# Inhibition of Scavenger Receptor-Mediated Modified Low-Density Lipoprotein Endocytosis in Cultured Bovine Aortic Endothelial Cells by the Glycoprotein Processing Inhibitor Castanospermine†

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ABSTRACT: Castanospermine (1,6,7,8-tetrahydroxyoctahydroindolizidine) is a plant alkaloid that inhibits α-glucosidases, including the glycoprotein processing glucosidase I. When endothelial cells were grown for 48 h, or longer, in the presence of this alkaloid, they produced scavenger receptors for modified low-density lipoproteins (LDL) that had mostly $Glc_3Man_{7-9}(GlcNAc)_2$ structures rather than the usual complex types of oligosaccharides. Furthermore, growth in the presence of castanospermine resulted in a substantial inhibition in degradation of endocytosed $^{125}$I-acetylated LDL, as well as a dose-dependent inhibition of $^{125}$I-acetylated LDL binding to these cells. Scatchard analysis of binding curves indicated that the diminished binding was due to a decrease in the number of scavenger receptor molecules at the cell surface rather than to a change in the affinity of the receptors for their ligand. Since castanospermine-treated cells had the same total number of cellular receptor molecules as did control cells, it seemed likely that castanospermine caused an alteration in receptor targeting, rather than an inhibition in receptor synthesis or a stimulation in receptor degradation. Density gradient fractionation of cell homogenates showed that castanospermine-treated cells did have a much greater percentage of scavenger LDL receptor molecules in the endoplasmic reticulum–Golgi fraction and fewer receptors in the plasma membrane fraction, whereas normal cells showed the opposite distribution.

Scavenger receptors, expressed exclusively on the surface of macrophages and endothelial cells, exhibit a high affinity for a diverse but limited number of anionic macromolecules, including oxidized and chemically modified low-density lipoproteins (LDL)[1] (Goldstein et al., 1979; Mahley et al., 1979; Stein & Stein, 1980; Fogelman et al., 1980). This high affinity, coupled with high levels of expression on arterial wall macrophages, has been linked to the uptake of modified LDL and the subsequent accumulation of large amounts of esterified cholesterol. In the presence of large amounts of available extracellular, modified LDL, this process of lipid accumulation culminates in the development of the macrophage into the lipid-laden foam cell, the early hallmark of atherogenesis (Schwartz et al., 1991). Further, scavenger receptors in large numbers and with high affinity for modified LDL have been identified *in vivo* on endothelial cells lining liver sinusoids and are postulated to play an important role, along with liver Kupffer cells, in removing modified lipoproteins from circulating blood (Pitas et al., 1985).

Though the functional characteristics of scavenger receptor were initially defined in 1979 (Goldstein et al., 1979; Mahley et al., 1979), only recently has this receptor been identified using molecular biological techniques. Cloning of the scavenger receptor cDNAs from three species (murine, bovine, and human) indicates that most of the structural characteristics of the receptor are conserved (Freeman et al., 1990). Among the six domains defined within these molecules is an intracellular α-helical–coiled coil (domain IV), containing seven potential N-glycosylation sites. This domain, along with domain V containing coiled collagen-like sequences, putatively houses the ligand binding capability of the receptor. In brief, the scavenger receptor appears to be an integral membrane glycoprotein exhibiting a short cytoplasmic domain, a transmembrane hydrophobic domain, and a large extracellular region comprising four domains.

Previous studies have demonstrated that the carbohydrate moiety of integral membrane glycoprotein receptors may play an important role in ligand binding and/or transport and plasmalemma expression (Elbein, 1991). Studies employing tunicamycin, an antibiotic that prevents addition of carbohydrate to N-glycosylation sites of receptors and other glycoproteins (Tkacz & Lampen, 1975), have been shown that the transport and/or ligand binding of the insulin receptor (Reed et al., 1981), the receptor for acetylcholine (Prives & Olden, 1980), and the LDL (Filipovic & Von Figura, 1980) receptor are all impaired by loss of the N-linked oligosac-

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[1] Abbreviations: Endo H, endoglucosaminidase H; ER, endoplasmic reticulum; LDL, low-density lipoprotein; AcLDL, acetylated low-density lipoprotein; TCA, trichloroacetic acid; BSA, bovine serum albumin; PBS, phosphate-buffered saline; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DMEM, Dulbecco's Eagle's medium; BCIS, bovine calf serum supplemented with iron; BAEC, bovine aortic endothelial cells; ApoB-LDL, apoprotein B component of LDL.

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charides. Since complete less of the carbohydrate component, as occurs with tunicamycin, may alter the solubility, stability, and/or conformation of these membrane proteins, such studies are difficult to interpret in terms of the role of carbohydrate in glycoprotein function. Recent studies have employed other types of inhibitory agents which allow the attachment of the N-linked oligosaccharides but interfere with their subsequent modification by the glycoprotein processing glycosidases of the endoplasmic reticulum (ER) and Golgi (Elbein, 1987). Since these inhibitors still allow glycosylation to occur, one would not expect the same problems with solubility or conformation.

Treatment of cells with castanospermine, a plant alkaloid that inhibits glucosidase I (Saul et al., 1983), results in the production of unprocessed glycoproteins containing $Glc_3$-$Man_{7-9}(GlcNAc)_2$ oligosaccharides (Pan et al., 1983). Previous studies from this laboratory have shown that pretreatment of fibroblasts or vascular smooth muscle cells and endothelial cells with this alkaloid results in a decreased cell surface expression of the LDL receptor. Further, these studies indicated that castanospermine did not alter the rate or extent of synthesis of the receptor, but it did change its distribution within the cell by causing a diminished rate of transport of receptor molecules to the plasmalemma (Edwards et al., 1989).

