Effect of Cobalt on Synthesis of Heme and Cytochrome P-450 in the Liver

STUDIES OF ADULT RAT HEPATOCYTES IN PRIMARY MONOLAYER CULTURE AND IN VIVO*

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Synthesis of heme and cytochrome P-450 have been studied in adult rat hepatocytes in primary monolayer culture. Incubation of cells with δ-aminolevulinic acid increases both cellular heme and cytochrome P-450 relative to that found in cells incubated under control conditions. Formation of heme is proportional to the concentration of δ-aminolevulinic acid in the culture medium and is not saturable. By contrast, formation of cytochrome P-450 is saturable; excess intracellular heme appears as a new absorption band at 420 nm in the carbon monoxide-reduced difference spectrum. We have studied the effect of cobalt on heme and hemoprotein formation in this cell system. The metal blocks formation of cytochrome P-450 but fails to affect heme synthesis. In contrast to previous findings with isolated mitochondria, no cobalt protoporphyrin formed in hepatocytes cultured in the presence of the metal. In studies of rats in vivo, it was confirmed that cobalt acts to reduce the amount of [14C]heme in the liver after administration of δ-aminolevulinic acid. However, the present findings suggest that this effect of cobalt represents accelerated breakdown of newly labeled hepatic heme rather than inhibition of synthesis. We conclude that cobalt interferes with formation of cytochrome P-450 not by direct inhibition of heme synthesis but most likely by blocking the association of heme and apocytochrome.

A group of microsomal cytochromes, known collectively as cytochrome P-450, are inducible in the liver by administration of various drugs, carcinogens, and environmental contaminants. Despite the importance of cytochrome P-450 in metabolism of these substances, regulation of this hemoprotein remains poorly understood. For example, it is not clear whether stimulated synthesis of heme or of apoprotein is the initial event of induction. Although previous work had suggested that availability of heme is rate-determining for cytochrome formation (1), evidence against this postulate has been adduced (2), and recent findings are consistent with the concept that synthesis of apocytochrome is the limiting factor (3, 4). In most of these studies, inhibitors of heme synthesis were used to dissociate the metabolism of heme and apocytochrome. Cobalt is particularly attractive in this regard, because available information suggests that the metal blocks heme synthesis selectively either by competing with iron for insertion into protoporphyrin (5), or possibly by decreasing enzymatic formation of heme precursors (6).

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subsequent to the synthesis of heme, possibly at the association of heme and apocytochrome P-450.

**Experimental Procedure**

**Materials**

Tissue culture media were prepared in our laboratory and sterilized by membrane filtration (Millipore, 0.45 μm pore size). Collagenase, type 1, was purchased from Sigma Chemical Co., St. Louis, Mo., Eagle's minimal essential vitamins (100 x) from Grand Island Biological Co., Grand Island, N.Y., and 5-aminolevulinic acid from ICN Pharmaceuticals, Inc., Irvine, Calif. Amino[5-14C]levulinic acid (25 Ci/mol), from Calbiochem, La Jolla, Calif., and ['Fe (2.5 Ci/mg) was obtained from Porphyrin Products, Logan, Utah.

**Preparation of Primary Cultures of Adult Rat Hepatic Parenchymal Cells**

Preparation of primary cultures of adult rat hepatic parenchymal cells was carried out as described previously (7). Cells were obtained from rat liver perfused in situ with buffered collagenase solution. The isolated hepatic parenchymal cells were separated cleanly from debris by centrifugation and were placed in plastic tissue culture dishes (60 mm) in a serum-free defined medium. With plating of cells at optimal density (5 x 10^6 cells/60-mm plate) and in a volume of 3.0 ml, a viable monolayer of hepatic parenchymal cells forms after 12 to 18 hours of incubation. The medium used in the present experiments, termed L-16, was slightly modified from that described previously (7), in that it contained 0.5% bovine serum albumin, 10% fetal bovine serum, and Amphotericin B were omitted. Substances to be added to the media were dissolved directly, and the solution was sterilized by filtration.

