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Biochemistry

Cobalt inhibition of synthesis and induction of δ-aminolevulinate synthase in liver

(trace metals/hepatic porphyria/hemoproteins/heme oxygenase/drug metabolism)

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ABSTRACT **Cobalt has complex actions on the metabolism of heme in the liver. In this organ the metal potently induces heme oxygenase (EC 1.14.99.3), and decreases cellular heme and hemoprotein content. The metal also displays biphasic effects on hepatic heme synthesis. These effects are reflected in the ability of cobalt to initially inhibit synthesis of δ-aminolevulinate synthase [succinyl-CoA:glycine *C*-succinyltransferase (decarboxylating), EC 2.3.1.37], the rate limiting enzyme of the heme pathway, following which a later enhanced rate of formation of this enzyme occurs. In this study, cobalt was shown to block almost entirely the ability of the barbiturate analogue allylisopropylacetamide to induce δ-aminolevulinate synthase in liver. The blocking effect of cobalt on the otherwise potent enzyme inducing action of this drug was time-dependent; if the metal was injected 30 min prior to allylisopropylacetamide, inhibition of enzyme induction was complete. When the metal was administered 1.5 or more hours after allylisopropylacetamide, inhibition of enzyme induction was incomplete. Cobalt did not block the ability of the drug to directly degrade heme to "green pigment"; thus the enzyme inducing action of allylisopropylacetamide and its degradative action on heme are separately mediated.**

The mechanism of early cobalt inhibition of δ-aminolevulinate synthase probably involves direct binding of the ionic metal to a regulatory site for this enzyme. It is suggested that the later increase in δ-aminolevulinate synthase formation is due to derepression of enzyme synthesis resulting from depletion of cellular heme content following metal induction of heme oxygenase.

Cobalt is a potent and rapidly acting inducer of heme oxygenase (EC 1.14.99.3) in liver and, concomitant with this enzyme-inducing action, the metal causes a profound depression of microsomal contents of heme and cytochrome *P*-450 (1–3). In addition, cobalt produces biphasic changes in δ-aminolevulinate synthase [ALAS, succinyl-CoA:glycine *C*-succinyltransferase (decarboxylating), EC 2.3.1.37] activity, with an initial depression of ALAS followed in several hours by a moderate increase in formation of the enzyme (2, 4).

A major class of drug inducers of hepatic ALAS in liver cells is represented by the barbiturate analogue allylisopropylacetamide (AIA). This drug not only induces high levels of ALAS, but also causes the production of a chemically undefined "green pigment" in liver cells (5, 6), an action which probably results from the direct breakdown of microsomal heme by an AIA metabolite, bypassing the usual route of heme oxidation and bile pigment formation.

The present report demonstrates that cobalt can markedly inhibit the ability of AIA to induce ALAS activity in liver cells without greatly altering the action of the drug in producing the hepatic "green pigment." The characteristics of the cobalt effect suggest that the trace metal blocks AIA induction of ALAS by interacting at the regulatory site that is involved in mediating the induction by the drug of this mitochondrial enzyme.

Abbreviations: ALAS, δ-aminolevulinate synthase; AIA, allylisopropylacetamide.

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MATERIALS AND METHODS

Male Sprague-Dawley rats, 120–160 g, were used. All chemicals were purchased from Sigma Chemical Co., St. Louis, Mo., unless otherwise indicated. Mesoheme IXα and mesoporphyrin IXα were prepared as described previously (7). Coproporphyrin III standard was purchased from Porphyrin Products Co., Logan, Utah. AIA was a gift of Hoffmann-La Roche, Inc., Nutley, N.J.

