Biochemical Pharmacology, Vol. 36, No. 16, pp. 2647–2652, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

CADMIUM INHIBITS PROTEIN SECRETION FROM CULTURED RAT LIVER PARENCHYMAL CELLS

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(*Received* 5 *January* 1987; *accepted* 3 *March* 1987)

Abstract—Depressive action of cadmium (Cd) to the protein secretion from liver was studied *in vitro* using primary cultures of rat liver parenchymal cells. The cells were cultured in the presence of 0.1–5 μM cadmium chloride. Over this concentration range Cd had no effect on protein synthesis, but caused a dose-dependent inhibition of protein secretion.

Although the metallothionein level was increased more than 4-fold by incubation with 10^{-6} M dexamethasone, the depressive action of Cd was not prevented by the induction of metallothionein.

Cadmium (Cd) is known to be a toxic element that accumulates preferentially in the liver and the hepatotoxicity of Cd has been intensively studied in both *in vivo* and *in vitro* experiments [1, 2]. During the survey of indices for the hepatotoxicity of Cd, we noticed that the secretion of serum proteins, one of the major functions of liver, was depressed in the rat administered a relatively small dose of Cd (1.5 mg Cd/kg body weight, subcutaneously) [3, 4]. The depressive action of Cd was not prevented by the pretreatment of rats with a small dose of Cd or zinc (Zn), suggesting that the sites of Cd action or mechanisms for the toxicity are different from those that can be protected by the induction of metallothionein (MT) [5].

The present study was intended to reveal the mechanism for the inhibitive action of Cd at the cellular level. For this purpose, we employed primary monolayer cultures of rat liver parenchymal cells since these cultured cells maintain differentiated functions of the intact liver for several days and seem to be suitable for the study of hepatic functions under controlled conditions [6, 7].

Although the Cd-induced hepatotoxicity has been investigated using isolated rat liver parenchymal cells (hepatocytes) increasingly [8–15], those were usually performed in suspension cultures. However, the cells cannot be maintained for a long time in the suspension culture and may be used for short term experiments [16]. As freshly isolated hepatocytes have impaired functions and one or more days are required for the recovery of the cells [6, 17], the limitation in the cultivation period is a disadvantage for studying liver functions. So far in many cases the cytotoxic action of Cd was investigated in a short term experiment at a high Cd concentration (about 50 μM or more) monitoring an enhancement of cell membrane permeability. On the other hand there have been only a few reports concerning Cd-induced alterations of metabolic functions. In those studies alterations of the energy level and the lactate-to-pyruvate ratio or inhibition of cellular protein synthesis were shown to be more susceptible to Cd than disturbance of membrane integrity. In the present study, it is demonstrated for the first time that protein secretion is effectively inhibited by Cd at lower concentrations where cellular protein synthesis is not disturbed.

MATERIALS AND METHODS

Preparation of monolayer cultures of rat liver parenchymal cells. Male Wistar strain rats (body weight, about 150 g) were given a commercial diet (CE-2, Clea Japan Co., Tokyo) and distilled water *ad libitum*. Liver cells were prepared by the collagenase perfusion technique [16]. Parenchymal cells were isolated by repeating centrifugation at 50 *g* for 1 min three times. After the last centrifugation the sedimented cells were resuspended in modified William's Medium E (Medium I) described previously [18]. In brief, Medium I consists of William's Medium E without $CaCl_2$, $CuSO_4$, $Fe(NO_3)_3$, $MnCl_2$ and $ZnSO_4$ and supplemented with 10^{-6} M insulin, 10% fetal calf serum, penicillin 100 U/ml, streptomycin 100 μg/ml and fungizone 0.25 μg/ml. Liver parenchymal cells were inoculated in 35-mm culture dishes (Falcon PRIMARIA®, Becton Dickinson, Oxnard) at a cell number of 0.85×10^6 in 1.7 ml of Medium I and allowed to attach to the dish for 4–6 hr. Then, the culture medium was changed to Medium II (1.7 ml), which was the same as Medium I except for supplements of 5% fetal calf serum and 10 mM sodium pyruvate. In some experiments dexamethasone (10^{-6} M) was added to Medium II. All monolayers were cultured at 37° under 5% CO_2 in air. The cell viability in the present study was determined to be about 90% by Trypan Blue exclusion.

