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Effect of cadmium on lung lysosomal enzymes in vitro

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Abstract Labilization of lysosomal enzymes is often associated with the general process of inflammation. The present study investigated the effect of the pneumotoxin cadmium on the release and activity of two lung lysosomal enzymes. Incubation of rat lung lysosomes with cadmium resulted in the release of β-glucuronidase but not acid phosphatase. The failure to "release" acid phosphatase appears to be the result of a direct inhibitory effect of cadmium on this enzyme. The K_I for cadmium was determined to be 26.3 μM. The differential effect of cadmium on these two lysosomal enzymes suggests that caution should be exercised in selecting the appropriate enzyme marker for assessing lysosomal fragility in the presence of this toxicant. Furthermore, the differential basal release rate of the two enzymes from lung lysosomes may reflect the cellular heterogeneity of the lung.

Key words Cadmium · Lung · Lysosomes · β-Glucuronidase · Acid phosphatase

Introduction

Cadmium is a potent metallic toxicant of continuing environmental and occupational concern. Potentially, as many as 1.5 million workers have been exposed to cadmium in the work place (National Institute of Occupational Safety and Health 1984; Waalkes and Oberdorster 1990) while cigarettes can also contribute to the body burden (Lewis et al. 1972). In situations of high dose, short inhalation exposure to cadmium produces toxicity predominately in the lung (Waalkes et al. 1992a). Experimental studies have demonstrated that intratracheal instillation of cadmium can result in pulmonary fibrosis (Dervan and Hayes 1974; Niewoehner and Hoidal 1982; Kutzman et al. 1986; Damiano et al. 1990), emphysema (Snider et al. 1973) and pulmonary carcinomas (Sunderman 1978, 1979; Waalkers and Oberdorster 1990). We have demonstrated that intraperitoneal administration of cadmium causes peritonitis which is accompanied by the release of histamine, lysosomal enzymes, prostaglandins and cyclic nucleotides (Giri et al. 1979).

The mechanism(s) by which cadmium produces the underlying inflammatory change preceding these sequelae is/are not well understood. However, one manifestation of the inflammatory process, in general, is the accumulation and labilization of lysosomes at sites of injury (Ignarro 1971). It is known that heavy metals such as cadmium can accumulate in lysosomes (Sato and Nagai 1980; Dallinger and Prosi 1988). In fact, the release of lysosomal enzymes has been used as an index of cytotoxicity. For example, one sign of cadmium nephropathy is an increased release of lysosomal enzymes β-galactosidase, β-*N*-acetyl glucosaminidase (Bernard et al. 1979) and alkaline phosphatase (Gompertz et al. 1983). The present study was designed to investigate the effect of cadmium on the in vitro release of two lung lysosomal enzymes, β-glucuronidase and acid phosphatase, in order to determine if lysosomal release is consistent with pneumotoxicity. Additional studies were carried out on the direct effect of cadmium on the activity of these enzymes.

Methods and materials

Lysosomal isolation

Male Sprague-Dawley rats, 250–300 g, were obtained from Simonsen Laboratories (Gilroy, Calif.) and utilized throughout this

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study. The rats were anesthetized with ether and exsanguinated. The lungs were perfused in situ with 0.18 M sucrose, 0.04 M TRIS acetate buffer, pH 7.4, removed with the heart en bloc, and dissected free.

The method for the isolation of lung lysosomes and measurement of free and total enzyme alteration was that of Ignarro (1972), with minor modifications. Lungs from one or two rats were homogenized in 7–10 ml 0.25 M sucrose, 0.02 M TRIS acetate buffer, pH 7.4, using a 15- ml capacity Dounce homogenizing vessel. Homogenization was conducted manually by executing 25 complete strokes with the standard loose clearance pestle. This method disrupts greater than 99% of tissue cells with minimal lysosomal damage. Homogenates were centrifuged at 600 *g* at 4°C for 5 min. The initial pellets were discarded and the supernatant fractions were diluted with an equal volume of 0.25 M sucrose, 0.02 M TRIS acetate buffer, pH 7.4, and re-centrifuged at 3500 *g* for 15 min at 4°C. The resulting supernatant fractions were collected and centrifuged at 25 000 *g* for 15 min at 4°C. Each of the 3500 *g* ("heavy") and 25 000 *g* ("light") pellet fractions was gently washed twice with 0.45 M sucrose, 0.04% glycogen, 0.02 M TRIS acetate buffer, pH 7.4, and re-centrifuged at their respective speeds. The washed pellet fractions were resuspended in 3–4 ml of 0.45 M sucrose, 0.04% glycogen, 0.02 M TRIS acetate buffer, pH 7.4. Resuspended pellet fractions containing either "light" or "heavy" lysosomes were incubated in glass tubes at 37°C under experimental conditions. The incubated fractions were subsequently transferred to polycarbonate tubes and centrifuged at 27 000 *g* for 15 min. The pellets were discarded and the supernatants assayed for β-glucuronidase and/or acid phosphatase activity.

