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THE ROLE OF LYSOSOMES IN THE HEPATIC ACCUMULATION AND RELEASE OF BERYLLIUM

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Abstract—The toxic metal beryllium (Be) can produce hepatic necrosis and is known to be concentrated by the liver after i.v. injection of particulate and soluble Be compounds. In the present study the accumulation and release of Be by a hepatic lysosome fraction of the rat has been examined after i.v. administration of sublethal doses of particulate Be phosphate (12.5 and 150 μmoles/kg) or the more hepatotoxic soluble $BeSO_4$ (12.5 and 25 μmoles/kg). Maximal lysosomal Be content is produced within 2 hr or 5 hr after the injection of Be phosphate or $BeSO_4$ respectively and in both cases this is followed by a gradual decrease in the lysosomal Be concentration of the next 7 days to 30–40 per cent of the maximal values, which is consistent with the loss of Be observed for whole liver. The release of Be from lysosomes has been examined by measurement *in vitro* of the liberation of Be into a suitable incubation medium from lysosomes prepared from Be treated animals. The results indicate that the liberation of Be was maximal for lysosomes isolated 5 hr after injection of either form of Be and was accompanied by a significant release of lysosomal β-glucuronidase activity, particularly at the higher doses of Be used, and therefore indicates that some loss of lysosomal integrity occurred. Rupture of the lysosomal membrane could not be demonstrated *in vitro* by incubation of lysosomes from untreated animals with externally added Be compounds. It is concluded, however, that any release of hydrolytic enzymes into the cytosol occurring *in vivo* may not be the major cause of cell necrosis produced by Be and it is suggested, therefore, that the role of lysosomes in Be hepatotoxicity is primarily in the intracellular accumulation and subsequent release of Be and that the main cytotoxic target for Be is probably extra-lysosomal.

Studies on the cellular toxicity of a wide variety of colloidal metal salts have demonstrated that after intravenous (i.v.) administration to experimental animals they are taken up from the blood into hepatic lysosomes [1–4]. The presence of the toxic metal beryllium (Be) can be shown also in rat hepatic lysosomes after i.v. injection of Be salts [5], but thus far a detailed study of lysosomal Be uptake has not been undertaken. Beryllium salts are known to generate particulate (Be phosphate) and colloidal (Be hydroxide) forms in the plasma [6] and hepatic accumulation of Be produces focal liver necrosis within 1–3 days [7, 8] probably after the cells have taken up a critical amount of Be [9]. Furthermore, injected soluble forms of Be (e.g. $BeSO_4$, LD_{50} ca. 30 μmoles Be/kg) have been found to be proportionately more toxic than performed particulate forms (e.g. Be phosphate, LD_{50} ca. 200 μmoles Be/kg) [9, 10]. However, the importance of any lysosomal damage produced by the Be both with respect to the chemical form administered and to the necrotic process itself is uncertain. We have therefore examined the time dependent accumulation and loss of Be by hepatic lysosomes after the i.v. administration of sublethal doses of either particulate Be phosphate or the more hepatotoxic Be salt $BeSO_4$. In addition, the discharge of Be by incubation *in vitro* of lysosomes prepared from treated animals has been measured, together with the appearance of β-glucuronidase (EC 3.2.1.31) activity in the incubation medium, a technique which has been used extensively by other workers to ascertain the extent of lysosomal membrane fragility [11, 12]. These experiments have lead us to conclude that whilst lysosomes play a role in the accumulation and subsequent release of Be, the metal induced cell necrosis is probably not the direct result of lysosomal rupture in the cell although after lethal doses of Be discharged hydrolytic enzymes might contribute to the production of hepatocyte damage.

MATERIALS AND METHODS

Estimation of beryllium (Be)

All Be compounds used for animal injections were labelled with radioisotope ^7Be (Radiochemical Centre, Amersham, U.K.) and administered i.v. (0.1–0.2 ml) at doses indicated in the text to male LAC P rats (200 ± 5 g) via a tail vein. Suspensions of particulate ^7Be phosphate (ca. 0.25 μCi/μmole) and $^7BeSO_4$ (ca. 0.5 μCi/μmole) solutions were prepared as previously described [9]. The Be content of the liver tissues and subcellular fractions after injection was estimated by measuring the ^7Be radiation of a sample in a Packard Auto Gamma Spectrometer, Model 5330, with a sample of the injected material as a reference standard.