The present study was designed to examine the influence of castanospermine on the function and expression of the endothelial cell scavenger receptor. These studies have shown that castanospermine strongly affects the receptor-mediated binding of acetylated LDL to the cell surface of endothelial cells, and this inhibition is dependent on the concentration of castanospermine in the medium. The inhibition is not due to a decrease in the synthesis of the receptor molecules, but is caused by a change in their distribution from the plasma membrane to the ER. The majority of receptor molecules synthesized in the presence of castanospermine have Endo H-sensitive oligosaccharides. These studies, and others cited in this article, indicate that the presence of glucose on the oligosaccharide chains, or the inability to remove this sugar, prevents the protein from being transported from the ER to the Golgi and, ultimately, to the plasma membrane.

## 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 using immunodiffusion, immunoelectrophoresis, and chemical composition (Kelley & Kruski, 1982) and was modified by acetylation (acetic anhydride) as described by Basu et al. (1976). This acetylated LDL (AcLDL) was radiolabeled with $^{125}I$ by the McFarlane iodine monochloride method (McFarlane, 1964) as modified by Bilheimer et al. (1972). The iodinated AcLDL was not used unless (1) more than 98% of the $^{125}I$ radioactivity was precipitable by trichloroacetic acid (TCA), (2) less than 5% of the radioactivity was present in the lipid portion as determined by Folch extraction (Folch et al., 1957), and (3) the specific radioactivity of the protein ranged from 100 to 300 dpm/ng of protein. Human lipoprotein-deficient serum (LPDS) was prepared by removal of lipoprotein by ultracentrifugation ($d = 1.21$ g/mL) and subsequent dialysis against PBS as previously described (Edwards et al., 1989). Castanospermine (1,6,7,8-tetrahydroxyoctahydroindolizidine) was isolated from the seeds of the Australian plant, *Castanospermum australe* (Hohenschutz et al., 1981). Deoxymannojirimycin was obtained from Genzyme, Inc. (Boston, MA), and swainsonine was isolated from *Astragalus lentiginosis* (Dorling et al., 1978; Molyneux & James, 1982). [2-$^3H$]-mannose was purchased from American Radiolabeled Chemicals, Inc., and endoglucosaminidase H (Endo H) was obtained from Miles Scientific Co. Pronase was from Calbiochem and Bio-Gel P-4 was from Bio-Rad. Bovine serum albumin (BSA) was obtained from Sigma Chemical Co., *n*-octylglucoside was from Boehringer Mannheim Biochemicals, egg phosphatidylcholine was purchased from Avanti Polar-Lipids, Inc., and cellulose acetate filters (0.45 μm, 25 mm diameter) were from Micro Filtration Systems.

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 medium (DMEM, Gibco Laboratories) containing 10% bovine calf serum supplemented with iron (BCIS) (Hyclone Laboratories, Inc., Logan, UT), and cells were used between passages 1 and 20. Unless otherwise specified, cell cultures were grown for 4 days in the presence or absence of inhibitor.

*Measurement of Surface Binding.* Cells, grown 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. DMEM, containing low bicarbonate (0.35 g/L) in 15 mM HEPES, 10% lipoprotein-deficient serum, and various amounts of [$^{125}I$]AcLDL as indicated below, was then added and the incubations were continued for 2 h at 4 °C. Control plates were incubated with the same concentrations of [$^{125}I$]-AcLDL in the presence of 500–2000 μg/mL unlabeled AcLDL. The binding reaction was terminated by removing the culture medium and rinsing the monolayers at 4 °C four times with 1% BSA in PBS and finally once with PBS alone. After rinsing, 1 mL of PBS was added to each plate and the plates were then transferred to a 37 °C incubator for 30 min to allow the cells to internalize surface-bound AcLDL. To remove any remaining labeled AcLDL nonspecifically bound to the cell surface or intercellular matrix, the cells were removed from the plastic dishes by the addition of 1 mL of 0.25% trypsin containing 0.05% EDTA and incubated for 10 min at 37 °C. Cells 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) and centrifuged (200*g*, 10 min) at 4 °C, and after careful removal of the supernatant, the cells were 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 receptor-mediated [$^{125}I$]AcLDL binding to the cell surface was calculated by subtracting the amount of radioactivity bound in the presence of labeled plus unlabeled AcLDL from the radioactivity bound to cell exposed only to labeled AcLDL. As a basis for normalization of the binding measurements, replicate cultures were carried through each experiment without adding radiolabeled AcLDL for determination of cell numbers using a Coulter Counter Model ZF.

*Measurement of [$^{125}I$]AcLDL Degradation.* BAEC were cultured in the presence or absence of inhibitor for 48 h, rinsed, and preincubated with PBS for 10 min at 37 °C. The cells were then incubated in medium containing 10% lipoprotein-deficient serum and 10 μg/mL [$^{125}I$]AcLDL at 37 °C. After periods ranging from 30 min to 4 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 (Henriksen et al., 1981) to

remove unbound labeled iodide ion. The cells were then rinsed with 1% BSA as above, and the cell pellets were dissolved in 1 mL of 0.1 N NaOH. The lysate plus three 1-mL PBS washes of the culture dishes were collected, pooled, and counted as a measure of cell-associated AcLDL.

