Comparative Study on in vitro Inhibitory Effects of Heavy Metals on Rabbit Drug-Metabolizing Enzymes

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The in vitro inhibitory effects of heavy metals, i.e., mercury, cadmium, lead, nickel and beryllium in their chloride forms, on rabbit pulmonary drug-metabolizing enzymes were studied comparatively. Microsomal and cytosolic fractions prepared from rabbit lungs were incubated in the presence of heavy metals prior to enzyme assays. Mercury was the most potent in reducing cytochrome P450 content and mixed-function oxidase activities including NADPH-cytochrome c reductase and benzo[a]pyrene hydroxylase. The addition of mercury to pulmonary microsomal preparations resulted in a spectral shift from 450 nm to 420 nm in an absorption maximum of cytochrome P450-carbon monoxide complex with mercury chloride being more potent than methylmercury. Cadmium was also inhibitory, while the effects of nickel were noted only at higher concentrations. Neither lead nor beryllium was inhibitory. Among second-phase drug-metabolizing enzymes, UDP-glucuronyltransferase and glutathione S-transferase were found to be susceptible to the adverse effects of mercury and lead, respectively. The extent of inhibition of the latter activity by mercury were highly dependent on the concentration of glutathione, implying the complex formation between them. Cadmium was slightly inhibitory to both enzyme activities, though the other metals had no effect. The results indicate that the pulmonary toxicities of airborne heavy metals could be inferred using simple in vitro assays.

Key words — heavy metal, drug-metabolizing enzyme, in vitro, lung, liver

INTRODUCTION

It is well known that heavy metals are widely distributed in an atmosphere and some of them can cause lung disorders in industrial fields.1) Cadmium (Cd),2,3 beryllium (Be),31 mercury (Hg) and other metals6) uptaken by inhalation have been reported to cause lung injuries in both human beings and experimental animals. Therefore, the evaluation of toxic potentials of metals is important for the risk assessment of human beings ordinarily exposed to these substances in an ambient air. Despite a number of studies concerning their toxicities, they have rarely been investigated comparatively. For this purpose and application to airborne chemicals in general, it is preferable to establish a simple in vitro method to estimate pulmonary toxicity. In the present study, the authors attempted to use in vitro biochemical parameters as surrogate markers for the toxicities on pulmonary cells or tissues.

Both phases 1 and 2 drug-metabolizing enzymes might play an important role in the protection of lung from dysfunctions caused by inhaled chemicals, as was the case of their hepatic counterparts. Therefore, the adverse effects on their activities in vitro could be candidate markers of pulmonary toxicity. Although drug-metabolizing enzymes of hepatic origin were proved to be suppressed by heavy metals,7,8 it remains to be determined whether the pulmonary enzymes are susceptible to the suppressive effects of heavy metals. In fact, the distinct properties of drug-metabolizing enzymes were observed between lung and liver.9

We demonstrated previously that Cd fumes exhibited an inhibitory action on the activity of mixed-function oxidases in rabbit lung under short-term inhalation conditions (Fukuhara et al., 1981),10 and these in vivo effects of Cd fumes were in good correlation with the in vitro inhibitory effects of Cd on mixed-function oxidase activities in isolated pulmo-
nary tissue fractions (Fukuhara and Takabatake, 1982).11)

In this paper, the in vitro effects of a series of heavy metals known as atmospheric pollutants on the activities of pulmonary drug-metabolizing enzymes were studied comparatively in order to estimate their relative potentials to cause pulmonary disorders in exposed human beings.

**MATERIALS AND METHODS**

**Chemicals** —— NADP, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, cytochrome c, UDP-glucuronic acid and glutathione were obtained from Boehringer-Mannheim (Mannheim, Germany). Benzo[a]pyrene, Cd chloride (CdCl2), Hg chloride (HgCl2), methyl-Hg chloride (MeHgCl), nickel (Ni) chloride (NiCl2), lead(Pb)chloride (PbCl2) and Be chloride (BeCl2) were products of Wako Pure Chemicals (Osaka, Japan). Aniline and aminopyrine were purchased from Sanko Pharmaceut.Indust. (Tokyo, Japan).

