Apparent Inhibition of Receptor-Mediated Endocytosis by Endogenous Ligands

Swainsonine and Castanospermine Blockade of Mannose Glycoprotein Uptake by Macrophages

Receptor-mediated uptake of mannose-terminated glycoproteins by macrophages is blocked by treating the cells with swainsonine, an inhibitor of α-mannosidase II, and by castanospermine, an inhibitor of the endoplasmic reticulum processing enzyme α-glucosidase. Both inhibitors are known to cause accumulation of unprocessed oligosaccharide chains terminating in mannose. Inhibition of ligand uptake by the drugs was time- and dose-dependent. Swainsonine produced a maximal effect after 2 h; castanospermine required 5–6 h. Following swainsonine treatment, complete recovery of mannose receptor activity required 24 h and was blocked by cycloheximide suggesting that new receptor synthesis was necessary. Tunicamycin, an inhibitor of oligosaccharide assembly, had no effect on uptake of mannosylated ligands, but tunicamycin pretreatment reduced the sensitivity to swainsonine. These effects of swainsonine and castanospermine appear to be specific, other macrophage pinocytosis receptors (e.g., mannose phosphate) or phagocytosis of yeast particles were unaffected. Moreover, swainsonine had no effect on the fibroblast mannose phosphate receptor. The ability of macrophages to process newly synthesized oligosaccharides was blocked following treatment with swainsonine. Normal processing was fully recovered 24 h after removal of the drug. Mannosidase II was partially inactivated by swainsonine treatment and only a portion was recovered after 24 h. Treatment of macrophages with swainsonine also resulted in an increase in net lysosomal enzyme secretion. Inhibition of mannose-specific receptor-mediated endocytosis in macrophages by swainsonine and castanospermine appears to be due to the formation of mannose-terminated membrane glycoproteins which engage the mannose receptor thereby preventing function. These results suggest a novel mechanism for regulation of receptor-mediated endocytosis.

Swainsonine is an indolizidine alkaloid which inhibits Golgi mannosidase II resulting in altered glycoprotein biosynthesis by cells (1–4). Oligosaccharide chains produced in the presence of swainsonine are truncated and contain terminal mannose residues (5, 6). Castanospermine has a structure similar to swainsonine but inhibits the α-glucosidase localized in the endoplasmic reticulum (7). Both drugs bring about the accumulation of unprocessed mannose-terminated oligosaccharide chains carried by both secretory and membrane glycoproteins. Treatment of animals with these agents brings about rapid changes in glycoprotein metabolism and lysosomal function. Plasma levels of lysosomal hydrolases have been shown to increase severalfold following swainsonine treatment (8).

Macrophages are known to express pinocytosis receptors at the cell surface which recognize and internalize mannose-terminated glycoproteins including a variety of lysosomal hydrolases (9, 10). The mannose receptor appears to follow a recycling pathway which takes it from the cell surface, where ligand is bound, to an intracellular, pre-lysosomal acidic compartment, where ligand dissociates (11–13). Unoccupied receptors then return to the cell surface. It is implicit that in order for the mannose receptor to operate efficiently in macrophages, newly synthesized membrane glycoproteins containing high-mannose chains must be sequestered away from the receptor until the oligosaccharide chains are processed; otherwise, the receptor might become permanently engaged with neighboring membrane glycoproteins. The question posed in this study is whether treatment of cells with swainsonine and castanospermine would affect mannose receptor activity. Our results show that both drugs are effective inhibitors of mannose receptor activity. The data indicate that the drugs have no direct effect on binding of ligand to the mannose receptor but suggest that inhibition is due to the formation of membrane glycoproteins containing unprocessed oligosaccharide chains which engage the receptor leading to inactivation.

EXPERIMENTAL PROCEDURES

Reagents—Swainsonine was generously provided by Dr. Peter Dorling, School of Veterinary Studies, Murdoch University, West Australia. Castanospermine was generously provided by Dr. Alan Elbein, University of Texas, San Antonio. The drugs were taken up into Hanks' balanced salt solution and stored frozen. Yeast mannan, tunicamycin, and enzyme substrates were obtained from Sigma. β-Glucuronidase was prepared from rat preplacental glands, acid β-Glucuronidase was isolated by the chloramine-T method (11). Na235I was obtained from Amersham Corp.

Cells—Rat bone marrow-derived macrophages were prepared by culture of marrow cells in α-minimal essential medium containing 10% fetal calf serum and 10% L-cell conditioned media (growth media) essentially as described by Lin and Gordon (14). After 4–5 days in culture, monolayers of well-differentiated macrophages were generated which expressed relatively high levels of mannose receptor activity.

