Biochemical Pharmacology, Vol. 35, No. 6, pp. 933–938, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

THE RELEASE OF INTRALYSOSOMALLY-STORED ^{125}I-TRITON WR-1339 AND LYSOSOMAL ENZYMES FROM THE ISOLATED PERFUSED RAT LIVER IN THE PRESENCE AND ABSENCE OF CYTOCHALASIN B

HELEN MICHELAKAKIS* and CHRISTOPHER J. DANPURE
Division of Inherited Metabolic Diseases, Clinical Research Centre, Watford Road, Harrow, Middlesex, HA1 3UJ, U.K.

(*Received* 30 *July* 1985; *accepted* 1 *October* 1985)

Abstract—The effect of *in vivo* loading of the lysosomotropic agent ^{125}I-Triton WR-1339 on the release of lysosomal enzymes in isolated perfused rat liver has been studied in the presence and absence of the microfilament poison cytochalasin B, as has the release of the ^{125}I-Triton WR-1339 itself.

Perfused isolated rat livers released all the enzymes studied (arylsulphatase, β-galactosidase and lactate dehydrogenase) and, when preloaded, ^{125}I-Triton WR-1339 was also released into the perfusate. The magnitude of the net release (after 5 hr perfusion) was in the order β-galactosidase = ^{125}I-Triton WR-1339 > lactate dehydrogenase > arylsulphatase. Preloading of the lysosomes with the detergent appeared to bring about an increase in the release of all the enzymes studied (3.5× for β-galactosidase, 2.6× for arylsulphatase and 1.7× for lactate dehydrogenase). The addition of the microfilament poison cytochalasin B into the perfusate of non-loaded livers significantly increased the release of the lysosomal enzymes but not that of lactate dehydrogenase. However in the ^{125}I-Triton WR-1339- loaded livers cytochalasin B had no effect on the release of lysosomal enzymes or detergent, but reduced the loss of lactate dehydrogenase by about 50%.

This failure of cytochalasin B to potentiate the exocytosis of lysosomal contents in ^{125}I-Triton WR-1339-loaded livers is similar to the effect found previously with ^{125}I-PVP-loaded livers and may be related to the already enhanced loss of lysosomal enzymes apparently caused by the loading.

It has been shown that Triton WR-1339 when administered *in vivo*, is incorporated into hepatic [1, 2] and aorta [2] lysosomes. The intralysosomal accumulation of the non-ionic detergent is associated with a reduction in the buoyant density of lysosomes [1], an effect which experimental evidence suggests is caused not just by the stored detergent [1] but also by the accumulation of lipids [3, 4] probably derived from serum very-low-density lipoprotein [5].

The uptake of Triton WR-1339 by liver has been shown to cause autophagy [6] which is followed by increased vacuolation and hypertrophy of the Golgi apparatus [1, 6]. The morphological changes in the lysosomal population brought about by Triton WR-1339 are similar to those observed in many lysosomal storage diseases, e.g. fucosidosis [7], GM_1-gangliosidosis [8] and mucolipidosis [9].

The role of the cytoskeleton in the extracellular release of secretory products and lysosomal enzymes has been extensively studied in a variety of systems. In particular the use of the microfilament poison cytochalasin B has shown microfilaments to be involved in many secretory processes including the exocytosis of lysosomal enzymes [10–12].

In the present study, we have investigated the extracellular release of ^{125}I-Triton WR-1339 and lysosomal enzymes from isolated pre-loaded rat livers kept in perfusion. The role of microfilaments in the above process was assessed using cytochalasin B.

* Present address: Institute of Child Health, "Aghia Sophia" Children Hospital, Athens GR-115 27, Greece.