*Assay for Total Cellular Scavenger Receptor Binding Activity.* Total cellular LDL receptor activity was measured as described by Schneider et al. (1980). Briefly, BAEC were cultured in the presence or absence of 250 μg/mL castanospermine, as described above. Cells from each treatment group (100-mm culture dishes) 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 6.0), containing 2 mM $CaCl_2$ 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 6.0), containing 2 mM $CaCl_2$, 40 mM *n*-octylglucoside, 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 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, either 10 μL in 0.15 M NaCl or 10 μL of 10 mg/mL unlabeled AcLDL, and 20 μL of [$^{125}$I]AcLDL (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) using cellulose acetate membrane filters, and the associated radioactivity was counted using a Beckman 310 gamma counter. Specific binding was calculated by subtracting the amount of [$^{125}$I]-AcLDL bound in the presence of labeled plus unlabeled AcLDL from that bound in the presence of labeled AcLDL alone. The protein content of the phosphatidylcholine/acetone precipitate was determined as described by Schneider et al. (1980).

*Characterization of Oligosaccharide Structure.* BAEC were plated into T-75 flasks (Corning) in normal growth media, and upon attaining confluence (4–5 days), cells were changed into fresh media in the presence or absence of castanospermine (0–250 μg/mL). In addition, 400 μCi of [2-$^3$H]mannose or [6-$^3$H]galactose was added to each flask, and the cells were then incubated for 48 h at 37 °C. After this incubation, the cells were removed by scraping and were disrupted by freezing and thawing. The homogenate was subjected to ultracentrifugation to obtain the membrane fraction, which was suspended in 0.5% SDS and electrophoresed. The radioactive band, labeled with either [$^3$H]mannose or [$^3$H]galactose, that migrated in the area reported for the scavenger receptor (260 kDa) was cut from the gels, homogenized with buffer, and digested with the proteolytic enzyme, pronase (1 g/100 mL), for 48 h at 37 °C to obtain glycopeptides. These glycopeptides were then isolated by gel filtration on columns (1.5 × 150 cm) of Bio-Gel P-4 (100-200 mesh). The entire glycopeptide peak (Fractions 35–60) was collected, concentrated to dryness, and dissolved in 500 μL of 0.1 M sodium citrate buffer (pH 5.5). Endoglucosaminidase H (Endo H, 2.5 munits in 5 μL of citrate buffer) was then added to the pooled material to digest high-mannose types of structures. After incubation at 37 °C for 24 h under a toluene atmosphere, an additional 2.5 μ of Endo H was added and the digestion was continued for an additional 24 h. These mixtures were then rechromatographed on the same Bio-Gel P-4 column (Elbein et al., 1991). The oligosaccharide peaks resulting from this treatment were pooled and subsequently characterized further by treatment with various exoglycosidases (i.e., jack bean α-mannosidase, glucosidase I, and glucosidase II), and the digestion mixtures were again chromatographed on the Bio-Gel P-4 columns.

*Subcellular Fractionation.* BAEC were cultured for 48 h in the presence or absence of 50 μg/mL castanospermine in DMEM containing 10% BCIS. For each treatment group, twelve 100-mm culture dishes were precooled and rinsed two times with ice-cold PBS at 4 °C. After mechanical detachment from the culture dishes in ice-cold 0.25 M sucrose, the cells 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). Fractions (1 mL) were collected for receptor analysis from the top of the tubes using a Beckman gradient collector.

The protein content of each fraction was measured 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 manner similar to that described for total receptors. Enzyme analyses were performed to confirm the identity of each fraction.

## RESULTS

*Influence of Castanospermine on the N-Linked Oligosaccharide Composition of the Scavenger Receptor.* To determine the effect of castanospermine on the structure of scavenger receptor N-linked oligosaccharides, confluent BAEC were incubated in the presence of [2-$^3$H]mannose for 48 h with or without the addition of 50 μg/mL of castanospermine. After this treatment period, cell membrane fractions were prepared and solubilized with octylglucoside, and membrane proteins were separated by SDS–PAGE electrophoresis. The separated proteins were electrophoretically transferred to nitrocellulose for detection of the scavenger receptor using Western blot analyses. Unlabeled AcLDL was employed as the primary ligand for Western blots, and its binding was detected using a polyclonal antibody to ApoB-LDL followed by a peroxidase conjugated anti-IgG. The area of the nitrocellulose corresponding to scavenger receptor (260 kDa), as evidenced by specific AcLDL binding, was excised and digested with pronase, and the radioactive glycopeptides were isolated on Bio-Gel P-4 columns. To determine the percentage of glycopeptides that contained high-mannose oligosaccharides, the entire glycopeptide peak was pooled, treated with Endo H, and rechromatographed on the Bio-Gel P-4 column.

As illustrated in Figure 1, the normal oligosaccharides associated with the scavenger receptor from control cells (i.e., not treated with castanospermine) emerged from the column in two broad peaks, suggesting that the receptor contains both complex and high-mannose types of oligosaccharide chains.

