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Effects of cadmium on heme oxygenase and hemoproteins in smooth and rough endoplasmic reticulum of rat liver

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Acute treatment with various toxic metals is associated with depression in certain components of the liver drug-oxidation system, namely cytochrome P-450 [1–6] and NADPH–cytochrome *c* reductase [2]. Characteristic mixed function oxidations coupled to cytochrome P-450 (e.g. ethylmorphine *N*-demethylase, aniline hydroxylase, and *p*-nitroanisole *O*-demethylase) are also decreased [2–6]. Recently, several investigators have also attributed a dramatic increase in liver microsomal heme oxygenase [6–8] to treatment with some toxic metals. However, there is an uncertainty as to what initiates the stimulation [9], and when this stimulation occurs relative to the change in cytochrome P-450 level [10, 11].

In this communication are reported the decrease in cytochrome P-450 and the increase in heme oxygenase which occur in smooth and rough endoplasmic reticulum of rat liver after the intraperitoneal injection of cadmium acetate [12, 13].

METHODS

Animals. Male Sprague–Dawley rats (200–250 g), from ARS/Sprague–Dawley, were used. Animals were pair-fed with Purina Laboratory Chow by restriction of feed consumption of control and treated rats (e.g. on day 1 after treatment, restricted to 6–8 g/day which was the approximate amount consumed by treated rats if fed *ad lib.*). The feed consumption by control, nontreated rats was approximately 20–22 g day if fed *ad lib.* All animals had free access to drinking water. The weight loss by control and treated rats did not differ.

Administration and dosage. All injections were made intraperitoneally. Cadmium acetate dihydrate (Fisher Scientific) was administered at 2.0 mg/kg, equivalent to 7.5 μmoles/kg, as a single injection at 72 hr (Table 1) or at 32 hr (Table 2) prior to isolation of the microsomal subfractions. Actinomycin D (CalBiochem) was administered four times at 8-hr intervals at 0.3 mg/kg in a solution containing 0.13 mg/ml; cycloheximide (Sigma Chemical) was injected four times at 8-hr intervals at 0.35 mg/kg in a solution containing 0.21 mg/ml. All compounds were dissolved in 0.9% NaCl.

Tissue preparation. Animals were sacrificed by cervical dislocation and their livers were quickly perfused *in situ* with cold 1.15% KCl. The method of Dallner [14], using density gradient centrifugation in the presence of Cs^+, was used to separate the microsomal fraction into smooth and rough subfractions as follows. Livers were homogenized in 0.25 M sucrose (3 g liver/9 ml of solution) using a Potter–Elvehjem type homogenizer. The homogenate was centrifuged at 10,000 g_{max} for 20 min. To 7.0 ml of the 10,000 g_{max} supernatant fraction, 105 μl of 1 M CsCl was added to yield a Cs^+ concentration of 15 mM. Six ml of this mixture was layered over 3.6 ml of 1.3 M sucrose containing 15 mM CsCl and centrifuged at 122,000 g_{av} for 120 min using a Beckman 50 rotor. After centrifugation, the clear upper layer was aspirated and discarded. The fluffy interface (smooth endoplasmic reticulum or SER) was collected and centrifuged in 1.15% KCl at 122,000 g_{av} for 60 min. The remaining supernatant was discarded and the pellet (rough endoplasmic reticulum or RER) was resuspended in 1.15% KCl and centrifuged at 122,000 g_{av} for 60 min. The final pellets of the SER and of the RER were resuspended in 1.0 ml of 0.1 M potassium phosphate (pH 7.4).

Enzyme assays. Cytochrome P-450 was analyzed by measuring the dithionite-reduced carbon monoxide difference spectrum (450–490 nm, $\epsilon = 91$ $mM^{-1}cm^{-1}$) [15]. Cytochrome b_5 was analyzed by measuring the NADH-reduced difference spectrum (424–490 nm, $\epsilon = 185$ $mM^{-1}cm^{-1}$) [16]. Heme oxygenase was assayed according to a modified method of Maines and Kappas [11]. The bilirubin formed, in the presence of excess biliverdin reductase, was measured by difference spectrum (468–530 nm, $\epsilon = 40$ $mM^{-1}cm^{-1}$). The reference and sample cuvettes contained 0.306 μmole hemin, 2.1 mg bovine serum albumin [17], 115 μmoles potassium phosphate (pH 7.4), cytosol (5.0 to 5.5 mg protein), and microsomes (1.2 mg protein); the volume was 2.8 ml. The cytosol, prepared from liver homogenates (in 0.1 M potassium phosphate, pH 7.4) of untreated rats, contained excess biliverdin reductase. Both cuvettes were preincubated for 5 min at 37° and the reaction was started with 1.5 μmoles 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 10 min. Protein was determined by the method of Lowry *et al.* [18], using bovine serum albumin as standard.

