Degradation of Endogenous Hepatic Heme by Pathways Not Yielding Carbon Monoxide

STUDIES IN NORMAL RAT LIVER AND IN PRIMARY HEPATOCYTE CULTURE

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ABSTRACT The conversion of endogenous hepatic heme to bilirubin and CO is established. However, it is unknown whether this process is quantitative or whether heme may be degraded to other products as well. To study this question, we administered the heme precursor, δ-amino-[5-^{14}C]levulinic acid to rats in vivo. In liver, [^{14}C]heme was predominately associated with microsomal cytochromes, and its degradation was examined over a period of 12–14 h; concurrently, excretion of labeled carbon monoxide ^{14}CO by the animal was measured. After correction for ^{14}CO derived from the breakdown of renal [^{14}C]heme, the rate of heme degradation calculated from the ^{14}CO excreted was substantially less than the rate of disappearance of hepatic [^{14}C]heme measured directly. The discrepancy between actual loss of labeled heme from the liver and generation of labeled CO was confirmed by direct study of endogenous [^{14}C]heme degradation in primary hepatocyte culture, in which only 25% of the labeled heme disappearing during the incubation was converted to ^{14}CO. By contrast, cultured cells converted exogenous [^{14}C]-heme nearly quantitatively to ^{14}CO. We conclude that heme associated with microsomal cytochromes in normal rat liver is degraded substantially by non-CO forming processes.

INTRODUCTION

Degradation of hemoglobin heme in vivo yields equimolar amounts of bile pigment and CO (1, 2). The process is mediated by microsomal heme oxygenase (3, 4), and the conversion to bile pigment or CO appears to be quantitative (5, 6). Similarly, catabolism of endogenous hepatic heme is known to give rise to bilirubin and CO (2, 7) and appears to be related to microsomal heme oxygenase in the liver (8). Because the latter is the only mechanism for heme breakdown of demonstrated physiological relevance, it has been assumed that endogenous hepatic heme, like exogenous heme, is degraded quantitatively to bile pigment and CO. This assumption, however, has not been verified experimentally, and *a priori* its validity may be questioned. Unlike hemoglobin, the heme proteins of the liver are heterogeneous, including the group known as cytochrome P-450, which consists of at least two (and probably several) distinct heme proteins (9). With regard to heme turnover, the latter are quantitatively the most important heme proteins in the liver (10), but whether or not each individual apoprotein of this group directs degradation of its respective heme moiety by way of microsomal heme oxygenase is conjectural at this time. Moreover, a very rough calculation of hepatic heme turnover and pigment production suggests that a discrepancy between these two processes exists. Hepatic heme turnover in a 300-g rat may be estimated at 620 nmol/d (10), while hepatic bilirubin production is only 250 nmol/d (10% of a total body bilirubin production of ~2,500 nmol/d) (1).

We recently examined quantitatively hepatic heme breakdown by correlating production of heme catabolites with rates of degradation of hepatic heme in rats given δ-amino[5-^{14}C]levulinic acid as the labeled heme precursor (8). One-eighth of the labeled carbons from this precursor are in the alpha methene bridge position of the tetrapyrrole, and conversion of the labeled heme to bile pigment is accompanied by

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stoichiometric release of ^{14}CO, which can be trapped and quantitated (2, 8). Our initial observations suggested that, with respect to catabolism of cytochrome P-450, ~70% of the heme degraded could be accounted for as CO (8). In this study, examining this process in greater detail, we find that the proportion of hepatic heme converted to CO was previously overestimated. Administered δ-amino [5-^{14}C]levulinic acid labels both hepatic and renal heme, and the latter contributes substantially to the basal rate of ^{14}CO excretion.[1] When this fact is taken into account, a sizeable discrepancy between heme disappearance from the liver and excretion of CO emerges. We have confirmed these findings in vivo with studies of heme degradation in rat hepatocytes in primary monolayer culture (11), and conclude that in normal rats, endogenous hepatic heme, in contrast to exogenous heme, is degraded predominately by non-CO forming pathways.