Rats were treated with AIA (400 mg/kg, in saline, subcutaneously) or cobaltous chloride (30 mg/kg, in saline, subcutaneously) according to the schedules indicated in the figures and table. Following sacrifice of the animals, the livers were perfused *in situ* with 0.9% NaCl until totally blanched, thereafter they were removed and homogenized in 0.05 M Tris·HCl, 0.25 M sucrose buffer (pH 7.4). Aliquots were taken for assays of ALAS (8), δ-aminolevulinate dehydratase (9), and ferrochelatase activities, and for measurements of total heme (10), protein (11), and porphyrins by fluorometric (12) and extraction techniques (13). Aliquots of the 10,000 × *g* supernatant were used for uroporphyrinogen I-synthase assays (14) and the remainder was used for the preparation of microsomal fractions in which the determinations of heme oxygenase activity (2), microsomal heme (10), cytochrome *P*-450 (13), NADPH-cytochrome *c* reductase (16), and ethylmorphine *N*-demethylation (7) activities were made.

Ferrochelatase activity was determined as follows: to 0.1 ml of homogenate suspension in Tris buffer (0.1 M, pH 7.8) containing 1 mM dithiothreitol (total volume, 1.2 ml), 0.1 ml of mesoporphyrin solution was added to a final concentration of 1 mM. The mesoporphyrin solution was prepared in KOH–methanol immediately before use. The tubes were sealed and a vacuum was established; then the atmosphere in the tubes was changed to nitrogen, followed by 2 min pre-incubation at 37°. Thereafter, 0.1 ml of ferrous citrate solution was injected into the tubes (final concentration, 50 mM). The tubes were then incubated for another 30 min. The reaction was stopped by addition of 0.5 ml of 0.1 mM iodoacetamide. The pyridine hemochromogen was measured following the addition of 1 ml of 1 M NaOH in 25% (vol/vol) pyridine. The extinction coefficient of mesoheme was measured following the addition of authentic mesoheme prepared as described earlier (7) to the enzyme preparation and the obtaining of the pyridine hemochromogen spectrum. The extinction coefficient of the absorption between 548 and 575 nm was 36.2 mM^{-1} cm^{-1}.

RESULTS

Fig. 1 shows the effect of cobalt pretreatment on the induction of ALAS by AIA. In agreement with previous data (17), 6 hr after injection of one dose of AIA, a substantial induction of

Table 1. Modifying effects of cobalt pretreatment on the allylisopropylacetamide-induced alterations and degradative pathways, and

Treatment	ALAD (nmol/mg·hr)	UROS (pmol/mg·hr)	Ferrochelatase (nmol/mg·hr)	Total porphyrin (nmol/mg)	Total heme (nmol/mg)
Control	6.06 ± 0.46	49.0 ± 5.0	4.30 ± 0.68	4.07 ± 0.30	6.15 ± 0.14
Co	5.54 ± 0.72	43.3 ± 3.9	6.14 ± 1.25	5.64 ± 0.34	5.62 ± 0.27
AIA	7.87 ± 0.35*	49.5 ± 5.0	6.12 ± 0.50*	13.72 ± 2.02*	4.29 ± 0.30*
AIA + Co	6.48 ± 1.06	48.8 ± 3.1	5.34 ± 0.65	5.06 ± 0.38	4.90 ± 0.30*

Rats were pretreated with cobalt and then with AIA as detailed in the caption to Fig. 1 and in *Materials and Methods.* Following the first injection the animals were starved. 12 hr after allylisopropylacetamide treatment, the animals were killed. The enzyme and other assays were carried out as described in *Materials and Methods.* The enzymatic activities are expressed as nmol of porphobilinogen produced/mg·hr for δ-aminolevulinate dehydratase (ALAD), pmol of coproporphyrin produced/mg·hr for uroporphyrinogen I synthase (UROS), nmol of cyto-

hepatic ALAS occurred and this elevated enzyme activity was maintained for at least 12 hr. With cobalt pretreatment (30 min), the induction of ALAS by the barbiturate was almost totally blocked at 6 hr. At 12 hr after injection of AIA + cobalt, there was an elevation of ALAS activity, which in degree exactly matched the late elevation of ALAS produced by cobalt alone that we demonstrated earlier (2, 4).

