EVIDENCE FOR THE CATABOLISM OF POLYCHLORINATED BIPHENYL-INDUCED CYTOCHROME P-448 BY MICROSOMAL HEME OXYGENASE, AND THE INHIBITION OF δ-AMINOLEVULINATE DEHYDRATASE BY POLYCHLORINATED BIPHENYLS

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Cobalt(ous) ion regulates cellular heme metabolism in the intact animal (1–7) as well as in cultured hepatocytes[1] through effects on the activities of microsomal heme oxygenase and on the mitochondrial enzyme δ-aminolevulinate synthetase (ALAS).[2] The metal effects on the latter enzyme result in an initial inhibition of ALAS activity which is then followed by induction of the enzyme (2, 5). The effect of cobalt on heme oxygenase, the enzyme that catalyzes degradation of heme compounds, is only that of induction (1–7 and footnote 2).

In the course of a number of studies we have noted that concomitant with the induction of hepatic heme oxygenase by cobalt there is a marked decrease in microsomal heme and cytochrome P-450 contents and prototypic P-450-dependent drug-metabolizing enzyme activities. These observations have led us to propose that the increased activity of heme oxygenase is responsible for the decrease in the cellular content of cytochrome P-450 and consequently the decrease in drug oxidative activities (3, 6, 8). Studies with the isotopically labeled heme precursor δ-aminolevulinate (ALA) have confirmed this idea, and supportive evidence for our proposal has also been recently presented by Guzelian and Bissell (9).

The tumorigenic agents, polychlorinated biphenyls (PCB) cause an induction of hepatic ALAS (10) and are potent porphyrinogenic compounds (11); as Alvares et al. have shown they also simultaneously cause the induction (12) of the microsomal CO-binding hemoprotein cytochrome P-448 or P$_1$-450 (13) having catalytic properties intermediate between those produced by the phenobarbital and the 3-methylcholanthrene (3-MC) or carcinogen types of inducers. Recently we have shown in cultured avian hepatocytes that the porphyrinogenic effects of several potent porphyria-inducing compounds such as allylisopropylacetamide (AIA), 3,5-dicarbethoxy-1,4-dihydrocollidine (DDC), and the 5β-steroid etiocholanolone can be blocked by pretreatment with cobalt chloride.[1] Moreover, when

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[1] Maines, M. D., P. Sinclair, and A. Kappas. 1976. Cobalt regulation of heme synthesis and degradation in avian liver cell culture. J. Biol. Chem. In press.

[2] Abbreviations used in this paper: AIA, allylisopropylacetamide; ALA, δ-aminolevulinate; ALAD, δ-aminolevulinic acid dehydratase; ALAS, δ-aminolevulinate synthetase; DDC, 3,5-dicarbethoxy-1,4-dihydrocollidine; PCB, polychlorinated biphenyls.

intact animals were pretreated with cobalt the metal was also able to block the induction of microsomal cytochrome P-450 by phenobarbital (3) in the liver.

In the present study the ability of cobalt to alter both the porphyrinogenic activity of PCB as well as the cytochrome P-448-inducing property of these agents was investigated. In addition, since the heme moiety of the microsomal hemoprotein cytochrome P-450 is known (8) to be metabolized by heme oxygenase, the possibility of degradation of PCB-induced cytochrome P-448 by this enzyme was also investigated. Finally, a new property of PCB was identified in its ability to depress hepatic activity of δ-aminolevulinate dehydratase (ALAD), the second enzyme in the heme biosynthetic pathway.

Materials and Methods

Materials. Male Sprague-Dawley rats (170–185 g) were used throughout the study. All chemicals were purchased from Sigma Chemical Co., St. Louis, Mo. The PCB, Aroclor 1254, was a gift of the Monsanto Corp., St. Louis, Mo.