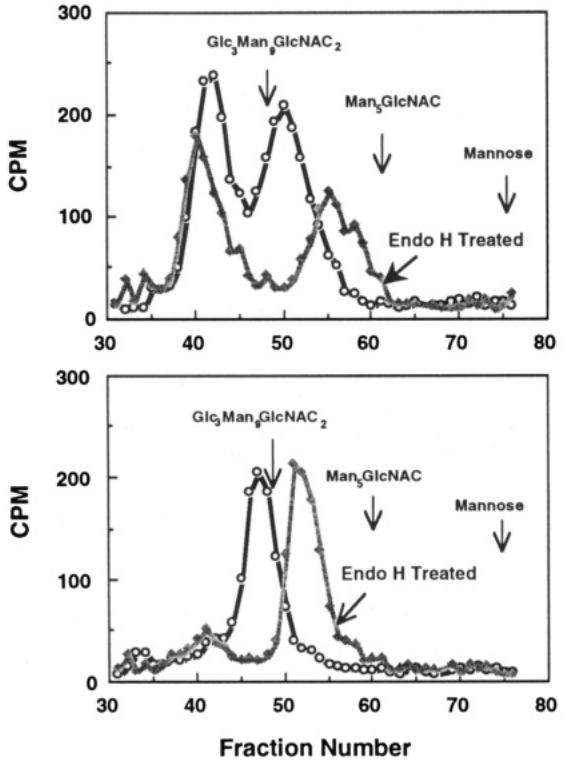


FIGURE 1: Effect of castanospermine on the composition of BAEC scavenger receptor N-linked oligosaccharides. Confluent cultures of BAEC were incubated in the presence of [2-$^3$H]mannose with or without the addition of castanospermine (50 $\mu$g/mL) for 48 h at 37 °C. After incubation, BAEC were scraped from the culture surface and disrupted by freezing and thawing. After ultracentrifugation to obtain a membrane fraction, this fraction was suspended in 5% SDS and electrophoresed. The band corresponding to the scavenger receptor (260 kDa) as detected by radioactivity was cut from the gel, homogenized with buffer, and digested with pronase to obtain glycopeptides which were then isolated on Bio-Gel P-4 columns (○). In addition, the entire glycopeptide peak (fractions 35–60) was pooled, digested with Endo H, and rechromatographed on the Bio-Gel column (●). Aliquots of each fraction were collected for liquid scintillation counting to determine their radioactive content.

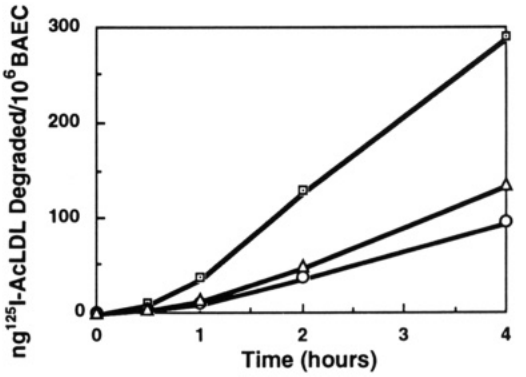


FIGURE 2: Influence of castanospermine and deoxymannojirimycin on receptor-mediated degradation of [$^{125}$I]AcLDL in cultured confluent BAEC. Cells were preincubated with culture medium alone (□) or medium containing either castanospermine (50 $\mu$g/mL, ○) or deoxymannojirimycin (400 $\mu$g/mL, △) for 48 h. After 48 h, the cells were rinsed and incubated with PBS for 10 min at 37 °C. BAEC were then incubated with medium containing 10% lipoprotein-deficient serum and 10 $\mu$g/mL [$^{125}$I]AcLDL in the presence or absence of a 20-fold excess of unlabeled AcLDL for times ranging from 30 min to 4 h at 37 °C. After incubation, the amount of specific receptor-mediated [$^{125}$I]AcLDL degraded was determined as described in Materials and Methods. Nonspecific values made up <10% of the total values for degradation in all treatment groups. Each point represents the mean of duplicated determinations from one of five separate experiments.

That the second peak (centered near elution fraction 50) is really a high-mannose oligosaccharide, probably containing some glucose [i.e., $Glc_{1-3}Man_9(GlcNAc)_2$], is indicated by the fact that this peak emerged near the standard $Glc_3Man_9(GlcNAc)_2$ and also by the fact that its migration was shifted to a smaller-sized oligosaccharide upon treatment with Endo H. In addition, this second peak also became labeled when cells were incubated in [$^3$H]galactose, and this label was released upon incubation with glucosidase II. On the other hand, peak I of the control cells was not susceptible to Endo H, indicating that it is composed of complex oligosaccharides. Since the receptor molecules isolated from normal cells would include those at the cell surface as well as those still being synthesized and processed in internal membranes, it is not surprising to find high-mannose oligosaccharides in the total receptor pool.

In contrast, as shown by the lower profile in Figure 1, the glycopeptides obtained from the scavenger receptor of castanospermine-treated cells eluted from the Bio-Gel P-4 column in a single major peak slightly before the $Glc_3Man_9(GlcNAc)_2$ standard, probably indicating that this peak is a glycopeptide and also contains some amino acids, i.e., $Glc_3Man_9(GlcNAc)_2Asn$. Thus, treatment of this peak with Endo H resulted in a shift in its mobility to a smaller-sized [$^3$H]mannose-labeled oligosaccharide that migrated like standard $Glc_3Man_9GlcNAc$. This oligosaccharide was treated with a partially purified preparation of glucosidase I and glucosidase II (Kaushal et al., 1991), and this treatment resulted in another shift of labeled oligosaccharide to a position corresponding to that of the standard, $Man_9GlcNAc$ (data not shown). In addition, this oligosaccharide could become labeled (but not nearly as well) when cells were incubated in [$^3$H]galactose, and this label was released by treatment with a mixture of glucosidases I and II. The initial oligosaccharide (and glycopeptide) was also susceptible to digestion by jack bean $\alpha$-mannosidase, and the migration of the resulting product indicated the loss of 3–4 hexoses (data not shown). Thus, the major oligosaccharide associated with the scavenger receptor produced in the presence of castanospermine is $Glc_3Man_9(GlcNAc)_2$.