All the following experiments were performed after preculture for 21–24 hr.

Accumulation of Cd *and induction of MT.* Parenchymal cells were exposed to Cd at concentrations of 0–2 μM as $CdCl_2$ in Medium II (1.7 ml). At the end of given cultivation periods, the medium was removed and the cells were washed twice with Hanks'

balanced salt solution and lysed in a 200 μl solution of 1% sodium deoxycholate and 1% Triton-X 100. The cell lysates were used for analyses of Cd and MT concentrations.

Synthesis and secretion of proteins in cultured liver parenchymal cells. The parenchymal cells were exposed to Cd at concentrations of 0–5 μM in Medium II for given periods. Then, the cells were washed twice with leucine-free Medium II with or without Cd and labeled for 0.5–2.5 hr with [^{14}C] leucine (0.1 μCi/ml) in a 0.85 ml of the same medium as used for washing. In some experiments the cells were labeled in a medium lacking both serum and Cd. After the labeling the cells and medium were separated and the cells were lysed as described above. Proteins synthesized in the cells and secreted into the medium were estimated by measuring radioactivities in acid-insoluble fractions of the cell lysate and the medium, respectively. Results were expressed as radioactivity per mg cellular protein.

Analysis. Concentration of Cd in the cell lysate was measured by polarized Zeeman flameless atomic absorption spectrometry (Hitachi Type 180-70 Spectrometer) after dilution with deionized water, while concentration of MT was determined by radioimmunoassay [19]. Protein concentration in the cell lysate was determined by the method of Lowry *et al* [20]. For measurement of radioactivity in the acid insoluble fraction a 20 μl of the cell lysate or 50 μl of the medium was precipitated with 5% of trichloroacetic acid and the acid insoluble materials were trapped on a glass fiber filter (Whatman GF/C®, Whatman International Ltd., Maidstone). When the acid insoluble fraction was obtained from the medium lacking serum, bovine serum albumin (50 μg) was added to the medium as a carrier protein. The radioactivity on the glass fiber filter was counted in a liquid scintillation counter (Packard 3255) using Aquasol-2® (New England Nuclear, Boston) or DPO-toluene solution (4 g of 2,5-diphenyloxazole/1 l. of toluene).

Statistical analysis. Statistical analysis was performed by Student's *t*-test. Significant differences compared to control ($P < 0.05$) were marked with asterisks.

Materials. The following reagents were used: as components of William's Medium E; amino acids (Wako Pure Chemical Industries Ltd., Osaka and Kyowa Hakko Kogyo Co. Ltd., Tokyo), methyl linoleate (Fluka, Bucho), menadion sodium bisulfate (Sigma Chemical Co., St. Louis, MO), inorganic salts, glucose, phenol red, sodium pyruvate, ascorbic acid, biotin, choline chloride, ergocholesterol, vitamin A acetate and vitamin B_{12} (Wako Pure Chemical Industries Ltd.), other vitamins (supplements as minimum essential medium, Flow Laboratories Inc., Road McLean), α-tocopherol phosphate (a gift from Dr. I. Hishinuma). Collagenase was obtained from Wako Pure Chemical Industries Ltd.; fetal calf serum from M.A. Bioproducts, Walkersville; insulin and dexamethasone from Sigma Chemical Co.; fungizone from Sankyo Co., Tokyo; penicillin and streptomycin from Meiji Seiyaku Co., Tokyo; Hanks solution from Nissui Pharmaceutical Co. Ltd., Tokyo. L-[^{14}C]-Leucine (348 mCi/mmole) was purchased from Amersham, Buckinghamshire.

The antibody against MT was raised in rabbits as described previously [19]. ^{125}I-Labeled rat MT-2 was provided by Dr C. Tohyama. Other chemicals used were of reagent grade.

RESULTS

Accumulation of Cd *and induction of MT in rat liver parenchymal cells*

Dexamethasone, a synthetic glucocorticoid, is known to induce MT and may influence the toxic action of Cd [21]. Therefore, although dexamethasone was reported to be important in maintaining the culture for a long time [17], parenchymal cells were cultured without adding the hormone at first. For the maintenance of the culture without dexamethasone, concentration of sodium pyruvate in the medium was increased and calcium was reduced as reported previously [18]. Under the present conditions rat liver parenchymal cells formed monolayers on the dishes and showed the flattened polygonal morphology characteristic of epithelial cells. There were no morphological differences between the cells exposed to 2 μM Cd and the control.