Treatment of Animals and Tissue Preparation. Rats were injected subcutaneously once with 25 mg/kg of PCB in corn oil. Cobalt(ous) chloride (250 μmol/kg in saline) was also injected subcutaneously 30 min before the injection of PCB. Control animals received corresponding volumes of corn oil or saline as required. In another series of experiments, the animals were treated with PCB for 24 h, then were injected with the above dose of cobalt, and were killed 24 h later. The livers and kidneys were perfused in situ with 0.9% NaCl. The livers were homogenized in 0.05 M Tris-HCl (0.1 M) buffer pH 7.4 containing 0.25 M sucrose. Aliquots of homogenate were removed for the assays of ALAS, δ-aminolevulinic acid dehydratase (ALAD), and total porphyrin content. The remainder was used for the preparation of the microsomal fractions as described previously (14). The kidneys were homogenized in potassium phosphate buffer, 0.1 M, pH 7.4, and microsomal fractions were prepared. The liver supernatant fraction was used as the source of biliverdin reductase for the assay of heme oxygenase both in the liver and the kidney.

Assays. The activity of ALAS was measured as described by Marver et al. (15) and that of ALAD by the method of Mauzerall and Granick (16) using dithiotriotol as the thiol activator. Total porphyrins were measured by the fluorometric method of Sassa and Granick (17) in a Hitachi Perkin-Elmer MPF-3 fluorescence spectrophotometer utilizing the extraction mixture of 1 N perchloric acid:methanol (1:1, vol/vol).

Microsomal content of the CO-binding hemoproteins was measured by the method of Omura and Sato (18). Total microsomal heme content was measured by the method of Paul et al. (19). Microsomal ethylmorphine N-demethylase activity was determined in the incubation medium described previously (14), measuring the amount of formaldehyde formed by the method of Nash (20). Microsomal heme oxygenase activity was assayed as described earlier (2) with the incubation being carried out aerobically in the dark. Protein content was determined by the method of Lowry et al. (21). The Student's "t" test was used as the means of statistical evaluation of the data, and the $P < 0.05$ denoted statistical significance.

Results

Fig. 1 shows the effect of cobalt and the combination of pretreatment with cobalt and one injection of PCB on the enzymes that catalyze the first and second steps in the heme biosynthetic pathway, ALAS and ALAD, as well as the cellular porphyrin content. After 24 h, cobalt alone produced a 50% increase in ALAS activity and simultaneously inhibited ALAD activity to 50% of the control value. There was a slight increase in the cellular porphyrin content. PCB by itself produced an increase in ALAS which was of the same magnitude as that produced by cobalt alone; in addition there was also a slight increase in hepatic porphyrin content. PCB treatment resulted in a significant inhibition of the ALAD activity. The combination treatment with cobalt and PCB had a marked
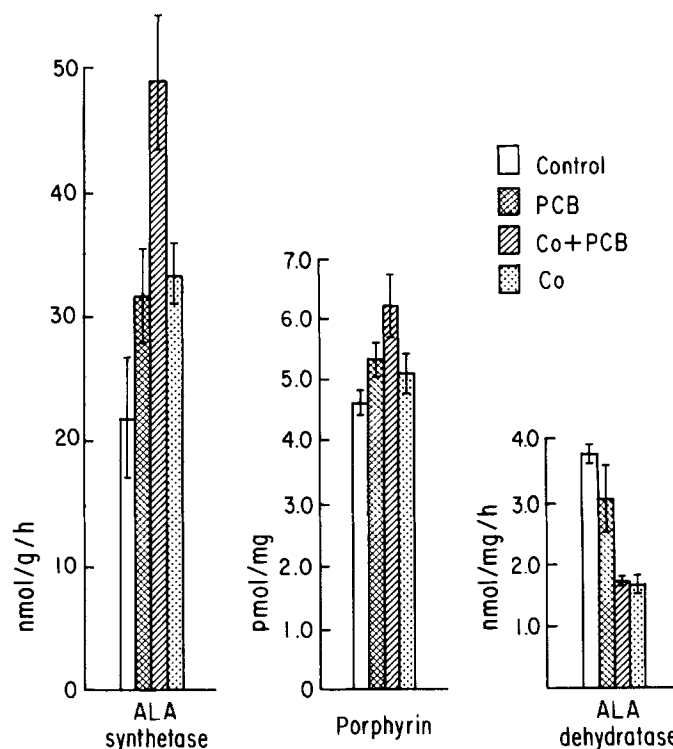
Fig. 1. Cobalt effect on alterations mediated by PCB in activities of the enzymes of heme biosynthetic pathway and porphyrin content in the liver. Rats were pretreated with cobalt (250 μg/kg, s.c.) and 30 min later treated with PCBs (25 mg/kg, s.c.). 24-h later the animals were killed. Assays were carried out as described in the Materials and Methods section.

