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IN VITRO INHIBITORY ACTION OF CADMIUM ON MICROSOMAL MONOOXYGENASES OF RABBIT LUNG

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Abstract—*In vitro* addition of cadmium (Cd) salts to pulmonary microsomes isolated from male rabbits decreased the cytochrome P-450 levels and the activity of benzo[*a*]pyrene hydroxylase and aminopyrine *N*-demethylase but not that of NADPH-cytochrome *c* reductase. The Cd-induced reduction of pulmonary monooxygenase activity was enhanced when microsomes were preincubated in the presence of Cd and inhibition increased as the time of preincubation progressed, attaining its maximum rate at 20 min of preincubation. When hepatic microsomes were used, this delayed effect of Cd on monooxygenase was less apparent. The presence of NADH and/or NADPH in the preincubation did not markedly enhance the Cd-induced inhibition rate of monooxygenase activity. The addition of Cd-acetate to pulmonary microsomes produced a concentration-dependent inhibition of the monooxygenase activity and the estimated IC_{50} values of Cd-acetate (i.e. the concentration required to inhibit control enzyme activity by 50%) were 3.8×10^{-5} M, 6.5×10^{-6} M and 5.3×10^{-5} M for cytochrome P-450, benzo[*a*]pyrene hydroxylase and aminopyrine *N*-demethylase, respectively. The inhibitory action of Cd-acetate on the monooxygenase activity was also observed with microsomes isolated from the lungs of male guinea-pigs and rats.

Cadmium (Cd) has been reported to be widely distributed throughout the environment and is found in the form of fumes in the general and industrial atmospheric environment [1–4]. Though the absorption of Cd when ingested with foods is very low, Cd is efficiently absorbed and retained in the body when inhaled with air [5–9]. It is well established that Cd fumes, when inhaled, elicit lung injury in both man and experimental animals [7, 8, 10–17].

Since the lung is the target organ of atmospheric pollutants involving carcinogenic chemicals in gaseous or aerosol forms, metabolism and disposition of chemicals in the lung has received much attention recently. Cd is known to be one of the potent inhibitors of the hepatic microsomal monooxygenases both *in vivo* and *in vitro* [18–24] but its effect on lung monooxygenases has been scarcely studied. In this regard, we have studied the effect of Cd fumes on the lung microsomal drug-metabolizing enzymes of rabbits and have found that the activity of the enzymes were reduced after the short-term inhalation of Cd-oxide fumes [25]. The same kind of study was reported recently using rat pulmonary microsomes [26]. These indicated that Cd fumes could modify the activity of xenobiotic enzymes in the lung and this may modify the metabolism or activity of other atmospheric pollutants inhaled simultaneously.

In an attempt to elucidate the mechanism by which Cd inhibits the pulmonary microsomal xenobiotic enzymes *in vivo*, the present study was designed to ascertain whether these findings *in vivo* could be explained by the direct *in vitro* effect of Cd salts on these enzymes. Pulmonary microsomes isolated from rabbits were mainly used since the monooxygenase activity of the lung in this species is higher than in other experimental animals [25].

We report here that Cd also reduced the activity of the monooxygenases in the pulmonary microsomes *in vitro* and that this reduction could be enhanced when microsomes were preincubated in the presence of Cd for certain lengths of time.

MATERIALS AND METHODS

Chemicals. All chemicals used were of reagent grade. Glucose 6-phosphate, NADP, NADPH, NADH and glucose 6-phosphate dehydrogenase were purchased from Boehringer–Mannheim–Yamanouchi Co. Ltd (Tokyo, Japan). Aminopyrine was obtained from Sanko Seiyaku Kogyo Ltd (Tokyo, Japan). Benzo[*a*]pyrene and Cd salts were purchased from Wako Pure Chemicals Ltd (Osaka, Japan).

Animals and preparation of microsomes. In most experiments, male albino rabbits (Japan Laboratory Animals Inc., Tokyo, Japan), weighing 2.0–2.5 kg were employed. In some experiments, male Wistar–Slc rats weighing 125–130 g and Hartley guinea-pigs weighing 210–230 g (Shizuoka Laboratory Animals Co. Ltd, Hamamatsu, Japan) were employed. Pulmonary microsomes were isolated from the non-treated animals as described previously [25]. Hepatic microsomes were also isolated in the same manner except that excised livers were directly homogenized in a Potter–Elvehjem glass homogenizer fitted with a Teflon pestle. The isolated microsomes were used immediately or stored at −80° until use. The microsomal protein was determined by a modification of Lowry's method [27] with bovine serum albumin as a standard.