**Experimental Approach**

All experiments with cultured adult rat hepatocytes were performed during the initial 24 hours after the plates had been plated. This period was selected for study because the rapid changes in cytochrome P-450, as well as the response of the cells to modified culture media, occur largely during this time. Thus, in a given experiment, cells from a single rat liver were apportioned according to the protocol and plated either in control or in modified media. After 20 to 24 hours of incubation, all cultures were terminated, and the cells were analyzed according to the procedures described below.

**Assay Procedures**

After removal of the medium and with washes with phosphate-buffered saline, pH 7.4, monolayer cells from two or more plates were combined, disrupted by sonication, and centrifuged, for preparation of a 10,000 x g supernatant or of microsomes, as described previously (7).

**Determination of Cytochrome P-450**

Suspensions of microsomes or 10,000 x g supernatant in 0.1 M potassium phosphate buffer were reduced with a few crystals of dithionite, and were placed in 1 ml of 0.1 M potassium phosphate, pH 7.4, minus tissue source. The extraction procedure for sample and blank was then carried out as described (16). Aniline hydroxylase activity in freshly isolated cells was 0.208 ± 0.115 nmol of aniline metabolized/mg protein, which did not differ significantly from that in donor liver in two experiments.

**Extraction of Labeled Heme**

Carrier heme was added to the sample in the form of 1 ml of harspinized whole rat blood, and heme was separated from the mixture by centrifugation (17). Comparative results were obtained when free heme was used as carrier, which indicates that free and cytochrome P-450 are extracted with equal efficiency from biological samples. For estimation of the radioactivity of the cytochrome from the heme, a precisely weighed amount of heme (0.5 to 0.8 mg) was dissolved in 0.5 ml of NaOH and added to the sample cuvettes. The concentration of heme in the CO complex absorbing at 420 nm was calculated using E_420 (7.4) = 110 (nmol/mg of protein).

**Protein**

The method of Lowry et al. (21) was used with crystalline bovine albumin as the standard.

**Results**

**Microsomal Function in Adult Rat Hepatocyte Cultures**

The evidence for sustained viability and the morphologic appearance of hepatocytes in this cell culture system have been presented in detail elsewhere (7, 22). Among microsome- associated processes, the most striking finding is an early decrease in the cellular level of cytochrome P-450. This change
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is evident, by comparison with the liver in vivo, after 4 hours in culture and after 20 hours is pronounced, with the level of cytochrome P-450 dropping to 10 to 20% of its concentration in freshly isolated hepatocytes. The selectivity of this change has been assessed by survey of other microsomal enzymes. Glucose-6-phosphatase activity is unchanged while the content of cytochrome b5 decreases moderately (Table I) over the first 24 hours of culture. The activity of various drug-metabolizing enzymes is variably affected, suggesting that the amount of cytochrome P-450 in the microsomes may be limiting for some of these activities but not for others. These results are reported in detail elsewhere. No evidence for the loss of a specific type of cytochrome P-450 (e.g., cytochrome P-450, as distinguished from cytochrome P-448) was found, in that the dithionite-reduced CO difference spectra of the hemoproteins in intact cells are identical. Furthermore, at no point during the first 24 hours of culture while cytochrome P-450 was falling rapidly, did the reduced CO difference spectrum reveal material absorbing at 420 nm.

Effect of \(\delta\)-Aminolevulinic Acid on Heme Synthesis and on Cytochrome P-450 in Cultured Hepatocytes—Addition of \(\delta\)-aminolevulinic acid to the culture medium increased the rate of heme formation by the cells in proportion to the concentration of \(\delta\)-aminolevulinic acid, as judged both by spectrophotometric studies (Figs. 1 and 2) and by incorporation of \(\delta\)-amino[\(14\)C]levulinic acid into cellular heme (Table II). When cells are plated in medium containing \(\delta\)-aminolevulinic acid, cytochrome P-450 also increases significantly after 24 hours, as compared to the concentration of this hemoprotein in cells incubated in control medium (Table I). The increase in cytochrome P-450 is dependent upon the concentration of added \(\delta\)-aminolevulinic acid, up to \(8 \times 10^{-4}\) M. At concentrations greater than this, the level of cytochrome P-450 fails to change, although the heme content of the cells continues to increase. The additional heme formed is proportional to the height of a peak at 420 nm in the reduced CO difference spectrum (Figs. 1 and 2). Although the absolute height of the 420 nm peak varied among batches of cells incubated with a given concentration of \(\delta\)-aminolevulinic acid (cf. Figs. 1 and 3), the "threshold" concentration of \(\delta\)-aminolevulinic acid for production of the 420 nm peak was relatively constant. The increase in cytochrome P-450 concentration, resulting from addition of \(\delta\)-aminolevulinic acid to the medium, was reflected in increased activity of the drug-metabolizing system in vitro (Table III), which indicates that the observed increment in hemoprotein represents functionally active material. Addition of \(\delta\)-aminolevulinic acid modified the composition of microsomal hemoproteins selectively, since the level of cytochrome b5 was unaltered under these conditions (Table I).

