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Castanospermine Inhibits the Function of the Low-Density Lipoprotein Receptor†

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Received January 25, 1989; Revised Manuscript Received June 5, 1989

ABSTRACT: Castanospermine, a plant alkaloid that inhibits the glycoprotein processing enzyme glucosidase I, has been used to inhibit N-linked oligosaccharide modification, resulting in the production of glycoproteins having $Glc_3Man_{7-9}(GlcNAc)_2$ oligosaccharides. This alkaloid caused a significant inhibition of LDL endocytosis in cultured primate smooth muscle cells and human skin fibroblasts. At an optimum concentration of 250 μg/mL, castanospermine caused a 40% decrease in cell surface receptor-mediated LDL binding at 4 °C, with no apparent change in affinity. Further, the inhibitor had no direct effect on LDL metabolism. This inhibition of LDL receptor expression and function occurred only when the drug was present during de novo receptor synthesis, i.e., during up-regulation. Although the number of cell surface LDL receptors was significantly reduced in the presence of castanospermine, the total number of receptors in the cell was only slightly reduced, indicating that castanospermine induced a redistribution rather than a reduction in the number of receptors. Similarly, subcellular fractionation studies confirmed that castanospermine treatment of fibroblasts results in an altered distribution of receptor activity compared with controls. These findings are consistent with the conclusion that the decrease in specific LDL binding to cells grown in the presence of castanospermine is due to intracellular redistribution of the LDL receptor so that more receptor remains in internal compartments as a result of a diminished rate of transport.

Many integral membrane proteins are glycoproteins that contain N-linked and O-linked oligosaccharides (Kornfeld & Kornfeld, 1985), and a number of these glycoproteins function as receptors for biologically important molecules such as transferrin (Omary & Trowbridge, 1981), insulin (Czech, 1982), acetylcholine (Prives & Bar-Sagi, 1983), and low-density lipoproteins (Brown & Goldstein, 1986). In each of these systems, the ligand–receptor complex is rapidly internalized through a process known as receptor-mediated endocytosis, which often, but not invariably, occurs in discrete clathrin-containing regions of the surface membrane called coated pits (Goldstein et al., 1976). At the present time, there is only preliminary and indirect evidence that suggests a role for the carbohydrate moiety in receptor recognition (Neufeld & Ashwell, 1981). However, much stronger evidence indicates that N-linked oligosaccharides are necessary for receptor function (Olden et al., 1978). Much of this evidence comes from the use of the antibiotic tunicamycin (Takatsu & Tamura, 1971), a compound which prevents N-linked glycosylation by inhibiting the formation of the first lipid-linked oligosaccharide intermediate, GlcNAc-PP-dolichol (Tkacz & Lampen, 1975). In the presence of tunicamycin, the insulin

† Supported by NHLBI Grants HL-26890 (to C.J.S.), HL-07446 (to C.J.S.), and HL-17783 (to A.D.E.).

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0006-2960/89/0428-7679$01.50/0

receptor (Ronnett & Lane, 1981), the acetylcholine receptor (Prives & Bar-Sagi, 1983), and possibly the LDL receptor (Chatterjee et al., 1981) fail to reach the plasma membrane and/or to function in a normal fashion. However, there are several difficulties associated with using tunicamycin to investigate the role of N-linked oligosaccharides in receptor function. First, many membrane glycoproteins become insoluble or have an altered conformation in the absence of carbohydrate, and, thus, may precipitate and be sequestered into vacuoles within the cell (Leavitt et al., 1977). Second, the nonglycosylated protein may be more susceptible to proteolysis, and therefore may undergo extensive degradation (Prives & Olden, 1980). Finally, under some circumstances, tunicamycin could inhibit protein synthesis in addition to N-linked glycosylation (Elbein, 1987). As a result the definitive role of N-linked oligosaccharides in receptor-mediated endocytosis has been difficult to assess with this inhibitor.

A number of compounds have been found that inhibit the normal processing of the oligosaccharide chains of the N-linked glycoproteins, after the oligosaccharide has been transferred to protein. One of these inhibitors is the plant indolizidine alkaloid castanospermine, a potent inhibitor of α-glucosidases, including the glycoprotein processing enzyme glucosidase I (Pan et al., 1983; Merkle et al., 1985). When mammalian cells are grown in the presence of this inhibitor, they are not able to process N-linked oligosaccharides beyond the glucosidase I stage, and the glycoproteins contain oligosaccharides with a $Glc_3Man_{7-9}(GlcNAc)_2$ structure (Pan et al., 1983). In the present studies, we have used castanospermine to determine how modifications in the oligosaccharide chain(s) of the LDL receptor influence its function and/or subcellular distribution.

The LDL receptor plays a key role in lipoprotein and cholesterol homeostasis (Brown & Goldstein, 1976, 1986; Goldstein et al., 1985). Because both its LDL binding kinetics and its endocytic pathway have been well-defined, this receptor represents an excellent model system to explore the role of carbohydrate in receptor function and targeting. Both the mature bovine and human LDL receptors have been shown to contain one or two N-linked oligosaccharides of the biantennary complex type, as well as numerous O-linked chains (Cummings et al., 1983; Kingsley et al., 1986). The experiments described here show that castanospermine has dramatic effects on the number of cell surface LDL receptors on both vascular smooth muscle and endothelial cells, and in human skin fibroblasts. Furthermore, these studies indicate that this decrease in cell surface LDL receptors is a result of a change in receptor distribution.

Materials and Methods

Materials. Human LDL (density 1.006–1.063 g/mL) was prepared by ultracentrifugation at 14 °C from the serum of fasting healthy human male volunteers (Kelley & Kruski, 1982). LDL was characterized by using immunodiffusion, immunoelectrophoresis, and chemical composition (Kelley & Kruski, 1982). LDL was radiolabeled with ^{125}I by the McFarlane iodine monochloride method (McFarlane, 1964) as modified by Bilheimer et al. (1972). The iodinated LDL was not used unless >98% of the ^{125}I radioactivity was precipitable by trichloroacetic acid (TCA), <5% of the radioactivity was present in the lipid portion as determined by Folch extraction (Folch et al., 1957), and the specific radioactivity ranged from 100 to 300 dpm/ng of protein. Human lipoprotein-deficient serum (LPDS) was prepared by removal of lipoproteins by ultracentrifugation (d = 1.21 g/mL) and subsequent dialysis against PBS as previously described (Sprague et al., 1987). Castanospermine (1,6,7,8-tetrahydroxyoctahydroindolizidine) was isolated from the seeds of the Australian plant *Castanospermum australe* (Hohenschutz et al., 1981), and swainsonine was isolated from *Astragalus lentiginosis* (Molyneux & James, 1982). [2-^3H]Mannose and [6-^3H]galactose were purchased from American Radiolabeled Chemicals, Inc., and endoglucosaminidase H (Endo H) was obtained from Miles Scientific. Pronase was purchased from Calbiochem, and Bio-Gel P-4 was obtained from Bio-Rad. Bovine serum albumin (BSA) and heparin were obtained from Sigma, *n*-octyl glucoside was from Boehringer Mannheim Biochemicals, egg phosphatidylcholine was from Avanti Polar-Lipids, Inc., and cellulose acetate filters (0.45 μm, 25-mm diameter) were from Micro Filtration Systems.