METHODS

Degradation of endogenous hepatic heme. Examination of this process was carried out according to published methods (8). In brief, male Sprague-Dawley rats (~250 g body wt) were used. Endogenous heme was labeled by intraperitoneal administration to the animals of 10 μCi δ-amino-[5-^{14}C]levulinic acid (New England Nuclear, Boston, Mass.; 50 mCi/mmol). After a period of 16 h, labeled heme had been discharged from rapidly turning over pools, leaving microsomal cytochromes as the major labeled heme proteins in the liver (8). At the start of the study period, the animal was placed under light ether anesthesia, and the anterior lobule of the right lateral hepatic lobe, weighing ~0.3 g, was removed through an abdominal incision. The [^{14}C]heme in this tissue sample was used to estimate the initial content of labeled heme in the liver. Preliminary studies had indicated that the amount of labeled heme, per milligram tissue, in the biopsy sample was 84±11% (mean±SD, n = 8) of that in the remainder of the liver. It also had been determined that total liver weight in male rats of the size used, fed ad lib., constituted 3.8±0.4% of body wt. Thus, from the labeled heme of the biopsy sample and the body weight of the animal, the initial [^{14}C]heme in the liver could be estimated. After this procedure, the animal was placed in the apparatus for trapping ^{14}CO (8), and total cumulative ^{14}CO was measured over a period of 12–14 h. At the end of the study period, the animal was sacrificed and the liver removed for measurement of residual [^{14}C]heme. The difference between the calculated initial and final extracted labeled heme was taken as total hepatic heme degradation; this value was compared to the measured total ^{14}CO production.

To test the possibility that the surgical procedure affected heme degradation in the remaining liver, control experiments were performed by selecting a pair of animals that, after administration of δ-amino [5-^{14}C]levulinate, excreted ^{14}CO at similar rates and thus presumably contained comparable amounts of hepatic [^{14}C]heme (8). One animal was killed for measurement of the "initial" hepatic [^{14}C]heme, and, after ^{14}CO had been collected for 14 h, the second animal was killed for determination of "final" hepatic [^{14}C]heme. Although interstudy variation was greater by this procedure than with that described above, results from the two approaches were not significantly different.

Primary hepatocyte culture. The methods for preparation and culture of adult rat hepatocytes have been reported (11). For studies of endogenous heme degradation in culture, a donor animal received δ-amino-[5-^{14}C]levulinic acid in vivo as described above. After 16 h, hepatocytes were isolated from the prelabeled rat and cultured in 100-mm plastic petri dishes. Part of the preparation was taken for determination of initial [^{14}C]heme content. Other plates from the same batch of cells were placed in airtight containers that had been equilibrated with the atmosphere of the culture incubator. After a period of 16–20 h in culture, the air from the container was flushed for 30 min into the trapping system for quantitation of ^{14}CO. Recovery of ^{14}CO in the container was >95% and was unaffected by the presence of cells or culture media. Cells and media from the same plates were combined, concentrated by lyophilization, and analyzed for labeled heme. Degradation of [^{14}C]heme and comparison with ^{14}CO production were calculated as described above for intact rats.

For examining degradation of exogenous heme in culture, [^{14}C]hemoglobin was prepared biosynthetically with δ-amino-[5-^{14}C]levulinic acid as precursor, and the labeled heme was extracted and crystallized (8). A quantity was dissolved in 0.2 N NaOH, mixed with an equimolar amount of rat serum albumin (fraction V, Sigma Chemical Co., St. Louis, Mo.), and adjusted to pH 7.4 with HCl. The labeled solution was diluted to a concentration of 5 μg heme/ml with complete culture medium and added to replicate plates of unlabeled cells. Measurement of [^{14}C]heme degradation and ^{14}CO production were carried out as for endogenously labeled cells. When [^{14}C]bilirubin was measured, this was carried out according to Ostrow et al. [13], after mixing the contents of culture plates with rat bile containing 60–100 μg bilirubin as carrier. To effect a complete separation of bilirubin and [^{14}C]heme, chloroform extracts were reduced to a small volume under nitrogen and applied to thin-layer plates (silica gel G, 0.25 mm), which were developed in chloroform containing 1% acetic acid. Heme remains at the origin in this chromatographic system. The recovery of labeled bilirubin was estimated from the recovery of carrier pigment eluted from the plates and measured at 454 nm.