MATERIALS AND METHODS

Materials

Bovine serum albumin fraction V powder, cytochalasin B, *p*-nitrocatechol sulphate, *p*-nitrophenyl-β-D-galactopyranoside were purchased from Sigma (London) Chemical Co. Ltd., (Poole, Dorset, U.K.), Leibovitz—L15 medium from Flow Laboratories (Irvine, Scotland) and Sephadex G-200 from Pharmacia (Great Britain) Ltd. (Hounslow, Middlesex). Triton WR-1339 came from Winthrop Laboratories (Newcastle-upon-Tyne, U.K.), and Na^{125}I from Radiochemical Centre (Amersham, U.K.). All the other chemicals were purchased from BDH (Poole, Dorset, U.K.).

The experimental animals used were Sprague–Dawley male rats weighing between 400 and 500 g. They were maintained on a "Spratts Rodent Breeding" diet, food and water being allowed *ad libitum*.

Methods

Enzyme assays. Arylsulphatase A+B was assayed by the method of Dodgson *et al.* [13], β-galactosidase and β-*N*-acetylglucosaminidase by the method of Barrett and Heath [14], lactate dehydrogenase by the method of Plummer and Wilkinson [15], and cytochrome-*c*-oxidase by a modification of the method of Cooperstein and Lazarow [16] similar to that of Appelmans *et al.* [17]. Protein was measured by the method of Lowry *et al.* [18].

Iodination of Triton WR-1339. The iodination of Triton WR-1339 was carried out using the chloramine T method as described by Michelakakis [19].

Injection solution. The animals were injected intraperitoneally with 85 mg/100 g body weight of a Triton WR-1339 solution (170 mg/ml in saline). When required 50 μCi of ^{125}I-Triton WR-1339 was simultaneously injected.

Subcellular distribution. Four days after the injection of Triton WR-1339 the rats were killed by dislocation of the cervical vertebrae. In order to remove the blood, the livers were perfused *in situ* with NaCl (0.9%) at 4° containing heparin (500 IU/ml) and were then minced and homogenised in 0.25 M sucrose using three up-and-down strokes of a Potter-Elvehjeim homogeniser. This step and all subsequent centrifugal steps were carried out at 4°. The homogenate was diluted with sucrose (0.25 M) to a final concentration of one gram wet weight of liver in 10 ml (10% w/v) and was spun at 500 *g* for 10 min to give a supernatant (cytoplasmic fraction) and a pellet (cell debris fraction). A mitochondrial-lysosomal pellet was obtained by spinning a cytoplasmic fraction at 30,000 *g* for 25 min. The pellet was washed once in sucrose (0.25 M) and the final pellet obtained was resuspended in the sucrose (0.25 M).

In order to investigate the intralysosomal distribution of the lysosomal enzymes and label, this mitochondrial-lysosomal fraction was sonicated and centrifuged at 75,000 *g* for 80 min to provide a pellet (membrane fraction) and a supernatant (luminal fraction).

Liver perfusion. The operation procedure followed for the removal of the livers was that described by Michelakakis [19] and Michelakakis and Danpure [20]. The livers were perfused (inflow via the hepatic portal vein and outflow via the vena cava) in a cabinet maintained at 37° using recycling, oxygenated Leibovitz L-15 medium supplemented with 5% bovine serum albumin and containing streptomycin and penicillin (both 100 units/ml). Cytochalasin B, dissolved in dimethyl sulphoxide, was added 90 min after the beginning of the perfusion to give a final concentration of 100 μg/ml (1% w/v DMSO). The control livers were treated with DMSO only. At 30-min intervals samples of the perfusate were assayed for lysosomal enzymes, lactate dehydrogenase and radioactivity. At the end of the perfusion the liver was homogenised in sucrose (0.25 M) and the homogenate was assayed for various enzymes.

Gel chromatography. Samples of the injection solutions, high-speed supernatants of sonicated mitochondrial-lysosomal fractions and perfusate were eluted through columns (1.5 cm × 80 cm) of Sephadex G200 with 10 mM Tris/HCl buffer pH 7.4 at 4°.