Lysosomal enzyme assays

β-Glucuronidase activity was determined with a modification of the method of Gianetto and DeDuve (1955). The formation of phenolphthalein from phenolphthalein glucuronide (Sigma, St Louis, Mo.) was measured. A 1-ml aliquot of "high-speed" supernatant and 1.0 ml distilled deionized water were combined with 1.0 ml 0.3 M citrate buffer, pH 4.8. This was preincubated for 5 min at 37°C after which 0.04 ml freshly prepared phenolphthalein glucuronide (10 mg/ml distilled deionized water) was added. The samples were incubated for 60 min at 37°C and the reaction terminated with the addition of 2.2 M glycine, 10 N sodium hydroxide buffer, pH 12. Optical density at 540 μ was measured with a Gilford spectrophotometer.

Acid phosphatase activity was determined with a modification of the method of Seligman et al. (1951). The formation of β-naphthol from β-naphthyl acid phosphate (Sigma) was measured. A 0.5 ml aliquot of "high-speed" supernatant was incubated for 30 or 60 min, at 37°C with 2.5 ml β-naphthyl acid phosphate which had been diluted 1:1 with 0.2 N acetate buffer, resulting in a substrate concentration of 0.004 M. The reaction was terminated with the addition of 0.08 ml 1.0 M sodium carbonate. Color was developed with 0.5 ml tetraotized dianisidine. Cold trichloroacetic acid (0.5 ml) was added, followed by color extraction with 6.0 ml ethyl acetate. The samples were centrifuged for 10 min at 2000 rpm. Optical density at 540 μ was measured with a Gilford spectrophotometer.

Effect of cadmium on stability of lysosomal membrane

The resuspended pellet fractions (0.2 ml, warmed to 25°C for 5 min) were incubated at 37°C in glass test tubes containing 1.7 ml 0.18 M sucrose, 0.04 M TRIS acetate buffer, pH 7.4, and 0.1 ml cadmium chloride in buffer. The final concentrations of cadmium tested were 10^{-3}, 5×10^{-4}, 10^{-4}, 5×10^{-5}, 10^{-5}, and 10^{-6} M. Immediately following incubation, the samples were transferred to polycarbonate tubes and centrifuged at 27 000 *g* for 15 min. The pellets were discarded and the supernatants were diluted 1:1 with 0.18 M sucrose, 0.04 M TRIS acetate buffer, pH 7.4, and assayed for activity of β-glucuronidase and acid phosphatase. Activity was expressed as percent of total enzyme activity. Total enzyme activity was determined by solubilizing 0.2 ml fractions of the resuspended pellet fraction in 0.2% (v/v) Triton X-100, 0.04 M TRIS acetate buffer, pH 7.4.

Inhibition of enzymes by cadmium

Enzyme assays were conducted by following the basic procedure outlined previously, except that the reaction mixture minus the substrate was incubated for 15 min at 37°C with cadmium. The reaction was then started with the addition of the substrate. The final concentrations of cadmium ($CdCl_2$ dissolved in 0.18 M sucrose, 0.04 M TRIS acetate buffer, pH 7.4) were based on the volume of the reaction mixture minus substrate plus the volume of cadmium. The concentrations tested for β-glucuronidase and acid phosphatase were 5×10^{-4}, 10^{-4}, 5×10^{-5}, 10^{-5} and 10^{-6} M.

