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Effects of Cadmium on Microsomal Hemoproteins and Heme Oxygenase in Rat Liver

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SUMMARY

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Various parameters of drug metabolism were measured in rats following a single intraperitoneal injection of cadmium acetate dihydrate (2.0 mg/kg; 7.5 μmoles/kg). Three days after treatment with Cd^{2+}, the hexobarbital-induced sleeping time was increased to 240% of control; the microsomal contents of cytochromes P-450 and b_5 were decreased by 44% and 27%, respectively. Seven days after treatment, the contents of cytochromes P-450 and b_5 had partially returned to normal but each was 15-20% below control levels. Aminopyrine demethylase activity in hepatic microsomes was decreased by 47% and 37% at 3 and 7 days, respectively, after treatment with Cd^{2+}; aniline hydroxylase was decreased by 32% and 23% at 3 and 7 days, respectively. Cadmium, given 3 days prior to injection of [^{3}H]δ-aminolevulinic acid, decreased the half-life of the heme in CO-binding particles (microsomes devoid of cytochrome b_5) from 8 hr to less than 2.5 hr in the fast-phase component, and from 60 hr to 32 hr in the slow-phase component. The greatest increase in microsomal heme oxygenase (to 350% of control) occurred 36-48 hr after treatment with Cd^{2+}, and the enzymatic activity returned essentially to normal at 7 days. The activity of biliverdin reductase of the cytosol was not altered 3 or 7 days after treatment with Cd^{2+}.

INTRODUCTION

The acute toxicity of cadmium in experimental animals has been the subject of recent studies (1, 2). One of the manifestations of toxicity is the depression of liver cytochrome P-450 (1). This hemoprotein is the terminal oxidase of the mixed-function oxidase system involved in the hepatic biotransformation of hormones, medicinal agents, and environmental chemicals. Such a decrease could be the result of an alteration in synthesis and/or degradation of the hemoprotein. Cobalt, tin (3, 4), and other transition metals (5) cause dramatic increases in the activity of heme oxygenase, a microsomal enzyme involved in the degradation of heme. However, the biological implications of changes in the activity of heme oxygenase and its possible role in the degradation of cytochrome P-450 and other hemoproteins are not clear.

In this paper we report on the effects of cadmium on the turnover of radioactive CO-binding particles (cytochrome P-450)

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and the temporal relationship between the increased activity of heme oxygenase and the depression in hemoproteins in rat liver microsomes. Preliminary reports of these studies have been presented (6, 7).

MATERIALS AND METHODS

Animals and treatment. Male Sprague-Dawley rats (200–250 g), obtained from ARS/Sprague-Dawley, were used in all experiments. Cadmium acetate dihydrate (Fisher), administered intraperitoneally as a single injection, was given at a dosage of 2 mg/kg of animal weight (7.5 μmoles/kg) in all experiments except for some studies on hexobarbital-induced sleeping times. Control animals received a corresponding volume of 0.9% NaCl. Animals were pair-fed with Purina laboratory chow following cadmium treatment; water was supplied ad libitum. The LD_{50} for cadmium acetate dihydrate administered intraperitoneally was determined by the method of Litchfield and Wilcoxon (8). Survival of animals was followed for a 14-day observation period. In studies of hexobarbital-induced sleeping time, sodium hexobarbital (Sterling) (100 mg/kg) was administered intraperitoneally 3 days following an injection of cadmium acetate dihydrate.

Subcellular fractions. Animals were killed by cervical dislocation. After perfusion with 1.15%KCl, livers were homogenized in 0.15 M KCl–50 mM Tris-HCl, pH 7.7, at 5°[3] (4 g of liver per 8 ml of buffer), using a Potter-Elvehjem homogenizer. The homogenate was centrifuged for 10 min at 24,000 × g_{max} in a Sorvall RC2-B centrifuge with SS-34 rotor. The resulting supernatant was then centrifuged for 30 min at 159,000 × g_{av} in a Beckman-Spinco centrifuge with type 65 or 50 rotor. The microsomal pellet was resuspended in Tris-HCl, pH 7.7, at 5°[3] and recentrifuged for 30 min at 159,000 × g_{av}.