Figure 1 shows the time course of Cd accumulation and MT induction in the parenchymal cells. Cadmium was accumulated in a dose dependent manner, but the rate of accumulation declined with the incubation period (Fig. 1a). Metallothionein concentrations in the cells increased after a lag time of about 6 hr (Fig. 1b). The cells accumulated Cd up to 186 ± 17 ng Cd/mg cellular protein by incubation with 2 μM Cd for 10 hr. This concentration corresponds to 24.0 μg Cd/g liver when concentration of protein in rat liver is assumed to be 129 mg/g tissue [22]. In our previous experiment Cd accumulated in the liver up to 19.5 ± 0.9 μg/g tissue when rats were injected with a single subcutaneous dose of 1.5 mg Cd/kg body weight once [5]. Thus, it can be estimated that the present condition for exposure to Cd resulted in a similar accumulation of Cd to the *in vivo* experiment.

Depression of protein secretion from parenchymal cells by Cd

Effects of Cd on the synthesis of total proteins and their secretion from the isolated liver parenchymal cells were examined by measuring incorporations of [^{14}C]-leucine into intracellular and extracellular proteins (Fig. 2). Radiolabeled intracellular proteins increased linearly with time from the beginning of the experiment (Fig. 2a). On the other hand, radioactive leucine was incorporated into extracellular proteins with a lag time (about 30 min) (Fig. 2b). The lag time is probably the time required for the newly synthesized proteins to migrate among cellular organelles and to be secreted into the medium [23].

Secretion of total proteins was depressed significantly by the exposure of parenchymal cells to 2 μM Cd for 3 hr before protein labeling (Fig. 2b), whereas cellular protein synthesis was not inhibited (Fig. 2a). When the exposure time to Cd was extended to 6 hr, protein secretion was further depressed (Fig. 2d) without disturbing the protein synthesis (Fig. 2c).

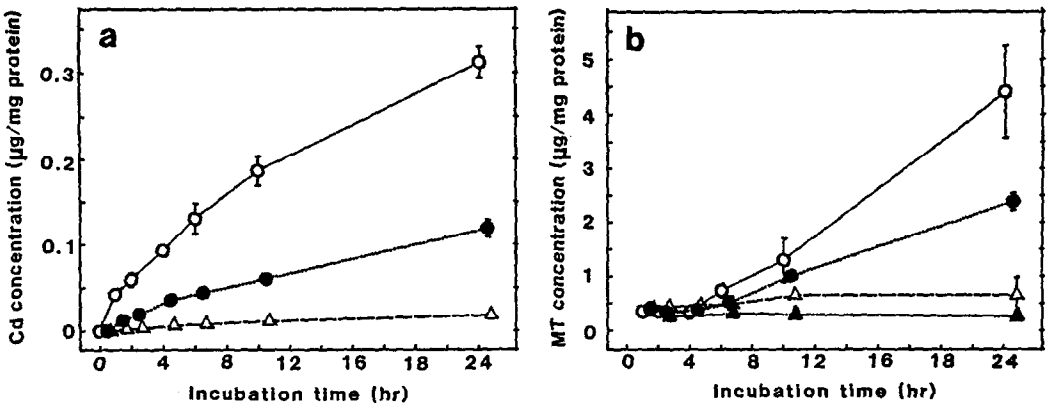

Fig. 1. Accumulation of cadmium (a) and induction of metallothionein (b) in rat liver parenchymal cells. Isolated rat liver parenchymal cells were cultured in a modified William's E Medium without dexamethasone. After pre-culture for 24 hr the cells were incubated in Medium II containing 0 (▲), 0.1 (△), 0.5 (●) and 2.0 (○) μM Cd for the indicated periods. Each point represents the mean and SD of three monolayers.