Measurement of β-glucuronidase activity

Estimation of the β-glucuronidase activity in the various liver preparations and subcellular fractions

was based on the measurement at 420 nm of *p*-nitrophenol liberated from *p*-nitrophenol–β-D-glucuronide [13]. Incubations (1 ml) were carried out at 37° with shaking in 10 ml conical flasks containing 2.5 mM *p*-nitrophenyl–β-D-glucuronide (Boehringer Corp. Ltd), 100 mM sodium acetate buffer, pH 5.0, 0.1 ml 10 per cent Triton X-100 (Koch-Light Ltd) and 0.05 ml test sample for periods of time (60–90 min) such that less than 10 per cent of the substrate was hydrolysed. The reaction was terminated by the addition of 2 ml ice cold 0.2 M glycine, pH 10.4, the mixture centrifuged at 2000 *g* for 10 min and the absorbance of the supernatant measured at 420 nm against an incubation blank stopped at zero time. When enzyme preparations were incubated in the presence of Be, no inhibition of β-glucuronidase activity could be demonstrated.

Isolation of hepatic lysosomes

Whole homogenates (10 per cent w/v) were prepared in 0.25 M sucrose (Analar) as previously described [5], filtered through nylon gauze and then centrifuged at 270 *g* for 10 min (4°) to remove cellular debris and nuclei [14]. The supernatant was then centrifuged at 30,000 *g* for 20 min (4°) to obtain a mitochondria/lysosome pellet (ML) which could be adequately used as a lysosome preparation in the present investigation since, as previously demonstrated [5], it was shown by a density gradient fractionation that Be was not associated with the mitochondrial fraction. Electron micrographic facilities to ascertain if Be was indeed present inside the lysosomes were not available in the present study but earlier ultrastructural studies [15] have shown that vacuolisation and dense deposits, presumably of Be, can be seen in hepatic lysosomes after i.v. treatment of rats with $BeSO_4$. The ML pellet was resuspended in 50 ml 0.25 M sucrose, 0.1 M Tris–HCl, pH 7.2 at 37° and a sample retained for protein determination [16]. The ML pellet prepared in this way would comprise a proportion of both heavy and light lysosomes derived from both liver parenchymal and non parenchymal cells [11, 14].

Incubation of lysosomes in vitro

Incubation of lysosmes *in vitro* usually in a hypoosomotic medium followed by measurement of the appearance of β-glucuronidase activity in the medium has been used by several workers to ascertain lysosomal membrane integrity [11, 12]. In the present study we have used a similar technique, but employed a 0.25 M sucrose buffer system which, although it elicited a slightly lower release of enzyme activity than 0.18 M sucrose [11], in our hands gave more reproducible results for hepatic lysosomes prepared from Be treated rats and induced less damage to control lysosomes. In addition, originally the release of lysosomal acid phosphatase (EC 3.1.3.2.) activity had been used as an index of lysosomal damage, but the results were found to be more variable than for β-glucuronidase. This may be explained by the recent findings that acid phosphatase activity appears to be closely associated with the lysosomal membrane structure [17].