Aminopyrine demethylase and aniline hydroxylase. The washed microsomes were resuspended in 0.1 M Tris-HCl, pH 7.7, at 5° for transfer to incubation mixtures (final pH, 7.6–7.7 at 37°). Glucose 6-phosphate, glucose 6-phosphate dehydrogenase (type XV), and NADP (all from Sigma) were used as the NADPH-generating system. Aminopyrine demethylase was measured (at 37° and 1.5–2.0 mg of microsomal protein per milliliter) by the formation of formaldehyde (Nash reaction) following a modified procedure of Dewaide and Henderson (9). Aniline hydroxylase was measured (at 37° and 1.5–2.0 mg of microsomal protein per milliliter) by the procedure of Imai *et al.* (10), modified by the addition of $HgCl_2$(11).

Cytochrome P-450 and cytochrome b_5. Microsomal cytochrome P-450 was analyzed by measuring the dithionite-reduced carbon monoxide difference spectrum (450 nm − 490 nm, ϵ = 91 mM^{-1} cm^{-1}) (12). Cytochrome b_5 was analyzed by measuring the NADH-reduced difference spectrum (424 nm − 409 nm, ϵ = 185 mM^{-1} cm^{-1}) (13).

Labeled cytochrome P-450 from CO-binding particles. Rats were injected intraperitoneally with [3,5-^{3}H(N)]δ-aminolevulinic acid (5.0 Ci/mmole; New England Nuclear) which had been diluted with unlabeled δ-aminolevulinic acid (Aldrich) (final specific activity, 268 mCi/mmole). All animals received 200 μCi and 0.125 mg of δ-aminolevulinic acid per kilogram of body weight in 0.9% NaCl.

Liver microsomes were washed once with 1.15% KCl–10 mM EDTA (14), followed by 0.1 M potassium phosphate, pH 7.4. Washed microsomes were incubated in 0.2% steapsin (Sigma) at 37° for 30 min under a nitrogen atmosphere (12). The mixture was centrifuged for 60 min at 159,000 × g_{av}, and the microsomal pellet (CO-binding particles) was solubilized for 30 min in 3% sodium deoxycholate (Sigma) at room temperature (13). The radioactivity of the solubilized CO-binding particles was measured by counting in a liquid scintillation spectrometer using Omnifluor (6.4 g/liter; New England Nuclear), 240 ml of xylene, 360 ml of toluene, 310 ml of Triton X-100, and 90 ml of water. Hemoprotein content (in the form of cytochrome P-420) in CO-binding particles was analyzed by measuring the dithionite-reduced carbon monoxide difference spectrum (420 nm − 490 nm, ϵ = 110 mM^{-1} cm^{-1}) (12).

Heme oxygenase and biliverdin reduc-

[3] In preparing microsomes for heme oxygenase assay, 0.1 M potassium phosphate, pH 7.4, was used as the homogenizing and resuspending medium.

tase. Microsomes were prepared using 0.1 M potassium phosphate, pH 7.4, as the homogenizing and washing medium. Microsomal heme oxygenase activity was assayed for bilirubin by a modified method of Maines and Kappas (15). The bilirubin formed, in the presence of excess biliverdin reductase, was measured by difference spectrum (468 nm − 530 nm, ϵ = 40 mM^{-1} cm^{-1}). The reference and sample cuvettes contained 0.306 μmole of hemin (Sigma), 2.1 mg of bovine serum albumin (Sigma) (16), 115 μmoles of potassium phosphate (pH 7.4), cytosol (5.0–5.5 mg of protein), and microsomes (1–2 mg of protein); the volume was 2.8 ml. Both cuvettes were incubated for 5 min at 37° before the reaction was started with 1.5 μmoles of NADPH in the sample cuvette and an equal volume (0.2 ml) of 0.1 M potassium phosphate, pH 7.4, in the reference cuvette. The assay was linear for at least 10 min.