RESULTS

Sephadex G200 chromatography of the ^{125}I-Triton WR-1339 injection solution (Fig. 1) showed that the radioactivity was divided into two peaks, one eluting just after the column void volume (V_o) and the other, smaller, peak at the column total volume (V_t). Four days after the administration of this material, the radioactivity was distributed throughout the body, the highest proportion being in the liver (11.1–15.8% of the injected dose) and the second highest in the thyroid (4.4–5.0% of the injected dose). About half of the liver radioactivity was recovered in the mitochondrial-lysosomal pellet along with about half of the lysosomal enzyme markers (Table 1). Investigation into the intralysosomal distribution of the radioactivity, as manifested by its distribution between the pellet and supernatant of a centrifuged sonicated mitochondrial-lysosomal fraction, showed that 90% was soluble (i.e. not membrane bound) compared to 70% for β-galactosidase, 40% for arylsulphatase and only 6% for β-*N*-acetylglucosaminidase (Table 1). The presence of the detergent did not bring about any change in enzyme distribution compared with the control (Table 1). When the centrifugal supernatant from a sonicated mitochondrial-lysosomal fraction was chromatographed on Sephadex G200, the radioactivity profile was similar to the high-molecular-weight component of the

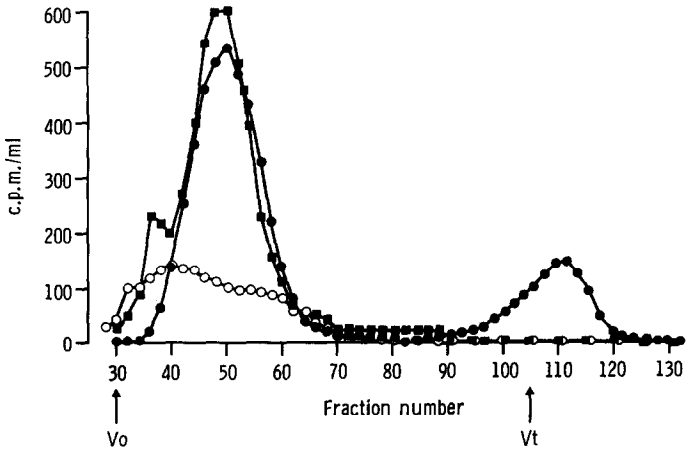

Fig. 1. The elution profiles obtained by passing down a Sephadex G-200 column a sample of the ^{125}I-Triton WR-1339 injection solution (●—●), a sample of the high-speed supernatant of a lysed mitochondrial-lysosomal pellet preparation obtained from a rat liver loaded with ^{125}I-Triton WR-1339 4 days earlier (■—■) and a sample of the perfusate of an isolated rat liver kept in perfusion in the absence of Cytochalasin B that was loaded with ^{125}I-Triton WR-1339 4 days earlier (○—○).

Table 1. The distribution of lysosomal enzymes, lactate dehydrogenase, protein and radioactivity in the mitochondrial-lysosomal (ML) pellet as a % of homogenate activity, and in the sonicated ML supernatant as a % of the activity in the ML pellet

	Activity in ML pellet (% of homogenate activity)		Activity in sonicated ML supernatant (% of ML pellet activity)	
	Loaded	Control	Loaded	Control
β-*N*-acetylglucosaminidase	44.7	60.0	6	6
Arylsulphatase	49.4	51.8	40	38
β-Galactosidase	51.0	55.0	70	70
^{125}I-Triton WR-1339	49.0	—	90	—
Protein	33.0	39.0	—	—
Lactate dehydrogenase	4.0	8.0	—	—

injection solution, with the addition of a small higher-molecular-weight peak or shoulder close to the V_0 (Fig. 1).

The presence of ^{125}I-Triton WR-1339 made little difference to the total liver activities of arylsulphatase, β-galactosidase or β-*N*-acetylglucosaminidase (data not shown).