Table 1 shows the effect of cobalt alone, AIA alone, and cobalt + AIA on enzymes and products of the heme pathway and the mixed function oxidase system at 12 hr. Cobalt and AIA alone produced small decreases and increases, respectively, of δ-aminolevulinate dehydratase activity; the combined treatment had an intermediate effect. Hepatic uroporphyrinogen I synthase and ferrochelatase activities were not altered by these treatments; NADPH-cytochrome *c* reductase was increased significantly by AIA, but not by other treatments. After treatment with cobalt or with cobalt + AIA, liver porphyrin contents were consistent with the hepatic levels of ALAS activity produced by such treatments (Fig. 1), and, as expected, liver porphyrin content in animals treated with AIA alone was substantially elevated. Total heme contents of livers after all treatments were lower than in control animals, with AIA alone

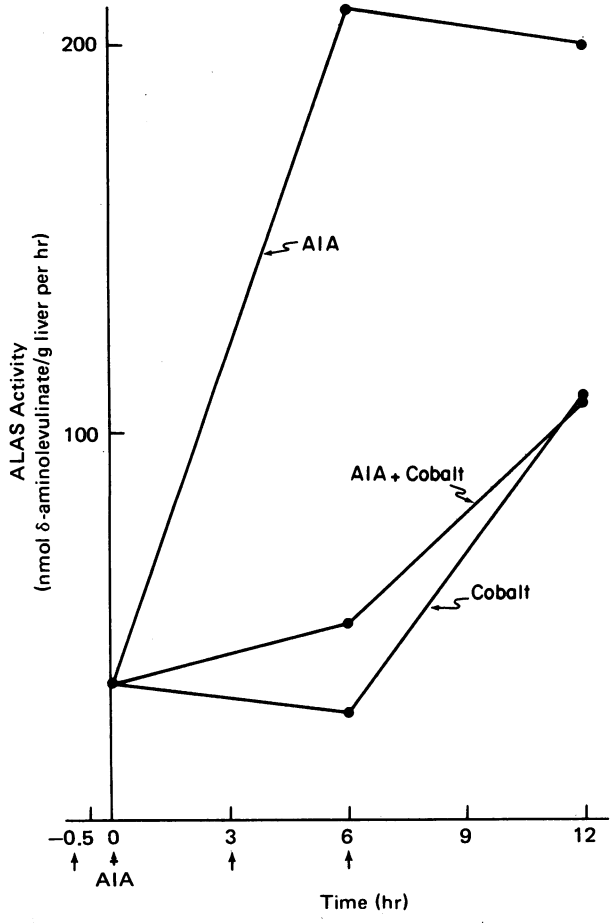

FIG. 1. Effect of cobalt pretreatment on the induction of ALAS by AIA. Fed rats were pretreated with cobalt 0.5 hr before AIA administration (↑). In order to maintain high plasma levels of the metal, the animals were injected twice more with cobalt, as indicated by the arrows. Following the first injection, food was withheld from the animals. At either 6 or 12 hr after AIA, the animals were killed and livers were perfused with 0.9% NaCl *in situ.* Whole-cell homogenate was used for the determination of δ-aminolevulinic acid synthase activity. Each point represents the mean value obtained for three rats.

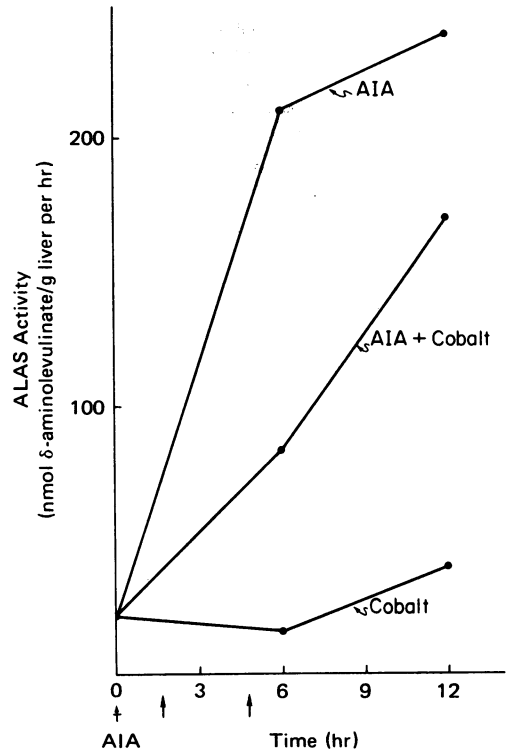

FIG. 2. Effect of delayed cobalt treatment on AIA induction of ALAS. Fed rats were treated with cobalt 1.5 hr subsequent to AIA treatment. The injection times of the metal (↑) and the AIA (‡) are indicated by arrows. The experimental details are indicated in the legend to Fig. 1 and in *Materials and Methods.* Each point represents the mean value obtained for three rats.