Cell Culture. Baboon aortic smooth muscle cells (SMC) were derived as explants from the aortic media and cultured as previously described (Sprague et al., 1982). Normal human diploid skin fibroblasts were obtained from the Human Genetic Mutant Cell Repository and maintained as described (Edwards et al., 1988). Both SMC and fibroblasts were grown in medium 199 (M199) containing 10% fetal calf serum (KC Biologicals), pH 7.4, 15 mM HEPES, 1.4 g/L bicarbonate, 120 units/mL penicillin, and 120 μg/mL streptomycin. Unless otherwise specified, cell cultures were grown for 4 days in the presence or absence of inhibitor. To maximize LDL receptor expression, preconfluent cells were incubated for an additional 24–48 h in 10% lipoprotein-deficient serum (receptor up-regulation) while maintaining inhibitor levels.

Bovine aortic endothelial cells (BAEC) were isolated from aortas obtained from a local slaughterhouse and cultured as previously described (Sprague et al., 1987). BAEC were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco Laboratories) containing 10% fetal calf serum and used between passages 1 and 20.

Measurement of ^{125}I-LDL Cell Surface Binding. (*A*) *Fibroblasts.* Cells, up-regulated in the presence or absence of inhibitor, were rinsed and preincubated for 10 min at 37 °C in Dulbecco's phosphate-buffered saline (PBS; Gibco Laboratories) and then precooled for 30 min at 4 °C. Medium 199, containing low bicarbonate (0.35 g/L) and 15 mM HEPES, 10% lipoprotein-deficient serum, and specified ^{125}I-LDL concentrations, was then added, and the incubation was continued for 2 h at 4 °C. Additional plates were incubated with the same concentrations of ^{125}I-LDL in the presence of 500–2000 μg/mL unlabeled LDL. The binding reaction was terminated by removing the culture medium and washing the monolayers as described previously (Goldstein et al., 1983). Heparin (10 mg/mL PBS) was then added, and the cells were incubated for 1 h at 4 °C to remove surface-bound ^{125}I-LDL. The heparin solution was collected and pooled with three 1-mL ice-cold PBS rinses, and ^{125}I-LDL radioactivity was determined as a measure of cell surface lipoprotein binding using a Beckman 310 γ counter. Specific receptor-mediated ^{125}I-LDL binding to the cell surface was calculated by subtracting the amount of radioactivity bound in the presence of labeled plus unlabeled LDL from the radioactivity bound to cells exposed only to labeled LDL. Replicate cultures were carried through each experiment without adding radiolabeled LDL for determination of cell numbers using a Coulter Counter Model ZF.

(*B*) *Smooth Muscle Cells (SMC).* Up-regulated SMC were incubated with ^{125}I-LDL in the presence or absence of unlabeled LDL as described for fibroblasts. Cultures were subsequently warmed to 37 °C for 30 min in the presence of M199 containing 10% lipoprotein-deficient serum (LPDS) to allow the bound ^{125}I-LDL to internalize, but not be degraded, ac-

cording to Kenagy et al. (1984). This was done to avoid measuring ^{125}I-LDL bound to the extracellular matrix and the plastic culture surface. The cells were then removed from the culture surface with 1 mL of 0.25% trypsin containing 0.05% EDTA and incubated for 10 min at 37 °C. SMC were combined with 1 mL each of 0.2% bovine serum albumin (BSA) in PBS (w/v) and 0.5% soybean trypsin inhibitor in PBS (w/v), centrifuged (200 g, 10 min) at 4 °C, and, after careful removal of the supernatant, resuspended in 1 mL of fresh 0.2% BSA. After recentrifugation, the ^{125}I activity in the cell pellet was counted as a measure of surface-bound lipoprotein. Specific LDL binding was calculated as described for fibroblasts, and replicate cultures were used to determine cell number. LDL binding studies using BAEC were conducted as described for SMC.

Measurement of ^{125}I-LDL Internalization and Degradation. SMC, fibroblasts, or BAEC were up-regulated in the presence or absence of inhibitor, rinsed, and preincubated with PBS as described above. Cells were then incubated in M199 containing 10% lipoprotein-deficient serum and 10 μg/mL ^{125}I-LDL at 37 °C. After periods ranging from 5 min to 24 h, 0.5 mL of culture medium was removed, and the release of TCA-soluble radioactivity into the medium was used as an estimate of lipoprotein degradation using modifications (Sprague et al., 1987) to remove unbound labeled iodide ion. The cells were then rinsed and treated with heparin to remove surface-bound ^{125}I-LDL as described above. The cells were dissolved in 1 mL of 0.1 N NaOH, and the lysate plus three 1-mL PBS washes of the culture dishes was collected, pooled, and counted as a measure of internalized LDL.

Measurement of ^{125}I-LDL Exocytosis. Cells up-regulated for the LDL receptor in the presence or absence of inhibitor were pulse-labeled by incubating cultures in M199 containing 10% LPDS and 10 μg/mL ^{125}I-LDL for 4 h at 37 °C. Cultures were then rinsed and treated with heparin to remove surface-bound ^{125}I-LDL as described above. Fresh medium was added, and the fate of the cell-associated ^{125}I-LDL was monitored during a subsequent 24-h chase. At specific times during the chase, the amount of ^{125}I-LDL bound to the cell surface, the amount degraded, and the amount remaining within the cell were measured. In addition, the amount of ^{125}I-LDL released intact, i.e., exocytosed, into the medium was determined by counting the initial TCA precipitate obtained during the processing of the conditioned media for degradation determination.