Effect of Cobalt on \(\delta\)-Aminolevulinic Acid-mediated Increase in Cytochrome P-450 in Cultured Hepatocytes—The decrease in cytochrome P-450 exhibited by cells cultured in control medium was unaffected by the presence of cobalt, so that concentrations of the hemoprotein at 24 hours of incubation were similar in cobalt-treated and control cultures (Fig. 3). However, cobalt blocked the increase in cytochrome P-450 associated with addition of \(\delta\)-aminolevulinic acid to the culture medium (Fig. 3), and the effect of cobalt was concentration-dependent (Table IV). The metal had no effect on incorpora-

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Table 1

Effect of \(\delta\)-aminolevulinic acid on microsomal hemoproteins in cultured hepatocytes during first 24 hours of incubation

Microsomal cytochromes were measured in 10,000 \(x\) g supernatant or in microsomes prepared from hepatocyte monolayers after 24 hours of incubation, either in control medium (see “Experimental Procedure”) or in medium supplemented with \(\delta\)-aminolevulinic acid (ALA). The values for nmol/mg of protein represent mean \(\pm\) S.D. The figures for percent change from zero time were calculated from measurement of cytochrome concentrations in freshly isolated cells from each experiment: in 10,000 \(x\) g supernatant, cytochrome P-450 was 0.140 \(\pm\) 0.052 nmol/mg of protein (mean \(\pm\) S.D., \(n = 14\)); in microsomes, cytochrome P-450 was 0.408 \(\pm\) 0.043 nmol/mg of protein (mean \(\pm\) S.D., \(n = 6\)) and cytochrome \(b_5\) was 0.234 \(\pm\) 0.0032 nmol/mg of protein (mean \(\pm\) S.D., \(n = 6\)).

| Incubation conditions | Cytochrome P-450 | Cytochrome \(b_5\) |
|-----------------------|------------------|-------------------|
|                       | nmol/mg protein  | Per cent of value at zero time | nmol/mg protein  | Per cent of value at zero time |
| 10,000 \(x\) g supernatant (\(n = 16\)) | | | | |
| Control medium        | 0.023 \(\pm\) 0.010 | 16.9 | 0.111 \(\pm\) 0.034 | 45.7 |
| + ALA (\(10^{-4}\) M)  | 0.040 \(\pm\) 0.017 | 29.8 | 0.106 \(\pm\) 0.027 | 43.6 |
| Microsomes (\(n = 3\)) | | | | |
| Control medium        | 0.048 \(\pm\) 0.025 | 11.8 | 0.106 \(\pm\) 0.037 | 24.5 |
| + ALA (\(10^{-4}\) M)  | 0.100 \(\pm\) 0.037 | 24.5 | 0.106 \(\pm\) 0.027 | 43.6 |

Fig. 1. Reduced CO difference spectra of 10,000 \(x\) g supernatants from cultured hepatocytes incubated in the presence of \(\delta\)-aminolevulinic acid (ALA). Isolated hepatocytes from a single rat liver were placed in L-16 culture medium containing various concentrations of \(\delta\)-aminolevulinic acid. After 24 hours of incubation, monolayers from two plates were pooled and homogenized and 10,000 \(x\) g supernatant was prepared. The dithionite-reduced CO difference spectrum was obtained. The average concentration of protein in the cuvette was 3.0 mg/ml. Quantitatively similar results were obtained in two repetitions of this experiment and in one experiment with microsomes. The concentrations of cytochrome P-450 (nanomoles mg per protein) were: ---, 0.015; ----, 0.035; ---, 0.044; --., 0.045.