of hepatic heme and hemoprotein content, and activities of the enzymes of the heme biosynthetic some microsomal enzymes

Microsomal heme (nmol/mg)	Cytochrome *P*-450 (nmol/mg)	NADPH-cytochrome *c* reductase (nmol/mg·min)	Heme oxygenase (nmol/mg·hr)	Ethylmorphine *N*-demethylase (nmol/mg·hr)
1.21 ± 0.18	0.66 ± 0.04	140 ± 12	2.93 ± 0.56	150.2 ± 25.0
0.81 ± 0.14*	0.42 ± 0.08*	125 ± 9	23.55 ± 3.08*	96.3 ± 18.1*
0.64 ± 0.10*	0.17 ± 0.06*	185 ± 10*	1.81 ± 0.30*	25.0 ± 14.0*
0.78 ± 0.10*	0.24 ± 0.11*	134 ± 18	18.14 ± 2.04*	22.5 ± 14.8*

chrome *c* reduced/mg·min for NADPH-cytochrome *c* reductase, and of formaldehyde produced/mg·hr for ethylmorphine *N*-demethylase. All assays were carried out using the procedures described in *Materials and Methods.*
* $P < 0.05$ when compared with the control group.

producing the greatest decrease in this parameter. It is of interest that cobalt completely blocked the porphyrinogenic effect of AIA, but not its ability to lower cellular heme content; as indicated below, this reflects the ability of the metal to dissociate AIA induction of ALAS from AIA degradation of heme.

A close correspondence was found between the depressing effects of all treatments on microsomal heme and *P*-450 contents and on total cell heme content, except that with each treatment the decrease in microsomal heme was proportionally greater than that of total cellular heme. This suggests the susceptibility of microsomal hemoproteins to the direct degrading effect of AIA and the enhanced heme oxygenase activity caused by cobalt treatment (18). The changes in microsomal heme and *P*-450 contents were reflected in marked impairments of ethylmorphine *N*-demethylation, as expected.

As this table also shows, heme oxygenase activity 12 hr after treatment was substantially elevated in both cobalt- and AIA + cobalt-treated rats, with the latter group exhibiting a greater elevation of activity of this enzyme. As we showed previously (18) hepatic heme oxygenase activity in animals treated with AIA alone was significantly decreased.

Fig. 2 shows the effect of cobalt on AIA induction of ALAS when the metal was administered after the AIA treatment. As indicated, when cobalt was first injected 1.5 hr after AIA, it was not able to block fully the induction of the enzyme; and, in animals who first received cobalt 6 hr after AIA, nearly full inducing potency of the AIA was expressed (data not shown).

One well-known effect of AIA on the liver is the production of "green pigment" (5), which presumably results from direct nonenzymatic degradation of heme by the barbiturate. Fig. 3 shows the effects of cobalt + AIA or AIA alone on the spectrum of pigments extracted from the livers of rats treated with these agents. As shown in the solid line of Fig. 3A, the initial 15% HCl extract from the AIA-treated animals, as well as the second acid extract (broken line), showed absorption maxima at 409 nm (porphyrins) and at 423 nm ("green pigment"). As expected, the absorbance at 409 was greater in the initial extract (which contains most of the extractable porphyrin), while the 423-nm absorption peak was higher in the second extract (which contains the residual extractable "green pigment"). In contrast (Fig. 3B), when rats were treated with cobalt + AIA the total amount of porphyrin extracted was less than in rats treated with AIA