In vitro *studies and enzyme assay.* For the study on cytochrome P-450, pulmonary microsomes were

suspended in Tris–Cl buffer solution (25 mM, pH 7.4) and incubated at 37° for different periods of time in the presence or absence of Cd. The final volume of incubation mixture was 5 ml. At the end of the incubation, the tube containing the microsomal suspension was transferred to ice and then analysed spectrophotometrically in a Shimazu MPS-5000 Spectrophotometer by the method of Johannesen and DePierre [28], the molar extinction coefficient being taken as $150\,mM^{-1}\,cm^{-1}$. In the experiment using hepatic microsomes, incubation was carried out in the same way as in case of pulmonary microsomes except that cytochrome P-450 was assayed according to the method of Omura and Sato [29].

For benzo[*a*]pyrene hydroxylase, pulmonary microsomes were suspended in Tris–Cl buffer solution (50 mM, pH 8.0) and preincubated for different periods of time in the presence or absence of Cd. Benzo[*a*]pyrene metabolism was initiated by the simultaneous addition of NADPH-generating system (final concentration: NADP, 0.5 mM, glucose 6-phosphate, 5 mM, glucose 6-phosphate dehydrogenase, 0.87 U/ml) and benzo[*a*]pyrene (0.08 mM). The final volume of reaction mixture was 2.5 ml. The metabolism of benzo[*a*]pyrene was terminated at 10 min and the activity was determined by the direct fluorometric method [30] by measuring the amounts of 3-hydroxy-benzo[*a*]pyrene with a Hitachi MPF-4 Fluorometric Spectrophotometer. In the experiments using hepatic microsomes, the enzyme assay was carried out in the same manner as pulmonary microsomes except that incubation in the presence of benzo[*a*]pyrene was done for 5 min in Tris–Cl buffer (50 mM, pH 7.4).

For the study on aminopyrine *N*-demethylase, pulmonary microsomes were suspended in Tris–Cl buffer solution (50 mM, pH 7.4) and preincubated in the presence or absence of Cd. Aminopyrine metabolism was then initiated by the simultaneous addition of the NADPH-generating system and aminopyrine (3 mM). The final volume of the incubation mixture was 2 ml. The reaction was terminated at 15 min and released formaldehyde was determined by the method of Nash [31].

For NADPH-cytochrome *c* reductase, the microsomes were suspended in phosphate buffer (30 mM, pH 7.4) and preincubated in the presence or absence of Cd. The NADPH-generating system and cytochrome *c* were then added and the activity was determined by measuring spectrophotometrically the rate of reduction of cytochrome *c* [32].

For the study of the effect of NADH and NADPH on Cd-induced decrease of microsomal monooxygenase activity, microsomes were preincubated with or without NADH (250 μM) and/or NADPH (250 μM) before the assay of the enzyme activity.

Cd *determination*. The contents of Cd in the microsomal protein fraction were determined with a Hitachi 207 Atomic Absorption Spectrophotometer after digestion of the particles with HNO_3–$HClO_4$.

RESULTS

Factors affecting Cd*-induced changes in monooxygenase activity*

Factors which might modify the *in vitro* action of Cd on the enzyme activity was examined.

First, the effect of the length of preincubation time in the presence of Cd was studied. Pulmonary

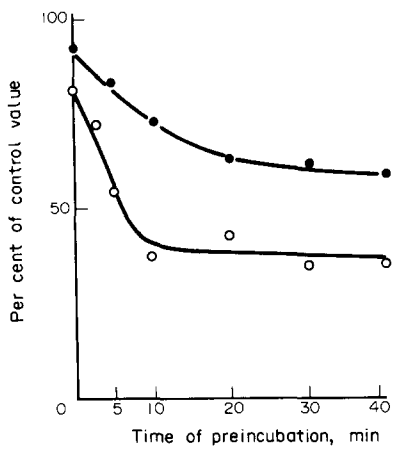

Fig. 1. Effect of preincubation time on Cd-induced changes in the activity of benzo[*a*]pyrene hydroxylase of lung (●) and liver (○) microsomes of rabbits. Preincubation of the microsomal suspensions was carried out in the presence or absence of Cd-acetate (4×10^{-6} M and 1.6×10^{-5} M for lung and liver enzyme, respectively). At the end of the indicated preincubation time, benzo[*a*]pyrene was added to the reaction mixture and enzyme assay was done for an additional 10 min. The concentration of microsomal proteins in the reaction mixture was 0.075 mg/ml and 0.01 mg/ml for lung and liver enzymes, respectively. Each point, expressed as the percentage of the corresponding control values at the same preincubation time, represents the mean of three determinations.