RESULTS

Effects of Cd^{2+} at 72 hr. In control animals, the activities of heme oxygenase and the contents of the cytochromes (per mg of protein) in the RER were 45–70 per cent of the levels in the SER (Table 1; also Table 2). At 72 hr after a single dose of Cd^{2+} (Table 1), the heme oxygenase in both the SER and RER subfractions of liver increased approximately 2- and 3-fold, respectively, while the cytochrome P-450 decreased by 25–30 per cent in both fractions. The cytochrome b_5 content was depressed by 15–20 per cent in both the SER and RER. These results reflect our findings [6, 12] in unfractionated liver microsomes.

Effects of Cd^{2+} and inhibitors of RNA and protein synthesis at 32 hr. At 32 hr after treatment with Cd^{2+} alone, the heme oxygenase in both the SER and RER was markedly increased and was higher at 32 hr (Table 2) than at 72 hr (Table 1) after treatment with Cd^{2+}. The majority (65–75 per cent) of the Cd^{2+}-induced increase in heme oxygenase in both the SER and RER did not occur if actinomycin D or cycloheximide was administered concurrently with the Cd^{2+} treatment. The heme oxygenase in those groups receiving Cd^{2+} plus one of the inhibitors was significantly less than the levels in the group receiving Cd^{2+} alone. The administration of cycloheximide alone did not cause an increase in heme oxygenase above control levels in either the SER or RER.

The content of cytochrome P-450 in both the SER and RER in the group receiving Cd^{2+} alone and in the groups receiving Cd^{2+} plus an inhibitor (actinomycin D or cycloheximide) was appreciably less than the level in the control

Table 1. Changes in heme oxygenase and cytochromes in the SER and RER of rat liver at 72 hr after injection of Cd^{2+}*

Fraction	Treatment	Heme oxygenase	Cytochrome P-450	Cytochrome b_5
SER	Control	98 ± 14	1359 ± 53	540 ± 26
	Cd^{2+}	176 ± 32†	995 ± 90‡	454 ± 29
RER	Control	69 ± 13	700 ± 59	344 ± 46
	Cd^{2+}	203 ± 22§	510 ± 67‖	273 ± 41

* Units: cytochromes P-450 and b_5, pmoles/mg of protein; heme oxygenase, pmoles bilirubin formed/min/mg of protein as assayed in the presence of excess biliverdin reductase. Values are the mean ± S. E. of eight rats each in the control and Cd^{2+}-treated groups. Cd^{2+}-treated rats were injected with cadmium acetate dihydrate (2.0 mg/kg) and controls with 0.9% NaCl. All animals were killed 72 hr later. Statistical analyses were by Student's *t*-test.

† $P < 0.05$.

‡ $P < 0.01$.

§ $P < 0.001$.

‖ $0.05 < P < 0.10$.

group. However, the cytochrome P-450 content in the SER of Cd^{2+}-treated rats did not differ from the levels observed in animals treated with Cd^{2+} plus actinomycin D or with Cd^{2+} plus cycloheximide. Treatment with cycloheximide alone decreased the cytochrome P-450 in the SER and RER by 30–40 per cent below control levels.

In the SER, the content of cytochrome b_5 was decreased at 32 hr after treatment with Cd^{2+} alone or Cd^{2+} and actinomycin D. In the SER and RER, the content of cytochrome b_5 was not decreased significantly by treatment with Cd^{2+} plus cycloheximide at this interval.

DISCUSSION

The administration of actinomycin D or cycloheximide to Cd^{2+}-treated rats prevents the majority of the increase in heme oxygenase in the SER and RER observed in rats treated with Cd^{2+} alone (Table 2). This finding implies that the mechanism by which Cd^{2+} increases the activity of heme oxygenase in liver involves the stimulation of the *de novo* synthesis of active enzyme proteins. A similar proposal has been made for the cobalt-induced increase in heme oxygenase in liver [11].