Duplicate samples (4 ml) of the resuspended ML pellet in 0.25 M sucrose, 0.1 M Tris–HCl buffer, pH 7.2 at 37° containing approximately 5 mg protein/ml were incubated in 10 ml conical flasks at 37° with gentle shaking for zero time, 15 min and 30 min. At the end of each time period, the incubation mixtures were transferred to 10 ml MSE polycarbonate tubes, immersed in ice and then immediately centrifuged at 30,000 *g* for 20 min (4°). The resultant supernatants were then removed and the pellet taken up in 4 ml of the 0.25 M sucrose buffer. The Be content and β-glucuronidase activity of the resuspended pellets and respective supernatants were then determined as described above. The loss of lysosomal Be is expressed in the text as pmoles/mg lysosomal protein and released β-glucuronidase activity as a per cent value of the total lysosomal activity, both being corrected for any small losses observed from the lysosomes at zero time of the incubation. In the case of β-glucuronidase activity measurements the values were also corrected for the per cent activity released from hepatic lysosomes of untreated rats under the same conditions (viz. 3–5 per cent). In this way, the rate of loss of both Be and enzyme from lysosomes was approximately linear over the 30 min incubation period after which the rates substantially declined. Additional experiments indicated also that approximately 60 per cent of the Be and 40 per cent of the β-glucuronidase activity could be released from Be containing lysosomes by a single freeze-thawing treatment. These values thus gave an index of the maximum lysosomal release of Be and β-glucuronidase that might be expected for the incubation conditions employed. The less than maximal liberation of Be from lysosomes after the freeze-thawing treatment either indicate incomplete lysosomal rupture or possibly non-specific association with lysosomal fragments of "colloidal" Be produced as a result of destruction of the internal lysosomal acid environment. The limited release of β-glucuronidase activity probably also indicates not all the enzyme is "unsedimentable" and that for the conditions used some degree of association of the enzyme with lysosomal membranes probably occurs similar to that observed for acid phosphatase [17].

RESULTS

Previous studies have shown that after i.v. injection into rats particulate Be phosphate is removed from the blood within 10–30 min almost entirely by the liver [9]. In contrast Be derived from injected $BeSO_4$, although also taken up principally by the liver, takes several hours to be removed from the circulation [9, 18]. The rate of disappearance of both forms of Be is dose dependent [9, 18] but the slower hepatic removal of soluble $BeSO_4$ has been attributed to the formation of both "particulate" and "diffusible"† forms of Be in the blood [6, 18]. Figures 1 and 2 illustrate that the dose-dependent uptake

† The "particulate" forms of Be formed in the blood after i.v. injection of $BeSO_4$ are believed to be Be phosphate [$Be_3(PO_4)_2$], Be hydroxide [$Be(OH)_2$] and possibly $Be_3(PO_4)_2$ $Be(OH)_2$ complexes, all probably associated with serum globulins [6]. The "diffusible" forms of Be are thought to be Be complexed predominantly with the plasma organic acid citrate [19].

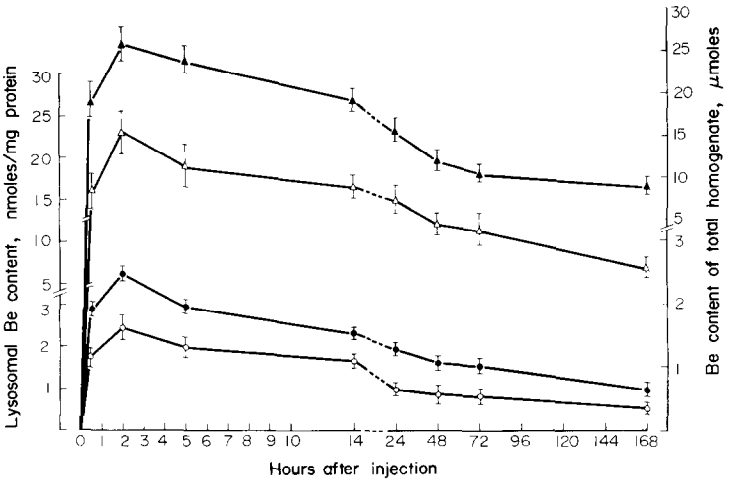

Fig. 1. Change of beryllium content with time of liver homogenates and a hepatic lysosome fraction isolated from rats injected i.v. with Be phosphate. Rats (200 ± 5 mg) were injected i.v. with Be phosphate (12.5 μmoles/kg or 150 μmoles/kg) killed at the time indicated and lysosome fractions (—○—; —△) isolated from liver homogenates (—●—; —▲—) for determination of Be content as described in Materials and Methods.