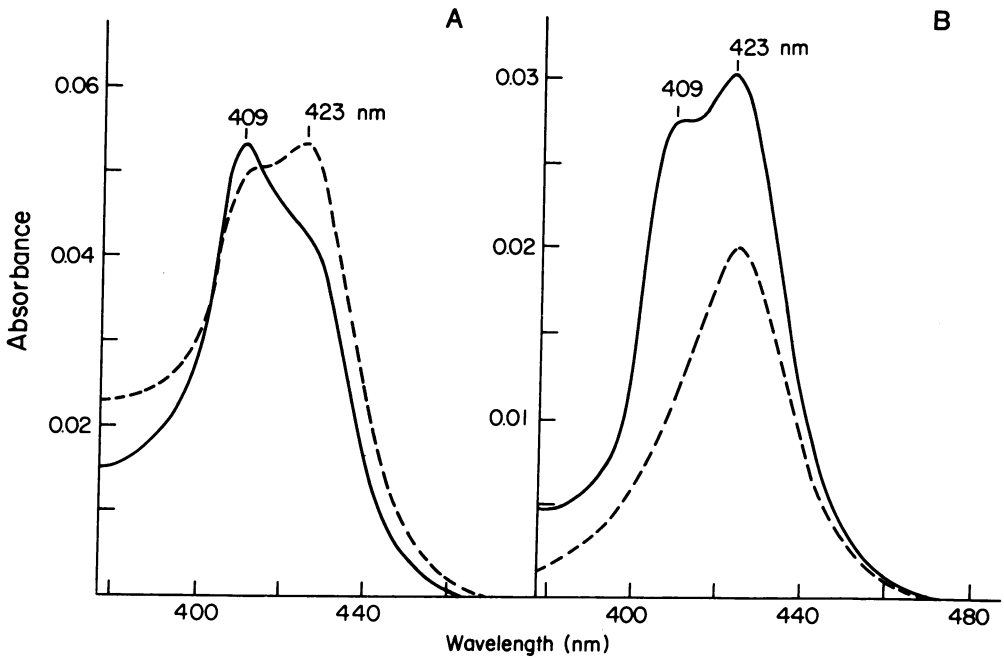

FIG. 3. Spectra of liver pigments. Liver homogenates from rats treated with AIA alone (A) or cobalt and AIA (B) as described in the legend to Fig. 1 were treated with sodium acetate:ethyl acetate (1:4) and the porphyrin and "green pigment" were extracted into 15% HCl (13). The solid lines depict the spectra of the initial HCl extract and the broken lines those of the second acid extract. The extracts were scanned in an Aminco DW2 spectrophotometer between 340 and 480 nm.

alone (absorbance scale in Fig. 3B is one-half that in Fig. 3A) and in the initial acid extract (solid line) the peak at 423 nm ("green pigment") was more prominent than that of the porphyrin peak at 409 nm. The second acid extract (broken line) exhibited no porphyrin absorbance peak at all, but only the "green pigment" peak; confirming an inhibition in these animals of porphyrinogenesis but not of the degradation of heme to "green pigment." Liver extracts from control animals or animals treated with cobalt alone showed only a porphyrin absorbance peak at 409 nm in the initial HCl extract and negligible amounts of absorbance at 409 nm in the second extract.

DISCUSSION

The present study reveals several new aspects of the action of the trace metal cobalt on heme metabolism in the liver. First, cobalt acts early after its administration to depress transiently the level of hepatic ALAS; second, the metal, when administered appropriately in time, showed an ability to block almost totally the potent ALAS induction action of AIA; and third, cobalt appeared to dissociate the ALAS-inducing properties of AIA from its ability to degrade heme directly to "green pigment," thus indicating that these effects of the barbiturate are unrelated and separately mediated.

The capacity of cobalt to block AIA action in the liver is striking in view of the great potency of this porphyrinogenic barbiturate analogue. Cobalt inhibition of AIA enhancement of ALAS formation is closely time-dependent; nearly complete blockade of the AIA induction effect occurred when the metal was administered 30 min before the drug. When the metal was first injected at 1.5 hr (Fig. 2) or more (data not shown) after AIA, the barbiturate retained a significant capacity to induce ALAS.