**Animals and Preparation of Subcellular Fractions of Lung and Liver** —— Male albino rabbits (Japanese White) weighing 2.5–3.0 kg were obtained from Japan Laboratory Animals Co. (Tokyo, Japan). Microsomal and cytosolic fractions were prepared from rabbit liver and lung as described previously.10) Briefly, homogenates of these organs were centrifuged at 9,000 \( g \) for 10 min and the pellets were further centrifuged at 105,000 \( g \) for 1 hr to give microsomal (precipitate) and cytosolic (supernatant) fractions. Protein concentrations of these fractions were determined by Lowry’s method12) using bovine serum albumin as a standard.

**Enzyme Assays and in vitro Studies** —— Amounts of cytochrome P450 proteins in microsomal fractions from liver and lung were determined by the method of Omura and Sato13) and Johannesen and DePierre,14) respectively. NADPH-cytochrome c reductase, aminopyrine N-demethylase, and aniline hydroxylase were assayed by the method of Mazel15) and NADH-cytochrome b5 reductase according to the method of Takesue and Omura.16) NADPH- and NADH-benzo[a]pyrene hydroxylases17) and UDP-glucuronyltransferase18) were also assayed in microsomal fractions. Cytosolic fraction was tested for glutathione S-transferase activity.19)

The effects of heavy metals were studied in vitro as described previously11) on the levels of cytochrome P450 proteins and the activities of NADPH-cytochrome c reductase and NADPH-benzo[a]pyrene hydroxylase. These enzymes were chosen as the representative enzymes of the mixed-function oxidase system. The effects of heavy metals on the activities of second-phase drug-metabolizing enzymes, UDP-glucuronyl transferase and glutathione S-transferase, were studied in the same manner. Briefly, the metals were added to the reaction mixtures consisting of pulmonary subcellular fractions, co-factors and substrates, and the whole was kept at 37°C for 20 min in advance of the enzyme reactions. The 20 min-preincubation was found to be long enough to obtain the maximum inhibition rates for most of the pulmonary drug-metabolizing enzymes.11)

**RESULTS**

**Comparison of the Activities of Drug-Metabolizing Enzyme in Rabbit Lung and Liver**

To characterize rabbit lung from a biochemical viewpoint, the basal levels of activities of mixed-function oxidases and second-phase drug-metabolizing enzymes as well as the protein content of cytochrome P450s were compared with those for liver. The enzyme activities were expressed on a protein basis and the protein levels of cytochrome P450s on the basis of wet organ weight. The results are summarized in Table 1. The protein content of cytochrome P450s in pulmonary microsomal fraction was approximately one third that of hepatic origin.

The specific activities of aminopyrine N-demethylase, aniline hydroxylase and NADPH-cytochrome c reductase activities in pulmonary microsomes were higher than 50% of their hepatic counterparts. Concerning NADH-cytochrome b5 reductase, there was no distinct difference between the enzyme activities of lung and liver. However, NADPH- and NADH-benzo[a]pyrene hydroxylases of pulmonary microsomes were of rather low specific activities compared with the respective hepatic enzymes. It is noteworthy that the activities of pulmonary second-phase drug-metabolizing enzymes were 2 to 3 orders of magnitude lower than the hepatic ones, as shown by the results for cytosolic glutathione S-transferase (1.2%) and microsomal UDP-glucuronyltransferase (0.2%) activities.
Table 1. Comparison of Drug-Metabolizing Enzymes in the Microsomal Fractions of Lung and Liver of Rabbit

