response to these stimuli is also an induction phenomenon. In addition, the skin heme oxygenase system exhibited a developmental pattern which closely resembled that of liver. The similarities of the response of skin and liver to different stimuli suggest that the enzymatic heme oxidation activity in these tissues are regulated by the same mechanisms.

The elevation of heme oxygenase activity in skin in response to non-injurious stimuli is most likely due to increased activity in fibroblasts which under normal conditions constitute the bulk of the skin. On the other hand, the increase in skin heme oxygenase activity after bruising and tissue injury could reflect increased activities of both fibroblasts and macrophages due to changes in the cell composition of bruised skin, through proliferation or migration of macrophages. The finding that skin heme oxygenase is locally elevated by the cellular constituents released during injury (such as myoglobin and hemoglobin) further emphasizes the physiological importance of skin heme oxidation activity in bile pigment formation in certain circumstances. This capability may, for example, eliminate or diminish the need after tissue injury for the transfer of released

tissue hemoproteins to other organs such as the liver or spleen for oxidative degradation. Moreover, the pattern of skin pigmentation after bruising or s.c. hemorrhage clearly implies that bilirubin and biliverdin accumulate locally.

The findings reported here that heme oxygenase activity in skin was also increased after s.c. administration of heme, cobalt ion, or cobalt-heme, but not of saline or protoporphyrin-IX, indicates that the elevation of enzyme activity in skin is a response to specific stimuli and not a manifestation of nonspecific injury. This view is supported by the similarity in the responses of hepatic heme oxygenase activity to each of these agents.

Finally, in the newborn, due to the proportionally large amount of skin in relation to body mass, elevated skin heme oxidation activity is probably a significant contributing factor to the development of postpartum jaundice. This factor could operate, in addition to the increased hepatic heme oxygenase activity (7, 13) and the depressed hepatic glucuronyl transferase activity (14), to exaggerate the degree of jaundice occurring at this stage of development. Increased skin heme oxygenase activity during the 1st wk postpartum may well be in fact a response to the elevated levels of heme in the general circulation which occur after parturition. Chemical methods of repressing cutaneous induction of heme oxygenase thus may offer a novel preventive therapeutic approach to the problems associated with neonatal jaundice.

Summary

Skin heme oxygenase is locally elevated by stimuli such as tissue injury and injections of whole blood, myoglobin, and hematin. The enzyme activity is also increased at the proximity of the injection site of chemicals such as cobalt and cobalt-protoporphyrin-IX (cobalt-heme). Protoporphyrin-IX, the tetrapyrrole nucleus of type-b heme compounds, was ineffective in altering the enzyme activity in vivo.

The developmental pattern of heme oxygenase in skin was compared to that of the enzyme in liver. The enzyme activity in both organs was greatest during the 1st postpartum wk and declined to adult levels after 2 wk.

The physiological implications of the increased activity of skin heme oxygenase are discussed, and it is concluded that the activity of the hepatic heme oxygenase system and that of the skin are regulated by the same mechanism.

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