The precise mechanism through which cobalt inhibits AIA induction of ALAS is not known, but the fact that the drug continues to produce some "green pigment" in the liver even when its capacity to induce ALAS is blocked suggests that the metal does not complex with AIA directly. Moreover, the allyl group of AIA is the structural feature of the molecule which is presumed to be necessary for ALAS induction (19), and cobalt would not be expected to complex with this group. The ability of cobalt to induce concurrently hepatic heme oxygenase to high levels (Table 1) while blocking AIA induction of ALAS indicates that the metal is not acting as a general inhibitor of nucleic acid or protein synthesis, or as a cellular toxin. It also supports the idea that cobalt and AIA do not interact directly, since complexed cobalt has been shown in our earlier studies to lose its ability to induce heme oxygenase (20). Finally, the continued production of "green pigment" in the livers of AIA + cobalt treated animals indicates that the metal does not block access of the drug to liver cells.

The close time-dependence of cobalt blockade of AIA induction of ALAS suggests that the metal and the barbiturate may exert their actions on some common regulatory step for ALAS production in the liver. This regulatory step cannot involve a single binding site for both compounds as the molecular configurations of the two agents indicate. Cobalt blockade of AIA induction of ALAS could, however, result from some metal-induced alteration in or near the binding site for the highly electrophilic allyl group of the drug. This binding site for AIA might, for example, be rendered inactive by changes in electron potential resulting from an irreversible reaction with the metal (cobaltous → cobaltic); or cobalt could, by complexing in the proximity of this regulatory site, lead to electronic displacements or conformational changes which would hinder AIA binding.

The second component of the biphasic effect of cobalt on ALAS—the late rise in this enzyme activity—can be attributed to the high levels of hepatic heme oxygenase which the metal induces. As we have shown earlier (4) endogenous heme serves as the substrate for the microsomal heme oxygenase system. Therefore depletion of cellular heme brought about by the high levels of this enzyme activity would lead to "derepression" of the structural gene coding for ALAS and an increased rate of synthesis of the enzyme. At the time this enhanced ALAS formation occurs, the concentration of cobalt in liver cells is greatly reduced (21) and its inhibitory effects on the enzyme are thus removed.

The possibility that cobalt inhibition of hepatic ALAS synthesis is not due to the ionic metal *per se* but to the formation *in vivo* of cobalt heme, after administration of the metal, may also be considered. This mode of action of the metal would require that the putative cobalt-protoporphyrin complex formed *in vivo* be able to bind at iron-heme binding sites involved in the feedback regulation of ALAS production (22), and presumably that the cobalt heme formed also be subject to metabolic degradation by heme oxygenase. There are no unequivocal data bearing on these possibilities at the present time. However, the capacity of cobalt both to enhance heme degradation by inducing heme oxygenase and to inhibit the synthesis of ALAS, the limiting enzyme in the heme pathway, mimics the ability of heme itself to regulate these enzymatic parameters. These resemblances in biological actions of heme and of cobalt on heme metabolism in liver would be consistent with the formation of cobalt-heme *in vivo* or with the idea that iron derived specifically from the iron-protoporphyrin complex is involved in the physiological regulation of ALAS and heme oxygenase (4). In unpublished studies (M. D. Maines, P. Sinclair, and A. Kappas, manuscript in preparation), cobalt has been shown to block the ability of AIA to induce ALAS in avian liver cells growing in primary tissue cultures. Thus the effect of the metal is a direct one in the liver and does not involve obligatory alterations in other organs or metabolic systems.

It is evident from the findings reported here and in earlier studies from these laboratories (2, 4, 18) that cobalt has complex actions on enzymes and chemical intermediates related to heme and heme-proteins and to their synthesis and degradation in the liver. These combined actions of the trace metal result in profound depression of hepatic cytochrome *P*-450 content and thus of *P*-450 dependent enzyme reactions involved in drug and foreign chemical biotransformations.

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