Other procedures

Bilateral nephrectomy was performed through 1–2-cm posterior paramedian incisions in rats anesthetized with ether. Each kidney was extruded through the incision with gentle pressure applied to the abdomen. The renal pedicle was ligated and the organ excised, care being taken avoid removal of the adrenals. The entire procedure took less than 5 min. Labeled heme was extracted from hepatic tissue or cultured cells with strontium chloride/acetic acid after addition of a known amount of carrier heme (as rat hemoglobin); recovery of [^{14}C]heme was calculated from the recovery of the carrier (8, 14). In several experiments, duplicate samples were assayed by a different procedure in which heme was extracted quantitatively without carrier into ether (15). Radioactivity was determined by liquid scintillation spectrometry, with counting efficiencies estimated by addition of a labeled internal standard. Results from the two extraction methods

[1] In a previous study, which was concerned with the effect of administered endotoxin on the degradation of [^{14}C]heme in rat liver to ^{14}CO (8), the contribution of renal [^{14}C]heme to basal ^{14}CO production was not appreciated. However, the disappearance of labeled heme from the kidney is unaffected by endotoxin (unpublished observations). Thus, the changes in ^{14}CO production caused by this agent appear to reflect changes in turnover of hepatic heme as reported (8).

varied <10%; with either method the percentage of total liver radioactivity extractable as heme was 70–75% both in the initial and final samples. By paper chromatography in a descending solvent system of 2,6-dimethylpyridine/water [10:7], the isolated [^{14}C]heme was 99% radiochemically pure, excluding significant contamination by δ-aminolevulinic acid, which migrates at about one-half the R_f of heme under these conditions (16).

Each study was conducted with culture plates prepared from a single rat liver. The results are expressed per plate, because protein content varied <5% among plates from the same batch of cells. The efficiency of the CO collection apparatus was 85–95%, as determined by recovery of ^{14}CO generated in the animal chamber from [^{14}C]formic acid dehydrated with hot (65°C) concentrated sulfuric acid. The data for ^{14}CO have been corrected for this efficiency factor.

RESULTS

Production of ^{14}CO from endogenous [^{14}C]heme in vivo: hepatic and renal components. The disappearance of labeled heme from the liver was measured and compared to the excretion of ^{14}CO over the same time period after administration of δ-amino-[5-^{14}C]levulinic acid. In intact animals, 65–70% of [^{14}C]heme disappearing during the period of study appeared to be accounted for as ^{14}CO (Table I), a figure not strikingly different from a previous estimate of the proportion of exogenous heme converted to bile pigment in vivo (17). However, this approach neglects a possible contribution of labeled renal heme to ^{14}CO production. After administration of δ-amino[5-^{14}C]levulinic acid, as described above (Methods), labeled heme is present in both kidneys and liver, in approximately equivalent amounts, with respect to the total labeled heme in the organs (Table II). Therefore, the kidneys are potentially a significant source of ^{14}CO. The renal component of total ^{14}CO excretion was studied first by comparing labeled CO production in an intact animal with that from an animal subjected to bilateral nephrectomy. As shown in Fig. 1, removal of the kidneys was followed by an average 30% decrease in ^{14}CO production.

If the renal contribution is considered and the data of Table I recalculated, the corrected figures suggest that ~50% of hepatic [^{14}C]heme disappearing during the period of observation can be accounted for by ^{14}CO. This inference was supported by a study in which the disappearance of labeled hepatic heme and production of labeled CO in vivo were measured by the usual protocol, modified to include a bilateral nephrectomy, which was carried out just before taking the initial liver specimen and placing the animals in the apparatus for CO collection. In these studies, ^{14}CO production accounted for 58±20% (mean±SD, n = 6) of [^{14}C]heme disappearing from the liver.