Fig. 2. Effect of \(\delta\)-aminolevulinic acid (ALA) concentration on the reduced CO difference spectrum of 10,000 \(x\) g supernatant from cultured hepatocytes at 24 hours. Experimental conditions were as described for Fig. 1.

that increased breakdown accounts fully for the reduced amount of \([^{14}C]\)heme in the liver of cobalt-treated animals. Differences in precursor pool size are unlikely to account for these results, because the effect of cobalt treatment on \([^{14}C]\)heme breakdown and on hepatic \([^{14}C]\)heme content are reciprocal. These data obtained in intact animals are consistent with the observations in cell culture, that cobalt has little, if any, direct effect on the conversion of \(\delta\)-aminolevulinic acid to heme in the liver.

Discussion

Adult rat parenchymal cells in culture respond to the addition of \(\delta\)-aminolevulinic acid by increasing the synthesis of both heme and cytochrome P-450. By contrast, hepatocytes in vivo react to administration of \(\delta\)-aminolevulinic acid by increasing only the synthesis of heme (2). The reason for this discrepancy remains unexplained; nevertheless, the present hepatocyte culture system provides a way to examine the relationship between the formation of heme and cytochrome P-450 in intact liver cells. With increasing amounts of \(\delta\)-aminolevulinic acid (up to \(8 \times 10^{-4}\) M) added to the culture medium at the time of cell plating, the concentration of cytochrome P-450 after 24 hours of incubation is increased proportionately. Over this concentration range of \(\delta\)-aminolevulinic acid, cytochrome P 450 responds selectively,
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TABLE II

| 10^6 x concentration of ALA | Cobalt (10^-5 M) | Total cellular ^14C|heme |
|----------------------------|-----------------|-------------------|
| ^m                         |                 |                   |
| 3                         | - 486           |                   |
| + 552                     |                 |
| 6                         | - 812           |                   |
| + 874                     |                 |
| 9                         | - 1047          |                   |
| + 1050                    |                 |
| 12                        | - 1397          |                   |
| + 1405                    |                 |

*ALA, ^6-aminolevulinic acid.

Table III

| Experiment | Additions to the L-16 Medium | Cytochrome P-450 | AP | ANL |
|------------|-----------------------------|-----------------|----|-----|
|            | None                         |                 |    |     |
| 1          | ALA (1 x 10^-5 M)            | 100             | 100| 100 |
| 2          | ALA (1 x 10^-6 M)            | 140             | 134| 134 |

Table IV

Effect of cobalt chloride on formation of CO-binding hemoproteins in hepatocyte monolayers at 24 hours

Table V

Formation of cobalt-protoporphyrin in vitro

Mitochondria, prepared from a fed 200-g male Sprague-Dawley rat, were washed twice and then incubated at 37° in glycine buffer with protoporphyrin (0.028 mg/ml), and CoCl_2 (0.012 mg/ml) as outlined by Tephy et al. The final concentration of protein in this mixture was 1.0 mg/ml. After 0, 15, 30 min of incubation, 1 ml of this mixture was transferred to the experimental cuvette in the split beam spectrophotometer and 1 ml of an identical mixture incubated without cobalt was transferred to the reference cuvette. Cobalt was then added to the reference sample and the difference spectrum was immediately recorded (oxidized-oxidized (ox-ox)), to measure the increase in the absorption peak at 438 nm (isosbestic point = 560 nm). The experimental and reference cuvettes were then reduced with a few crystals of dithionite, and, after obtaining a base-line, the experimental sample was saturated with CO, and the new spectrum was recorded (CO/Reduced-Reduced (CO/Red-Red)).
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Isolated hepatocytes from a single rat liver were plated in L-16 medium containing 55Fe and cobaltous chloride, δ-aminolevulinic acid (ALA), or their combination. The final concentration of 55Fe in the medium in Experiment 1 was $1 \times 10^{-6}$ M (specific activity $= 11.4$ Ci/mg), and in Experiment 2 was $1 \times 10^{-7}$ M (specific activity $= 2.5$ Ci/mg). After 24 hours incubation, monolayers in each sample (six plates in Experiment 1, eight plates/sample in Experiment 2) were scraped, and pooled, and microsomes were prepared as described under "Experimental Procedure." Heme was then extracted from the microsomal pellet and counted.