Biliverdin reductase was measured in the same manner as heme oxygenase. The reference and sample cuvettes contained 0.060 μmole of biliverdin (Sigma), 2.1 mg of bovine serum albumin, 115 μmoles of potassium phosphate (pH 7.4), and cytosol (1.0–1.5 mg of protein); the total volume was 2.8 ml. Both cuvettes were incubated for 5 min at 37° before the reaction was started with 1.5 μmoles of NADPH in the sample cuvette. An equal volume (0.2 ml) of 0.1 M potassium phosphate, pH 7.4, was added to the reference cuvette. The assay was linear for at least 30 min.

Metal content of tissue. Each sample of liver was dried, subjected to low-temperature ashing, and dissolved in 6 M nitric acid. Atomic absorption analysis (Perkin-Elmer model 306), using the flame technique, was performed on samples diluted to 3 M nitric acid. A commerical standard $CdCl_2$ solution (Fisher) was used.

Protein determination. Protein was determined by the method of Lowry *et al.* (17), using bovine serum albumin as standard.

Statistics. Student's *t*-test was used for statistical analyses.

RESULTS

The LD_{50} for cadmium acetate dihydrate was 7.2 mg/kg (95% confidence limit, 5.7–9.0 mg/kg). Cadmium acetate dihydrate, injected 3 days prior to administration of sodium hexobarbital (100 mg/kg intraperitoneally), caused increases in sleeping time of 242% and 211% with respect to controls at 2.0 and 2.5 mg of salt per kilogram, respectively. These results confirmed similar findings of Hadley *et al.* (1). In all subsequent experiments a dose of 2.0 mg/kg of cadmium acetate dihydrate, equivalent to 7.5 μmoles/kg, was used. This dose was less than the $LD_{1\%}$, based on extrapolation of the lethal dose curve (8). The median contents of metallic cadmium in the livers of experimental animals 3 and 7 days after Cd^{2+} injection were 8.6 and 7.0 μg/g, wet weight, respectively. The cadmium content in livers of control animals was less than 0.05 μg/g, wet weight.

Treatment with the Cd^{2+} salt markedly decreased the levels of cytochrome P-450 and cytochrome b_5, the two hemoproteins which occur in liver microsomes (Table 1). Three days after Cd^{2+} treatment, microsomal cytochrome P-450 and cytochrome b_5 were decreased by 40–50% and 25–30%, respectively. Seven days after treatment, the levels of both cytochromes were 15–20% below controls and thus were returning toward control levels.

Aminopyrine and aniline give type I and type II binding spectra, respectively, upon binding to microsomes. The activities of microsomal aminopyrine demethylase and aniline hydroxylase were reduced markedly 3 days following treatment with Cd^{2+} (Table 1). At 7 days, the percentage decreases were less than those found at 3 days.

Cadmium-treated rats showed a reduced intake of food offered ad libitum for several days after injection, and control rats were pair-fed with the Cd^{2+}-treated animals. Levels of cytochrome P-450, cytochrome b_5, and aniline hydroxylase were lower in 3-day than in 7-day controls, and this finding was attributed to the restricted feed intake for several days preceding the isolation of the microsomes in the 3-day controls.

Figure 1 shows the rate of disappearance of radioactive heme from the hepatic CO-binding particles (microsomes devoid of cy-

TABLE 1

Effect of Cd^{2+} treatment on microsomal enzymes and cytochromes

Each value (mean ± standard error) was derived from 8–12 animals injected intraperitoneally with a single dose of Cd^{2+}.