Cd-induced depression of protein secretion was dependent on Cd concentration in the medium (Fig. 3). After exposure to 5 μM Cd for 7 hr protein secretion was descreased to about 30% of the control (Fig. 3b). However, protein synthesis was not inhibited even at the highest dose in the present experiment (Fig. 3a).

Effect of dexamethasone on Cd*-induced depression of protein secretion*

The MT concentration in the parenchymal cells was increased 4-fold by pretreatment of cultures for 18 hr with 10^{-6} M dexamethasone (Fig. 4b). Whereas, accumulation of Cd was little stimulated by the presence of dexamethasone (Fig. 4a). After

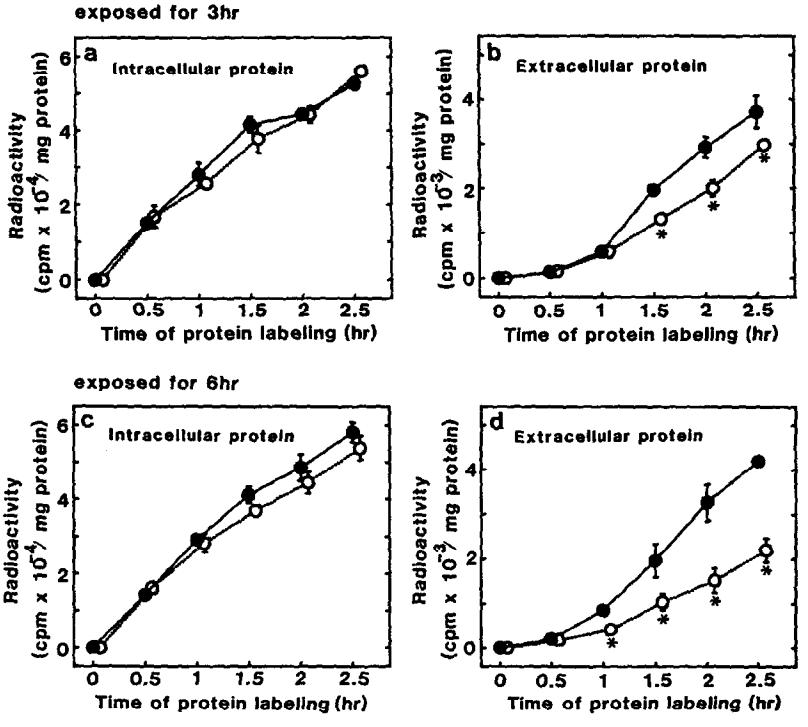

Fig. 2. Effects of cadmium on the synthesis and secretion of total proteins in rat liver parenchymal cells. After pre-culture for 22 hr the cells were exposed to 0 (●) or 2 μM (○) Cd for 3 (a, b) and 6 hr (c, d). Then, the cells were incubated with [^{14}C]-leucine (0.1 μCi/ml) in the absence or presence of Cd for the given periods. Incorporation of the radioactive leucine into intracellular (a, c) and extracellular (b, d) proteins was determined as described in Materials and Methods. Each point represents the mean and SD of three monolayers.

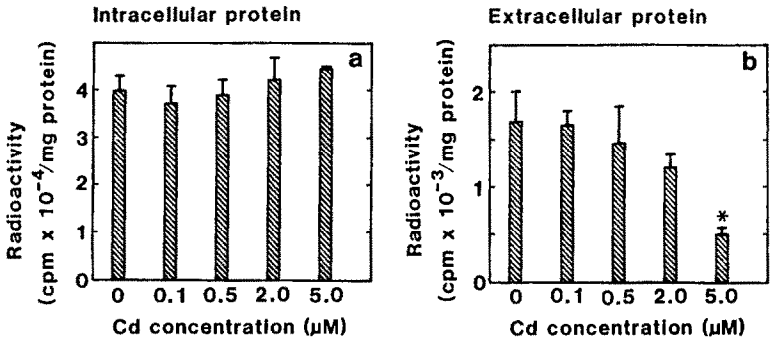

Fig. 3. Dose–response in Cd-induced depression of protein secretion from rat liver parenchymal cells. After pre-culture for 21 hr the hepatocytes were exposed to 0, 0.1, 0.5, 2.0 and 5.0 μM Cd for 7 hr and subsequently incubated with [^{14}C]-leucine (0.1 μCi/ml) in the presence of same concentrations of Cd for 2 hr. Incorporations of radioactive leucine into intracellular (a) and extracellular (b) proteins were measured as described in Materials and Methods. Each point represents the mean and SD of three monolayers.

exposure of the cells to Cd for 12 hr, molar ratios of the accumulated Cd to MT were 7.0 and 3.1 in the absence and presence of dexamethasone, respectively (assuming the molecular weight of MT as 6700).