Uptake and Enzyme Assays—For uptake, cells were incubated in buffered Hanks' balanced salt solution (10 mM TES, 1 mM Hepes, 100 nM L-glutamate, 100 nM L-glutamine). Following a 2-h incubation, the uptake media were removed and the cells were washed four times with fresh solution prior to solubilization of the cells and determination of radioactivity. Enzyme assays were performed essentially as described by Lin and Gordon (14).
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RESULTS

Inhibition of β-Glucuronidase Uptake by Swainsonine and Castanospermine: Concentration and Time Dependence

Rat bone marrow-derived macrophages were incubated with increasing concentrations of swainsonine (2 h) and castanospermine (6 h) at 37°C in standard growth medium. The medium was removed and uptake assays were carried out in Hanks' bovine serum albumin with 125I-β-glucuronidase as ligand. The results in Fig. 1A show the concentration-dependent inhibition of 125I-β-glucuronidase uptake by swainsonine and castanospermine pretreatment. Swainsonine is a potent inhibitor of β-glucuronidase uptake requiring less than 0.01 μg/ml (0.06 μM) to produce 50% inhibition. Swainsonine had no effect on cell viability, even after overnight incubation, or on cellular protein content. Castanospermine was less potent than swainsonine in producing the inhibitory effect; approximately 5 μg/ml of drug (25 μM) was required to produce 50% inhibition. Castanospermine was likewise nontoxic to the cells. The time-dependence (Fig. 1B) of inhibition of β-glucuronidase uptake was studied by preincubating cells with 1 μg/ml of swainsonine in standard growth medium for 2, 4, and 6 h, or with 10 μg/ml of castanospermine for 2, 4, 7, and 11 h. The cells were then washed free of drug, and uptake was determined by incubation with 125I-β-glucuronidase. The results show (Fig. 1B) that within 2 h of swainsonine addition, most of the uptake activity was lost. Castanospermine produced a slower response requiring more than 10 h to reach maximal effects. In a related study, both ligand and swainsonine (1 μg/ml) were added simultaneously to the cells at 37°C (data not shown). Uptake of ligand into the cells was linear over the first 30 min after which the rate fell precipitously. These results indicate that some period of pretreatment is required for maximal inhibition of uptake.

FIG. 1. Inhibition of mannose-specific endocytosis of 125I-β-glucuronidase by macrophages: concentration- and time-dependent effects of swainsonine and castanospermine. A, concentration dependence: rat bone marrow-derived macrophage cultures were incubated with increasing concentrations of swainsonine (2 h) and castanospermine (6 h), after which the cells were washed free of drug and assayed for uptake of 125I-β-glucuronidase as described under "Materials and Methods." B, time dependence: macrophages were incubated with swainsonine (1 μg/ml) or castanospermine (10 μg/ml) for the times indicated. The cells were then washed free of drug and assayed for uptake of 125I-β-glucuronidase. Results are the average of three experiments and are expressed as a percent of untreated control cultures.

Recovery of Receptor Activity following Inhibitor Treatment

The dramatic loss of mannose receptor activity observed after a brief treatment with swainsonine or castanospermine is followed by recovery of receptor activity after removal of the drug. This result is shown in Fig. 2. Cells were incubated with or without swainsonine (1.0 μg/ml) or castanospermine (25 μg/ml) for 2 and 6 h, respectively. The cells were washed free of drug and allowed to recover. Complete recovery of mannose receptor activity required about 24 h. If mannose receptors were inactivated by drug treatment and if recovery over 24 h required de novo synthesis of receptor molecules, recovery should be impaired by cycloheximide. The results in Fig. 3 indicate that cycloheximide alone (0.5 μg/ml) over 12 h had negligible effects on uptake. However, in the presence of cycloheximide, recovery of mannose receptor activity following swainsonine pretreatment was significantly impaired.

Possible Role of Endogenous Glycoconjugates in Mediating the Response to Swainsonine

Effect of Tunicamycin—If the response to swainsonine and castanospermine were due to the generation of mannoseterminated protein-bound oligosaccharides which bind up available receptor molecules, the inhibitory response to swainsonine should be abrogated by tunicamycin. Tunicamycin, by inhibiting transfer of lipid-linked oligosaccharides to nascent polypeptides, should reduce the intracellular levels of glycoproteins containing high-mannose chains. Preincubation of cells with 10 μg/ml of tunicamycin for 4 h alone had no effect on mannose receptor activity (data not shown). The results
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FIG. 2. Recovery of mannose-specific endocytosis following treatment with swainsonine and castanospermine. Following incubation of bone marrow macrophages with swainsonine (1.0 μg/ml) or castanospermine (25 μg/ml) for 2 and 6 h, respectively, the cells were washed free of drug and allowed to recover for 12 and 24 h. Recovery was carried out in growth media. Control dishes were incubated under the same conditions but without drugs added. Zero recovery is referred to as pulse. The data are presented as per cent control. In a typical control experiment, 2-4 ng of 125I-β-glucuronidase were taken up per μg of protein/h. Cellular protein content was unaffected by treatment with either drug.