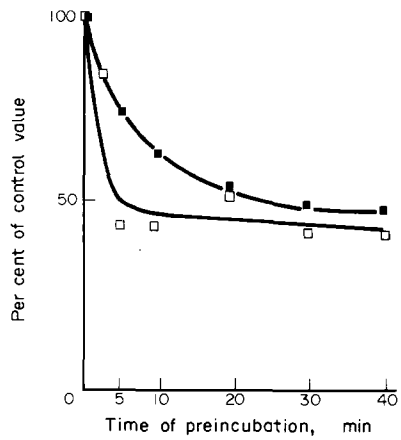

Fig. 2. Effect of preincubation time on Cd-induced changes in cytochrome P-450 levels of lung (■) and liver (□) microsomes of rabbits. Preincubations of both microsomal suspensions were carried out in the presence or absence of Cd-acetate (4×10^{-5} M). The cytochrome P-450 levels were determined immediately after the end of the indicated preincubation time. The concentrations of microsomal proteins in the reaction mixture were 0.430 mg/ml and 0.405 mg/ml for lung and liver enzymes, respectively. Each point, expressed as percentage of corresponding control values at the same preincubation time, represents the mean of three determinations.

Table 1. Effect of NADH and NADPH on Cd-induced decrease of monooxygenase activity of rabbit lung microsomes

	Cd-acetate	Cytochrome P-450 (nmole/mg protein)	Benzo[*a*]pyrene hydroxylase (pmole/min/mg protein)
Control	−	0.235 ± 0.003	30.7 ± 2.4
	+	0.143 ± 0.003* (39.2%)	20.8 ± 3.1* (32.1%)
+NADH	−	0.307 ± 0.007	34.5 ± 1.7
	+	0.149 ± 0.011* (51.5%)	26.4 ± 2.6 (16.5%)
+NADPH	−	0.237 ± 0.010	29.7 ± 2.8
	+	0.122 ± 0.006* (48.6%)	24.8 ± 2.0 (16.5%)
+NADH	−	0.257 ± 0.020	33.2 ± 0.7
+NADPH	+	0.158 ± 0.002* (38.5%)	24.0 ± 0.6 (27.7%)

Microsomes were preincubated for 20 min before enzyme assay in the presence of Cd-acetate at concentrations of 4×10^{-5} M and 4×10^{-6} M for cytochrome P-450 (0.416 mg protein/ml reaction mixture) and benzo[*a*]pyrene hydroxylase (0.023 mg protein/ml), respectively. NADH (250 μM) and/or NADPH (250 μM) were included in the reaction mixture. Values given are the means ± S.E. of three determinations with significant differences from corresponding control values at $P < 0.01$ (*). The values in parentheses are the percentage inhibition rates compared with corresponding controls.

microsomes of rabbits were preincubated for various times in the presence of Cd-acetate before the addition of enzyme substrates. Similar experiments were carried out using hepatic microsomes isolated from rabbits.

As illustrated in Fig. 1, benzo[*a*]pyrene hydroxylase activity of pulmonary microsomes was reduced when assayed after the preincubation of microsomes in the presence of Cd-acetate (4×10^{-6} M) and the inhibition increased as the time of preincubation progressed and attained its maximum reduction rate at about 20 min. In the similar manner (Fig. 2), cytochrome P-450 levels in the pulmonary microsomes were reduced when microsomes were preincubated in the presence of Cd-acetate (4×10^{-5} M).

The delayed action of Cd on monooxygenase activity of hepatic microsomes was less pronounced and a marked inhibition of the enzyme activity by Cd occurred within 10 min of preincubation.

Secondly, the effect of the presence of NADH and/or NADPH in the preincubation was studied. Microsomes from rabbit lungs were preincubated for 20 min with or without NADH and/or NADPH in the presence of Cd-acetate before the enzyme assay. As shown in Table 1, Cd-induced inhibition of cytochrome P-450 levels was slightly enhanced and that of benzo[*a*]pyrene hydroxylase activity was slightly ameliorated by NADH and NADPH, but these differences were not statistically significant.

Thirdly, the effect of various forms of Cd salts on the enzyme activity was compared. Cytochrome P-450 levels and the activity of benzo[*a*]pyrene hydroxylase and aminopyrine *N*-demethylase were determined in the presence of Cd-acetate, bromide and chloride. These Cd salts were equally effective in reducing the activity of the enzymes. In addition, these Cd salts did not markedly alter the pH of the assay system used in the concentrations of 1×10^{-4} to 1×10^{-2} M, indicating that the inhibition of the enzyme activity is not attributed to the changes in pH of the assay system.