When livers previously loaded with ^{125}I-Triton WR-1339 were perfused with recirculating medium, significant amounts of β-galactosidase, arylsulphatase, lactate dehydrogenase and radioactivity were released into the perfusate (Fig. 2). Although the spread of the values was large, ^{125}I and β-galactosidase were released to similar extents but much

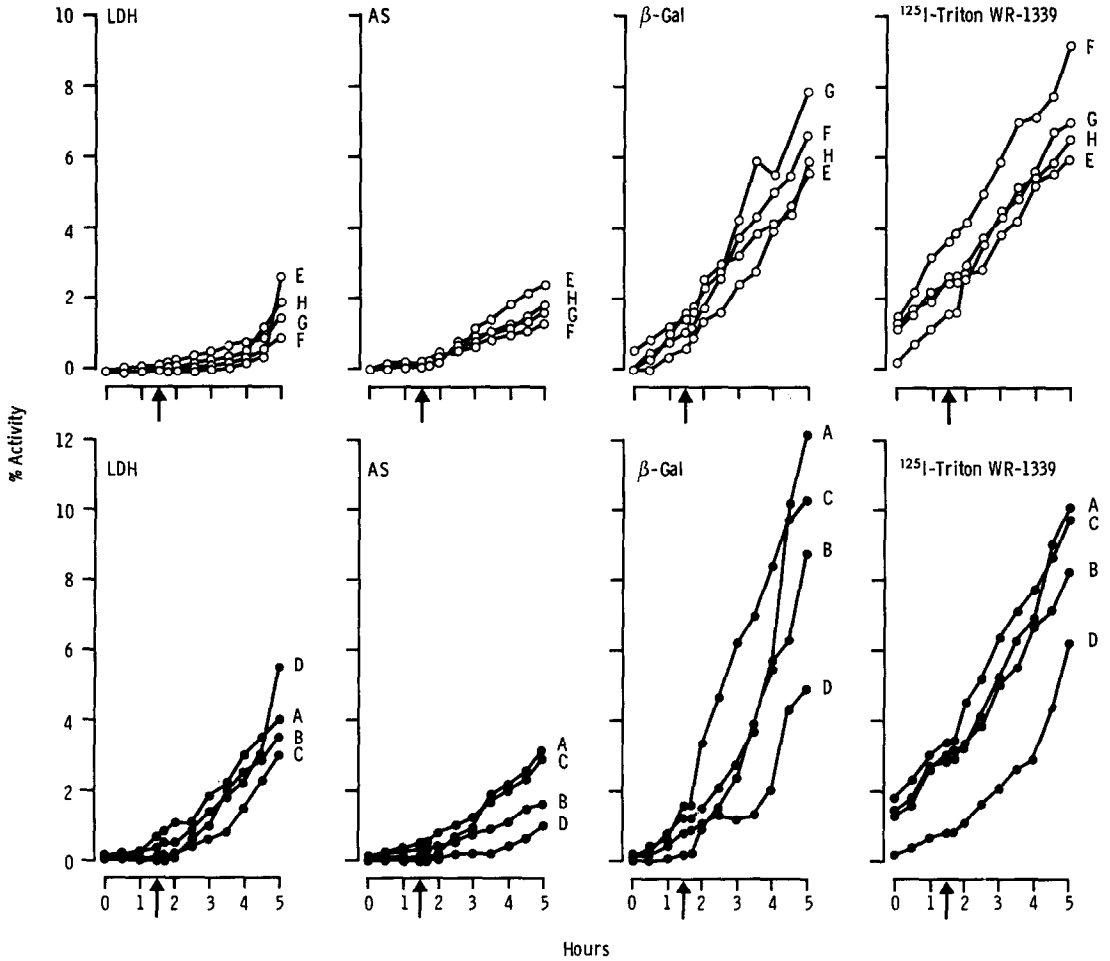

Fig. 2. The release of lactate dehydrogenase (LDH), arylsulphatase (AS), β-galactosidase (β-Gal) and label from controls (●—●) and cytochalasin B treated (○—○) isolated perfused rat livers loaded with ^{125}I-Triton WR-1339 4 days earlier. The arrows represent the time points at which cytochalasin B or DMSO were added. The letters correspond to different experiments.