synergistic inductive effect on ALAS activity and increased the total cell porphyrin content as well. These values were both significantly greater than those produced by PCB or cobalt alone. ALAD activity was also depressed by the combination treatment, but this enzyme depression was not significantly different from that observed with cobalt treatment alone.

Fig. 2 shows the effects of the above treatments on microsomal enzymic parameters. Consistent with our previous findings (1-6) the microsomal contents of CO-binding hemoprotein P-450 and heme and ethylmorphine N-demethylation activity were decreased by cobalt treatment to the extent of 60-70%, and there was a 10-14-fold increase in microsomal heme oxygenase activity. PCB alone produced a nearly 300% increase in microsomal CO-binding hemoprotein content with the hemoprotein being of the cytochrome P-448 type as earlier described by Alvares et al. (12). There also was a major increase produced by PCB in microsomal heme content and a similar increase in microsomal ethylmorphine N-demethylase activity. Microsomal heme oxygenase activity was somewhat increased in these animals, a response which may be due to the secondary effect of treatment, since it is known that starvation induces heme oxygenase activity. The refusal to consume food and the weight loss which was noted in these animals were most likely responsible for this slight increase in heme oxygenase activity. When cobalt was administered 30 min before the PCB
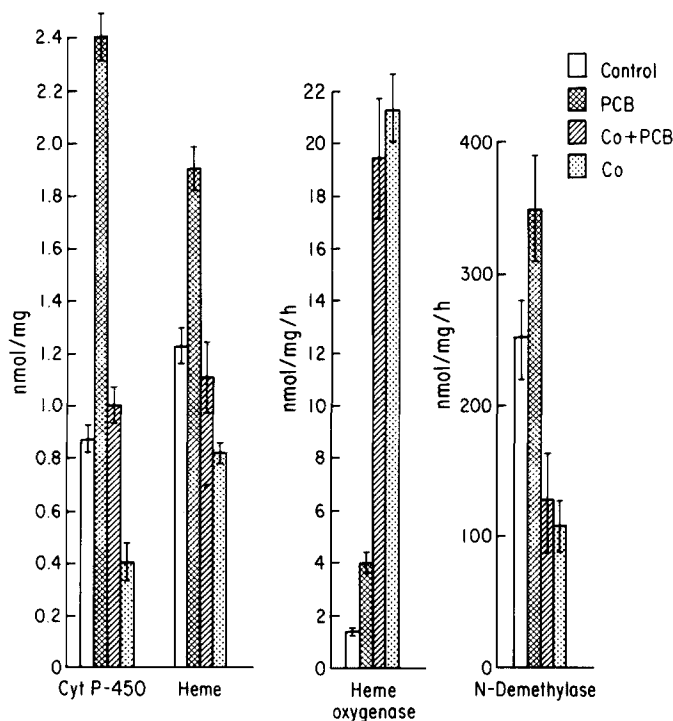
FIG. 2. Cobalt effect on alterations mediated by PCB in hepatic microsomal enzyme activities and heme and hemoprotein contents. Hepatic microsomal fractions were prepared from rats treated as described in the legend in Fig. 1. The hepatic heme and hemoprotein content, and the oxidative activities of the microsomal fractions for heme and for ethylmorphine, were measured as described in the Materials and Methods section. Microsomal heme oxygenase activity is expressed as the nanomoles of bilirubin produced per milligram protein per hour and that of ethylmorphine N-demethylase activity is expressed as the nanomoles of formaldehyde produced per milligram protein per hour. Cytochrome P-450 refers to the CO-binding hemoproteins, P-450 and P_1-450 (448).