It is possible that, in the presence of castanospermine, a small number of cell surface receptors still contain complex oligosaccharides. However, on the basis of the data presented in Figure 1, these structures could not account for any more than 5% of the total oligosaccharides. Thus, they are not likely to significantly influence the binding quantity or affinity of acetylated LDL.

*Influence of Castanospermine on Scavenger Receptor-Mediated AcLDL Endocytosis.* On the basis of previous observations indicating that the intracellular degradation and release of TCA-soluble AcLDL peptide fragments into the media reflected primarily the receptor-mediated endocytosis of AcLDL, initial studies focused on the effect of castanospermine and other glycoprotein processing inhibitors on the cumulative degradation of [$^{125}$I]AcLDL as a function of time. In these studies, confluent BAEC were pretreated with castanospermine (50 $\mu$g/mL) or deoxymannojirimycin (400 $\mu$g/mL) for 48 h prior to being incubated with [$^{125}$I]AcLDL (20 $\mu$g/mL) for various times up to 4 h. We then measured the amount of TCA-soluble, [$^{125}$I]labeled, degradation products released into the culture medium. As shown in Figure 2, castanospermine pretreatment of BAEC is associated with an approximate 65% inhibition of AcLDL degradation, relative to that of control (i.e., untreated) cells. Similar to castanospermine, deoxymannojirimycin pretreatment also inhibited

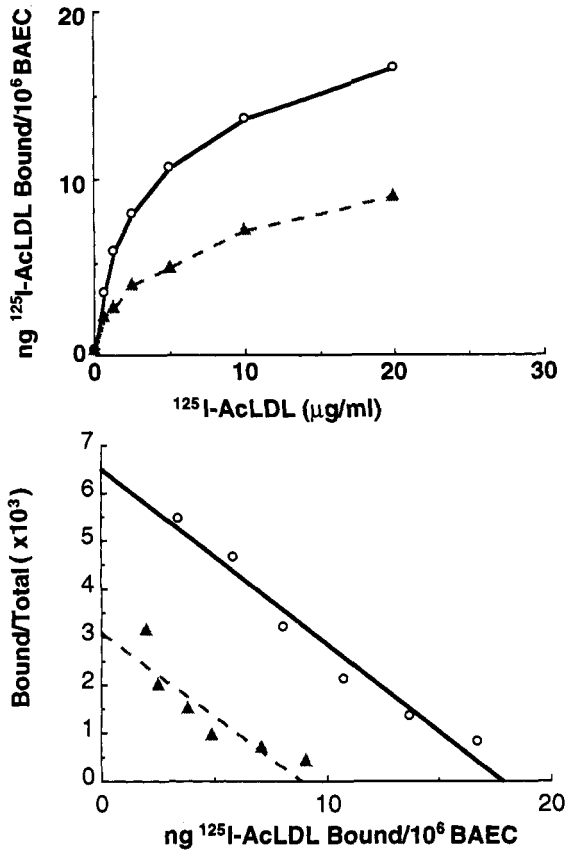


FIGURE 3: Effect of castanospermine pretreatment on specific receptor-mediated binding of [$^{125}$I]AcLDL to BAEC (upper panel) and Scatchard plot (lower panel) of the same data. Confluent BAEC in 35-mm culture dishes were preincubated for 48 h at 37 °C in normal culture medium with or without the addition of 250 μg/mL castanospermine. BAEC were then rinsed, precooled to 4 °C for 30 min, and incubated at 4 °C for 2 h with medium containing increasing concentrations of [$^{125}$I]AcLDL in the presence or absence of a 50-fold excess of unlabeled AcLDL. BAEC were then rinsed thoroughly, warmed to 37 °C for 30 min to allow internalization of ligand, and finally removed by trypsinization and rinsed for radioactive counting as described in Materials and Methods. The specific binding data presented for control (○) and castanospermine-treated (▲) BAEC were obtained by the net difference between total binding and nonspecific binding which did not exceed 10% of the total binding. Each point represents the mean specific binding of duplicate measurements from one of five separate experiments.

[$^{125}$I]AcLDL endocytosis as measured by degradation, although it was somewhat less effective as indicated in Figure 2. Measurements performed in the presence of a 50-fold excess of unlabeled AcLDL indicated that >90% of the degradation was specific or receptor-mediated.