Assay for Total Cellular LDL Receptor Binding Activity. Total cellular LDL receptor activity was measured as described by Schneider et al. (1985). Briefly, fibroblasts were up-regulated for the LDL receptor in the presence or absence of 250 μg/mL castanospermine as described above. Cells from eight 100-mm culture dishes from each treatment group were then rinsed once with ice-cold PBS. All subsequent procedures were conducted at 4 °C. Cells were mechanically detached from the culture dishes in 0.5 mL of 25 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES) buffer, pH 6, $CaCl_2$, and 80 mM NaCl and centrifuged at 800*g* for 5 min. The cell pellet was solubilized by resuspension in 1.2 mL of 50 mM Tris–maleate buffer, pH 6, containing 2 mM $CaCl_2$, 40 mM *n*-octyl glucoside, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 0.01 mM leupeptin and incubated for 10 min at 0–4 °C. After centrifugation at 100000*g* for 1 h, the solubilized receptors were precipitated from the supernatant with phosphatidylcholine/acetone (Schneider et al., 1985). After further centrifugation at 20000*g* for 20 min at 4 °C, the pellet (phosphatidylcholine/acetone precipitate) was resuspended in 0.2 mL of 20 mM Tris–chloride buffer, pH 8, containing 1 mM $CaCl_2$ and 50 mM NaCl by aspiration through a 22-gauge needle. Receptor binding activity was determined by using an assay mixture (100 μL) composed of 40 μL of resuspended phospholipid/acetone precipitate, 30 μL of 200 mM Tris–chloride buffer, pH 8, containing 50 mM NaCl, 1 mM $CaCl_2$, and 80 mg/mL BSA, 10 μL of 0.15 M NaCl or 10 μL of 10 mg/mL unlabeled LDL, and 20 μL of ^{125}I-LDL (100 μg of protein/mL of 0.15 M NaCl). After incubation for 1 h at room temperature, 80-μL aliquots of each reaction mixture were filtered (Schneider et al., 1985) with cellulose acetate membrane filters, and the associated radioactivity was counted by using a Beckman 310 γ counter. Specific binding was calculated by subtracting the amount of ^{125}I-LDL bound in the presence of labeled plus unlabeled LDL from that bound in the presence of labeled LDL only. The protein content of the phosphatidylcholine/acetone precipitate was determined as described by Schneider et al. (1980).

Characterization of Oligosaccharide Structure. SMC were plated into T-75 flasks (Corning Glass Works) in normal growth media, and 1 week later, cells were changed into media containing 10% LPDS in the presence or absence of castanospermine (0–250 μg/mL). After a 12-h incubation, 400 μCi of [2-^{3}H]mannose or [6-^{3}H]galactose was added to each flask, and the incubation was continued for an additional 12 h at 37 °C. The cells were then removed by scraping and disrupted by freezing and thawing. The cell residue was digested with 1 mL of Pronase (5 mg/cell pellet) for 48 h at 37 °C to obtain glycopeptides which were isolated on columns of Bio-Gel P-4. The entire glycopeptide peak was collected, dried using a rotary evaporator, and dissolved in 500 μL of 0.1 M sodium citrate buffer, pH 5.5. Endoglucosaminidase H (Endo H, 2.5 milliunits in 5 μL of citrate buffer) was added to each glycopeptide peak. The samples were subsequently incubated at 37 °C under a toluene atmosphere. After 24 h, an additional 2.5 milliunits of Endo H was added, and the digestion was continued for an additional 24 h. These mixtures were then chromatographed on the Bio-Gel P-4 column (Pan et al., 1983).

Determination of Cell Replication Rate. The influence of castanospermine on total [^{3}H]thymidine incorporation into both normal human fibroblasts and SMC was determined. Two to three days after subculture, cells were receptor up-regulated for 48 h in the presence or absence of 250 μg/mL castanospermine as described above. Cultures were then incubated with 1 μCi/mL [^{3}H]thymidine (53 Ci/mmol, ICN) in M199 containing 10% LPDS. After 2 h at 37 °C, the medium was aspirated, and the cells were rinsed twice with Hank's balanced salt solution (HBSS) containing 0.05% EDTA and removed by trypsinization at 37 °C. Duplicate aliquots were removed for cell counts and measurement of incorporated [^{3}H]thymidine by TCA extraction as described by Osborne et al. (1976). The [^{3}H]thymidine activity was counted in a Beckman liquid scintillation spectrometer (Model LS-5801), and the results are expressed as dpm of [^{3}H]thymidine/10^6 cells.

The cell replication rate was also examined by [^{3}H]thymidine autoradiography. After receptor up-regulation in the presence or absence of castanospermine, cells were incubated with 1 μCi/mL [^{3}H]thymidine for 2 h at 37 °C. The cultures were then cooled to 4 °C and rinsed once with PBS and twice with methanol before fixation with methanol for 15 min at 4 °C. The fixed cells were rinsed 5 times with water at 4 °C, air-dried, layered with NTB-2 emulsion (Eastman Kodak, Rochester, NY) in a darkroom, and sealed in a light-proof

container over Drierite. After 7–10 days, the emulsion was developed with a Kodak D19 developer for 3 min, and the cells were stained with Giemsa. The cell replication rate was measured as the percentage of radiolabeled nuclei.

Subcellular Fractionation. Normal human skin fibroblasts were up-regulated for 18 h in the presence of absence of 250 μg/mL castanospermine in M199 containing 10% LPDS. For each treatment group, 12 100-mm culture dishes were precooled and rinsed 2 times with ice-cold PBS at 4 °C. After mechanical detachment from the culture dishes in ice-cold 0.25 M sucrose, the fibroblasts were centrifuged at 200*g* for 5 min to obtain cell pellets. Cells were homogenized in 2 mL of 0.25 M sucrose using a Dounce homogenizer at 4 °C. Nuclei and debris were removed by centrifugation at 800*g* for 5 min. Approximately 1.5 mL of homogenate was separated into subcellular fractions on a Percoll gradient (Percoll–2.5 M sucrose–water, 4.9:1:3 v/v) and centrifuged opposite density marker beads (1.035–1.135 g/mL; Pharmacia Fine Chemicals) at 23 000 rpm for 150 min in a Beckman L5-65 centrifuge using a Beckman type 30 rotor (26° angle). One-milliliter fractions were collected for receptor analysis from the top of the tubes using a Beckman gradient collector.

The protein content of each fraction was measured by using the bicinchoninic acid (BCA) protein assay reagent (Pierce Chemical Co.) and the Percoll–0.25 M sucrose gradient mixture as a blank. Receptor activity in subcellular fractions was measured in a similar manner to that described for total receptors.