Degradation of endogenous or exogenous [^{14}C]heme in primary hepatocyte culture. The experimental data may be corrected for the renal component of ^{14}CO production, with the assumption that labeled hepatic heme is the source for the remaining observed ^{14}CO. However, organs other than liver and kidney form small amounts of [^{14}C]heme (Table II), and these may contribute to ^{14}CO production, spuriously elevating the apparent conversion of hepatic heme to CO. Moreover, interstudy variation with the intact animals is considerable as a result of the various factors that enter into the calculation of initial hepatic [^{14}C]heme (Methods). For these reasons, studies with hepatocytes in culture were undertaken as a means of directly correlating degradation of labeled heme and production of labeled CO or bile pigment in hepatic parenchymal cells. To ensure that the labeled endogenous heme fraction studied in culture was similar to that in vivo, hepatocyte cultures were prepared from animals that had received δ-amino[5-^{14}C]levulinic

TABLE I

Degradation of Endogenous Hepatic [^{14}C]Heme and Production of ^{14}CO in Intact Rats

Study	Hepatic [^{14}C]heme		^{14}CO production		Apparent fractional conversion of [^{14}C]heme to ^{14}CO
	Initial (A)	Final (B)	Total (C)	Heme equivalent* (D)	(D/A − B) × 100
	10^{-3} × dpm		10^{-3} × dpm		%
1	1,770	912	73	584	68
2	1,772	1,064	62	496	70
3	1,968	818	95	760	66

Animals of comparable age and weight received 10 μCi of δ-amino[5-^{14}C]levulinic acid. 16 h later, ~3% of the liver was removed for estimating the initial hepatic[^{14}C]heme, as described in Methods. The animals then were placed in metabolic chambers and ^{14}CO continuously collected for the subsequent 12 h. At the end of this period, the animal was killed, the liver excised, and the remaining labeled heme quantitated.

* Represents (C × 8); with δ-amino[5-^{14}C]levulinic acid as precursor, one-eighth of the ^{14}C in newly synthesized heme is in the *α-meso* bridge carbon.

TABLE II
Distribution of [^{14}C]Heme in Rats Given δ-Amino[5-^{14}C]Levulinic Acid

Tissue	Amount measured	[^{14}C]heme
		dpm, Mean±SD
Brain	Total	481±300
Spleen	Total	1,479±436
Duodenum + jejunum	30 cm	15,019±3,096
Lungs	Total	3,972±2,894
Adrenals	Total	389±118
Muscle, skeletal	g	1,371±898
Kidneys	Total	720,237±122,689
Blood	ml	502±51
Liver	Total	565,314±198,236

Rats weighing 275–315 g (n = 4) received δ-amino[5-^{14}C]levulinic acid, 5 μCi, according to the protocol described in Methods and 16 h later were sacrificed by exsanguination under ether anesthesia. Organs were excised, homogenized in isotonic saline and analyzed for labeled heme without calculation of residual blood within the tissue; the segment of small intestine was flushed thoroughly with iced saline before homogenization.

acid in vivo, 16 h before the preparation of isolated hepatocytes, as described in Methods. As shown in Table III, ~25% of the labeled endogenous heme disappearing in culture during the study period could be accounted for as ^{14}CO, and this was unaffected by the presence of albumin and unlabeled heme (5 μg/ml) in the culture medium. By contrast, exogenous [^{14}C]-heme (presented to the cells as a complex with albumin) underwent essentially quantitative conversion to ^{14}CO (Table III). In other studies, [^{14}C]bilirubin was extracted and quantitated; it was always less (on a molar basis) than the amount of CO produced under similar conditions, regardless of the addition of serum albumin to the culture medium. This finding is consistent with partial spontaneous degradation of the pigment during incubation in culture which was confirmed in control studies. When culture media containing [^{14}C]bilirubin were added to plates without cells and incubated at 37°C for 20 h, only 25% of the initial [^{14}C]bilirubin was recovered; in the presence of 10 mg/ml bovine serum albumin, 75% was recovered.