Figure 5 shows the effect of dexamethasone on the Cd-induced depression of protein secretion from the parenchymal cells. In this experiment the cells were labeled in the medium without serum and Cd. Cellular protein synthesis was not inhibited by exposure to 2 μM Cd for 3–24 hr both in the absence (Fig. 5a) and in the presence (Fig. 5b) of dexamethasone except for one of the data (incubation for 3 hr) in Fig. 5b. Rather the protein synthesis showed a tendency to increase in response to Cd exposure (Fig. 5a). On the other hand, depression of protein secretion by Cd was still observed even after renewal of the medium to Cd-free medium (Fig. 5c). The result shown in Fig. 5d suggests that dexamethasone stimulated protein secretion in all experimental groups. However, depression of protein secretion by Cd was not protected by dexamethasone.

DISCUSSION

In the present study rat liver parenchymal cells in monolayer culture accumulated Cd in a similar time course to that reported by Failla *et al.* [24]. After exposure to 2 μM Cd cellular concentration of Cd was almost identical to that in the liver of rat which was injected with Cd at a dose that depressed secretion of serum protein from liver significantly [5]. Thus, from the present and previous results Cd was shown to depress the protein secretion at a similar concentration both *in vivo* and *in vitro*, and the concentration of Cd examined in the present experiment was adequate to study the mechanism in *in vitro* system.

Cadmium did not inhibit the synthesis of cellular proteins including MT at the present concentrations (0.1–5 μM). Rather the cellular level of MT was increased in response to the incorporation of Cd (Fig. 1b). After exposure to Cd for 12 hr the molar ratio of accumulated Cd to MT was 7.0 (Fig. 4a and

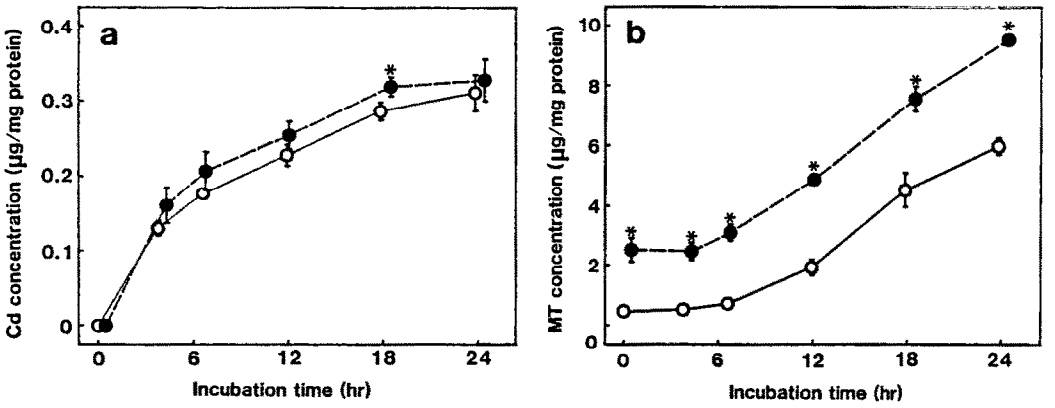

Fig. 4. Effects of dexamethasone on cadmium accumulation (a) and metallothionein induction (b) in rat liver parenchymal cells. The cells were cultured with (●) or without (○) dexamethasone (10^{-6} M) for 18 hr after 4 hr-culture in Medium I. Then, the cells were exposed to 2 μM Cd for the indicated periods with or without dexamethasone. Each point represents the mean and SD of three monolayers.