Results

Influence of Castanospermine on the Structure of Cellular N-Linked Oligosaccharides. To determine the effect of castanospermine on the structure of N-linked glycoproteins, smooth muscle cells were incubated in the presence of castanospermine (0-250 μg/mL) and [2-^{3}H]mannose, prior to the isolation of the glycopeptides on columns of Bio-Gel P-4. The entire glycopeptide peak was pooled, treated with Endo H, and rechromatographed on the Bio-Gel P-4 column in order to determine the percentage of glycopeptides that were susceptible to this enzyme (i.e., high-mannose structures). Figure 1 shows the elution profiles of the glycopeptides and oligosaccharides at the various castanospermine concentrations. Prior to Endo H treatment, the glycopeptides isolated from control cells emerged from the column in a broad area from fractions 30 to about 55 [control profile (A)]. In this case, it was difficult to distinguish clearly the complex types of oligosaccharides from high-mannose or other types of chains. However, after treatment with Endo H (A′), the glycopeptides and oligosaccharides could be resolved into an initial shoulder and two discrete peaks (marked I, II, and III in control profile).

On the other hand, in the presence of castanospermine, the profiles were considerably altered compared to those of the controls. With increasing concentrations of castanospermine (10–250 μg/mL, panels B′–D′ of Figure 1), the amount of radioactivity in the first two peaks (indicated as I and II) was markedly reduced after Endo H treatment, whereas a large peak of radioactivity appeared (peak III) that migrated slightly faster than peak III of the control cells. When the castanospermine-induced oligosaccharide (peak III; castanospermine 250 μg/mL; panel D′, Figure 1) was chromatographed on the longer, standardized column of Bio-Gel P-4 (1.5 × 200 cm) to improve resolution, it eluted in the area indicative of hexose$_{10-11}$GlcNAc, whereas peak III from the control cells eluted in the area of the column expected for a hexose$_{8-9}$GlcNAc structure. Peak III from the castanospermine-treated cells was only partially susceptible to digestion

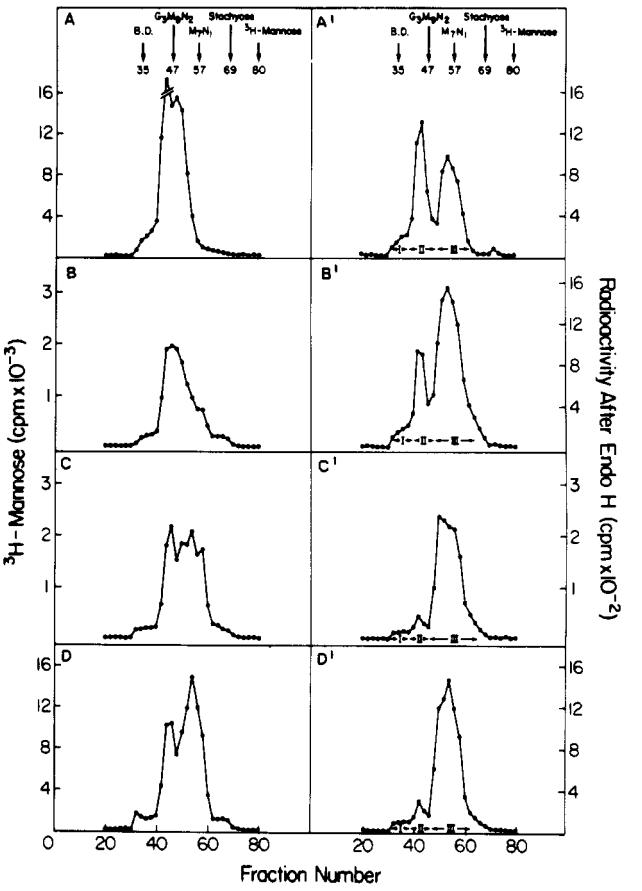

FIGURE 1: Bio-Gel P-4 column chromatography of glycopeptides and oligosaccharides from SMC exposed to increasing concentrations of castanospermine. [2-^{3}H]Mannose-labeled glycopeptides were prepared from SMC glycoproteins as described under Materials and Methods and loaded on a 1.5 × 100 cm Bio-Gel P-4 column, equilibrated with 0.35% acetic acid (v/v). Fractions of 3 mL were collected, and radioactivity was determined, as shown by the profiles on the left (A–D). The entire glycopeptide peak was then digested with endoglucosaminidase H (Endo H) and rechromatographed on the Bio-Gel column. These profiles are shown on the right (A′–D′). Each set of profiles was obtained from SMC exposed for 24 h to the following castanospermine concentrations: 0, panels A, A′; 10 μg/mL, panels B, B′; 50 μg/mL, panels C, C′; 250 μg/mL, panels D, D′.

by jack bean α-mannosidase and gave rise to a small peak of radioactive mannose and a large oligosaccharide peak that migrated near the original oligosaccharide. On the other hand, peak III from control cells was almost completely susceptible to α-mannosidase and gave rise to a major peak of free mannose and a small peak of ManGlcNAc.

The oligosaccharide from castanospermine-treated cells could be labeled in the glucose portion by growing the cells in the presence of [6-^{3}H]galactose as described under Materials and Methods. Treatment of this glucose-labeled oligosaccharide with a partially purified glucosidase I, free of glucosidase II (Szumilo et al., 1986), released approximately one-third of the radioactivity as free glucose, whereas oligosaccharide exposure to both glucosidase I and glucosidase II liberated essentially all of the radioactivity as free glucose (data not shown). These data indicate that the major oligosaccharide formed in the presence of castanospermine is a Glc$_3$Man$_{7-9}$GlcNAc. The radioactive mannose incorporated into peaks I and II by control cells was shown to be present in complex oligosaccharides on the basis of their binding (or lack of binding) to concanavalin A–Sepharose as described previously (Cummings & Kornfeld, 1982), as well as by treatments with various enzymes (data not shown). Thus, peak I appears to represent mostly tri- and tetraantennary chains,

which do not bind to concanavalin A, whereas peak II is composed of biantennary chains which bind weakly to the lectin columns. Endo H profiles from castanospermine-treated cells indicate that as the inhibitor concentration was increased, smaller amounts of radioactive mannose were found in these peaks. Thus, at 250 μg/mL castanospermine, less than 5% of the total radioactivity was present in peaks I and II, indicating an almost total inhibition of glucosidase I activity.