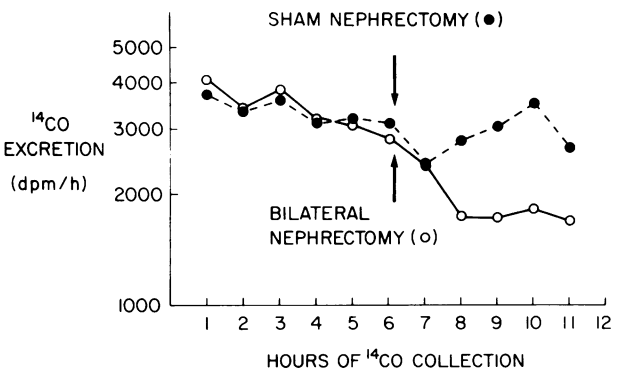

FIGURE 1 Effect of bilateral nephrectomy on ^{14}CO excretion in vivo. A pair of rats were labeled with δ-amino[5-^{14}C]-levulinic acid, and 16 h later, hourly collections of ^{14}CO were begun. After measurement of base-line ^{14}CO excretion in both animals, one animal was subjected to bilateral nephrectomy and the other to a sham operation as outlined in Methods. From the difference in ^{14}CO excretion rates 1 h after the operations (between 8 and 11 h), the renal contribution to total ^{14}CO excretion was calculated to be 33%. A repetition of the experiment gave a value of 28%.

DISCUSSION

These studies in vivo and in primary hepatocyte culture demonstrate that endogenous heme in normal rat liver is degraded to a significant extent by pathways not yielding CO and bile pigment. The rationale for measuring production of ^{14}CO in vivo rather than the appearance of labeled bilirubin in bile, has been discussed (8). Bile bilirubin and exhaled CO are equivalent parameters of heme degradation in the rat, as shown by the studies of Landaw and co-workers (2). In hepatocyte culture, measurement of [^{14}C]bilirubin underestimates the conversion of heme to bile pigment because of the instability of bilirubin in culture medium, even in the presence of added serum albumin. Therefore, measurement of ^{14}CO appeared to be superior to that of [^{14}C]bilirubin in this setting also. Possible methodological artifacts associated with the studies in culture include metabolism of CO (pre-

TABLE III
Degradation of Exogenous or Endogenous [^{14}C]Heme and Production of ^{14}CO by Rat Hepatocytes in Primary Culture

	Conversion of [^{14}C]heme to ^{14}CO*	
	Mean percent±SD	Range
		%
Exogenous [^{14}C]heme (n = 5)	90±11	72–99
Endogenous [^{14}C]heme (n = 5)	25±7	18–35

Cultures from untreated animals were prepared as described in Methods. Exogenous [^{14}C]heme was added to culture medium as a complex with rat serum albumin, at a final concentration of 5 μg/ml. Cultures with endogenous [^{14}C]heme were from animals that had received δ-amino[5-^{14}C]levulinic acid 16 h before hepatocyte preparation (see Methods).
* Conversion calculated as in Table I.

sumably to CO_2), its binding to tissue components, or leakage of the gas from the incubation containers, any of which would result in a low recovery of ^{14}CO. With regard to the oxidation of CO or its binding to tissue components, it should be noted that the degradation of exogenous heme in culture was accompanied by a nearly stoichiometric release of CO. Thus, if metabolism or tissue binding occur, it appears to involve selectively CO derived from endogenous, rather than exogenous, heme. Although theoretically possible, subcellular compartmentalization of a gas would be unprecedented. Leakage of CO from the incubation containers and binding of exogenous ^{14}CO to culture components were examined in preliminary experiments and found to be negligible. We have previously reported that degradation of cytochrome P-450 heme is accelerated in cultured hepatocytes (14, 18, 19), which raises the possibility that heme catabolism is altered. However, the accelerated degradation of cytochrome P-450 heme in cultured hepatocytes is accompanied by a striking increase in heme oxygenase activity (18), which suggests at least that there is an ample enzymatic capacity in the cells for the conversion of heme to CO.