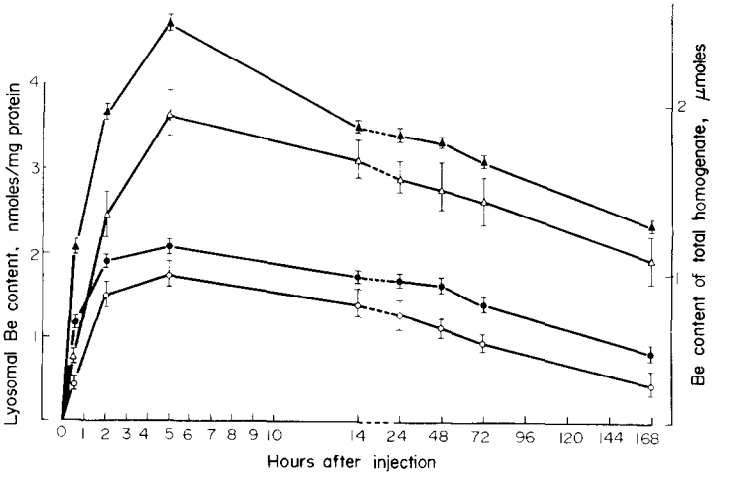

Fig. 2. Change of beryllium content with time of liver homogenates and a hepatic lysosome fraction isolated from rats injected i.v. with $BeSO_4$. Details as given in Fig. 1 but animals given $BeSO_4$ (12.5 μmoles/kg, —●— (homogenate), —○— (lysosomes) or 25 μmoles/kg, —▲— (homogenate), —△— (lysosomes).

and loss of Be by hepatic lysosomes from non lethal doses of Be phosphate or $BeSO_4$ closely follows the pattern of the accumulation and subsequent loss of Be observed in whole liver. The data indicate that following i.v. administration of Be phosphate (12.5 and 150 μmoles Be/kg) maximum Be content of the lysosome fraction is reached within 2 hr, after which there is a gradual fall in the lysosomal Be concentration over the next 7 days to about 30 per cent of the value at 2 hr (Fig. 1). In the case of rats treated with $BeSO_4$ (12.5 and 25 μmoles Be/kg) the maximum lysosomal Be content was not achieved until about 5 hr after injection and then this also was followed by a 60–75 per cent decrease in the lysosomal Be over the next 7 days (Fig. 2).

The gradual decrease after 2–5 hr in the values of the lysosomal Be content (Figs. 1 and 2) could be due either to release of the Be from the lysosomes *per se*, or, be a result of some loss of integrity of the lysosomal membrane. During the course of the experiments both the specific activity of the enzyme β-glucuronidase in the lysosome fractions and the total β-glucuronidase in the corresponding liver homogenates remained more or less constant, which might suggest any marked Be induced lysosomal damage was improbable.

A technique which has been used by other workers as an index of lysosomal damage *in vivo* has been to measure the latent release of lysosomal β-glucuronidase or other enzyme activities from isolated lysosomes into a suitable incubation medium *in vitro* [11, 12]. Therefore, this approach was employed to monitor both release of β-glucuronidase activity and the simultaneous appearance of Be in an incubation medium for hepatic lysosomes prepared at various times after i.v. injection of rats with Be phosphate

or $BeSO_4$. The data indicate that, although no information could be gathered regarding its chemical form, the liberation of Be into the incubation medium was greatest for lysosomes isolated from the livers of rats injected with Be 2–14 hr previously (Table 1). The loss, however, was more marked the greater the lysosomal Be concentration, and estimated never to exceed 5–7 per cent of the initial Be content of the lysosomes compared with 60 per cent which could be obtained by a single freeze-thawing treatment (see Materials and Methods). The extent of the lysosomal rupture as indicated by β-glucuronidase activity release showed also that hepatic lysosomes were most susceptible to damage when prepared from rats treated with Be 2–5 hr previously being particularly marked at the higher doses of Be used. The release of enzyme activity then declined to almost insignificant levels by 2 days after Be treatment (Table 1). It is interesting to note that lysosomes derived from animals treated with $BeSO_4$ showed the more marked fragility which is consistent with the greater hepatotoxicity of this form of administered Be.