Influence of Castanospermine and Swainsonine on Smooth Muscle Cell (SMC) Proliferation. These studies were designed to determine whether any effects of either castanospermine or swainsonine on LDL receptor function or distribution might be related to direct effects on cell proliferation. The inhibitors were added to the SMC 2–3 days after subculture. Under these conditions, SMC cultures exposed to 250 μg/mL castanospermine for up to 72 h attained 103.4% ± 2.6% (n = 15) of control culture density, while cultures treated with 250 ng/mL swainsonine for the same periods contained 97.3% ± 2.0% (n = 13) of the number of cells observed in controls. In contrast, when castanospermine was added to SMC cultures within 24 h after plating at concentrations of 100 μg/mL or greater for 5 days, an almost 50% reduction in cell density was observed relative to control cells. Swainsonine at 200 μg/mL similarly induced a 20% reduction in cell density using the same treatment regimen. As a result of these studies, we exposed the cells to castanospermine only after they were established in culture, i.e., 2 or more days after subculturing, and limited the times of exposure to inhibitor to a maximum of 72 h for all experiments. To confirm these observations, the incorporation of [^{3}H]thymidine into cellular DNA in the presence of various amounts of castanospermine was measured. These studies indicated that under the conditions described above, i.e., adding inhibitor 2–3 days after plating, castanospermine had no cytotoxic effects on either SMC or fibroblasts and did not inhibit DNA synthesis or cell growth.

Influence of Castanospermine on Receptor-Mediated ^{125}I-LDL Internalization and Degradation. The effect of castanospermine on the degradation of ^{125}I-LDL was examined by exposing SMC to 0 or 250 μg/mL castanospermine for periods of 30 min–72 h. For 24 h prior to measurement of LDL endocytosis (the final 24 h for those castanospermine treatments that exceed 24 h), cells were incubated in media containing 10% lipoprotein-deficient serum in order to induce LDL receptor synthesis and expression (up-regulation). After each castanospermine preincubation, degradation was measured at the end of a 4-h incubation with ^{125}I-LDL (5 μg/mL) in the presence or absence of a 40-fold excess (200 μg/mL) of unlabeled LDL as described under Materials and Methods. As presented in Figure 2, SMC exposed to castanospermine for times exceeding 2 h exhibited greater than 40% reduction in receptor-mediated ^{125}I-LDL degradation.

To determine whether inhibitor treatment also affected the rate of lipoprotein internalization, the time course of LDL internalization and degradation was examined in control and castanospermine-treated cells using the incubation conditions described above. The results indicated that castanospermine did not alter the rate of internalization of ^{125}I-LDL but it significantly reduced the total amount internalized (data not shown). As expected, this decreased internalization was also reflected in a parallel decrease of lipoprotein degradation for inhibitor-treated cells. These data suggest that although castanospermine-treated SMC are able to internalize and catabolize the lipoprotein, there is less LDL being internalized, perhaps due to a decrease in total LDL receptors. Furthermore, these results suggest that castanospermine had to be present in the medium during receptor up-regulation in order to show these effects. This conclusion was confirmed in experiments in which castanospermine was added to the medium of cells after up-regulation (receptor synthesis) had occurred. In this case, castanospermine did not affect lipoprotein internalization or degradation. These experiments further indicate that castanospermine did not directly affect lipoprotein metabolism. Interestingly enough, both LDL internalization and degradation were still inhibited when castanospermine was present during up-regulation but was removed during the incubation with LDL, indicating that LDL metabolism is not rapidly recovered after the removal of castanospermine.

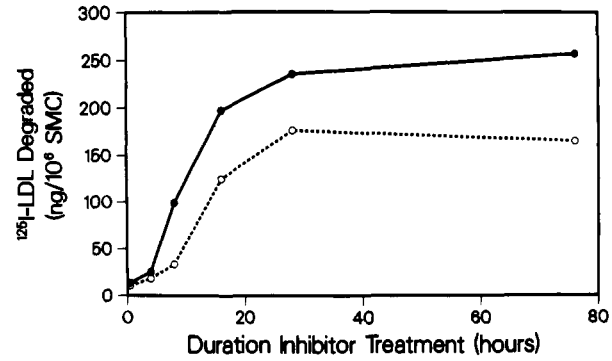

FIGURE 2: Influence of castanospermine on receptor-mediated degradation of ^{125}I-LDL in smooth muscle cells. Cells were exposed to 250 μg/mL castanospermine for periods ranging from 30 min to 72 h. During the final 24 h, the medium was changed to medium containing 10% lipoprotein-deficient serum and inhibitor (where appropriate). After the indicated preincubation, each culture received 1 mL of castanospermine and ^{125}I-LDL (5 μg of protein/mL). Replicate treatment cultures received labeled LDL plus a 40-fold excess of unlabeled LDL (200 μg of protein/mL). After a 4-h incubation of 37 °C, the amount of specific ^{125}I-LDL degraded by castanospermine-treated (○) and untreated control cells (●) was determined as described. The nonspecific values comprised <10% of the total values from both treatment groups. Each *point* represents the mean of duplicate determinations from one of two separate experiments.

Effect of Castanospermine on the Fate of Internalized LDL. To further rule out the possibility that castanospermine might directly influence LDL degradation, the effect of castanospermine on the fate of internalized ^{125}I-LDL was examined as shown in Figure 3. SMC were induced to synthesize LDL receptors in the presence or absence of 250 mg/mL inhibitor and then pulse-labeled with ^{125}I-LDL (10 mg/mL) for 4 h at 37 °C. After heparin removal of the surface-bound ^{125}I-LDL, the amount of ^{125}I-LDL degraded, exocytosed, and remaining within the cell was periodically determined during a subsequent 24-h chase as described under Materials and Methods. As shown in Figure 3A, the cell-associated ^{125}I-LDL decreased in both the control and castanospermine-treated cultures during the first 8 h with little change thereafter. Initially, the amount of intracellular ^{125}I-LDL was 24% less in inhibitor-treated cells as compared to control cells. Both ^{125}I-LDL degradation (Figure 3B) and exocytosis (Figure 3C) were decreased by castanospermine treatment to a degree commensurate with the lesser initial amount of ^{125}I-LDL present within the inhibitor-treated SMC (Figure 3A) at the end of the LDL pulse period. The results of these pulse–chase experiments are consistent with the idea that the major effect of castanospermine is on LDL receptor function, resulting in decreased LDL uptake.