injection, the metal completely inhibited the induction of CO-binding hemoprotein and of microsomal heme produced by PCB. However, microsomal drug metabolizing activity, as measured by the rate of ethylmorphine N-demethylation, decreased to less than 50% of that in the control animals. The induction of hepatic heme oxygenase activity by cobalt was not altered in the presence of PCB (Fig. 2). Fig. 3 shows the effect of two (24-h intervals) sequential injections of PCB alone on hepatic ALAD activity. PCB potently inhibited ALAD, reducing this enzymic activity by 50% in 48 h.

The absorption spectrum of the CO-binding hemoprotein from the livers of animals treated with cobalt alone exhibited an absorption maximum at 450 nm, the same as in control animals. However, the absorption spectrum of microsomes from the cobalt plus PCB-treated animals exhibited an absorption maximum at 448 nm as reported for PCB treatment alone (12).

In another set of experiments the sequence of administration of the metal and of PCB was reversed. Animals were pretreated with PCB for 24 h before cobalt administration, in order to increase the hepatic content of microsomal cyto-
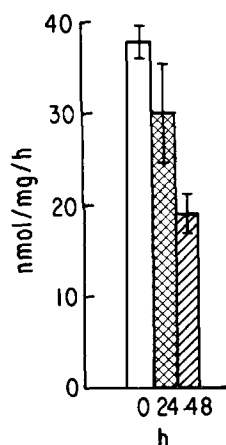
FIG. 3. Inhibition of hepatic δ-aminolevulinate dehydratase by PCB. Rats were injected subcutaneously once or two times with 25 mg/kg of Aroclor 1254. They were killed 24 h after the last injection, and ALAD activity was measured in the whole homogenate as described in the Materials and Methods section.

chrome P-448 before heme oxygenase was induced. 24 h later the animals were killed. As Table I shows, cobalt administered after PCB treatment caused a substantial inhibition of the induction of ALAS elicited by PCB. This was in contrast to the synergistic inductive effect produced when cobalt was administered before PCB. The sequence of treatment had no effect on the inhibition of ALAD which was produced by both compounds (data not shown). PCB significantly induced hepatic microsomal hemoprotein (P-448 type) and heme contents, as noted earlier (12). Cobalt administration to PCB-pretreated animals markedly diminished microsomal hemoprotein content and substantially decreased total microsomal heme and ethylmorphine N-demethylase activity. The sequence of cobalt and PCB treatments did not alter the level of heme oxygenase activity, and this enzyme was as highly induced in these experiments as in the experiments shown in Fig. 2. Cobalt administration after PCB treatment shifted the PCB-induced microsomal hemoprotein absorption peak to a wavelength somewhat longer than 448 nm in these animals.

The effects of PCB alone, and of PCB on cobalt-induced changes in renal heme metabolic activity were also examined. The heme oxygenase system in kidney is highly responsive to induction by trace metals such as cobalt, tin, and related transitional elements (2, 6, 22); and concomitant with the alterations of this enzyme activity, there is a decrease not only in renal heme and cytoplasmic hemoprotein contents and drug oxidative ability, but also in the contents of mitochondrial cytochromes involved in oxidative phosphorylation. PCB, as shown in Fig. 4, only increased renal heme content slightly as compared with liver (Fig. 2) and did not alter heme oxygenase activity at all. Cobalt substantially enhanced renal levels of heme oxygenase (Fig. 4), but the administration of PCB 30 min after cobalt administration almost entirely blocked the ability of the metal to induce heme oxygenase. PCB simultaneously prevented the decrease in renal heme content produced by the metal.