FIG. 3. Effect of cycloheximide on the recovery of mannose receptor activity following swainsonine treatment. Cells were incubated with swainsonine (0.3 μg/ml) as described in the legend to Fig. 2. The cells were washed free of drug and allowed to recover in the presence or absence of cycloheximide (0.5 μg/ml) for 12 h. Uptake of 125I-β-glucuronidase was then measured. The effect of cycloheximide without swainsonine pretreatment is shown. In typical control experiments 2-4 ng of ligand was taken up per μg of cellular protein/h. Results are the average of three experiments.

FIG. 4. Effect of tunicamycin on the cellular response to swainsonine. Cells were pretreated with tunicamycin (10 μg/ml) for 4 h. A dose-response to swainsonine was then initiated by addition of the drug in the presence of tunicamycin. After an additional 2 h in the presence of various concentrations of swainsonine, the cells were assayed for 125I-β-glucuronidase uptake. Results are expressed as the per cent of the maximal inhibition obtained with swainsonine alone. In Fig. 4 show the dose-dependent response to swainsonine with and without tunicamycin pretreatment. Preincubation of cells with tunicamycin led to a substantial shift in the dose-response curve to swainsonine, approximately 20-fold to the right.

Effects of Swainsonine on Membrane Glycopeptide Processing—To confirm that membrane oligosaccharide processing was blocked by swainsonine treatment, cells were pulsed with swainsonine for 3 h followed by a 24-h recovery period without drug. Following a pulse of [3H]mannose incorporation, crude membranes were isolated, digested with Pronase, and chromatographed on ConA-Sepharose. Cells treated with swainsonine (0.3 μg/ml for 3 h) produced a greater percentage of glycopeptides which bound to ConA-Sepharose compared to control cells (Table I). This effect was completely reversed in cells which recovered for 24 h after drug removal.

Effect of Swainsonine Treatment on Lysosomal Enzyme Secretion by Macrophages—Rat bone marrow-derived macrophages were cultured with or without 0.1 μg/ml of swainsonine for 16 h and the media were tested for β-hexosaminidase activity: swainsonine treatment increased net β-hexosaminidase secretion by 25% over the test period. Fibroblast β-hexosaminidase secretion was unaffected by swainsonine treatment.

Effect of Swainsonine on Acid α-Mannosidase and α-Mannosidase II: Inhibition and Recovery

The delayed recovery of mannose receptor activity appears to be due to the degradation of receptor molecules whose replacement requires de novo synthesis. Additionally, the delayed recovery could be due to inactivation of the Golgi
TABLE I
Chromatography of macrophage-derived 3H-glycopeptides on ConA-Sepharose following swainsonine treatment and recovery

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Eluted radioactivity</th>
<th>Bound radioactivity</th>
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<tr>
<td></td>
<td>% of total</td>
<td></td>
</tr>
<tr>
<td>3-h pulse</td>
<td>Control 35</td>
<td>66</td>
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<tr>
<td></td>
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</tr>
<tr>
<td>24-h recovery</td>
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<td>59</td>
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<tr>
<td></td>
<td>Swainsonine 35</td>
<td>66</td>
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TABLE II
Effect of swainsonine treatment on macrophage acid α-mannosidase and pH 6.0 α-mannosidase (α-mannosidase II)

Bone marrow-derived macrophages were treated with 0.3 μg/ml of swainsonine and allowed to recover as described in the legend to Fig. 2. The cells were washed free of media and suspended in 0.1% Triton X-100. The cell extracts were assayed at pH 4.0 and 6.0 in the presence or absence of added swainsonine (0.3 μg/ml) and the activity remaining after addition of swainsonine under these conditions is referred to as acid α-mannosidase and mannosidase II. The data are the average of three experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Acid α-mannosidase</th>
<th>Mannosidase II % control</th>
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</thead>
<tbody>
<tr>
<td>Swainsonine</td>
<td>3 h 87</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>Recovery 12 h 112</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>Recovery 24 h 110</td>
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DISCUSSION

