Inhibition by Swainsonine of the Degradation of Endocytosed Glycoproteins in Isolated Rat Liver Parenchymal Cells*

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Swainsonine, an α-mannosidase inhibitor found in the Australian plant *Swainsona canescens* and in spotted locoweed, was tested for its effect on the degradation of endocytosed proteins in rat liver cells. The compound inhibited the release of proteolytically derived breakdown products from endocytosed glycoproteins but not from nonglycoproteins. Fifty per cent inhibition of the degradation of 125I-asialofetuin occurred at a concentration of swainsonine in the medium of 6 × 10⁻⁷ M. In the presence of the inhibitor, there was an increased cellular accumulation of the glycoproteins corresponding quantitatively to the decreased degradation, so that total uptake was the same in the presence or absence of the inhibitor. The excess 125I-labeled material which accumulated within cells under these conditions was found entirely in the lysosomal fraction on Percoll density gradient centrifugation and migrated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a mobility indistinguishable from that of the original material. Thus, the inhibition of breakdown occurs prior to any conversion of the protein to intermediates of detectably smaller size. In contrast to the results with swainsonine, degradation of both glycoproteins and nonglycoproteins was inhibited by chloroquine and by 3-methyladenine. The findings with 3-methyladenine are in conflict with earlier reports in the literature on the effect of this compound on degradation of endocytosed proteins.

Evidence from a number of laboratories has indicated that deglycosylated forms of a number of glycoproteins are degraded at substantially greater rates than the corresponding holoproteins (1–9). These findings suggest that the carbohydrate moieties of glycoproteins serve to protect them against proteolytic degradation. In order to test this hypothesis further, we have examined the effect of the α-mannosidase inhibitor swainsonine on the intracellular degradation of several glycoproteins and nonglycoproteins taken up by rat hepatocytes in culture.

Swainsonine is an alkaloid present in the Australian plant *Swainsona canescens*. It was first isolated in the laboratory of Dorling in Murdoch, Australia and shown to be responsible for the mannosidosis of animals that graze on the plant (10–12). More recently, it has been isolated from spotted locoweed by Molyneux and James (13). The compound inhibits both lysosomal α-mannosidase and Golgi α-mannosidase II (14) and has been reported to inhibit glycoprotein processing (14–17).

We report here that swainsonine is a potent inhibitor of the degradation by cultured rat liver cells of endocytosed glycoproteins but that it has no effect on the degradation of endocytosed nonglycoproteins. The inhibitor led to an accumulation of the glycoprotein markers in the lysosome fraction of cells in a form with electrophoretic mobility indistinguishable from that of the original material. In contrast to the results with swainsonine, the degradation of both glycoproteins and nonglycoproteins was inhibited in this system by chloroquine and 3-methyladenine. The latter has been reported by Seglen's laboratory to be a specific inhibitor of autophagy and to have no effect on the degradation of exogenously introduced proteins (15–20). A possible basis for the discrepancy between their findings and ours is discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**—Male Sprague Dawley rats (250–400 g), obtained from Holtzman Co., were maintained on rat chow ad libitum in a temperature-controlled chamber at 26 °C with a 12-h light/dark cycle. Medium 199 (with Earle's salts and glutamine), horse serum (not heat-inactivated), and the penicillin/streptomycin solution were purchased from Gibco, Grand Island, NY. Swainsonine was a gift from Dr. A. D. Elbein, University of Texas Health Center, San Antonio. Asialofetuin and 125I-asialo-hCG (1.1 atoms of 125I/molecule) were gifts from Dr. O. P. Bahl of this Division. Asialofetuin was iodinated (2.2 atoms of 125I/molecule) in the laboratory of Dr. H. Baumann, Roswell Park Memorial Institute, Buffalo. All iodinations were by the chloramine-T method (21). 125I-Insulin (75.6 μCi/μg) was obtained from New England Nuclear. [3H]Leucine-labeled rat liver cytosol proteins were obtained as previously described (22). After passage through a concanavalin A-Sepharose column, the final preparation contained 0.2% carbohydrate (23) relative to protein (24). 3-Methyladenine (6-amino-3-methylpurine) was purchased from Fluka, Buchs, Switzerland. Type II collagenase was obtained from Worthington. Carrier-free 125I, as the sodium salt, was obtained from Amersham/Searle. Aquasol II and [3H]leucine were purchased from New England Nuclear. Percoll was purchased from Pharmacia Fine Chemicals. Acrylamide, bisacrylamide, Tris, sodium dodecyl sulfate, glycine, Coomassie brilliant blue R-250, and molecular weight standards for gel electrophoresis were from Bio-Rad. Kodak type BB1 autoradiographic film was used for autoradiography. All other biochemicals were from Sigma.