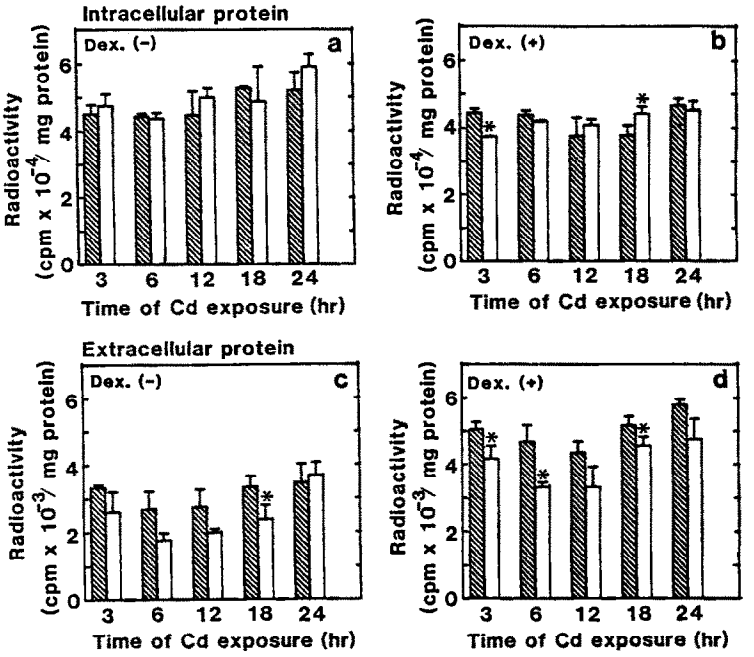

Fig. 5. Effects of dexamethasone on cadmium-induced depression of protein secretion from rat liver parenchymal cells. The cells were cultured in the absence (a, c) or in the presence (b, d) of dexamethasone (10^{-6} M) for 18 hr after 4 hr culture in Medium I. Then, the cells were exposed to 0 (shaded bar) and 2 μM (open bar) of Cd for the indicated periods followed by incubation with [^{14}C]-leucine in Cd-free medium for 2 hr. Radioactivities in the intracellular (a, b) and extracellular (c, d) proteins were determined as described in Materials and Methods. Each point represents the mean and SD of three monolayers.

4b). Considering that 1 mole of MT can bind to 7 g atom of Cd [25], enough MT was induced to bind the accumulated Cd after incubation for 12 hr and more. Nevertheless, depression of protein secretion was observed throughout the incubation period in the present experiment (Fig. 5c). When dexamethasone was added to the culture medium, it showed an additive effect on MT induction by Cd but preinduced MT did not stimulate Cd uptake, which was similar to the report by Kobayashi *et al.* [26]. At the beginning of Cd exposure 2.5 μg (0.37 nmole) MT/mg cellular protein (Fig. 4b) was sufficient to bind as much as 0.29 μg Cd (on the assumption of Cd to MT molar ratio of 7) and molar ratio of Cd to MT did not exceed 7 throughout Cd exposure (Fig. 4a and 4b). Thus, in the presence of dexamethasone MT induced in the cells was enough to sequester incorporated Cd. However, MT did not work as a widely recognized protective protein [27] in the process of protein secretion. This result correlated well with our previous observation that MT induction by pretreatment with a small dose of Cd or zinc was not effective on Cd-induced inhibition of protein secretion *in vivo* [5]. These observations suggest that the target sites of Cd may not be intracellular components in the depressive action of Cd to the secretory process. The sites might be the plasma membrane; secretory proteins are synthesized on the ribosome bound to the rough endoplasmic reticulum, transported through the Golgi apparatus to the plasma membrane and then secreted by exocytosis [28]. Cadmium may adversely affect the plasma membrane and inhibit the exocytotic process when the metal ions are incorporated into the cells.

In summary depression of protein secretion by Cd was first observed in cultured rat liver parenchymal cells at relatively low levels of Cd which do not inhibit protein synthesis. This depressive action of Cd is probably an early toxic effect to the liver. Monolayer culture of rat parenchymal cells was demonstrated to be a useful technique to clarify the mechanism of the inhibitory action of Cd to the secretory function of the liver.

Acknowledgement—The authors express their thanks to Dr C. Tohyama for his helpful instructions to radioimmunoassay of MT and to Dr I. Hishinuma for his kind gift of α-tocopherol phosphate. Thanks are also due to Dr A. Koizumi for his encouragement.

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