Treatment	Cytochrome P-450		Cytochrome b_5		Aminopyrine demethylase		Aniline hydroxylase	
	3 days	7 days	3 days	7 days	3 days	7 days	3 days	7 days
	nmoles/mg protein		*nmole/mg protein*		*nmoles/mg protein/min*		*nmole/mg protein/min*	
Control	0.998 ± 0.038	1.260 ± 0.036	0.517 ± 0.022	0.582 ± 0.011	5.22 ± 0.49	5.70 ± 0.22	0.74 ± 0.04	0.61 ± 0.03
Cadmium acetate·$2H_2O$ (2.0 mg/kg)	0.562 ± 0.080[a]	1.022 ± 0.030[a]	0.379 ± 0.022[a]	0.495 ± 0.010[a]	2.75 ± 0.57[b]	3.60 ± 0.42[a]	0.50 ± 0.06[b]	0.47 ± 0.03[b]
Decrease	44%	19%	27%	15%	47%	37%	32%	23%

[a] $p < 0.001$.
[b] $p < 0.01$.

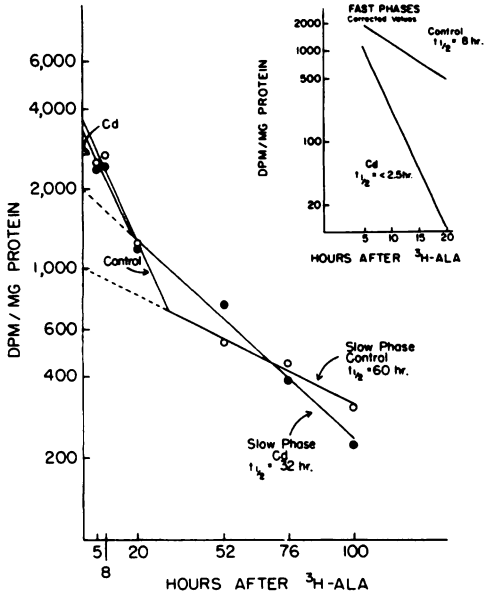

FIG. 1. *Disappearance of labeled cytochrome P-450 heme from CO-binding particles obtained from control and Cd^{2+}-treated rats*

[^{3}H]δ-Aminolevulinic (^{3}H-ALA) acid was injected intraperitoneally into animals 3 days after treatment with Cd^{2+}. Animals were killed 5–100 hr after injection of the radioactive precursor. Each point represents the mean of four to eight (mean, 5.5) animals at each time interval. The half-lives of the fast-phase fractions (after correction of total incorporation for the contribution of the slow-phase fraction) (18) were calculated from data at 5, 8, and 20 hr, and the half-life of the slow-phase fraction in control animals was calculated from data at 52, 76, and 100 hr. However, in Cd^{2+}-treated animals, because the fast-phase fraction contributes to little of the radioactivity at the 20-hr point, the slow-phase half-life was calculated from data at 20, 52, 76, and 100 hr.

tochrome b_5) of controls and rats treated 3 days earlier with Cd^{2+}. The disappearance of cytochrome P-450 heme was biphasic, indicating the existence of at least two forms or states of the hemoprotein, as previously reported (18). The half-life of the control fast-phase component, corrected for contribution of the slow phase, was 8 hr. In Cd^{2+}-treated rats, the half-life of the corrected fast-phase fraction was decreased to less than 2.5 hr. The half-life of the slow-phase component was 60 hr in control animals, whereas the half-life was

reduced to 32 hr in Cd^{2+}-treated rats.[4] The ratio of the fast-phase to the slow-phase component was 2.6 in the control animals.

Liver microsomal heme oxygenase, the degradative enzyme that converts heme to biliverdin (19), doubled in activity at 3 days following Cd^{2+} treatment (Table 2). At 7 days, the enzyme level of treated animals had returned almost to the control level. Biliverdin reductase, which converts biliverdin to bilirubin (16) in the heme degradative pathway, was apparently not altered 3 or 7 days after Cd^{2+} treatment (Table 2). The temporal relationship of changes in heme oxygenase and the cytochromes following Cd^{2+} treatment is shown in Fig. 2. Trends of the changes in microsomal parameters were evident by 12 hr after Cd^{2+} treatment, and the microsomal levels of treated rats were generally significantly changed by 24 hr.