TABLE I

Effect of Cobalt on Various Cellular Heme-Related Parameters in PCB-Pretreated Rats

Treatment	ALA synthetase	Microsomal heme	Cytochrome P-450*	Ethylmorphine N-demethylase
	nmol/g per h	*nmol/mg*	*nmol/mg*	*nmol/mg per h*
Control	22.6	1.32	0.79	245.6
PCB	58.5	2.12	1.81	275.2
PCB + Co	38.1	0.86	0.39	101.1
Co	47.1	0.95	0.35	60.4

Rats were treated with PCBs (25 mg/kg, in corn oil, i.p.). 24-h later the animals were treated with cobalt (60 mg/kg, s.c.), they were then killed 24 h later. The enzyme determinations were made on appropriate cellular fractions as described in the Materials and Methods section.
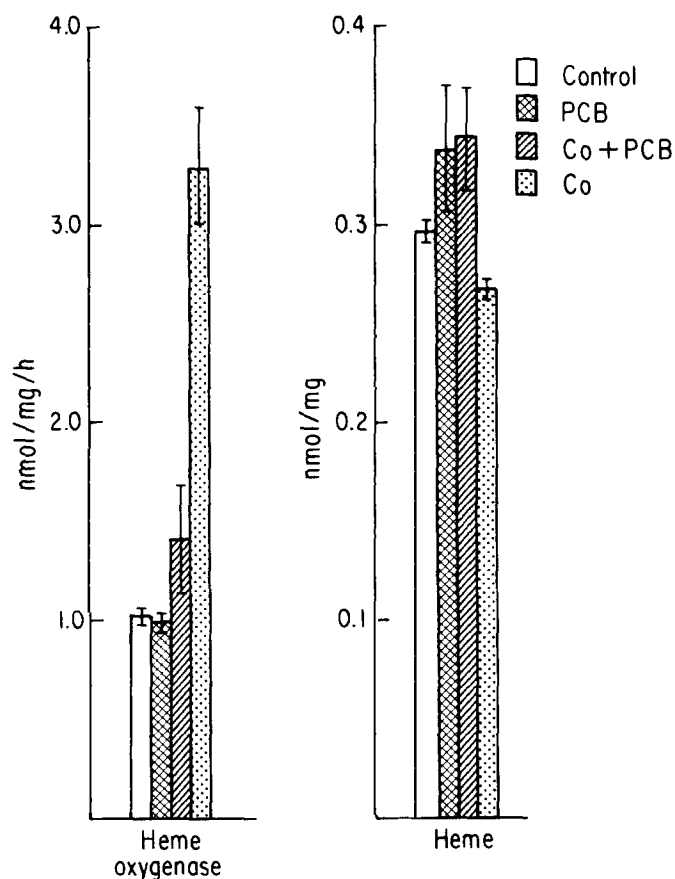* CO-binding hemoprotein cytochrome P-450 and P_1-450 (448).

FIG. 4. Effect of PCB on renal microsomal heme oxygenase activity and microsomal heme content and on the influence of cobalt on these parameters. The kidney of rats treated as in the legend of Fig. 1 were utilized for the preparation of the microsomal fractions in which heme oxygenase activity and heme content were measured. Heme oxygenase activity is expressed as the nanomoles of bilirubin formed per milligram protein per hour.

