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BUTANEDIONE TREATMENT REDUCES RECEPTOR BINDING OF A LYSOSOMAL ENZYME TO CELLS AND MEMBRANES

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Summary: Treatment of the lysosomal enzyme, α-L-iduronidase, with 2,3 butanedione, an arginine modifying reagent, under conditions where enzyme activity was unaffected, reduced by 50% the internalization of the enzyme into cultured human fibroblasts. The lowered rate of internalization was a result of a reduced binding of the enzyme to cell surface receptors. The butanedione treatment of α-L-iduronidase caused a 90% reduction of binding when isolated fibroblast membranes were used as the source of receptor. This marked reduction of binding was also seen when membranes from a rat chondrosarcoma were examined. Although there is ample evidence that the receptor recognizes mannose 6-phosphate residues on the enzyme, the results suggest that other structural features, such as arginine moieties, may also be important in iduronidase binding.

Introduction

Cultured human fibroblasts have cell surface receptors for α-L-iduronidase, a lysosomal hydrolase (1). The binding is specific for the high uptake form of the enzyme and is competitively inhibited by mannose 6-phosphate. Once bound, the enzyme is endocytosed and incorporated into lysosomes (2,3). Internalization experiments with several other lysosomal hydrolases implicate receptor binding as a general mechanism for enzyme uptake (4-6). There is now considerable evidence that the cell surface receptors "recognize" mannose 6-phosphate moieties attached to the enzyme (2,4-11). Recent studies suggest that multiplicity of receptor sites may contribute to the effectiveness of binding (1,7-9); however, model compounds have been prepared with up to 25 molecules of mannose 6-phosphate per molecule of protein and these inhibited no better than would be expected by their

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mannose 6-phosphate residues alone (12). Binding of the enzymes therefore may depend on the exact position of attached mannose 6-phosphate residues or on other structural characteristics.

Like certain lysosomal enzymes, low density lipoprotein (LDL) is specifically bound, internalized, and packaged into lysosomes of human fibroblasts (for review see 13). Mahley et al. proposed that arginine groups were part of the recognition marker for LDL since selective modification of these residues abolished LDL binding to cell surface receptors (14). Filipovic and Buddecke concluded from studies utilizing chemical modification of LDL and enzymatic removal of sialic residues that it is the net charge of the LDL rather than the arginine groups per se that regulates its binding characteristics (15). These studies prompted us to examine the role of arginine moieties in α-L-iduronidase binding.

Materials and Methods

Enzyme Modification: High uptake α-L-iduronidase, prepared as described (1), was incubated with 50 mM 2,3 butanedione (Aldrich) in 75 mM borax-phosphate buffer, pH 7.0 (4.8 g borax, 10.2 g KH_2PO_4/liter). After 2 h at ca. 25°, the solution was diluted 5-fold with cold 75 mM borax-phosphate buffer, pH 6.5 (4.8 g borax, 10.35 g $NaH_2PO_4 \cdot H_2O$/liter adjusted to pH 6.5) containing 1 mg/ml bovine serum albumin and dialyzed overnight against the pH 6.5 borax-phosphate buffer (1 liter, three changes). As a control, enzyme was subjected to identical conditions, only without the 2,3 butanedione ("mock treated enzyme")

Membranes: α-L-iduronidase deficient fibroblasts were labeled, harvested, and broken as described (1-3). The post nuclear supernatant was centrifuged at 12,000 x g for 20 min (Sorvall SM24 rotor). The supernatant was decanted and subjected to a 100,000 x g centrifugation for 1 h. The resulting membranous pellet was suspended by sonication in 0.01 M sodium phosphate buffer, pH 6.8, containing 0.15 M NaCl. The high speed membrane fraction contained approximately 90% of the total α-L-iduronidase binding activity that was recovered from whole cells (the remaining 10% was in the nuclear and 12,000 x g pellets).

Swarm rat chondrosarcoma tissue (obtained from Dr. J. Kimura of the National Institute of Dental Research) was cleaned and pressed through a 1 mm^2 mesh stainless steel sieve (16). The minced tumor was suspended in two volumes of the sodium phosphate buffer, homogenized 5 min in a Waring blender and passed through a French pressure cell (American Instrument Co.) at a pressure of 10,000 psi. The thick suspension was subjected to a centrifugation at 12,000 x g for 20 min and the resulting supernatant was recentrifuged for 1 h at 100,000 x g. The high speed membranes were either resuspended by sonication as above or extracted with acetone at -10° to -20° (17); acetone extracted membranes were dissolved in 0.01 M HCl before they were used.