*Effect of Castanospermine on Binding of [$^{125}$I]AcLDL to Cell Surface.* To determine whether this decreased degradation of modified LDL was associated with a corresponding decrease in either the number or the affinity of cell surface scavenger receptors, binding studies were performed at 4 °C. As indicated above, BAEC were preincubated for 48 h (at 37 °C) in the presence or absence of 50 μg/mL castanospermine. After this preincubation, the cells were cooled to 4 °C for 30 min prior to a 2-h incubation at 4 °C with various concentrations of [$^{125}$I]AcLDL, in the presence or absence of a 50-fold excess of unlabeled AcLDL. One of five binding experiments is shown in Figure 3. As demonstrated in the top panel, BAEC pretreated with castanospermine exhibit an almost 50% decrease in specific binding of [$^{125}$I]AcLDL to the cell surface. Scatchard analyses of these binding results (bottom panel) yielded two parallel lines for the control and

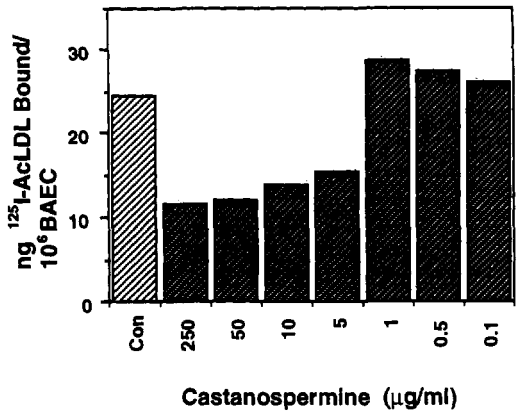


FIGURE 4: Influence of pretreatment of BAEC with increasing castanospermine concentrations on [$^{125}$I]AcLDL receptor-mediated binding performed at 4 °C. BAEC were preincubated for 48 h in the absence (Con) or presence of increasing concentrations of castanospermine. Cells were then rinsed and precooled to 4 °C, and binding studies were performed in medium containing 10 μg/mL [$^{125}$I]AcLDL in the presence or absence of excess unlabeled AcLDL. After 2 h at 4 °C, specific receptor-mediated binding was performed as described in the legend for Figure 3 and Materials and Methods. Each bar represents the mean specific binding of duplicate determinations from one of three separate experiments.

the castanospermine-treated BAEC. These results indicate that the decrease in receptor-mediated [$^{125}$I]AcLDL binding is related to a significant decrease ($p < 0.01$, $n = 5$) in the number of available and specific endothelial cell surface binding sites. However, this analysis also indicates that receptors synthesized in the presence of castanospermine show no decrease in affinity for their ligand ($K_d = 1.2 \times 10^{-8}$ M).

To determine the optimum concentration of the alkaloid necessary to alter receptor binding of AcLDL to endothelial cells, the cells were incubated with various amounts of castanospermine ranging from 0.1 to 250 μg/mL for 48 h, and the subsequent ability of the cells to bind [$^{125}$I]AcLDL was determined. The results of these experiments are shown in Figure 4. It can be seen that, when castanospermine was added to cells at levels of up to 1 μg/mL, it did not inhibit AcLDL binding and, in fact, may have caused a slight stimulation in binding. This could be due to an increase in the rate of transport of the glycoprotein if one or a few of the oligosaccharide chains are not processed while most of the chains still do undergo processing. At this stage, we cannot account for this increase in binding at low castanospermine concentrations. However, the data do indicate that, at concentrations of alkaloid of 5 μg/mL or higher, there is a substantial inhibition in specific binding, and this inhibition increases as the amount of castanospermine is increased. Thus, at the highest concentration tested (i.e., 250 μg/mL) there is a 52% inhibition in AcLDL binding.

Previous studies from this laboratory have demonstrated a differential influence of several glycoprotein processing inhibitors on cell surface LDL receptor expression in several cell types, including BAEC (Edwards et al., 1989). Figure 5 shows the results of an experiment comparing the effect of various processing inhibitors on the specific binding of [$^{125}$I]-AcLDL to endothelial cells at 4 °C. The results are similar to previous studies on the endocytosis of LDL and demonstrate that swainsonine has no significant effect on [$^{125}$I]AcLDL binding when cells are preincubated in 100 μg/mL of this alkaloid for 48 h. However, previous studies did show that this amount of swainsonine completely inhibits mannosidase II and leads to the formation of glycoproteins with hybrid types of oligosaccharides. Similar to the degradation studies

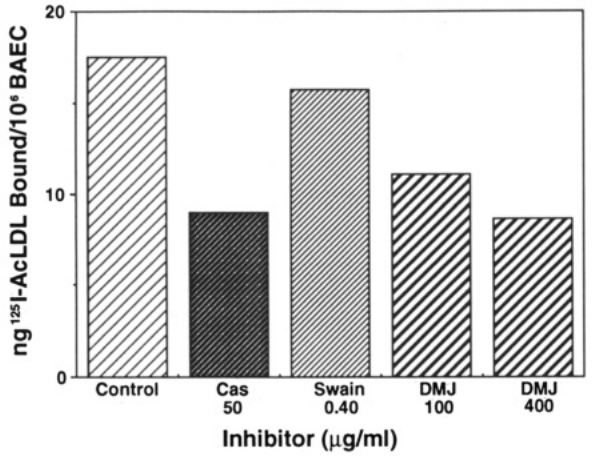


FIGURE 5: Relative influence of different glycoprotein processing inhibitor pretreatment of BAEC on receptor-mediated [$^{125}$I]AcLDL binding. Confluent BAEC were preincubated for 48 h at 37 °C with designated concentrations of castanospermine (Cas), swainsonine (swain), or deoxymannojirimycin (DMJ). After preincubation, cells were rinsed, precooled to 4 °C, and incubated at 4 °C for 2 h with 10 μg/mL [$^{125}$I]AcLDL in the presence or absence of a 50-fold excess of unlabeled AcLDL. Binding studies were performed as described in the legend for Figure 3 and Materials and Methods. Each bar represents the mean specific binding of duplicate determinations from one of four separate experiments.