**Cell Isolation and Culture**—Rat liver parenchymal cells were obtained by a modification of the procedure described by Seglen (25). All perfusion solutions were sterile and contained 100 units/ml of penicillin and 100 μg/ml of streptomycin. They were maintained at 37 °C and were well aerated with 95% O₂, 5% CO₂. The liver was perfused in situ with 250 ml of Ca²⁺-free perfusion buffer plus EGTA (0.5 mM), followed by 250 ml of the same buffer without EGTA. The liver was removed and perfused with 80 ml of collagenase buffer (20) containing 50 mg of collagenase for about 12 min. It was then washed and gently homogenized in 80 ml of collagenase buffer with a Teflon pestle. The homogenate was centrifuged at 800 × g for 10 min to remove cellular debris. The supernatant was recentrifuged at 12,000 × g for 10 min to yield a pellet containing the liver parenchymal cells. The cells were suspended in Medium 199 (with Earle's salts and glutamine) and horse serum, and the final cell concentration was adjusted by volume to reach at least 2 × 10⁷ cells/ml.

1 The abbreviations used are: hCG, human chorionic gonadotropin; EGTA, ethyleneglycol bishydroxyethylamine-N,N,N',N'-tetraacetic acid; Hapes, N-(2-hydroxyethyl)-l-piperazine-N,2-ethane sulfonic acid.

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dispersed by gentle mechanical agitation with a comb in ice-cold Medium 199, pH 7.4, containing the antibiotics. The suspension was then passed through a series of filters as described by Seglen (25). Parenchymal cells were separated from nonparenchymal cells by differential centrifugation in a fixed angle rotor at 100 g for 30 min. The resulting parenchymal cell pellet was washed twice at 4 °C with 1 ml of Medium 199 containing the antibiotics. The parenchymal cells thus obtained were suspended at a concentration of 2.5 × 10^6 cells/ml in the same medium plus 20% horse serum (v/v). Cell viability was greater than 95% in all cases as determined by 0.3% trypan blue exclusion. Five ml of the cell suspension were pipetted onto 60-mm Falcon tissue culture plates and incubated in a CO_2 incubator (Forma Scientific) at 37 °C, 5% CO_2, 95% air. Cells remained nonadherent under these conditions.

Cell Incubations—After 20 h in culture, the cells were removed from the plates and resuspended in an equal volume of Medium 199 plus antibiotics at 4 °C. The cells were washed twice with this medium in a fixed angle rotor at 100 g, min. The final pellet was resuspended in Medium 199 containing 1% bovine serum albumin, antibiotics, 10 mM Hepes, pH 7.4, and 5 g/liter of glucose at 4 °C to a concentration of 2.5 × 10^6 cells/ml (containing 1.5-1.7 mg of protein/ml), unless otherwise noted.

Five ml of the cell suspension were preincubated for 30-45 min, with or without inhibitors, in 60-mm Falcon tissue culture plates on a shaker table in the CO_2 incubator or in 50-ml siliconized Erlenmeyer flasks in a 37 °C gyratory bath with gassing with 95% O_2, 5% CO_2 at half-hour intervals. The reaction was initiated by the addition to the suspension of labeled marker protein. One ml aliquots were removed from the culture plates or flasks at the appropriate times, added to equal volumes of ice-cold PBS solution (10 mM potassium phosphate, 0.9% NaCl, pH 7.4), and centrifuged at 600 g for 5 min. The supernatant layer was removed and 1 ml was added to an equal volume of 40% trichloroacetic acid. After 20 h on ice, it was centrifuged at 60,500 g for 30 min, and then 1.0 ml of the supernatant solution was removed for counting in a Beckman γ counter or mixed with 10 ml of Aquasol I for counting in a Beckman scintillation counter.

RESULTS

Fig. 1 shows the effect of swainsonine on the degradation of asialo-hCG. As may be seen, swainsonine at a concentration of 10^{-8} M inhibited the rate of production of acid-soluble counts over 50%. Nearly complete inhibition was observed at 10^{-7} M swainsonine (not shown).