Uptake and Binding: Measurements of α-L-iduronidase uptake and binding to cultured cells in suspension were carried out as described (1). Binding to membrane preparations was carried out using a modification of the method of Basu et al. (18). Membranes, enzyme, and other additives were mixed in serum free medium in a final volume of 75 µl in 1.5 ml Brinkman polypropylene

tubes. After 90 min on ice, the mixtures were layered onto 90 µl of 100 mg/ml bovine serum albumin in serum free medium. The tubes were subjected to centrifugation in a Beckman Airfuge (100,000 x g, 20 min, 4). The supernatant was removed by aspiration and the pellet resuspended using a thin steel wire in 170 µl of 1 mg/ml bovine serum albumin in serum free medium. The tube was again subjected to centrifugation. After removal of the supernatant, the pellet was dispersed in 30 µl of 0.8% Triton X-100 in 0.9% NaCl. A 25 µl aliquot was used for assay of α-L-iduronidase activity (1).

Results

Exposure of urinary high uptake α-L-iduronidase to 50 mM 2,3 butanedione caused no detectable reduction in enzyme activity compared either to the starting enzyme activity or a mock treated control. However, the rate of internalization of the treated enzyme by human diploid fibroblasts was reduced by approximately 50% over the mock treated control (Fig. 1a). Uptake of both enzyme preparations was blocked by mannose 6-phosphate. The effect of butanedione treatment on enzyme internalization appeared to be a result of a reduction in binding (Fig. 1b). The lowered binding probably did not result

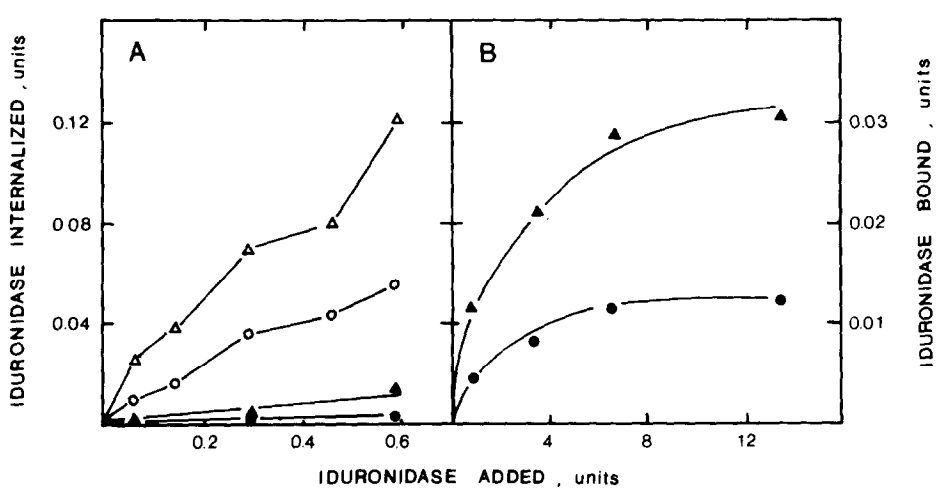

Figure 1

Effect of butanedione treatment on the internalization and binding of α-L-iduronidase with intact cells.
Mixtures contained ~4 x 10^5 cells and the indicated amount of enzyme. Internalization was at 37° for 30 min. Binding was carried out at 0° for 90 min.

A. Internalization; o high uptake enzyme treated with 2,3 butanedione; Δ mock treated enzyme; ● butanedione treated enzyme plus 6.7 mM mannose 6-phosphate; ▲ mock treated enzyme plus 6.7 mM mannose 6-phosphate.
B. Binding; ● high uptake enzyme treated with 2,3 butanedione; ▲ mock treated enzyme. Values represent specific binding (i.e. inhibitable by mannose 6-phosphate.

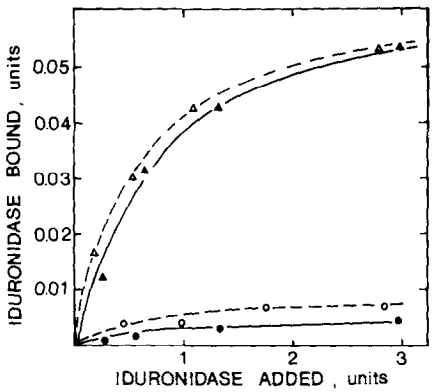

Figure 2

Effect of butanedione on the binding of α-L-iduronidase to isolated membranes. Assays contained either 35 μg of fibroblast membranes or 5 μg of Swarm rat chondrosarcoma membranes and the indicated amount of enzyme. Incubations were for 90 min at 0°. ●—● fibroblast membranes with 2,3 butanedione treated enzyme; ▲—▲ fibroblast membranes with mock treated enzyme; o---o tumor membranes with 2,3 butanedione treated enzyme; Δ---Δ tumor membranes with mock treated enzyme. Values represent specific binding (i.e. inhibitable by mannose 6-phosphate.)

from an altered affinity of treated enzyme for receptors since analysis of the binding curves by the method of Scatchard (19) revealed the same binding affinities for the treated and untreated enzymes (Kd $\cong$ 2 x 10^{-9} M). The lowered binding appeared kinetically to result from a reduction in the number of binding sites available to the treated enzyme (~7,000 sites/cell for the treated enzyme versus ~15,000 sites/cell for the untreated enzyme).