TABLE VI

| Sample | Additions to L-16 Medium | Microsomal radioactivity in heme | Total recovered微somal protein |
|--------|--------------------------|---------------------------------|-----------------------------|
|        |                          | Experiment 1 | Experiment 2 | Experiment 1 | Experiment 2 |
| 1 None |                         | 13.9         | 4.36         | 10.56       | 15.58       |
| 2 CoCl₂ ($1 \times 10^{-3}$ M) | 2.01 | 4.41 | 10.01 | 15.25 |
| 3 ALA ($1 \times 10^{-3}$ M) | 9.63 | 17.02 | 10.98 | 15.99 |
| 4 CoCl₂ ($1 \times 10^{-5}$ M) + ALA ($1 \times 10^{-5}$ M) | 12.14 | 17.94 | 11.10 | 16.41 |

TABLE VII

| Time | Treatment | Homogenate | Microsomal | Total 55Fe in enzyme-treated microsomes |
|------|-----------|------------|------------|---------------------------------------|
| min  |           | [14C]heme | Cytochrome P-450 | dpm/mg protein | dpm/mg protein |
| 30   | Saline    | 149,360    | 0.78       | 4155       | 3443       |
|      | CoCl₂     | 90,688     | 0.76       | 2610       | 2211       |
| 60   | Saline    | 190,148    | 0.77       | 5700       | 4772       |
|      | CoCl₂     | 95,280     | 0.85       | 2840       | 2379       |
| 90   | Saline    | 268,800    | 0.82       | 6430       | 5357       |
|      | CoCl₂     | 110,400    | 0.48       | 2480       | 2291       |

of heme several times greater than that in cytochrome P-450. Also, cells cultured in control medium failed to exhibit an absorption band at 420 nm, despite substantial loss of cytochrome P-450 associated with the initial period of culture. For these reasons, we believe that the 420 nm absorption, observed after incubation of the cells with high concentrations of δ-aminolevulinic acid, does not represent "cytochrome P-420," i.e. denatured cytochrome P-450 (24), but rather reflects nonspecific binding of "excess" intracellular heme to microsomes (25).

The mechanism of the effect of δ-aminolevulinic acid on cytochrome P-450 in cultured hepatocytes cannot be stated with certainty. Heme from added δ-aminolevulinic acid may stimulate new formation of apocytochrome P-450. Alternatively, newly produced heme may combine with pre-existing apocytochrome to form the holoenzyme, a possibility suggested by recent studies in vitro (3, 4, 26). The experimental means for distinguishing between these two possible mechanisms is not currently available, since we lack direct methods for measuring the amount of apocytochrome in the cells. Moreover, inhibitors of protein synthesis, such as cycloheximide, provide no help in this regard, because they block expression of the culture-related perturbation of cytochrome P-450 and the response to δ-aminolevulinic acid.

Addition of cobalt to the culture medium completely blocked the increase in cytochrome P-450 which occurs in cells incubated with δ-aminolevulinic acid alone. This effect of cobalt in culture would be predicted, because cobalt inhibits induction of cytochrome P-450 by phenobarbital in vivo (27) and because the mechanism for inhibition of cytochrome formation by cobalt in vivo has been ascribed to blocked hepatic heme synthesis (5, 6). However, the metal clearly had no inhibitory effect on heme synthesis in the hepatocyte culture system. Therefore, we examined the previous evidence in support of the concept that cobalt blocks heme synthesis. We have confirmed observations which indicated that the metal inhibits heme synthesis in isolated mitochondria by causing formation of cobalt protoporphyrin (23). However, we have found no evidence that this metalloporphyrin is formed in cultured hepatocytes. Thus, the effect of cobalt on heme metabolism in isolated mitochondria appears to be irrelevant for events occurring in intact liver cells.