In the presence of the inhibitor, there was an increased cellular accumulation of the marker quantitatively corresponding to the decreased degradation, so that total uptake was unaffected by the inhibitor.

Results similar to those with asialo-hCG were obtained with asialofetuin (Fig. 2). Again there was a quantitative correspondence between diminished degradation and increased cellular accumulation at each inhibitor concentration, so that total uptake was the same in all cases.
Swainsonine Inhibits Glycoprotein Degradation

The effect of swainsonine on the degradation and uptake of nonglycoproteins is shown in Fig. 4. For this purpose, both \(^{125}\)I-insulin and \([\text{H}]{\text{H}}\)leucine-labeled proteins of the cytosol fraction of rat liver, which had been rendered carbohydrate-free by passage through a concanavalin A-Sepharose column, were used. As may be seen, swainsonine at \(10^{-3}\) M, which inhibited glycoprotein breakdown nearly completely, had no effect on the breakdown of the nonglycoproteins under any of the conditions tested. These included concentrations of insulin at which uptake would be expected to depend upon both high affinity and low affinity binding sites, respectively (29).

In order to determine the intracellular site of the swainsonine-induced accumulation of endocytosed glycoproteins, cell homogenates were fractionated on Percoll density gradients. As may be seen from Fig. 5, the endocytosed marker protein appeared virtually entirely in the lysosomal peak. As also noted previously in the experiment described in Fig. 3, the marker reached a steady state level by 3 h in the control cultures and continued to increase throughout the 5-h period in the inhibitor-treated cultures.

To obtain information on the nature of the material which accumulates in the cells in the presence of swainsonine, we compared its electrophoretic mobility with that of unincubated \(^{125}\)I-asialofetuin. As may be seen from Fig. 6, the material re-extracted from the cell migrated in a manner indistinguishable from unincubated \(^{125}\)I-asialofetuin, with the bulk of...
Swainsonine Inhibits Glycoprotein Degradation

FIG. 5. Subcellular localization of 125I by Percoll density gradient centrifugation of cell homogenates after endocytosis of 125I-asialofetuin. A, N-acetylhexosaminidase activity expressed in arbitrary fluorescence units (100 units = 34 nmol of β-methylumbelliferone/assay tube); B, C, and D, distribution of 125I after 1, 3, and 5 h, respectively, of marker uptake. ○, no swainsonine; ●, 10⁻³ M swainsonine.

FIG. 6. Electrophoretic mobility of 125I-re-extracted from cells after endocytosis of 125I-asialofetuin. Lysosomes were isolated from cell homogenates as described in the text and illustrated in Fig. 5. The pooled lysosomal fractions were extracted, electrophoresed, stained, and radioautographed as described in the text. The radioautogram is shown together with the locations of the Coomassie blue-stained molecular weight markers (92.5 K, for example, M₉ = 92,500). Lane A, unincubated 125I-asialofetuin. Lane B, the marker was added in the cold to cells, which were then immediately homogenized. A postnuclear supernatant was prepared, which was then treated in the same way as the pooled lysosomes in lanes C-H. Lanes C, E, and G, re-extraction was after 1, 3, and 5 h, respectively, of marker uptake in the absence of swainsonine. Lanes D, F, and H, re-extraction was after 1, 3, and 5 h, respectively, of marker uptake in the presence of 10⁻³ M swainsonine.

the radioactivity in both cases at a location consistent with the reported molecular weight for fetuin of 92,500 (30).

Two other inhibitors of degradation were also tested for their effect on these markers (Table I). Chloroquine, a well established inhibitor of lysosomal proteolysis, inhibited nearly completely the degradation of all markers tested.

3-Methyladenine has been found in Seglen's laboratory to inhibit endogenous protein degradation, apparently by interfering with the formation of autophagic vesicles (18–20, 31). This group claimed that 3-methyladenine was ineffective in inhibiting the breakdown of exogenously introduced asialofetuin. However, as may be seen from Table I, in our hands inhibition of degradation of all three exogenous markers tested was obtained. Total uptake of the markers was unaffected by the inhibitor.

DISCUSSION

Since lysosomes appear to lack endoglycosidases (32, 33), the removal of sugar residues from glycoproteins in their degradative breakdown in these organelles evidently depends upon the sequential action of a series of exoglycosidases. Thus, swainsonine, as an inhibitor of lysosomal α-mannosidase (14), would be expected to prevent not only removal of the mannose residues in this process, but of the remainder of the carbohydrate chain proximal to the mannose residues as well. We propose that the swainsonine inhibition of proteolytic degradation of endocytosed glycoproteins reported here is a consequence of this block in deglycosylation. Furthermore, since the inhibition observed approaches 100% of both glycoproteins tested, it follows, if this mechanism is correct, that there is a virtually absolute dependence of the proteolytic breakdown of these substances to acid-soluble products on the prior removal of the oligosaccharide chains.