When isolated membrane preparations were examined, the effect of butanedione treatment was even more dramatic. The binding of α-L-iduronidase to fibroblast membranes was reduced 90% following modification (Fig. 2). The modified enzyme binding was too low for an accurate measurements of constants. The effect was identical at 37° and 0° (not shown). Similar results were obtained when membranes were isolated from Swarm rat chondrosarcomas, an animal source found in a preliminary screen to be particularly rich in iduronidase receptors (Fig. 2).

A detailed comparison of isolated membranes and whole cells was carried out to see if there were additional differences in binding characteristics. The affinity of untreated α-L-iduronidase for fibroblast and chondrosarcoma

TABLE I

Characterization of the Binding of α-L-Iduronidase to Fibroblast and Chondrosarcoma Receptors

Source of Receptor	K_d (M)	Sites/Cell*	Enzyme Bound (units/mg protein)	Ki mannose 6-PO_4 (M)
Human fibroblasts† (intact cells)	2×10^{-9}	1.5×10^4	—	1×10^{-4}
Human fibroblasts† (membranes)	5×10^{-9}	3×10^4	0.5	1×10^{-3}
Swarm rat chondrosarcoma (membranes)	5×10^{-9}	—	4.3	5×10^{-4}

*There was some variation in the number of sites per cell among different preparations of fibroblasts; however, the 2:1 ratio between membranes and intact cells was consistent.

†α-L-iduronidase deficient fibroblasts were used due to the absence of background enzymatic activity. A limited number of studies have been carried out with membranes derived from normal human diploid fibroblasts with identical results.

membranes was slightly less than that seen for intact cells (Table I). The total fibroblast membrane fraction had approximately twice the number of receptors as the whole cells from which they were prepared. Swarm rat chondrosarcoma membranes contained approximately 8 times the number of receptor sites as fibroblasts per mg of membrane protein. As is seen in whole cells, the binding to both membrane preparations is competitively inhibited by mannose 6-phosphate; however, the Ki for mannose 6-phosphate inhibition of binding to membranes is almost an order of magnitude higher than the value measured with whole cells.

Discussion

Butanedione is one of a number of reagents that has been used to probe the role of arginine residues in catalytic activity (for review see 20). Although this reagent did not block the enzymatic activity of α-L-iduronidase (21), it markedly affected receptor binding. Based on the specificity of butanedione (22,23) and enhancement by borate (23), it is likely that this effect is due to modification of arginine residues, although more direct

means to demonstrate modified arginine groups have not been employed due to the lack of sufficient quantities of pure high uptake α-L-iduronidase.

Treatment of α-L-iduronidase with butanedione caused a significant reduction in the rate of enzyme internalization into cultured diploid fibroblasts. Studies with intact cells at 0° indicated that the binding of the treated enzyme remained specific and saturable; however, the maximal amount of enzyme that could be bound was reduced by approximately 50%. Binding of α-L-iduronidase to isolated membrane preparations was reduced by nearly 90% following enzyme modification. This effect appears to be independent of the source of membrane selected since similar results were obtained for membranes of human fibroblasts and Swarm rat chondrosarcomas.

Although explanations for the observed effects would be speculative at best, there are some mechanisms that should be considered. In addition to mannose 6-phosphate, the recognition marker may contain essential arginines. These residues could either have a distinct role in binding or potentiate the function of mannose 6-phosphate moieties. It is interesting that the binding of α-L-iduronidase to isolated membranes is less sensitive to mannose 6-phosphate and more sensitive to arginine modification than the binding to whole cells. This could be due to the disruption upon membrane isolation of some cooperative interaction between mannose 6-phosphate and some key portion of the polypeptide.

An alternative explanation would be that untreated enzyme binds to receptors as a dimer and that charge interactions via arginine residues are responsible for keeping the enzyme in this multimeric form. Modification of arginine residues with butanedione could prevent dimerization without altering the receptor recognition site and thus reduce the maximum amount of binding. Although this model does not readily explain the decreased binding of modified enzyme to isolated membranes, it is supported by the known aggregation of urinary α-L-iduronidase in low salt (24). A third possibility that butanedione treatment removes phosphate groups was considered; however,

chemical dephosphorylation by butanedione seems improbable and removal of phosphate by endogenous phosphatases was prevented by inhibitory levels of inorganic phosphate. Furthermore, the kinetics of binding and the differences between membranes and intact cells make such an explanation unlikely.

Previous studies of lysosomal enzyme receptors have been limited to intact cells. The development of a membrane binding assay for α-L-iduronidase has enabled us to measure receptors in cells and tissues containing endogenous enzyme activity. Membranes of Swarm rat chondrosarcoma appear to be a rich source of receptors and can be used for receptor purification.

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