Preceding studies in intact rats also seemingly supported the postulate that cobalt blocks hepatic heme synthesis. After administration of a tracer pulse of δ-aminolevulinic acid, the liver of cobalt-treated animals was found to contain less labeled heme than the liver of control rats (5, 6). Although these data were interpreted as an inhibition of heme synthesis by cobalt, an effect of the metal on the breakdown of newly

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Fig. 4. After an overnight fast with free access to water, two 200-g male Sprague-Dawley rats were given either CoCl₂ dissolved in saline (60 mg/kg), by two 0.1-ml subcutaneous injections on either side of the back or equal volumes of isotonic saline. Six hours later each rat received 5 μCi of δ-aminolevulinic acid (5-14C-ALA) in 0.5 ml of saline intraperitoneally placed in a metabolic cage. The expired air was collected for sequential 30-min periods, and the CO₂ content was determined as outlined under "Experimental Procedure."
formed hepatic heme was not excluded. Since it is known that breakdown products of labeled heme (bilirubin or carbon monoxide) normally appear as early as 30 min after injection of \(\delta\)-amino\[^{14}\text{C}\]levulinic acid (28), it seemed important to re-examine the studies of \[^{14}\text{C}\]heme incorporation in cobalt-treated and control animals, with particular attention to degradation of newly labeled heme. When such studies were carried out, it was found that degradation of newly synthesized heme was strikingly accelerated in cobalt-treated animals (Fig. 4). Indeed, accelerated degradation may account entirely for the decreased content of \[^{14}\text{C}\]heme in the liver of these animals. These findings in \textit{vivo} corroborate the studies in \textit{culture}, with the conclusion that cobalt has little direct effect on synthesis of heme in the liver.

An unexpected finding in these studies was that incubation of hepatocytes with cobalt failed to reduce the basal level of cytochrome P-450 over and above the reduction associated with adaptation of cells to culture. By contrast, administration of cobalt \textit{in vivo} causes a marked drop in the level of cytochrome P-450 in the liver (5). A possible explanation for this discrepancy is that the conditions of cell culture may perturb hemoprotein metabolism in a manner similar to that produced by cobalt \textit{in vivo}. If this is correct, the effects of cobalt in the hepatocytes would not be additive with the culture-related perturbation under standard conditions of incubation. However, when the availability of endogenous heme for synthesis of cytochrome P-450 was increased by addition of \(\delta\)-aminolevulinic acid, the effect of cobalt in preventing new formation of cytochrome P-450 became apparent.

The present observations suggest a new concept for the effect of cobalt on the synthesis of cytochrome P-450. Not only does the metal fail to block heme synthesis, it also has no inhibitory effect on the synthesis of microsomal protein, either in cell culture or \textit{in vivo} (5), which suggests that synthesis of apocytochrome is unaffected by cobalt. Therefore, cobalt may act on a step in the formation of cytochrome P-450 which follows synthesis of the heme moiety, as for example, by blocking the translocation of heme from mitochondria to endoplasmic reticulum or by interfering with the association of heme and its specific receptor in the membrane (apocytochrome P-450). In hepatocytes cultured in the presence of \(\delta\)-aminolevulinic acid and cobalt, the reciprocal relationship of cytochrome P-450 and of the 420 nm complex (heme bound to apocytochrome) is unaffected by cobalt. Therefore, cobalt may act on a step in the formation of cytochrome P-450 which follows synthesis of the heme moiety, as for example, by blocking the translocation of heme from mitochondria to endoplasmic reticulum or by interfering with the association of heme and its specific receptor in the membrane (apocytochrome P-450). In hepatocytes cultured in the presence of \(\delta\)-aminolevulinic acid and cobalt, the reciprocal relationship of cytochrome P-450 and of the 420 nm complex (heme bound “nonspecifically” to microsomes) suggests that cobalt prevents the normal association of heme and apocytochrome P-450, displacing cytochrome P-450 heme. This mechanism would imply that cobalt administered \textit{in vivo} diverts heme destined for assembly with apocytochrome P 450, presumably into degradative channels. Consistent with this postulate is the fact that cobalt has been shown to stimulate hepatic heme oxygenase activity in the liver (29), and in the present studies, to accelerate heme breakdown.

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