Enzyme kinetics of acid phosphatase

Optimal conditions for ascertaining enzyme activity during a specified amount of time (15 min) with varying amounts of enzyme was determined. This was accomplished by isolating the heavy lysosomal fraction and effecting total enzyme release, determining the protein content of this fraction (Lowry et al. 1952), and assaying for acid phosphatase using aliquots of enzyme that contained 12.5, 25, 50 or 100 μg protein. The next step was to vary the incubation time of the acid phosphatase assay with a constant quantity of protein (50 μg). The procedure was the same as described above, except that incubation times were 15, 30, 45, and 60 min. Having determined the desired protein content of the enzyme fraction (50 μg protein), incubation time (30 min), and cadmium concentration (5×10^{-5} M), we wanted to vary substrate (β-naphthyl acid phosphate) concentration with and without the presence of cadmium in order to obtain V_{max}, K_m, and K_I values for the system.

Results

The effect of varying concentrations of cadmium on the release of β-glucuronidase from lung "heavy" and "light" lysosomes is shown in Fig. 1. Cadmium had a similar effect on both the "heavy" and "light" fractions. In both cases, cadmium produced no significant effect at the lower two concentrations. However, cadmium did cause a statistically significant increase in the release of β-glucuronidase at concentrations of 50 μM and 100 μM. These results also indicate that cadmium at the highest concentration (500 μM) had an inhibitory effect on enzyme release. However, we believe this to be an artifact, since lysosomes were found to form a cloudy precipitate, in the presence of this relatively large concentration.

Figure 2 shows the effect of the same concentrations of cadmium, as used above, on the release of acid phosphatase from lung lysosomes. In contrast to the results obtained with β-glucuronidase, acid phosphatase release was not found to be increased. The significant reduction in release at the higher concentration was again attributed to lysosome precipitation.

One possible explanation for cadmium appearing to cause the release of β-glucuronidase, but not acid

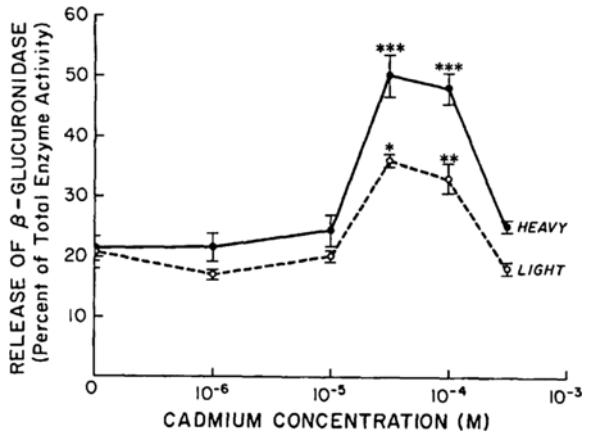

Fig. 1 The effect of varying concentrations of cadmium on the in vitro release of β-glucuronidase from lung "heavy" and "light" lysosomes

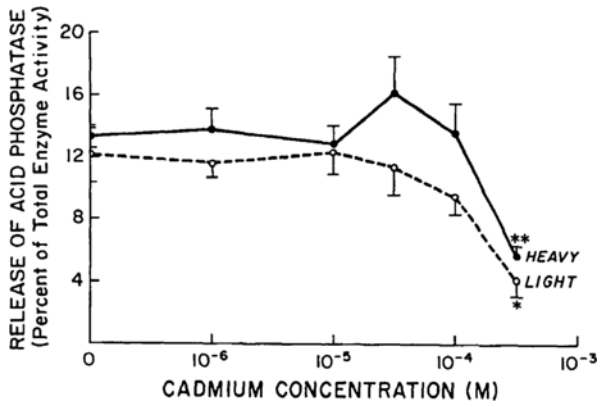

Fig. 2 The effect of varying concentrations of cadmium on the in vitro release of acid phosphatase from lung "heavy" and "light" lysosomes

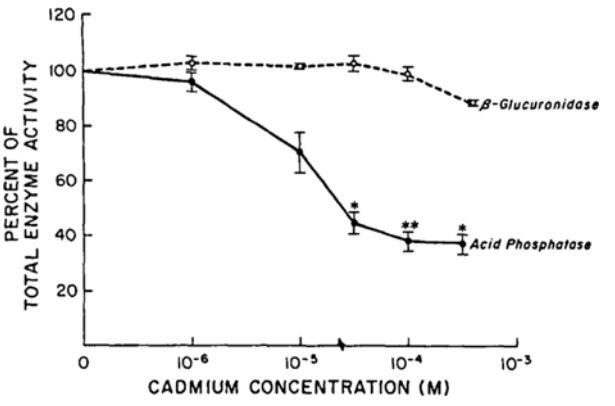

Fig. 3 The in vitro effect of cadmium on lung lysosomal β-glucoronidase and acid phosphatase activity