Consequently, the subsequent studies were done with 20 min of preincubation in the presence of Cd-acetate and in the absence of NADH and NADPH.

Dose-related inhibitory action of Cd *on pulmonary monooxygenase activity*

Cytochrome P-450 levels and the activity of benzo[*a*]pyrene hydroxylase, aminopyrine *N*-demethylase and NADPH-cytochrome *c* reductase were determined in the presence of Cd-acetate at various concentrations in the incubation mixture. As shown in Fig. 3, the addition of Cd-acetate to pulmonary microsomes isolated from rabbits produced a concentration-related inhibition of cyrochrome P-450 levels and of the activity of aminopyrine *N*-demethylase and benzo[*a*]pyrene hydroxylase. In

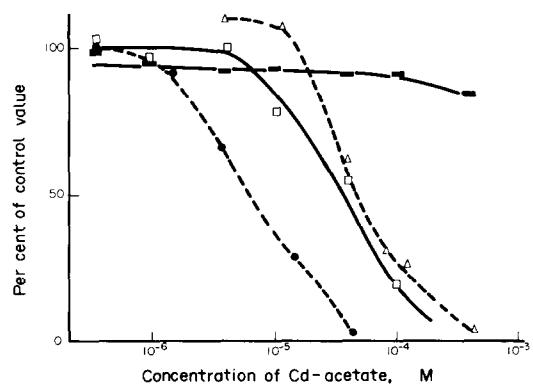

Fig. 3. Dose-related inhibitory action of Cd-acetate on monooxygenase activity of pulmonary microsomes of rabbits. Preincubation of microsomes was carried out for 20 min in the presence of Cd-acetate. The microsomal protein concentrations for the assays of cytochrome P-450 (□), benzo[*a*]pyrene hydroxylase (●), aminopyrine *N*-demethylase (△) and NADPH-cytochrome *c* reductase (■) were 0.43 mg/ml, 0.75 mg/ml, 0.97 mg/ml and 0.27 mg/ml, respectively. Each point, expressed as percentage of control values, represents the mean of three determinations.

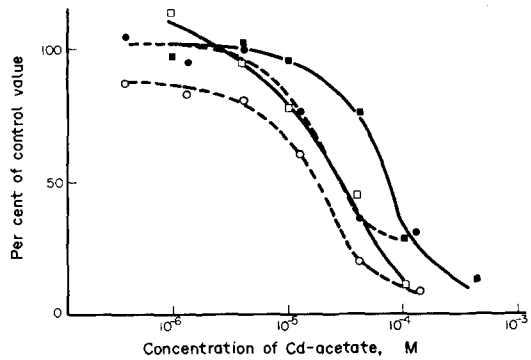

Fig. 4. Dose-related inhibitory action of Cd-acetate on monooxygenases of lung microsomes isolated from rats and guinea-pigs. Experiments were carried out in the same manner as in the experiments described in Fig. 3. In the cytochrome P-450 assays, the concentrations of guinea-pig (□) and rat (■) pulmonary microsomal proteins were 0.764 mg/ml and 1.36 mg/ml; in the benzo[*a*]pyrene hydroxylase measurements, the protein concentrations of guinea-pig (○) and rat (●) microsomes were 0.046 mg/ml and 0.275 mg/ml, respectively.

contrast, the activity of NADPH-cytochrome *c* reductase was not affected by Cd-acetate at the concentrations studied (4×10^{-7} to 4×10^{-4} M). Estimated IC_{50} values of Cd-acetate (i.e. the concentration required to inhibit the control enzyme activity by 50%) were about 6.5×10^{-6} M, 3.8×10^{-5} M and 5.3×10^{-5} M for benzo[*a*]pyrene hydroxylase, cytochrome P-450 and aminopyrine *N*-demethylase, respectively.

To determine whether Cd was equally inhibitory to pulmonary monooxygenases of other species of animals, microsomes were isolated from the lungs of male guinea-pigs and rats and assayed for cytochrome P-450 and benzo[*a*]pyrene hydroxylase activity in the presence and absence of Cd-acetate. As shown in Fig. 4, the addition of Cd-acetate to microsomes from the lungs of these animals also produced a concentration-related inhibition of the monooxygenase activity. Estimated IC_{50} values of Cd-acetate were about 3.1×10^{-5} M and 6.2×10^{-5} M on rat enzymes and 1.8×10^{-5} M and 2.6×10^{-5} M on guinea-pig enzymes for benzo[*a*]pyrene hydroxylase and cytochrome P-450, respectively.