Discussion

It has been demonstrated that cobalt (1–7), as well as other transition elements and heavy metals (3, 4, 6, 22), are potent regulators of cellular heme metabolism. Cobalt is a highly active inducer of heme oxygenase, the microsomal enzyme which degrades heme; it also regulates heme synthesis through its biphasic (inhibition followed by induction) actions on the activity of ALAS (2, 5, 8), the rate-limiting enzyme in the heme biosynthetic pathway (23). Furthermore, as shown here, cobalt can also significantly inhibit ALAD, the second enzyme in the heme biosynthetic sequence. One consequence of the cobalt effect on heme synthetic activity is the modifying influence which this metal exerts on hepatic cell responses to porphyrinogenic compounds (5 and footnote 1). Recently, we have shown that in whole animals (5), as well as in cultured avian hepatocytes,[1] pretreatment with cobalt was an extremely effective means of blocking the potent porphyrinogenic action of compounds such as AIA, DDC, and 5β-steroids. Also cobalt inhibits the induction of microsomal cytochrome P-450 produced by barbiturates (3, 24). PCB are known porphyrinogens which manifest their effects mainly through the induction of ALAS as well as the inhibition of uroporphyrinogen decarboxylase activities (11). However, the PCB possess a property that is quite distinct from that of other porphyrinogenic compounds – this is their ability to cause the induction of a novel type of hepatic microsomal hemoprotein, cytochrome P-448 (12). This cytochrome, which is also induced by polycyclic hydrocarbon carcinogens, is distinct from the native CO-binding hemoprotein P-450 not only in its spectral properties but in its catalytic activities as well (25).

Previous studies have led us to the conclusion that the microsomal hemoproteins are degraded in vivo by the action of cellular heme oxygenase system (3, 6, 8). However, these studies were limited to experiments with hepatocytes containing only cytochrome P-450. The unique capacity of the PCB both to alter heme synthesis, as well as to induce cytochrome P-448, provided the opportunity to examine the ability of cobalt to modify the effects of PCB on heme synthesis and to study the possibility of degradation of microsomal hemoprotein of the P-448 type by the heme oxygenase system.

The data presented in this report indicate that cytochrome P-448 is indeed degraded by the microsomal heme oxygenase system. As Fig. 2 shows the cellular contents of heme and PCB-induced cytochrome P-448 in cobalt-pretreated animals were greatly reduced in comparison to the animals treated with PCB alone. Since heme production, as judged by the activity of ALAS and the total porphyrin content, was not impaired in the cobalt-pretreated animals (Fig. 1), the only plausible explanation for the depletion of cytochrome P-448 (Fig. 2) is the presence of highly elevated heme oxygenase activity in the cobalt-pretreated animals. This conclusion is further supported by the finding (Table I) that when cobalt was administered to animals in which the hemoprotein content had already been elevated by prior treatment with PCB, the microsomal hemoprotein content was subsequently greatly reduced. The degradation of pre-existing heme by the rapidly increasing heme oxygenase activity elicited by cobalt most likely was the explanation for the heme and hemoprotein depletions observed in this experiment. Thus it can be concluded that cytochrome P-448 formed after cobalt treatment, as well as the P-448 formed before cobalt treat-

ment, are both degraded by the microsomal heme oxygenase system, as is the case with cytochrome P-450 (6, 8).

In earlier studies it was proposed that an initial step in the oxidative degradation of cytochrome P-450 heme involves its endogenous conversion to the catalytically inactive P-420 form, a species of the cytochrome in which the heme moiety is especially labile, as earlier studies of Maines and co-workers have shown (26, 27). The lability of the P-420 heme moiety permits its transfer and binding to the heme oxygenase complex in microsomes, where its oxidative metabolism takes place.

The results of the studies reported in Fig. 1 and Table I established that the heme of cytochrome P-448 is also metabolized by cobalt-induced microsomal heme oxygenase. It is not clear whether the P-448 species of microsomal hemoprotein undergoes transition to the P-420 form before serving as substrate for heme oxygenase as does P-450, but it is not unreasonable to believe that this is the case. Thus cytochrome P-420 may be considered to be the species through which all types of cytochrome P-450's are transformed before metabolic degradation of their hemes to biliverdin and bilirubin.