Table 2. The enzyme activities and ^{125}I-Triton WR-1339 found in the perfusate at the end of the perfusion period in the presence or absence of cytochalasin B expressed as the percentage of total recovered activity and the statistical significance of their difference, obtained by Students *t*-test

	Activity in perfusate		
	Controls(DMSO) Mean (Range, N)	Cytochalasin B Treated Mean (Range, N)	P values
Non-Loaded			
Arylsulphatase	0.84 (0.4–1.4,4)	1.9 (1.3–2.7,4)	$P < 0.025$
β-Galactosidase	1.6 (0.8–2.3,4)	3.0 (1.8–5.0,4)	$P < 0.05$
Lactate dehydrogenase	1.7 (1.2–2.0,4)	1.8 (1.0–2.0,4)	NS
^{125}I-Triton WR-1339-Loaded			
Arylsulphatase	2.2 (1.0–3.2,4)	1.9 (1.3–2.4,4)	NS
β-Galactosidase	9.1 (5.0–12.2,4)	6.6 (5.6–7.9,4)	NS
^{125}I-Triton WR-1339	8.6 (6.2–10.1,4)	7.5 (6.0–9.3,4)	NS
Lactate dehydrogenase	4.0 (3.0–5.5,4)	1.8 (0.9–2.6,4)	$P < 0.005$

The livers were either not loaded or loaded with ^{125}I-Triton WR-1339 4 days previously.

more so than arylsulphatase and lactate dehydrogenase (Fig. 2, Table 2). Gel chromatography of a sample of the perfusate showed that the radioactivity released by the liver was of a molecular weight similar to the high-molecular-weight component of the injected dose, but skewed towards even higher molecular weights and the V_0 (Fig. 1). In the absence of the microfilament poison cytochalasin B, all the enzymes were released to a greater extent from the ^{125}I-Triton WR-1339—loaded livers than from the non-loaded controls (Table 2). In the presence of cytochalasin B the pattern of enzyme and radioactivity release was very similar to the controls except that the release of lactate dehydrogenase was significantly reduced (Fig. 2, Table 2).

DISCUSSION

The uptake by the liver and the intralysosomal localization of Triton-WR-1339, as well as ^{125}I-Triton WR-1339, has been well documented previously [1, 21].

The elution profile of the injected material on Sephadex G-200 showed two peaks of radioactivity. Henning and Plattner [22] managed to fractionate the commercially-available Triton WR-1339 into high and low molecular weight components. Although it is not clear to what extent the second peak obtained in the present study is microtriton or free iodide, since they should both have the same elution volume on Sephadex G-200, the fact that 4–5% of the injected radioactivity was associated with the thyroid suggests that at least part of the second peak was free iodide.

Previous studies have demonstrated rather contradictory effects of Triton WR-1339 on liver lysosomal enzyme activities. Some are increased [23], others decreased [24], while most appear to be unaltered [1, 23, 24]. In our study, the detergent did not alter the total liver activities of β-galactosidase, β-*N*-acetylglucosaminidase or arylsulphatase.

Lysosomal enzymes and intralysosomally-stored ^{125}I-Triton WR-1339 could be released during perfusion by exocytosis or cell death. The clearance of "tritosomes" into the bile canaliculi via an exocytic mechanism has been suggested by De Duve and Wattiaux [25]. Trout and Viles [6, 26] have obtained morphological evidence for the clearance of "tritosomes" both into the bile canaliculi and the space of Disse, whereas La Russo *et al.* [24] obtained biochemical evidence for the clearance into the bile. Despite all the above findings the mechanism of the release is still unknown. In the present study the fact that not only the intralysosomal contents but also lactate dehydrogenase were released into the perfusate makes the assessment of the relative contributions of exocytosis and cell death in the release of the lysosomal contents difficult.

The fact that the release of β-galactosidase and radioactivity into the perfusate exceeded that of lactate dehydrogenase might suggest that at least part of their release was not due to cell death. Net secretion of lysosomal enzymes into the perfusate is likely to be a result of the difference between exocytosis and re-endocytosis [27], whereas lactate dehydrogenase is unlikely to be significantly taken back up under the conditions of the present experiment.