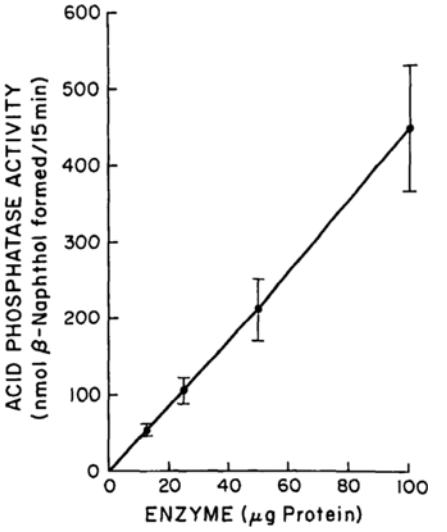

Fig. 4 The relationship of lung lysosomal protein concentration to in vitro acid phosphatase activity

phosphatase, is that cadmium might directly inhibit the activity of acid phosphatase once the enzymes are released from the lysosomes. In order to test this possibility, both β-glucuronidase and acid phosphatase were released from lysosomes with Triton X-100 and incubated with various concentrations of cadmium. The results are shown in Fig 3. Over the concentration range studied, cadmium had no significant effect on β-glucuronidase activity. However, cadmium did produce a dose-dependent inhibition of acid phosphatase activity being statistically significant at concentrations of 50 μM and above.

In order to determine the kinetic parameters of cadmium's inhibitory effect on acid phosphatase, a number of preliminary studies were carried out in order to establish optimum assay conditions. Figure 4 demonstrates the effect of protein concentration on enzyme activity. The results indicate that enzyme activity is linear with lysosomal protein content up to 100 μg. In addition, Fig. 5 demonstrates that enzyme activity was essentially linear for 45 min in the presence of a constant quantity of lysosomal protein (50 μg).

The effect of cadmium on in vitro kinetic parameters of lung lysosomal acid phosphatase is shown in Table 1. In the presence of 50 μM cadmium, V_{max} was reduced by 66% while the Michaelis constant decreased from 46.5 to 15.3 μM (67%). Lineweaver-Burk analysis of the double-reciprocal equation revealed a K_I of 26.3 μM for cadmium with a profile suggesting uncompetitive inhibition (the abscissal intercept was changed without affecting the slope; data not shown).

Discussion

The results of the present study indicate (1) that cadmium induced inflammatory changes in the lung may partly be attributed to the release of lysosomal enzyme(s), as indicated by elevated release of

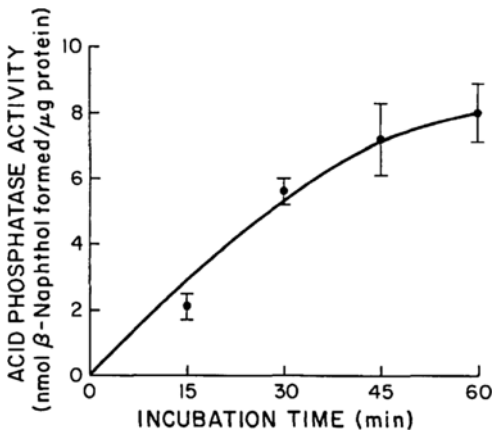

Fig. 5 Effect of incubation time on in vitro lung lysosomal acid phosphatase activity

β-glucuronidase, (2) that the basal release rate for β-glucuronidase and acid phosphatase differ from lung lysosomes and (3) that cadmium can inhibit acid phosphatase directly in an uncompetitive manner thus nullifying the value of acid phosphatase as an indice of cadmium-induced lysosomal fragility. Uncompetitive inhibition can, under certain circumstances, represent a special case of heterotropic-cooperative noncompetitive inhibition where binding of substrate increases the affinity of binding of the inhibitor from near zero to its final value (Matthews 1993).

Cadmium has previously been demonstrated to have the ability to inhibit certain enzyme activity. For example, Kinne-Saffran et al. (1993) have reported that cadmium chloride can inhibit both dogfish rectal gland and rabbit kidney medullar Na, K-ATPase with an I_{50} of 13 and 19 μM, respectively, as well as K-dependent *p*-nitrophenylphosphatase (pNPPase; I_{50} 9.4 μM). These concentrations compare reasonably well with the observed K_I for acid phosphatase of 26.3 μM in the present study. Akerman et al. (1985), has found that Cd also inhibits red cell Ca-ATPase non-competitively at micromolar concentrations, whereas Visser et al. (1993) report that cadmium inhibits Ca-ATPase in the nanomolar range.