Similar results were obtained when the above experiments were repeated using normal human skin fibroblasts, which have been shown to possess a larger number of LDL receptors (Goldstein et al., 1983). In fibroblasts, even greater differences

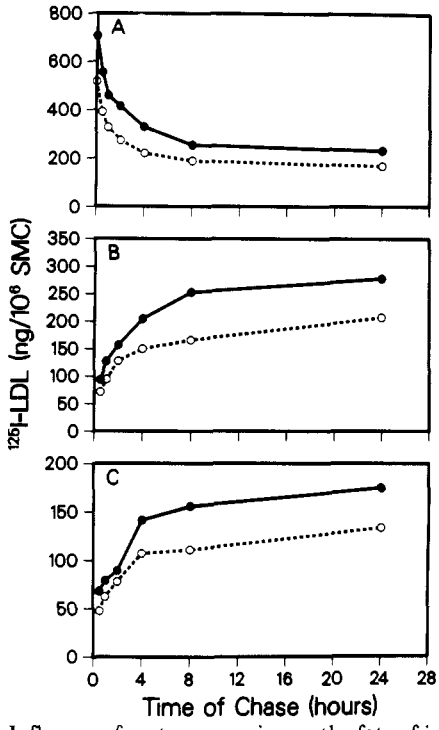

FIGURE 3: Influence of castanospermine on the fate of intracellular ^{125}I-LDL in smooth muscle cells. After incubation for 24 h in medium containing 10% lipoprotein-deficient serum in the presence or absence of 250 μg/mL castanospermine, each culture received 1 mL of medium containing 10% lipoprotein-deficient serum and ^{125}I-LDL (10 mg of protein/mL), maintaining the inhibitor concentration where appropriate. After incubation for 4 h at 37 °C, cells were rinsed and treated with heparin to remove surface-bound ^{125}I-LDL as described under Materials and Methods. Each culture then received 2 mL of fresh medium, with or without inhibitor, and at specific times during the subsequent 24-h chase, the amount of ^{125}I-LDL bound to the cell surface (not shown), cell-associated (A), degraded (B), and exocytosed (C) into the medium was determined for both castanospermine-treated (○) and control cells (●) as described under Materials and Methods.

were observed, especially in degradation, between control and inhibitor-treated cells when LDL receptor synthesis occurred in the presence of castanospermine (data not shown).

Effect of Castanospermine on the Binding of ^{125}I-LDL to the Cell Surface at 4 °C. On the basis of the above results, 4 °C binding studies were conducted to determine whether receptor synthesized in the presence of castanospermine had reduced affinity for LDL or whether the number of functional LDL receptors expressed on the cell surface was reduced. SMC were up-regulated in the presence or absence of 250 μg/mL castanospermine, and LDL binding was measured as described under Materials and Methods. The results in Figure 4 show that the specific binding of ^{125}I-LDL to the surface of SMC was reduced by a mean of 37% when compared with controls (Figure 4, upper panel). The parallel slopes of the Scatchard plots (Figure 4, lower panel) indicate that the binding affinity was unaltered but that castanospermine treatment resulted in about a 40% reduction in the capacity of SMC to bind LDL relative to control cells. Normal human skin fibroblasts were also examined in these studies. When up-regulated in the presence of 250 μg/mL castanospermine, the binding of ^{125}I-LDL was about 60% of control fibroblasts, and, again, Scatchard analysis confirmed a reduction in the number of specific cell surface receptor sites.

We examined the effects of various castanospermine concentrations on LDL binding to fibroblasts to determine the optimum inhibitor concentration. Cells were up-regulated in the presence or absence of 10–750 μg/mL castanospermine, after which 4 °C binding studies were performed using 10

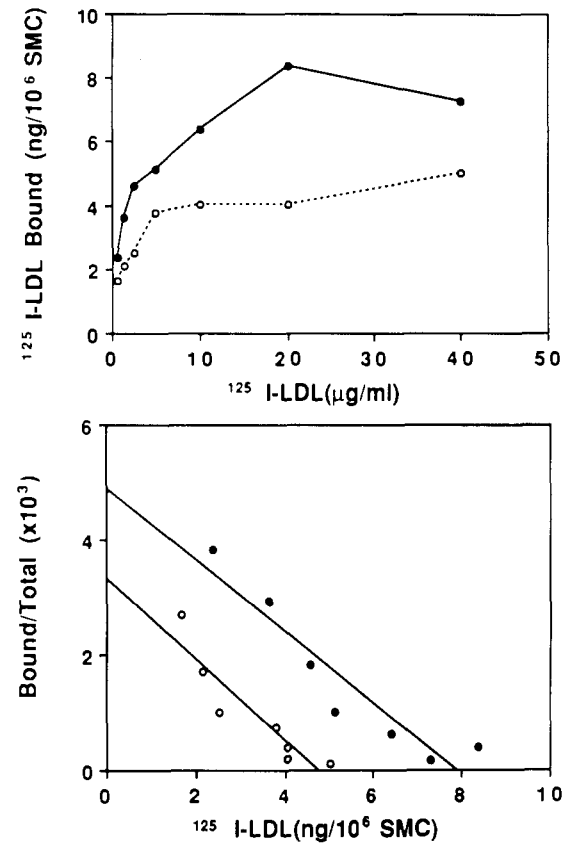

FIGURE 4: Influence of castanospermine on receptor-mediated binding of ^{125}I-LDL to the cell surface of smooth muscle cells (upper panel) and Scatchard plot of 4 °C binding data (lower panel). After incubation for 48 h in medium containing 10% lipoprotein-deficient serum, in the presence or absence of 250 μg/mL castanospermine, SMC were rinsed, precooled to 4 °C, and incubated with 1 mL of medium containing 10% lipoprotein-deficient serum and the indicated concentration of ^{125}I-LDL. Replicate cultures received the medium containing the labeled lipoprotein plus 500–4000 μg of protein/mL unlabeled LDL. After incubation for 2 h at 4 °C, receptor-mediated ^{125}I-LDL binding was determined for both castanospermine-treated (○) and control (●) SMC as described under Materials and Methods. The nonspecific value comprised <10% of the total values for cells from both treatment groups. Each *point* represents the mean specific binding of duplicate determinations from one of three separate experiments.

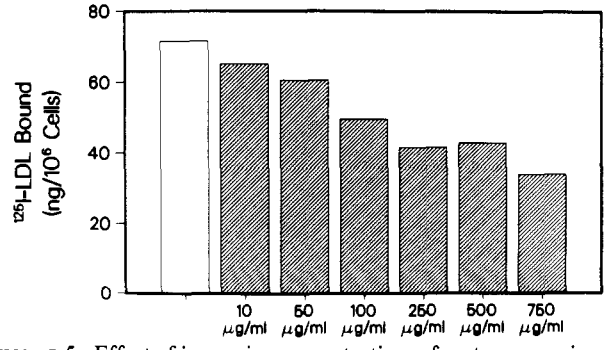

FIGURE 5: Effect of increasing concentrations of castanospermine on receptor-mediated binding of ^{125}I-LDL to the surface of normal human skin fibroblasts at 4 °C. Cells were incubated for 48 h in medium containing 20% lipoprotein-deficient serum in the absence (open bars) or presence (hatched bars) of the indicated concentrations of castanospermine and then rinsed and precooled for 30 min at 4 °C. One milliliter of medium containing 10% lipoprotein-deficient serum and ^{125}I-LDL (10 μg of protein/mL) was then added. Replicate cultures received the medium containing labeled LDL plus 500 μg of unlabeled LDL. After 2 h at 4 °C, receptor-mediated binding was determined as described under Materials and Methods. Each *bar* represents the mean specific binding of duplicate determinations from one of three separate experiments.