The radioactivity released into the perfusate as well as that in the mitochondrial-lysosomal pellet was entirely in a high-molecular-weight form, with little or no degradation and release of low-molecular-weight ^{125}I. The portion of the radioactivity of molecular weight apparently greater than the injected "macrotriton" could correspond to Triton WR-1339 associated with α-lipoprotein as suggested previously [22, 28].

Compared to non-loaded livers ^{125}I-Triton WR-1339-loaded livers released higher amounts of all the enzymic parameters measured (Table 2 and [20]). Triton WR-1339 has been shown to increase the release of lysosomal enzymes into the bile without any increase in the release of lactate dehydrogenase [24]. In the present study although the detergent increased both the release of lysosomal enzymes and lactate dehydrogenase, the former was greater than the latter. It could be argued therefore that the detergent can induce the release of lysosomal enzymes both into the space of Disse, as it is the case in this study, and the bile canaliculi through a

mechanism other than cell death, possibly exocytosis. Assuming that such an exocytic mechanism does occur, Triton WR-1339 could have brought it about by modifying the fusion characteristics of hepatocyte lysosomes, probably by altering the fluidity properties of the hepatocyte membranes [29].

In order to investigate the mechanism of the release of the intralysosomal contents of livers kept in perfusion, cytochalasin B, an agent known to promote exocytosis [10, 11], by interfering with microfilaments, was added in the perfusate. Whereas in non-loaded livers (Table 2 and [20]) cytochalasin B did stimulate the release of lysosomal enzymes, in loaded livers the addition of the microfilament poison did not bring about any change in the amounts of the parameters measured in the perfusate apart from lactate dehydrogenase, which was reduced to about 50%.

Although it has been shown that there exist high affinity binding sites for cytochalasin B in human, bovine and rabbit red blood cells located on membrane proteins exposed to the cytoplasmic side of the cell membrane [30], as well as in hepatocyte plasma and intracellular membranes [31], no evidence is available for a stabilising effect of cytochalasin B on cellular membranes. However, the approximately 50% reduction in the release of lactate dehydrogenase from Triton WR-1339 loaded livers, brought about by the addition of cytochalasin B, suggests that the microfilament poison could have such an effect in the presence of the detergent.

The higher release of lactate dehydrogenase in the absence of cytochalasin B than in its presence makes the interpretation of the absence of cytochalasin B effect on the release of lysosomal enzymes difficult. It is not clear therefore to what extent microfilaments are involved in the secretion of the lysosomal contents from ^{125}I-Triton WR-1339-loaded livers. Nonetheless the fact that the release of lysosomal enzymes and ^{125}I-Triton WR-1339 was not reduced in the presence of cytochalasin B, as did that of lactate dehydrogenase, could indicate that at least some of the lysosomal contents were released via an exocytic mechanism.

As found in a previous study [20], the net release of the intralysosomally-stored material (in that case ^{125}I-PVP) and β-galactosidase was greater than that of arylsulphatase. Whether this is associated with the greater apparent intralysosomal solubility of the former two is not clear. But nevertheless, the increased lysosomal enzyme release brought about by the detergent in the present study was not related to intralysosomal solubility, as the latter did not change.

Although loading of lysosomes with ^{125}I-PVP, another non-digestible material, has been shown to increase the release of lysosomal enzymes from isolated rat livers kept in perfusion as compared to non-loaded livers [20], the increase observed in the present study was far greater. Also cytochalasin B, although failing to increase the release of lysosomal contents from both ^{125}I-PVP and ^{125}I-Triton WR-1339-loaded livers, in the case of the latter also brought about a significant reduction in the release of lactate dehydrogenase. It seems possible that, in the case of loaded livers, the release of the lysosomal contents and the response of the process to cytochalasin B is determined not only by the intralysosomal accumulation of non-digestible material but also by the nature of the stored materials.

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