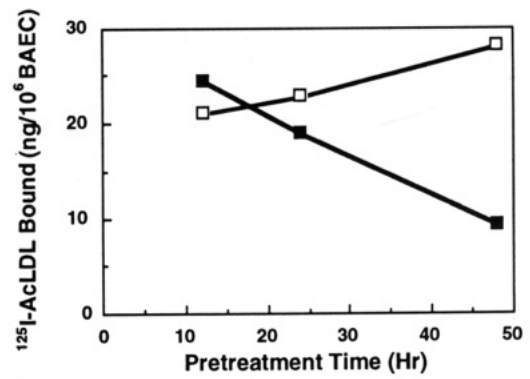


FIGURE 6: Determination of the castanospermine pretreatment time required to inhibit receptor-mediated binding of [$^{125}$I]AcLDL. Confluent BAEC were preincubated with culture medium containing 50 μg/mL castanospermine (■) or culture medium alone (□) for 12, 24, or 48 h at 37 °C, and BAEC were then rinsed, precooled to 4 °C, and incubated with 10 μg/mL [$^{125}$I]AcLDL in the presence or absence of a 50-fold excess of unlabeled LDL prior to measurement of specific binding as described in Figure 3 and Materials and Methods. Each point represents the mean specific binding of duplicate determinations from one of three separate experiments.

described above, deoxymannojirimycin also inhibited the binding of AcLDL to the cells, although it also showed a dose-dependent inhibition. In a recent report, we demonstrated that pretreatment of BAEC with kifunensine, another inhibitor of mannosidase I, resulted in a decrease in receptor-mediated endocytosis of AcLDL, which is also due to a decrease in scavenger receptor sites at the cell surface (Elbein et al., 1991).

Since the above studies were all performed by incubating cells for 48 h in the presence of inhibitor, it was of interest to determine how long the cells had to be incubated with inhibitor to affect the scavenger receptor function. Such studies should give information about the half-life or time of cell surface turnover of this receptor. To address this question, two sets of experiments were performed. In one experiment, BAEC were preincubated either with castanospermine (50 μg/mL) or in normal (inhibitor-free) culture medium for 12, 24, and 48 h. The ability of these cells to bind [$^{125}$I]AcLDL was then tested. As shown in Figure 6, significant inhibition of specific [$^{125}$I]AcLDL binding was not achieved until cells had been in castanospermine for 48 h, when an almost 65% inhibition in binding was observed. Although these results suggested that the inhibitory effects of castanospermine were not likely to be due to the inhibitor directly competing with AcLDL for specific cell surface binding sites, we felt it necessary to perform another competition study where castanospermine was added at various concentrations immediately before the addition of [$^{125}$I]AcLDL to determine whether it would affect specific binding. In these experiments, castanospermine, regardless of the concentration used, failed to compete with [$^{125}$I]AcLDL for cell surface receptor sites, even though the inhibitor was added immediately prior to addition of the labeled lipoprotein. On the other hand, unlabeled AcLDL did yield the expected competition (~90%) with labeled AcLDL under similar conditions.

Although the above data clearly demonstrate that castanospermine treatment is associated with a decrease in cell surface receptors for modified LDL, these data do not address the cellular mechanisms involved in this inhibitory effect. For this reason, studies were performed to examine the effect of the inhibitor on the total number of scavenger receptors within the cell and, further, to attempt to determine the cellular compartmentalization of those receptors. Using the receptor solubilization assay described by Schneider et al. (1980), these experiments indicated that castanospermine (50 μg/mL for 48 h) influenced the number of AcLDL receptors at the cell surface, but did not affect the total number of AcLDL receptors in the cells. To further define the cellular localization of these modified LDL receptors, control and castanospermine-treated BAEC were gently homogenized, and the homogenate was subjected to subcellular fractionation using a Percoll density gradient. Binding of AcLDL by each fraction was then determined using the solubilization assay to measure receptor activity, and various enzyme assays were performed to verify the identity of each cellular fraction. Figure 7 shows that there was a much lower number of AcLDL receptors in the plasmalemma fraction (fraction 6, identified by 5′-nucleotidase activity) of castanospermine-treated cells, as compared to normal cells. In contrast, castanospermine-treated cells showed a large relative increase in the specific AcLDL binding within both the cellular fractions corresponding to the endoplasmic reticulum–Golgi (fraction 9, identified by glucosidase I and mannosidase I activity) and the lysosomes (fraction 11, identified by β-*N*-acetylglucosaminidase activity) as compared to untreated cells. Thus, these data indicate that castanospermine treatment alters the distribution of AcLDL receptors but does not affect the total number of receptors within endothelial cells.

It seems likely, on the basis of earlier studies by Lodish and Kong (1984) and others (Elbein, 1987), that the transport of receptors from the ER to the cell surface is inhibited or slowed when the oligosaccharide portion of the receptor molecule contains glucose.