In an attempt to determine if direct action of Be compounds with the lysosomal membrane could bring about damage of the organelle, hepatic lysosomes from untreated animals were incubated *in vitro* with various externally added Be compounds (0.1–1.0 mM). No release of β-glucuronidase activity could be detected under these conditions (Table 1) and therefore the results indicate that either the lysosomal rupture observed for Be treated animals might be brought about by some other action of Be or that the lysosomal membrane is susceptible to attack by intralysosomal Be incorporated by endocytosis whilst remaining relatively unaffected by Be applied to the face of the membrane *in vitro*.

DISCUSSION

The data presented shows that there is a good correlation between the time course of the accumulation and loss of Be by the liver and that observed in hepatic lysosomes from rats injected with a single i.v. dose of Be phosphate or $BeSO_4$. These results illustrate the central role played by lysosomes in the cellular uptake of Be and are consistent with the recent indications from experiments with liver cells in culture that Be probably enters the cells mainly by endocytosis of a particulate or colloidal form rather than as a soluble complex [20]. Earlier work has shown that for the doses used in the present studies, after hepatic accumulation, Be is redistributed to the bone tissue or the spleen [9], and therefore the mechanism of hepatic Be release is of particular interest. It has been established also that

Table 1. Liberation of beryllium and β-glucuronidase activity from hepatic lysosomes of beryllium treated rats after incubation *in vitro*

Dose of Be to rats	Time after injection of Be: 30 min	2 hr	5 hr	14 hr	1 day	2 day	3 day	7 day	
	Liberation of Be from lysosomes in 30 min (pmol/mg lysosomal protein)								
Be phosphate									
12.5 μmoles/kg	22 ± 7	71 ± 19	141 ± 25	101 ± 24	65 ± 17	44 ± 22	23 ± 13	18 ± 9	
150 μmoles/kg	49 ± 10	506 ± 96	993 ± 124	706 ± 92	103 ± 30	56 ± 12	54 ± 11	41 ± 8	
$BeSO_4$									
12.5 μmoles/kg	15 ± 4	52 ± 14	105 ± 18	46 ± 17	41 ± 14	22 ± 6	25 ± 5	12 ± 2	
25.0 μmoles/kg	27 ± 5	60 ± 10	109 ± 16	65 ± 16	60 ± 15	47 ± 10	42 ± 8	40 ± 7	
	Liberation of β-glucuronidase activity from lysosomes in 30 min (%)								
Be phosphate									
12.5 μmoles/kg	4.5 ± 2.5	4.5 ± 2.1	6.0 ± 1.8	3.4 ± 1.4	2.5 ± 1.0	2.5 ± 1.0	3.2 ± 1.2	2.6 ± 0.8	$<1.0^a$
150 μmoles/kg	6.7 ± 2.6	9.0 ± 2.8	12.2 ± 3.1	5.8 ± 1.7	4.5 ± 1.5	4.0 ± 1.3	5.9 ± 2.0	4.6 ± 1.1	1.0 ± 0.2^b
$BeSO_4$									
12.5 μmoles/kg	2.6 ± 0.7	6.0 ± 2.2	8.5 ± 2.7	4.4 ± 1.6	4.5 ± 1.5	3.2 ± 1.1	2.7 ± 0.9	3.3 ± 1.2	$<1.0^c$
25.0 μmoles/kg	4.0 ± 1.4	9.7 ± 3.2	13.2 ± 3.4	6.6 ± 2.0	5.2 ± 1.6	4.1 ± 1.5	3.4 ± 1.3	3.7 ± 1.4	0.8 ± 0.1^d

Rats (200 ± 5 g) were injected i.v. with either Be phosphate or $BeSO_4$, killed at the times indicated and a lysosome fraction prepared from a liver homogenate as described in the Materials and Methods section. The isolated lysosomes were incubated at 37° in 0.25 M sucrose 0.1 M Tris, pH 7.2 for 30 min and then the β-glucuronidase activity and Be released into the medium was measured. All values are quoted as Mean ± S.D. (four determinations) after correction for any small loss of Be or enzyme activity from the lysosomes observed at zero time of the incubation and for β-glucuronidase activity released from hepatic lysosomes of untreated animals under the same conditions (see Materials and Methods).