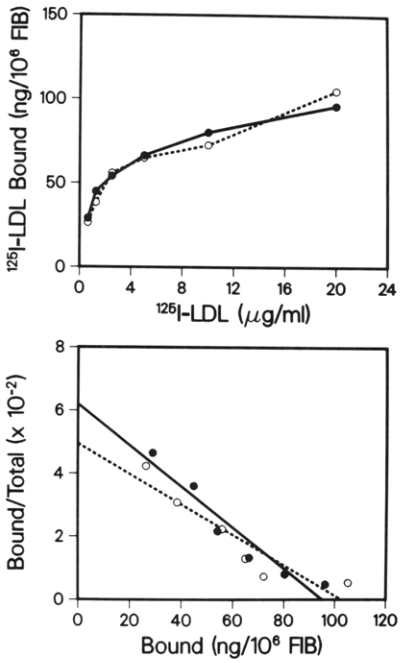

FIGURE 6: Effect of swainsonine on receptor-mediated binding of ^{125}I-LDL to the cell surface of normal human skin fibroblasts (upper panel) and Scatchard plot of 4 °C binding data (lower panel). The experiment was conducted as described in the legend to Figure 7. ^{125}I-LDL binding was determined as described under Materials and Methods for swainsonine-treated (○) and control (●) fibroblasts. Each *point* represents the mean specific binding of duplicate determinations from one of three separate experiments.

μg/mL ^{125}I-LDL in the presence or absence of 500 μg/mL unlabeled LDL as described above. As indicated in Figure 5, castanospermine concentrations of up to 250 μg/mL resulted in a dose-dependent decrease in specific receptor-mediated ^{125}I-LDL binding to the cell surface. However, increasing the inhibitor concentration to 500 μg/mL, or even to 750 μg/mL, did not produce any further significant decrease in ^{125}I-LDL binding.

To be certain that this influence on LDL receptor mediated binding was due to specific changes in oligosaccharide structure caused by castanospermine, we conducted similar binding studies in fibroblasts induced to synthesize LDL receptors in the presence of 250 μg/mL swainsonine. Unlike the modification produced by castanospermine, swainsonine causes the production of hybrid types of N-linked oligosaccharides in the glycoproteins of treated cells (Elbein, 1987). A representative experiment is illustrated in Figure 6. Clearly, exposure of fibroblasts to this alkaloid during receptor up-regulation did not significantly affect the receptor-mediated binding of ^{125}I-LDL. As shown in the lower graph, Scatchard analyses revealed that neither the binding affinity nor the receptor number was significantly altered.

Measurement of Total Cellular LDL Receptor Binding Activity and Localization in Subcellular Fractions. In order to determine whether the observed decrease in LDL binding in castanospermine-treated cells was due to a decreased synthesis, an increased degradation, or an altered targeting of LDL receptors, we compared total (surface plus intracellular) LDL receptor binding in control and castanospermine-treated fibroblasts. Fibroblasts were used due to the large number of receptors present in up-regulated cells. Schneider et al. (1985) previously demonstrated that total cellular LDL binding activity can be measured by solubilizing cells in a buffer containing 40 mM octyl glucoside. The solubilized receptors were then reconstituted into phosphatidylcholine liposomes, the complex was precipitated with acetone, and the

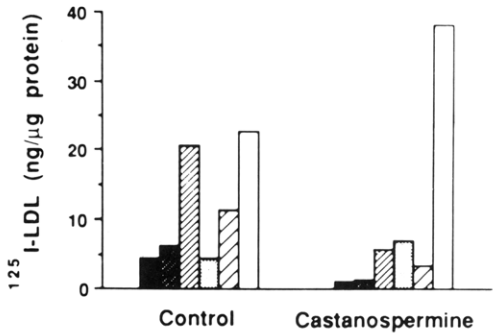

FIGURE 7: LDL receptor activity in subcellular fractions from castanospermine-treated and untreated control normal human skin fibroblasts. Fibroblasts were incubated for 18 h in medium containing 10% lipoprotein-deficient serum in the presence or absence of 250 mg/mL castanospermine. After precooling and rinsing 2 times with PBS at 4 °C, cells were mechanically detached from the culture surface in ice-cold 0.25 M sucrose. Fibroblasts were homogenized, and subcellular fractions were separated on a Percoll–0.25 M sucrose gradient as described under Materials and Methods. One-milliliter fractions were collected from the top of the tube, and those containing a detectable amount of protein were assayed for LDL receptor activity as described under Materials and Methods. Each *point* represents the mean specific binding of duplicate determinations from one of two separate experiments. Fraction densities as determined with density marker beads (Pharmacia Fine Chemicals) ranged from about 1.03 [fraction 2 (■)] to 1.09 g/mL [fraction 7 (□)].

^{125}I-LDL binding activity was measured in the presence or absence of unlabeled LDL, using a solid-phase filtration assay. In contrast to the difference in LDL receptor numbers observed on the cell surface, the number of total cellular LDL receptors observed in normal fibroblasts, up-regulated in the presence of 250 μg/mL castanospermine, was not significantly different from the number in control cells. For example, in one of two separate experiments, a total of 0.43 vs 0.49 ng of ^{125}I-LDL bound/mg of receptor precipitate was measured in castanospermine-treated cells relative to control cells. As a test of the reliability of this assay, we found essentially no binding activity when LDL receptor-negative mutant fibroblasts were processed in the same way. These results indicate that the decrease in cell surface LDL binding is not due to a change in the total number of LDL receptors but instead is due to an alteration in targeting of these receptors to the cell surface.

Since castanospermine appeared to affect the targeting of receptors, we examined the subcellular location of LDL receptors in cells up-regulated in the presence or absence of inhibitor. After receptor up-regulation in the presence or absence of 250 μg/mL castanospermine, cell homogenates were subjected to subcellular fractionation on a Percoll-0.25 M sucrose gradient as described under Materials and Methods. Fractions of 1 mL were collected, and those containing a detectable amount of protein were examined for LDL receptor activity. The results from a representative experiment are depicted in Figure 7. The LDL receptor activity profile in the subcellular fractions from castanospermine-treated cells is strikingly different from that observed in control cells and suggests that the drug may cause an intracellular sequestration of receptors, probably in the endoplasmic reticulum. Although it was not possible to examine marker enzymes in this experiment due to the small amounts of material, it seems likely that fraction 7 does represent the endoplasmic reticulum fraction.