Amounts of Cd *bound to microsomes*

Microsomes that had been incubated for 20 min in the presence of Cd-acetate (4×10^{-5} M) under the same conditions as for the assay of cytochrome P-450, when recovered by centrifugation (105,000 *g*, 60 min) and washed twice with Tris (50 mM)—KCl (150 mM), were found to contain 0.523 ± 0.007 μg Cd/mg protein.

DISCUSSION

The present study has shown that the *in vitro* addition of Cd-salts to pulmonary microsomes of rabbits caused a decrease in the cytochrome P-450 levels and in the activity of benzo[*a*]pyrene hydroxylase and aminopyrine *N*-demethylase. These observations are in good agreement with the results of our previous *in vivo* study where microsomal drug-metabolizing enzymes were found to be reduced in the lungs of rabbits exposed to Cd-oxide fumes [25]. The *in vitro* inhibitory action of Cd on the monooxygenase was also observed on the pulmonary microsomes isolated from guinea-pigs and rats. Thus Cd has a general inhibitory action on the pulmonary monooxygenases both *in vivo* and *in vitro*. Though the mechanism by which Cd reduces cytochrome P-450 levels and cytochrome P-450 dependent oxidation activity is not known at present, the inhibition of these enzyme activities apparently was not a result of impairment of the electron transporting capacity of NADPH-cytochrome *c* reductase which was not inhibited by Cd as shown by the present study.

A number of studies have been devoted to the *in vitro* effect of Cd on the monooxygenase activity of the liver [19, 22–24]. According to these reports, the concentrations of Cd that inhibited the activity of the hepatic enzymes by 50% ranged from 5×10^{-6} to 1×10^{-3} M. These concentrations were relatively high in comparison with those found in the present study to produce similar inhibition of the pulmonary enzymes. This might be, in part, due to the length of preincubation time and, in part, to the differences in the animal species and organs used. As shown by the current study with pulmonary microsomes, the time of preincubation with Cd is important. The preincubation for at least 20 min in the presence of Cd was necessary to obtain a maximum reduction of the pulmonary enzyme activity.

The concentrations of Cd that caused 50% inhibition of monooxygenase activity in rabbit lung microsomes *in vitro* ranged from 4×10^{-6} to 5.3×10^{-5} M. These concentrations correspond to 0.51–5.9 μg Cd/ml incubation mixture. In the *in vivo* study [25], the levels of Cd in the pulmonary tissues of rabbits in which microsomal enzyme activity was inhibited by 50% at 2 days after inhalation of Cd-oxide fumes were approximately 3–5 μg Cd/g tissue. The overall concentrations of Cd that caused inhibition of the enzyme activity were approximately of the same order both *in vivo* and *in vitro*, indicating that Cd-induced reduction of the enzyme activity *in vitro*, as observed in the present study, reflects that of the *in vivo* study [25]. However, when Cd concentrations are expressed as the amounts of bound Cd per unit of microsomal proteins, the 50% inhibition level of Cd is much higher *in vitro* than *in vivo*. In our *in vivo* study [25], the amount of Cd found in the microsomal fraction of the lungs of rabbits that inhaled Cd fumes (12.6 ± 0.4 mg Cd/m^3 air for 15 min) was 0.027 ± 0.003 μg Cd/mg microsomal protein (unpublished). In the present study, the microsomes contained 0.523 ± 0.007 μg Cd/mg protein. Thus the concentrations of Cd required to inhibit the enzyme activity *in vitro* are approximately 20 times greater than the amount of Cd found in the microsomes in the *in vivo* study, i.e. higher amounts of bound Cd were required *in vitro* than *in vivo* to produce similar inhibition of the enzyme activity. Whether this signifies indirect action of Cd on the enzyme activity or not remains to be elucidated. In this regard, it should be first elucidated whether the

Cd-induced decrease in the enzyme activity is the result of direct binding of Cd to active sites or of other indirect causes evoked through the changes in cell functions of the lungs of Cd-exposed animals. On the other hand, it should be noted that the changes in the activity of pulmonary enzymes and other biochemical parameters occurred two or three days after the inhalation of Cd fumes [15, 25]. This time frame is considerably longer than one would expect if Cd was exerting a direct inhibitory action *in vivo* on pulmonary monooxygenase, since Cd gains rapid access into pulmonary cells [8, 16, 33].

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