A number of points support this proposed mechanism of action of swainsonine on glycoprotein degradation. It is consistent with the frequently reported observation that deglycosylated forms of glycoproteins are degraded more rapidly than the corresponding holoproteins both within cells and in cell-free systems (1–9), indicating that deglycosylation of glycoproteins is the rate-limiting step in their overall breakdown. In addition, the inhibition by swainsonine in this system was specific for glycoproteins with no effect on the breakdown of nonglycoproteins. The following evidence indicates that the swainsonine inhibition is on a lysosomal process rather than a prior one such as endocytosis or intracellular translocation. Swainsonine had no effect on uptake of any of the markers tested. Delivery of both types of markers was to a degradative compartment in which proteolysis was inhibited by chloroquine (Table I) and which sedimented with lysosomal markers in a Percoll density gradient (Fig. 5).

Degradation of both glycoproteins and nonglycoproteins was by a process sensitive to 3-methyladenine (Table I) which, according to Seglen and co-workers (18–20, 31), acts by inhibiting translocation from vesicles to lysosomes. Thus, this step in the process appears to be similar for both types of markers. The intracellular accumulation of glycoprotein marker in the presence of swainsonine was entirely in the lysosomal compartment, further indicating that the inhibition is on a process subsequent to the delivery of the marker to the lysosomes.

The radioactivity that accumulates in cells incubated with 125I-asialofetuin in the presence of swainsonine is associated with a species indistinguishable in size from that of the original marker (Fig. 6). This result rules out the possibility

TABLE I

Inhibition by chloroquine and 3-methyladenine of degradation of endocytosed proteins

Incubations were as described in the text, except as noted, for a period of 3 h. 125I radioactivity is expressed as counts/min and 3H radioactivity is expressed as disintegrations/min. Numbers in parentheses are per cent inhibition.

<table>
<thead>
<tr>
<th>Marker protein</th>
<th>Control</th>
<th>+10⁻⁷ M chloroquine</th>
<th>+10⁻⁵ M 3-methyladenine</th>
</tr>
</thead>
<tbody>
<tr>
<td>125I-Asialo-hCG*</td>
<td>2753</td>
<td>0 (100)</td>
<td>100 (56)</td>
</tr>
<tr>
<td>125I-Asialo-hCG</td>
<td>6976</td>
<td>3667 (47)</td>
<td>764 (56)</td>
</tr>
<tr>
<td>125I-Asialofetuin</td>
<td>1720</td>
<td>249 (86)</td>
<td>764 (56)</td>
</tr>
<tr>
<td>3H-labeled rat liver cytosol</td>
<td>233</td>
<td>31 (87)</td>
<td>148 (36)</td>
</tr>
</tbody>
</table>

a In this experiment, 0.75 mg of cell protein/ml were present.

b In this experiment, 0.3 mg of cell protein/ml were present.
that the swainsonine-induced block in breakdown is at a step subsequent to an initial proteolytic cleavage to large peptide fragments and indicates instead that no proteolysis of asialofetuin, or at most loss of only a few terminal amino acids, occurs when demannosylation is prevented. In this connection, Aronson and de Duve (34) similarly found that in the digestion of submaxillary mucin by lysosome extracts, no proteolysis took place until a large portion of the carbohydrate chains had been removed. In contrast, Bernard et al. (9) have reported that only the carbohydrate-rich domains of fibronectin were protected by the polysaccharide chains from proteolytic digestion in vitro.

Our findings on the ability of 3-methyladenine to inhibit degradation of endocytosed proteins differ from those of Seglen’s group (18–20, 31). We believe that the basis of this discrepancy lies in the fact that, in the experiments of the latter group, the inhibitor was not added until some time after uptake of the exogenous marker was completed, so that all steps prior to lysosomal digestion had already occurred. From our finding that overall degradation, but not uptake of exogenous markers, was inhibited by 3-methyladenine and the finding of Seglen’s group that lysosomal proteolysis was not affected, it appears that the site of action of the inhibitor is on a process between endocytic vacuole formation and degradation within the lysosome.

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