Influence of Castanospermine on LDL Receptor Activity in Bovine Aortic Endothelial Cells. Studies were also done in bovine aortic endothelial cells to determine if the effects of castanospermine were similar in other cell types. Cells were exposed to increasing concentrations of inhibitor ranging from

0.1 to 400 μg/mL during LDL receptor up-regulation. Lipoprotein degradation was measured after a 3-h incubation with 10 μg/mL ^{125}I-LDL in the presence or absence of a 50-fold excess of unlabeled LDL, and results were compared with untreated controls. The results showed a dose-dependent inhibition in ^{125}I-LDL degradation as castanospermine concentrations increased from 0.1 to 25 μg/mL, where a maximal inhibition of about 70% was observed. Increasing the castanospermine concentration to 50, 100, 250, or even 400 μg/mL did not produce any greater inhibitor in lipoprotein degradation (data not shown). Binding studies conducted at 4 °C using a castanospermine concentration of 25 μg/mL exhibited an even greater inhibition of binding than that observed with either smooth muscle cells or fibroblasts. These data suggest that there is a differential sensitivity of cell types to castanospermine.

Discussion

We have established that the plant alkaloid castanospermine, an inhibitor of the N-linked glycoprotein processing enzyme glucosidase I (Elbein, 1987), causes a significant decrease in the binding and endocytosis of LDL when cultured arterial smooth muscle cells are allowed to synthesize LDL receptor in the presence of inhibitor. Moreover, these results indicate that castanospermine treatment is associated with a significant reduction in the number of cell surface LDL receptors. A similar inhibition of ^{125}I-LDL endocytosis and specific LDL binding sites was observed in cultured normal human skin fibroblasts and in bovine aortic endothelial cells. However, in the latter cells, inhibition was seen at one-tenth the concentration of castanospermine used for fibroblasts and smooth muscle cells. These studies indicate that the effect of castanospermine on the function and metabolism of the LDL receptor occurs in all cells expressing this receptor, but with different sensitivities.

It is important to point out that castanospermine had no direct effect on LDL metabolism within the 24-h incubation period used in these studies. Thus, LDL metabolism is apparently affected only when the drug is able to influence receptor synthesis. One likely explanation for these results is the prevention of modification of the N-linked oligosaccharide chains on the immature LDL receptor, causing them to remain as $Glc_3Man_{7-9}(GlcNAc)_2$ structures, rather than being processed to the complex types found on the mature receptors (Cummings et al., 1983). In this regard, the failure of swainsonine to influence receptor-mediated binding of LDL is of some significance. Swainsonine inhibits mannosidase II (Broquist, 1985; Elbein, 1987), a step much later in the processing pathway of N-linked oligosaccharides. This results in the accumulation of glycoproteins containing hybrid-type structures that more closely resemble the complex structure found on the mature receptor. Apparently, such hybrid chains allow the receptor molecules to be expressed on the cell surface.

It is possible that a longer exposure to castanospermine might result in direct effects on LDL metabolism. For example, many, if not all, lysosomal enzymes are N-linked glycoproteins. These proteins are targeted to the lysosomes via a mannose 6-phosphate signal on some of the high-mannose oligosaccharides (Kornfeld & Kornfeld, 1985). This signal is added to certain mannose residues in the Golgi apparatus by the transfer of a GlcNAc-1-P to specific mannose residues, and then removal of the GlcNAc. Thus, if castanospermine prevented the removal of glucose residues from the high-mannose chains, it could prevent the transfer of GlcNAc-1-P, and thus block targeting to the lysosomes. Such an effect could cause these cultured cells to resemble cells from patients with I cell disease that are missing the phospho-*N*-acetylglucosaminyl transferase (Bach et al., 1979).

However, because the influence of castanospermine is dependent upon receptor synthesis, an alternative explanation of the reduction in cell surface LDL receptors is due to a recompartmentalization of receptors. If the oligosaccharides normally play a role in targeting of the receptor to the plasma membrane, then a structural alteration in the N-linked oligosaccharides either might retard the receptor in its movement from the endoplasmic reticulum through the Golgi to the cell surface or cause the receptor to become sequestered internally during recycling. In fact, studies by Lodish and Kong (1984), using the glucosidase I inhibitor deoxynojirimycin, have shown that the presence of glucose on some of the glycoproteins produced by hepatocytes markedly retarded their secretion from the cells. The proteins appeared to be sequestered in the ER. It is possible that it is the movement of the altered receptor from the endoplasmic reticulum to the Golgi that is markedly impaired in the presence of castanospermine.

Accordingly, the results from the studies in which we measured total cellular LDL receptor binding demonstrated only a small difference in the total number of LDL receptors between castanospermine-treated and control fibroblasts, indicating that decreased receptor synthesis or increased degradation cannot account for the decrease in cell surface ^{125}I-LDL binding. Cell surface lipoprotein binding at 4 °C was inhibited approximately 40%, with no apparent change in affinity, while total cellular LDL binding activity was not significantly reduced relative to controls. Furthermore, the subcellular fractionation studies illustrate that LDL receptor activity in fractions from castanospermine-treated cells is markedly different from that observed in untreated fibroblasts. These results suggest and, again, support the hypothesis that inhibitor exposure during receptor synthesis leads to a perturbation in normal LDL receptor distribution. Thus, incomplete processing of the oligosaccharide moiety of the LDL receptor could either delay transport through the synthetic cellular compartments or impair the insertion of receptor into the plasma membrane. Such is consistent with the spectrum of mutant Chinese hamster ovary cell lines, which exhibit varying degrees of glycosylation defects in both N- and O-linked oligosaccharide chains (Kingsley et al., 1984, 1986). Mutants exhibiting dramatic modifications in both N- and O-linked oligosaccharide structures have markedly diminished LDL receptor activity (5–10% of normal) while others with less dramatic structural modifications possess substantial levels of LDL receptor activity, possibly similar to the observed influence of castanospermine on LDL receptor mediated endocytosis described in these studies.

Acknowledgments

We gratefully acknowledge the excellent technical contributions of Dr. C. Alan Suenram and Peter Binkley in lipoprotein preparation and Yolanda Villarreal-Acosta and Pam Keister in cell culture and the expert typing of Peggy Pegg and Laida Garcia in preparation of the manuscript.

Registry No. Castanospermine, 79831-76-8.

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