* Data for β-glucuronidase liberation from lysosomes isolated from untreated animals and incubated *in vitro* under the same conditions but in the presence of either 10 μM and 1.0 mM Be phosphate (*a* and *b*) or 10 μM and 1.0 mM $BeSO_4$ (*c* and *d*). Similar values (not shown) were obtained when lysosomes were incubated in the presence of the model soluble Be complex Be sulphosalicylate (1:2) (20).

The release of β-glucuronidase activity which could be obtained by a single freeze-thawing treatment was 40 per cent (see Materials and Methods).

whilst the form of Be derived from injected $BeSO_4$ is taken up by both the parenchymal and sinusoidal cells of the liver, injected Be phosphate is accumulated primarily by the sinusoidal (Kupffer) cells [9] and this has helped explain the relatively greater hepatotoxicity of $BeSO_4$ *in vivo*. In the present investigation, therefore, the changes in lysosomal Be content observed after Be phosphate treatment will reflect essentially the behaviour of Be in Kupffer cell lysosomes, whereas the corresponding changes measured for $BeSO_4$ treated animals will be that of Be in lysosomes of mixed hepatocellular origin However, the similar time course patterns in the disappearance of Be from lysosomes observed for both Be phosphate and $BeSO_4$ treated rats might suggest a similar process occurs in respect of the mechanisms of release of Be from the lysosomes. Since it is known that lysosomes possess an acid internal environment [21], it follows from chemical considerations that Be should be readily liberated from both Be phosphate or Be hydroxide, the forms believed to be actually endocytosed into the cell [20]. The chemical form in which the Be might be released into the cell is uncertain, although in view of the propensity of Be to form its phosphate in the presence of inorganic phosphate or hydroxides at neutral pH, it is likely that these chemical forms could be regenerated in the cytosol in addition to Be complexed with certain carboxylic acids (e.g. citrate). Furthermore, in view of the liberation of both Be and β-glucuronidase activity from lysosomes incubated *in vitro*, particularly 2–14 hr after injection of the animals with Be, this suggests that the gradual decrease in lysosomal Be content observed *in vivo* may result from some degree of lysosomal rupture rather than passage of Be *per se* through the lysosomal membrane. In addition the apparent loss of Be from lysosomes could in part also reflect either the normal or an enhanced cellular lysosomal turnover [21] during which Be becomes liberated from the lysosomal population; but more definitive experiments would need to be done to ascertain if this were the case.

The present results obtained after injection of sublethal doses of Be compounds contrast with the earlier study using lethal doses of $BeSO_4$ in which a massive release of acid phosphatase into the liver cytosol was observed 24 hr after administration of the Be [8]. However, these authors concluded that this lysosomal release of hydrolytic enzyme activity probably reflected the aftermath rather than the initiation of the hepatic necrosis. These conclusions raise the general question of the relevance of the lysosomal release of hydrolytic enzymes to the cytotoxicity of Be, since this appears to be important after the uptake of some substances by cells [21, 23]. However severe hepatic lysosomal damage together with liberation of hydrolytic enzymes can be produced, for example, by injection of 1/20 LD_{50} doses of the dye neutral red (400 mg/kg i.p.) to rats [24]. In the present study also, hepatic lysosomes isolated after 1–4 hr from similarly treated rats showed a release of 20–30 per cent β-glucuronidase activity when incubated *in vitro* for 30 min (unpublished data), which was more marked than that observed for Be treated animals. Thus it is suggested that the cytotoxic target for Be is probably extra-lysosomal, and that a primary role of hepatic lysosomes in Be toxicity is in the intracellular accumulation and subsequent release of Be. It is known that Be can be found associated with cell nuclei after hepatic accumulation [5], but preliminary experiments indicate that after non lethal doses of Be this may be only a small proportion of the Be initially taken up into lysosomes. These results might suggest therefore, that under these conditions hepatic lysosomal Be is released, probably to be relocated mainly in the spleen and skeletal tissue [9] rather than being retained within the hepatocytes.

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