## DISCUSSION

The results of these studies indicate that pretretament of BAEC for at least 48 h results in a significant reduction in both specific receptor-mediated binding (4 °C) and degradation (37 °C) of AcLDL. Moreover, these results indicate that the decrease in specific AcLDL endocytosis observed in these studies is related to a reduction in available cell surface receptors for the modified LDL. The data indicate that, while castanospermine does not affect the synthesis or turnover of

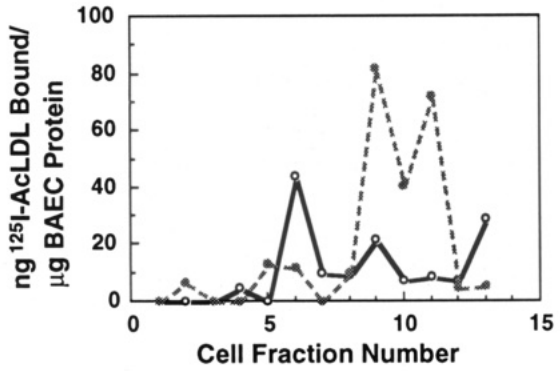


FIGURE 7: Specific scavenger receptor-mediated binding activity associated with different subcellular fractions prepared from untreated (○) and castanospermine-treated (●) BAEC. Confluent BAEC were pretreated with 50 μg/mL castanospermine for 48 h at 37 °C, rinsed, and precooled to 4 °C. After rinsing, the cells were mechanically detached from the surface using ice-cold 0.25 M sucrose and homogenized, and the subcellular fractions were separated on a Percoll gradient. Samples were centrifuged opposite density marker beads, and enzyme analyses were performed to confirm the identity of each subcellular fraction. The peak 5′-nucleotidase activity was located in cell fraction 6 (plasmalemma), the peak glucosidase I and mannosidase I activity was associated with cell fraction 9 (endoplasmic reticulum–Golgi), and the peak β-*N*-acetylglucosaminidase activity was associated with cell fraction 11 (lysosomes). Specific scavenger receptor-mediated binding of [$^{125}$I]AcLDL of each 1-mL fraction containing measurable protein was assayed as described for total cellular receptor binding activity in Materials and Methods. Each point represents the mean specific binding of duplicate determinations from one of two separate experiments.

the scavenger receptor, it does alter the distribution of receptor molecules within the cell. Thus, when endothelial cells were incubated for 48 h in the presence of this drug, there was a great decrease in the number of receptor molecules in the plasma membrane and a considerable increase in receptors in internal cellular compartments. Although we were not able to separate the ER from the Golgi membranes in this experiment, the data clearly demonstrate a large number of receptors in the cell fractions corresponding to these compartments. Thus, the presence of glucose in the receptor oligosaccharides apparently slows the rate of transport of these molecules through internal cell membranes. Such results are similar to those of Lodish and Kong (1984) and others (Elbein, 1987) and suggest that many receptors are actually in the ER. However, immunocytochemical studies will likely be required to definitely prove the site of receptor localization.

Castanospermine is a potent inhibitor of glucosidases including the N-linked glycoprotein processing enzyme, glucosidase I (Pan et al., 1983). In the presence of this drug, N-linked oligosaccharides are not processed to complex chains and remain as glucose-containing high-mannose structures, i.e., $Glc_3Man_9(GlcNAc)_2$. Such has been shown to be the case for the AcLDL receptor, as demonstrated in the current study.

Thus, in Hep G-2 cells grown in the presence of deoxymannojirimycin, the rate of secretion of $\alpha_1$-antitrypsin decreased significantly, but only marginal effects were observed on other liver glycoproteins such as ceruloplasmin or the C3 component of complement or on non-glycoproteins such as albumin (Lodish & Kong, 1984). When castanospermine was used with cultured IM-9 lymphocytes to determine the effect of alteration of carbohydrate structure on the function of the insulin receptor, these cells showed a 50% decrease in cell surface insulin receptors as measured by the binding of radiolabeled insulin or other measurements (Arakaki et al., 1987). The presence of glucose on the α-subunit of the sodium channel of rat brain neurons appeared to prevent the incorporation of most of the sialic acid into the molecule, and also inhibited sulfation but did not affect palmitylation. Interestingly enough, although castanospermine caused extensive changes in the structure of these glycoproteins, it did not prevent the covalent assembly of α- and $\beta_2$-subunits nor the transfer of $\alpha\beta_2$ complexes to the cell surface. Furthermore, the sodium channels synthesized under these conditions still showed normal affinity for binding saxitoxin, indicating that they functioned normally (Schmidt & Catkerall, 1987).

It is not clear how the presence of glucose could affect the rate of transport of the scavenger receptor from the ER to the Golgi, but understanding this step could provide valuable information about protein targeting. One possible explanation could be that there is a glucose receptor in the ER and that this receptor binds and retains glucose-containing glycoproteins. Thus, N-linked glycoproteins synthesized in the presence of deoxymannojirimycin or castanospermine would be bound by this hypothetical receptor. The problem with this hypothesis is that the secretion of some hepatocyte N-linked glycoproteins is slowed down by these inhibitors whereas that of other proteins is not. Thus, a more likely explanation is that the removal of glucose from some proteins such as the scavenger receptor alters the conformation of the protein to one that is recognized by a "transport protein", possibly a chaperone-like molecule. Other proteins may be in the appropriate conformation even with the glucose, and therefore, the rate of transport is not changed by the removal of glucose. Thus, studies with these and other specific inhibitors may provide clues as to the mechanism of transport for this receptor important in the binding and endocytosis of modified LDL.

In conclusion, our results indicate that intracellular processing of the endothelial cell scavenger receptor, at least to the stage of glucose removal, is critical to the rate of transport through the ER–Golgi complex to the cell surface. This knowledge of the role that the carbohydrate moiety of the scavenger receptor may play in governing the kinetics of cell surface expression has potential important implications for possible regulation of this receptor.

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