The maximum change in heme oxygenase activity occurred 36–48 hr following Cd^{2+} treatment and was returning toward the control level at 72 hr. The greatest change in microsomal content of cytochrome P-450 occurred by 36 hr and remained constant up to 72 hr after Cd^{2+} injection (Fig. 2). The microsomal content of cytochrome b_5 decreased to its lowest level at 72 hr. At 168 hr (7 days), heme oxygenase returned essentially to normal (Table 2), and cytochromes P-450 and b_5 approached control levels but did not reach them (Table 1).

DISCUSSION

The data indicate that cadmium acetate dihydrate significantly depresses both cytochrome P-450 and cytochrome b_5 levels. The effect on the former is of considerable importance in view of the part that cytochrome P-450 plays in the NADPH-supported metabolism of endogenous substances such as steroids and exogenous substances such as drugs and environmental toxicants (20). The percentage reduction of cytochrome b_5 content was not as large as that of cytochrome P-450. However, the possible importance of this effect on metabolism in the liver should be considered. Previous investigation has shown that cytochrome b_5 may be a donor of the second electron necessary in mixed-function oxidative reactions (21). However, Lu *et al.* (22) have also shown that, although cytochrome b_5 may participate in NADPH-dependent hydroxylation reactions, these reactions are dependent on the particular hemoprotein catalyzing the reaction as well as the specific reaction studied.

Cadmium also decreased, by percentages comparable to the percentage decrease in cytochrome P-450, the metabolism of aminopyrine and aniline *in vitro*. Aminopyrine demethylase and aniline hydroxylase require cytochrome P-450 to complete their respective oxidative reactions. It is possible, therefore, that the reduction in metabolism of these substances by their respective microsomal enzymes is a direct result of the depression of cytochrome P-450.

Since cytochrome P-450 has been shown to turn over biphasically *in vivo* with half-lives of 7–10 hr (fast phase) and 45–65 hr (slow phase) (18, 23), it was of interest to determine the effect of Cd^{2+} on the turnover of the two observed forms of cytochrome P-450. In studies of the incorporation of the heme precursor [^{3}H]δ-aminolevulinic acid into the liver microsomal hemoprotein(s), Cd^{2+} was found to alter the turnover of the fast- and slow-phase components of CO-binding particles (cytochrome P-450) of liver by decreasing the half-life of each. Another metal of recognized toxicity, methyl mercury hydroxide, likewise caused a reduction in cytochrome P-450 content of hepatic microsomes and a major decrease in the half-life of the fast-phase component (23).

It was observed (Fig. 2; Tables 1 and 2) that the following changes occurred in livers of Cd^{2+}-treated rats: (a) an increase in the microsomal activity of heme oxygen-

[4] Figure 1 and the values in the text were calculated from the pooled data at each time interval. If the data from the individual experiments (three sets) are used, the half-lives of the slow-phase components were (mean ± SE) 63 ± 11 hr and 35 ± 4 hr ($0.05 < p < 0.10$) in control and Cd^{2+}-treated rats, respectively. The half-lives of the fast-phase components were 6.3 ± 1.8 hr and 2.4 ± 0.3 hr ($0.05 < p < 0.10$) in control and Cd^{2+}-treated animals, respectively.

TABLE 2

Effect of Cd^{2+} treatment on levels of heme oxygenase and biliverdin reductase in rat liver

Each value (mean ± standard error) was derived from 8–12 animals injected intraperitoneally with a single dose of Cd^{2+}.