The time-dependent relationship between the actions of cobalt and PCB on the activity of ALAS (Fig. 1 vs. Table I) suggests that the porphyrinogen and the metal exert their effects on some common regulatory site for ALAS in hepatocytes. Cobalt administration before PCB synergized the inductive effect of PCB on ALAS activity. This synergism is probably composed of two separate components. One is the direct induction of ALAS by PCB and the other is the secondary induction of ALAS produced by cobalt. As was noted cobalt regulates heme synthesis in a biphasic manner (initial inhibition of ALAS followed by induction of the enzyme [2, 5, 8]). The initial inhibition of the enzyme is due to a direct action of the metal on ALAS synthesis; the secondary increase of ALAS results from the derepression of enzyme synthesis which follows depletion of cellular heme brought about by the increase in activity of heme oxygenase (5). ALAS formation is known to be regulated by cellular heme content (23); since the latter is determined by microsomal heme oxygenase activity, a regulatory relationship between this enzyme and ALAS must exist.

The finding that cobalt administered after PCB treatment did not synergize the inductive effect of PCB on ALAS reflects the direct inhibitory effect of cobalt on ALAS production. In this instance it can be postulated that cobalt directly inhibited PCB-induced formation of ALAS; however, as the cellular content of heme was depleted the derepression of the enzyme, as was detailed above, would cause the induction of ALAS which in this experiment was of the same magnitude as that observed with cobalt alone.

The differing effects of PCB on the heme synthesizing activity of liver and kidney are of interest with respect to control of the activity of this pathway in these organs. As Fig. 3 shows, PCB in the kidney did not produce a major increase in cellular content of heme, in contrast to the effect of PCB on this parameter in hepatocytes. This finding is consistent with other evidence which indicates that the mechanism by which heme synthesis is regulated in these two tissues is quite different.[3]

Finally, it is apparent from the inhibition of ALAD activity reported here

(Fig. 3) that PCB alter enzymic activities in the heme pathway at more sites than previously recognized. The finding that the activity of hepatic ALAD is inhibited by PCB and is a time- and dose-dependent phenomenon is novel and would explain the excessive urinary excretion of ALA in PCB poisoning.

Summary

Polychlorinated biphenyls (PCB) are potent inducers of hepatic microsomal CO-binding hemoprotein P-448 (P_1-450) and of δ-aminolevulinate synthetase (ALAS) activity. Inorganic cobalt was able to block PCB induction of cytochrome P-448 and to modify the PCB effect on ALAS activity in a time-dependent manner. PCB were also found to decrease the activity of δ-aminolevulinic acid dehydratase (ALAD) in liver.

Pretreatment of rats with cobalt (30 min) produced the following changes in PCB actions on heme metabolism in liver: (a) augmentation of the porphyrinogenic effect of PCB, as determined by the total porphyrin content and ALAS activity; (b) augmentation of PCB inhibition of ALAD activity; and (c) blockade of induction of microsomal hemoprotein (cytochrome P-448). PCB did not interfere with cobalt induction of hepatic heme oxygenase activity.

The sequence of administration of the metal and the PCB was important in relation to the changes produced in hepatic ALAS activity and microsomal hemoprotein and heme contents. When cobalt was administered 24 h after PCB treatment, the magnitude of induction of ALAS by PCB was lowered, and there was a great reduction in microsomal hemoprotein and heme contents. The renal response to PCB was different than that of the liver. In the kidney, PCB blocked the induction of heme oxygenase and depletion of cellular heme produced by cobalt. Furthermore, renal microsomal heme content was increased by PCB treatment alone or in combination with cobalt.

It is concluded that (a) the heme moiety of microsomal cytochrome P-448 is metabolized by the heme oxygenase system, and it is suggested that for this catabolism to take place, the hemoprotein must be first converted to the denatured form of the hemoprotein, cytochrome P-420; (b) that the synthesis of heme in the kidney and the liver are regulated through different mechanisms; and (c) that ionic cobalt controls activity of ALAS by first inhibiting synthesis of the enzyme followed by the indirect induction of the enzyme as a result of the catabolism of heme, the physiological repressor of ALAS, by the metal-induced heme oxygenase. Thus microsomal heme oxygenase may be viewed as having an overall regulatory role in relation to mictochondrial ALAS by virtue of its ability to catabolize endogenous heme.

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