This protocol was devised to provide information largely about heme pools associated with the group of microsomal cytochromes P-450 (and, perhaps, cytochrome b_5, although this heme protein turns over relatively slowly). The fractional conversion of non-microsomal heme to CO and bile pigment may differ from that reported here. While this possibility should be examined, the degradation of cytochrome P-450 heme holds particular interest since these hemoproteins account for well over 50% of total hepatic heme turnover (10). Moreover, unlike the mitochondrial (*a* and *c* type) cytochromes, the prosthetic group for cytochrome P-450 is the same as that of hemoglobin, i.e., protoheme (20). The latter fact invites a comparison between the degradation of microsomal and exogenous heme in rat liver, particularly since circulating hemoglobin (either free or complexed with haptoglobin), methemalbumin, and heme-hemopexin are taken up and degraded in the parenchymal cells of the liver (21). Ostrow and colleagues (17) showed several years ago that the fractional conversion of administered hemoglobin to bile pigment was variable, ranging as low as 55%, and they raised the possibility of non-bilirubin pathways for heme degradation. Snyder and Schmid (22) found a similarly reduced recovery in administered [^{14}C]hematin as [^{14}C]bilirubin. However, with the smallest doses of labeled hemoglobin, the recovery of labeled bilirubin approached 100% (17), and Landaw (6), in a more recent study of transfused labeled erythrocytes, has reported quantitative conversion of circulating heme to CO. Likewise, these studies indicate that hepatic parenchymal cells in primary culture convert exogenous heme nearly quantitatively to CO. In sharp contrast, catabolism of endogenous (microsomal) heme in hepatic parenchymal cells appears to proceed to only a minor extent by way of bile pigment formation. If degradation of both endogenous and exogenous heme takes place in the same hepatic cells, these findings suggest that compartmentation of heme catabolism must exist, supporting conclusions from earlier studies of bilirubin kinetics in liver and plasma (21, 23–25).

We had previously postulated the existence of a non-CO forming pathway for degradation of endogenous hepatic heme in vivo (26). In those preliminary studies, treatment of rats with carbon tetrachloride or with the porphyrinogenic agent, allylisopropylacetamide, was found to accelerate the degradation of hepatic heme in the absence of a concomitant increase in CO formation (26). Although it was assumed that the non-CO forming pathway was an "abnormal" one, elicited under conditions of a toxic insult to the liver, the present studies in normal rats emphasize that a quantitatively important route for degradation of hepatic (microsomal) heme also does not involve CO. Carbon tetrachloride or allylisopropylacetamide may directly attack the heme molecule, as recently reported for the latter agent (27). However, it is also possible that these compounds accelerate the breakdown of hepatic heme in part by stimulating normal degradative pathways.

The physiologic process responsible for heme catabolism by the non-CO forming pathway(s) in liver remains to be identified. By extrapolation from in vitro studies, it is possible to speculate that formation of lipid peroxides may be involved. The heme moiety of cytochrome P-450 is susceptible to peroxidative attack in vitro, yielding unknown products that do not include CO (28, 29). Studies of the effects of preformed lipid peroxides have shown that cytochrome P-450 exhibits peroxidase activity and, under some circumstances, may undergo peroxidative "autodegradation" (30). Jeffery and co-workers (31), extending these observations, have reported that destruction of cytochrome P-450 in vitro by lineoleic hydroperoxide is biphasic. About 50% of the cytochrome exhibited a relatively high susceptibility to peroxidative attack, and this fraction appeared to be increased in microsomes from animals pretreated with phenobarbital. The implication of these data for the present study is that heme undergoing degradation by non-CO forming pathways may be associated with a subspecies of cytochrome P-450, some fractions of microsomal heme being degraded by heme oxygenase, others by the non-CO forming route. By simple modification of the present approach, it should be possible to measure the fractional conversion to CO of endogenous heme in various pools—the heme associated with subspecies of cytochrome P-450 or the heme precursor of the "early labeled" bile pigment fraction in liver (7).

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