Receptor-mediated endocytosis commences with high-affinity interaction of ligands with cell surface receptors. Receptor-ligand complexes are rapidly internalized into an acidic compartment, where receptor-ligand dissociation occurs. Active receptors appear to operate in the plasma membrane, prelysosomal acidic compartments (or CURL) and some elements of the Golgi apparatus (19). However, newly synthesized membrane receptors for glycoconjugates, such as those specific for mannose- or galactose-terminated oligosaccharides, must be initially transported from the rough endoplasmic reticulum to the cell surface through intracellular vesicles (e.g. Golgi apparatus) where they come into contact with assorted membrane glycoproteins. It must be assumed that the membrane glycoproteins along this pathway are poorly enriched in oligosaccharides terminating in mannose or galactose, otherwise, movement of receptors out of these compartments and to the cell surface would be impaired. Alternatively, receptors, such as those mentioned above, could be made as inactive precursors. With the availability of the alkaloids swainsonine and castanospermine, inhibitors of the Golgi processing enzyme α-mannosidase II and the ER processing enzyme α-glucosidase, respectively, it became possible to alter the oligosaccharide composition of newly synthesized membrane glycoproteins. With this information in hand, we set out to determine whether the generation of mannoseterminated membrane glycoproteins in a cell that normally expresses a mannose-specific pinocytosis receptor, would interfere with the activity of the receptor. Our results indicate that swainsonine and castanospermine are rapid and potent inhibitors of mannose receptor activity in macrophages. The effects appear to be highly specific. The mannose-phosphate receptor was not affected by swainsonine treatment in macrophages or fibroblasts, nor was the phagocytosis of yeast particles inhibited. The inhibitory effect appeared to be dependent on the formation of membrane proteins with mannose-terminated oligosaccharide chains since (i) tunicamycin reduced the sensitivity of cells to the swainsonine and (ii) the loss of uptake activity correlated with an increase in cell surface high-mannose chains, as revealed by the binding of a soluble mannose-binding protein (data not shown). Similar observations have been reported for the binding of ConA to the surface of cells treated with swainsonine (3). The synthesis of aberrant (i.e. inactive) receptor molecules is not a suitable explanation of this effect because the mannose receptor has been shown to have a rather long survival time (t1/2 > 16 h) (15) and the swainsonine effect occurred in 60-90 min. It seems more likely that mannoseterminated membrane glycoproteins, formed in the presence of swainsonine and castanospermine, interact with the mannose receptor. Because of the proximity of this "endogenous" ligand to the receptor, the latter may become permanently occupied; the receptor-ligand complex, unable to separate, would lead to
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receptor degradation (Fig. 5). While we have no specific information on receptor turnover in the presence of swainsonine, it appears that drug treatment leads to receptor destruction. Recovery of activity requires new protein synthesis and is effectively blocked with cycloheximide. (The kind of inhibition found above has been observed previously in studies with membranes containing the galactose receptor. Treatment of such membranes with neuraminidase led to exposure of the penultimate sugar galactose and inactivation of receptor activity (20).)

Mannose receptor activity recovered in 24 h following treatment with swainsonine. However, the Golgi processing enzyme, α-mannosidase II, which is responsible for trimming of the high-mannose chains, recovered only slightly following swainsonine removal for 24 h. To test whether the cells actually recover the ability to process their oligosaccharides during a 24-h recovery period, biosynthetic experiments were carried out with membrane glycopeptides. Treatment of cells with swainsonine led to the production of membrane glycopeptides which bind more avidly to ConA-Sepharose. After the drug was removed for 24 h, the cells fully recovered their ability to process oligosaccharides. This recovery of processing correlates well with the recovery of mannose receptor activity. The reason for the lack of correlation with the recovery of Golgi α-mannosidase remains obscure especially given the recent observation that the mannosidase II has a half-time of about 20 h (21). Studies of α-mannosidase II biosynthesis following swainsonine treatment should resolve this issue.

Recently, Touster and colleagues (22) showed that treatment of rats for short periods of time with swainsonine resulted in an increase in plasma lysosomal enzyme levels. This increase may be due to depressed clearance of secreted hydrolases or enhanced secretion of enzymes; our results, coupled with those of Touster’s, suggest that both of the above may be altered. Since plasma lysosomal hydrolases are normally cleared via the mannose receptor, the clearance of rat preputial β-glucuronidase was examined by Touster and colleagues (22) in swainsonine-treated rats. The clearance of β-glucuronidase was found to be substantially reduced in the swainsonine-treated animals. The effect was reversible. These in vivo data coupled with the in vitro data presented here suggest that the mannose receptor of mononuclear phagocytes normally plays a role in the regulation of extracellular levels of lysosomal hydrolases.

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REFERENCES