An interest exists in the possible functional and temporal relationships between the increase in heme oxygenase and the decrease in cytochrome P-450, since the heme moiety of the latter is a possible substrate of heme oxygenase. In those animals which received Cd^{2+} plus actinomycin D or cycloheximide, there is relatively little increase in heme oxygenase. In contrast with the pattern observed for heme oxygenase, the administration of actinomycin D or cycloheximide with the Cd^{2+} does not alter the decreases, or slightly accentuates the decreases, in cytochrome P-450 in the SER and RER. Thus, it is not essential that a decrease in cytochrome P-450 be accompanied by an increase in heme oxygenase in liver. Treatment with cycloheximide alone (i.e. no Cd^{2+}) also causes a decrease in cytochrome P-450 but no increase in heme oxygenase. Consequently, in rats treated with both Cd^{2+} and cycloheximide, the de-

Table 2. Effects of actinomycin D and cycloheximide on 32-hr cadmium-induced changes in heme oxygenase and cytochromes of the SER and RER*

Fraction	Treatment	Heme oxygenase	Cytochrome P-450	Cytochrome b_5
SER	Control A	148 ± 17†	1244 ± 45†	530 ± 25‡
	Cd^{2+}	337 ± 44§	896 ± 79§	455 ± 22‖
	Cd^{2+} + actinomycin D	204 ± 44¶	948 ± 76§	390 ± 37§
	Cd^{2+} + cycloheximide	196 ± 19‡,**	889 ± 85§	491 ± 65
	Control B	138 ± 90	1202 ± 124	488 ± 77
	Cycloheximide	86 ± 12	734 ± 85‖	406 ± 36
RER	Control A	72 ± 16††	708 ± 73¶	332 ± 35
	Cd^{2+}	318 ± 45‡‡	543 ± 50**	300 ± 13
	Cd^{2+} + actinomycin D	131 ± 21†,**	449 ± 98**	255 ± 24**
	Cd^{2+} + cycloheximide	149 ± 51‡	387 ± 40‡,§	308 ± 31
	Control B	66 ± 14	507 ± 86	281 ± 29
	Cycloheximide	52 ± 17	354 ± 34	254 ± 12

* Units: cytochromes P-450 and b_5, pmoles/mg of protein; heme oxygenase, pmoles bilirubin formed/min/mg of protein as assayed in the presence of excess biliverdin reductase. Cd^{2+}-treated rats were injected with cadmium acetate dihydrate (7.5 μmoles/kg or 2.0 mg/kg) at 0 hr and with 0.9% NaCl at 8, 16 and 24 hr; rats receiving Cd^{2+} and actinomycin D were treated with Cd^{2+} (7.5 μmoles/kg) at 0 hr and actinomycin D (0.3 mg/kg) at 0, 8, 16 and 24 hr; rats receiving Cd^{2+} and cycloheximide were treated with Cd^{2+} (7.5 μmoles/kg) at 0 hr and cycloheximide (0.35 mg/kg) at 0, 8, 16 and 24 hr; rats receiving cycloheximide alone were treated with cycloheximide (0.35 mg/kg) at 0, 8, 16 and 24 hr; control animals received injections of 0.9% NaCl at 0, 8, 16 and 24 hr. All animals were killed at 32 hr. Values are the mean ± S. E. of eight rats each in control A and Cd^{2+}-treated groups, five rats each in the Cd^{2+}-treated rats receiving actinomycin D or cycloheximide, four rats in control B, and six rats in the cycloheximide group. Statistical analyses were by Student's *t*-test.

†–‡‡ Superscripts **, ‖, § or ‡‡ are used for statistical comparisons vs controls; superscripts ¶, ‡, † or †† are used for comparisons vs Cd^{2+} (alone): ** or ¶, $0.05 < P < 0.10$; ‖ or ‡, $P < 0.05$; § or †, $P < 0.01$; and ‡‡ or ††, $P < 0.001$.

crease in cytochrome P-450 may be caused by the Cd^{2+}-induced degradation of this hemoprotein and/or the cycloheximide-induced inhibition of synthesis of new cytochrome P-450. In the first, a Cd^{2+}-induced breakdown of cytochrome P-450 (perhaps due to increased activity of microsomal proteolytic enzymes or lipid peroxidation) may occur and is followed by a subsequent induction of heme oxygenase by the released heme or heme-related compounds. In liver cells treated with non-heme inducers of heme oxygenase, the decrease in cytochrome P-450 precedes the induction of heme oxygenase [10]. Furthermore, the injection of heme into rats causes an induction of heme oxygenase in liver [19]. Alternatively, the decrease in cytochrome P-450 may be due to a normal degradative rate of this hemoprotein by the basal level of heme oxygenase and the inhibition by the cycloheximide of the synthesis of sufficient new cytochrome P-450 to maintain a steady-state control level.

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