Aerosol administration of cadmium to rats has been reported to produce inhibition of both lung mitochondrial succinic dehydrogenase and cytochrome C oxidase activity (Rao and Gardner 1986). In an osteoblastic cell culture (MC3T3-E_1), 10 μM cadmium was found to inhibit alkaline phosphatase by 95% after 7 days exposure (Iwami and Moriyama 1993). Other studies (Palmer et al. 1987) report an LC_{50} for cadmium chloride in pulmonary macrophages, in vitro, of 28 μM while 30 μM cadmium chloride produces significant depression of myocardial mechanical activity (Kopp et al. 1978).

In the present study, cadmium (50 μM) was found to decrease the V_{max} of acid phosphatase for β-naphthol phosphate from 10.75 to 3.7 μM β-naphthol formed/mg protein per hour. At a similar concentration (40 μM), cadmium has been reported to produce no significant effect on the V_{max} of pNPPase (Kinne-Saffran et al. 1993). In addition, we observed a statistically significant reduction in the apparent K_m of acid phosphatase for β-naphthol, in the presence of 50 μM cadmium, while Kinne-Saffran et al. (1993) reported a significant increase in the apparent K_m of pNPPase for Mg. These data suggest that the mechanism of action of cadmium on enzyme activity varies with the enzyme in question as well as its source. Differences in sensitivity of the enzyme may be related to variations in protein structure. For example, lysosomal acid phosphatases are known to differ from prostatic acid phosphatases by the substitution of Lys and Gly for Tyr and Arg at the active site (Schneider et al. 1993).

Acid phosphatases are widely distributed in both the animal and plant kingdoms and are divided into three groups (Vincent et al. 1992). One such group is the high molecular weight phosphatase (45–60 KDa per subunit) found in lysosomes which do not utilize metal ions for catalysis (Schneider et al. 1993). Therefore, it is unlikely that the inhibitory effect of cadmium is related to displacement of metal co-factors as occurs with Na, K-ATPase (Kinne-Saffran et al., 1993). An alternative explanation could conceivably involve the chloride anion of cadmium chloride since small monovalent anions are weak inhibitors of acid phosphatase (Yam 1974). However, this is unlikely, since antagonism studies with zinc acetate (data not shown) demonstrate substantial reversal of cadmium's inhibitory effect on acid phosphatase, suggesting involvement of the cadmium cation. Zinc's reversal of cadmium's inhibitory effect is consistent with its reported stimulatory effect on the activity of acid phosphatase in the femoral epiphysis (Yamaguchi et al. 1983).

Table 1 Effects of inorganic cadmium on kinetic parameters of lung acid phosphatase in vitro. The double reciprocal plot of Lineweaver and Burk was employed to determine the kinetic parameters.

Treatment	Mean ± SE of four animals		
	V_{MAX} (μM/mg protein per hour)	K_M(μM)	K_I(μM)
Control (saline)	10.75 ± 0.59	46.5 ± 11.1	–
Cadmium (50 μM)	3.70 ± 0.09	15.3 ± 0.6	–
P Values	< 0.05	< 0.001	–
Cadmium			26.3 ± 18.3

The lung is an organ composed of some 40 different cell types. This heterogeneity may offer a partial explanation for the variability in basal release rate of lung lysosomes for the two enzyme studied (23 versus 12%). That is, some cells may contain different enzyme levels in their lysosomes which themselves may vary in fragility. This difference in lung cell population may also partially explain the higher basal fragility of lung lysosomes vis-a-vis liver lysosomes (7–8%) for these two enzymes (Ignarro 1972). Therefore, our results should be viewed as representing the totality of the lung lysosome response to cadmium.

In conclusion, the results of the present study suggest that (1) lung lysosomal β-glucuronidase and acid phosphatase can respond differentially to the effect of cadmium, in vitro, and (2) the basal release rate of the two enzymes differ from lung lysosomes. Therefore, care should be exercised in drawing conclusions related to lysosomal mediated effects of cadmium induced inflammation based solely upon the measurement of acid phosphatase and that the variation in basal release of lysosomal enzymes need also be taken into consideration.

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