Treatment	Heme oxygenase		Biliverdin reductase	
	3 days	7 days	3 days	7 days
	nmole/mg protein/min		*nmole/mg protein/min*	
Control	0.064 ± 0.004	0.067 ± 0.006	0.33 ± 0.02	0.38 ± 0.02
Cadmium acetate·$2H_2O$ (2.0 mg/kg)	0.128 ± 0.014[a]	0.075 ± 0.009	0.34 ± 0.02	0.34 ± 0.02
Percentage of control	200%	112%	103%	89%

[a] $p < 0.001$.

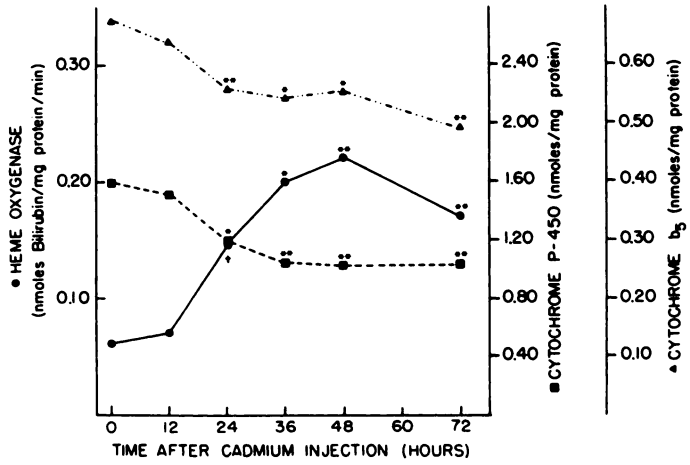

FIG. 2. *Variations in levels of heme oxygenase and cytochromes P-450 and b_5 at various times after treatment with Cd^{2+}*

Each value was derived from six animals. In this experiment, the food intake was restricted for 72 hr (8 g/day/rat), and the weight loss per animal did not differ in all control and Cd^{2+}-treated rats. Consequently the observed changes were not due to differences in food intake during any of the time intervals.

** $p < 0.01$.

* $p < 0.05$.

† $0.05 < p < 0.10$.

ase, the first enzyme in the heme degradative pathway, and (b) marked decreases in the microsomal content of cytochrome P-450 and cytochrome b_5. However, the order and mechanism of these changes were not established.

The following mechanisms have been proposed to relate changes in the parameters in various tissues and the effects of other modifiers (usually toxic agents) of xenobiotic metabolism. (a) The modifier may initially induce the activity of microsomal heme oxygenase. The increased enzymatic activity then causes a decrease in the microsomal content of the hemoproteins (cytochromes P-450 and b_5) which are potential substrates of the oxygenase. Such a mechanism would be consistent with the instance in which the cobalt-induced increase in heme oxygenase occurred prior to decreases in cytochromes P-450 and b_5 or total heme in microsomes of liver (15). (b) Alternatively, the modifier may cause the breakdown of the microsomal membrane (e.g., via lipid peroxidation or release of proteolytic enzymes) or of cytochrome P-450, and the heme or hemoprotein released may then induce an increase in the activity of heme oxygenase. Such a mechanism is consistent with the

induction of heme oxygenase in liver by the injection of heme into intact rats (24) and with the finding that non-heme inducers of heme oxygenase cause a marked decrease in microsomal cytochrome P-450 of liver cells before the induction of heme oxygenase (25). (c) In addition, results were obtained in nonhepatic tissues which indicated that the increase in heme oxygenase may not be related (as cause and effect) in all tissues to the decrease in cytochrome P-450 (26). Such a pattern is observed when cobalt increases both heme oxygenase and microsomal cytochrome P-450 in intestinal mucosa (26).

In subsequent studies in this laboratory, it was observed that the injection of actinomycin D or cycloheximide into Cd^{2+}-treated rats blocks most of the induction of heme oxygenase but does not prevent a decrease in the microsomal content of cytochromes P-450 and b_5.[5]

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[5] Manuscript in preparation.