<table>
<thead>
<tr>
<th>Enzyme activity (nmol/min/mg-protein)</th>
<th>Lung</th>
<th>Liver</th>
<th>Ratio (Lung/liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organ wet weight (g/100 g-body.weight)</td>
<td>0.38 ± 0.03</td>
<td>2.30 ± 0.20</td>
<td>(0.16)</td>
</tr>
<tr>
<td>Cytochrome P450 (nmol/mg-protein)</td>
<td>0.33 ± 0.03</td>
<td>1.00 ± 0.10</td>
<td>(0.33)</td>
</tr>
<tr>
<td>Aminopyrine N-demethylase</td>
<td>2.9 ± 0.3</td>
<td>4.9 ± 0.3</td>
<td>(0.59)</td>
</tr>
<tr>
<td>Aniline hydroxylase</td>
<td>0.26 ± 0.05</td>
<td>0.40 ± 0.03</td>
<td>(0.65)</td>
</tr>
<tr>
<td>NADPH-cyt. c reductase</td>
<td>19.2 ± 1.6</td>
<td>33.8 ± 2.1</td>
<td>(0.57)</td>
</tr>
<tr>
<td>NADH-cyt. b5 reductase</td>
<td>2.8 ± 0.2</td>
<td>2.9 ± 0.2</td>
<td>(0.96)</td>
</tr>
<tr>
<td>Benzo[a]pyrene hydroxylase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADPH-dependent</td>
<td>0.09 ± 0.01</td>
<td>1.60 ± 0.10</td>
<td>(0.06)</td>
</tr>
<tr>
<td>NADH-dependent</td>
<td>0.06 ± 0.01</td>
<td>0.40 ± 0.05</td>
<td>(0.15)</td>
</tr>
<tr>
<td>Glucuronyl transferase</td>
<td>0.03 ± 0.01</td>
<td>17.2 ± 1.3</td>
<td>(0.002)</td>
</tr>
<tr>
<td>Glutathione S-transferase</td>
<td>67 ± 5</td>
<td>5660 ± 150</td>
<td>(0.012)</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.E.M. of 5 animals.

Fig. 1. In vitro Effects of Heavy Metals on Cytochrome P450 Content and the Enzyme Activities of Mixed-Function System in Pulmonary Microsomal Fractions

Effects of Heavy Metals on the Cytochrome P450 Content and the Enzyme Activities of Mixed-Function System in Pulmonary Microsomal Fractions

Fig. 2. Spectral Shift of Maximum Absorption of Cytochrome P450-CO Complex in the Presence of Hg

Spectra of the reduced form of cytochrome P450-CO complex were determined after the incubation of pulmonary microsomes at 37°C for 20 min in the presence of different concentrations of Hg.
concentrations, whereas Be and Pb did not affect cytochrome P450 content.

For NADPH-cytochrome c reductase, the highest inhibition of enzyme activity was recognized in the presence of Hg (Fig. 3). The inhibitory effect of Cd was observed only at higher concentrations and the other metals were substantially of no effect on the enzyme activity.

Likewise, Hg was the most potent inhibitor of NADPH-benzo[a]pyrene hydroxylase (Fig. 4). Cd and Ni were also inhibitory but less potent than Hg, and the other metals had no effect on this enzyme activity. Since Hg was proved to be the most potent inhibitor of NADPH-benzo[a]pyrene hydroxylase among the metals tested, the effect of MeHg on the same enzyme activity was tested to see whether the observed inhibitory effect of Hg was dependent on the chemical form. The 50% inhibitory concentration (IC₅₀) of MeHg at 20 × 10⁻⁷ M was found to be higher than that of Hg at 8 × 10⁻⁷ M, suggesting that inorganic Hg was more potent than the organic form.

Effects of Heavy Metals on the Activities of Second-Phase Drug-Metabolizing Enzymes in Pulmonary Microsomal and Cytosolic Fractions

Among second-phase drug-metabolizing enzymes, UDP-glucuronyltransferase activity in the microsomal fraction and glutathione S-transferase in the cytosolic fraction were tested for their susceptibilities to heavy metals. In parallel with the results for the first-phase drug-metabolizing enzymes, Hg was the top-ranked inhibitor of UDP-glucuronyltransferase, followed by Cd and Ni (Fig. 5). No marked effects were noted for Be or Pb. In contrast, Pb and Cd were equally potent in the reduction of glutathione S-transferase activity (Fig. 6), while Be and Ni did not show any effect. The results for Hg are not included in Fig. 6 because the effect of Hg depends on the concentration of glutathione in the reaction mixture due to the complex formation with Hg₂⁺.

DISCUSSION

In the present study, it was shown that some of heavy metals present in the atmospheric environ-
ment were inhibitory against pulmonary drug-metabolizing enzymes *in vitro*. The potentials of individual metals are variable. However, Hg was generally the most potent in the inhibition of tested enzyme activities. Although acute exposure to a high level of Hg vapor may cause pneumonitis and other symptoms of pulmonary dysfunction,6) few biochemical studies have been done on the pulmonary toxicity of this metal. Based on the results obtained in this study, Hg might exert a more potent inhibitory effect on a pulmonary drug-metabolizing function than the other heavy metals simultaneously tested when inhaled through respiratory tracts. The *in vitro* inhibitory action of Hg on pulmonary drug-metabolizing enzymes was partly explained by the direct degradation of cytochrome P450s as can be seen by a marked shift in the absorption maximum of cytochrome P450-carbon monoxide complex from 450 to 420 nm in the presence of Hg at concentrations inhibitory to most enzymes (Fig. 2). Atmospheric Hg was reported to be at least 90% in the form of Hg vapor and the remainder consisted of inorganic Hg and MeHg compounds,69 which were released from incinerators, extraction processes for gold, mining, etc. Furthermore, concern has recently focused on the Hg exposure caused by dental amalgam, which is believed to be the major background source of human exposure to this metal.6,22) Due to its highly diffusible and lipophilic nature, about 80% of inhaled Hg is estimated to be retained in the body.6) It is thus important to evaluate human risks to exposure to atmospheric Hg.

For the other metals, there have been a few stud-
ies demonstrating the inhibitory actions on pulmonary enzymes *in vivo* (inhalation experiment) and *in vitro*. In the present study, Cd was shown to be another potent inhibitor of pulmonary enzymes *in vitro*, though it could not exceed Hg, in good accord-
ance with our published findings as well as those of other groups using a series of animals exposed to metals in aerosol10,23) and an *in vitro* study.11) Although the toxicities of Be and Pb to lungs have been reported in animal experiments,17) they were found to be less potent than Hg and Cd in terms of the suppression of enzyme activities. The discrepancy in the results obtained *in vitro* and *in vivo* imply that Be and Pb might exert their *in vivo* effects by different mechanisms from those of Hg and Cd.

Concerning the effects of heavy metals on the hepatic drug-metabolizing enzyme activities, Cd was reported to be suppressive on mixed-function oxidas-
es.24–29) Hg,30,31) Pb,30–34) Be35) and Ni7) are also able to reduce the hepatic enzyme activities. Based on those findings and our new results, the organ specificities shown by liver and lung in the susceptibili-
ties of drug-metabolizing enzymes to several heavy metals were attributable partly, if not entirely, to the organ specific nature of enzymes. The basal activities of mixed-function oxidases and second-phase drug-metabolizing enzymes in lung were generally lower than those in liver in terms of a specific activ-
ity (Table 1). The activities of second-phase en-
zymes, which play a crucial role in the detoxifica-
tion of xenobiotics, were extremely poor in lung, indicating that the toxic metabolites resulting from first-phase oxidative reactions could confer more severe damage to lung than liver. The toxicities or pharmacological activities of some chemicals are known to depend largely on the characteristics and contents of metabolizing enzymes in target organs. This indicates that the biochemical background of pulmonary toxicities of certain chemicals could be estimated by the activities of drug-metabolizing en-
zymes of lung origin. The metals uptaken via respira-
tory tracts should be given attention because they would affect the functional integrity of drug-metabo-
lizing enzymes as environmental factors modifying the toxicities or pharmacological actions of xenobiotics or drugs.

Since there were few studies on the correlation between pulmonary toxicities of metals6) and their inhibitory activities on drug-metabolizing enzymes, the authors refrain from concluding that the inhibi-
tory potentials of metals on enzyme activities shown in the present study represent their ability to cause
pulmonary toxicity. However, based on the results of our previous studies comparing the in vivo and in vitro activities of Cd in lung in rats exposed to cadmium chloride aerosol. Archives of Environmental Health, 25, 145–148.


