Defective nitric oxide-dependent, deaminative cleavage of glypican-1 heparan sulfate in Niemann–Pick C1 fibroblasts

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Exit of recycling cholesterol from late endosomes is defective in Niemann–Pick C1 (NPC1) and Niemann–Pick C2 (NPC2) diseases. The traffic route of the recycling proteoglycan glypican-1 (Gpc-1) may also involve late endosomes and could thus be affected in these diseases. During recycling through intracellular compartments, the heparan sulfate (HS) side chains of Gpc-1 are deaminatively degraded by nitric oxide (NO) derived from preformed S-nitroso groups in the core protein. We have now investigated whether this NO-dependent Gpc-1 autoprocessing is active in fibroblasts from NPC1 disease. The results showed that Gpc-1 autoprocessing was defective in these cells and, furthermore, greatly depressed in normal fibroblasts treated with U18666A (3-[2-(diethylamino)ethoxy]androst-5-en-17-one), a compound widely used to induce cholesterol accumulation. In both cases, autoprocessing was partially restored by treatment with ascorbate which induced NO release, resulting in deaminative cleavage of HS. However, when NO-dependent Gpc-1 autoprocessing is depressed and heparanase-catalyzed degradation of HS remains active, a truncated Gpc-1 with shorter HS chains would prevail, resulting in fewer NO-sensitive sites/proteoglycan. Therefore, addition of ascorbate to cells with depressed autoprocessing resulted in nitration of tyrosines. Nitration was diminished when heparanase was inhibited with suramin or when Gpc-1 expression was silenced by RNAi. Gpc-1 misprocessing in NPC1 cells could thus contribute to neurodegeneration mediated by reactive nitrogen species.

Key words: cholesterol/glypican-1/heparan sulfate/Niemann–Pick C/nitric oxide

Introduction

Niemann–Pick type C (NPC) is an autosomal, recessive neurovisceral lipid storage disease resulting in fatal neurodegeneration. At the cellular level, the disorder is characterized by the accumulation of cholesterol and glycosphingolipids in late endosomes and lysosomes. Approximately 95% of the patients have mutations in the NPC1 gene that encodes a large membrane glycoprotein primarily located to late endosomes. The remainder have mutations in the NPC2 gene that encodes a small, soluble, endosomal/lysosomal cholesterol-binding glycoprotein. Despite extensive investigations conducted in various animal models, the precise functions of NPC1 and NPC2 at the molecular level, as well as the pathophysiological effects of their dysfunction, have remained elusive (for reviews, see Vanier and Millat, 2003; Liscum and Sturley, 2004; Mukherjee and Maxfield, 2004; Chang et al., 2005; Ioannou, 2005).

Cholesterol is delivered to cells by uptake of low-density lipoproteins via the low-density lipoprotein receptor and released in lysosomes (Brown and Goldstein, 1986). Exogenously as well as endogenously synthesized cholesterol, together with sphingolipids and glycosphingolipidinositol (GPI)-anchored proteins, is enriched in plasma membrane microdomains called lipid rafts and in the raft-derived, caveolin-coated membrane invaginations called caveolae. Clustering of lipid raft components can trigger caveolaemediated endocytosis, whereby cholesterol-, sphingolipid-, and GPI-protein-enriched vesicles move along microtubules to caveosomes. Caveolin-free vesicles can exit from caveosomes and reach late endosomes and then return to the cell surface via the Golgi (for reviews, see Nichols, 2003; Parriton and Richards, 2003; Pelkmans and Helenius, 2003; Soccio and Breslow, 2004). The sorting of GPI-linked, cell-surface proteins to recycling endosomes appears extremely sensitive to the extent of clustering (Sharma et al., 2004).

The GPI-anchored, heparan sulfate (HS)-substituted and recycling proteoglycan glypican-1 (Gpc-1) is abundant in the brain and potentially self-associating (for review, see Fransson et al., 2004). Gpc-1 localizes to lipid rafts (Watanabe et al., 2004) and is internalized via vesicles that contain caveolin-1 and react with cholera toxin B (Cheng et al., 2002). During recycling via endosomes and the Golgi, Gpc-1 undergoes modifications of both the core protein and the HS chains as well as degradations of the HS chains (Figure 1). Removal of the N-substituent on some of the glucosamine residues in HS, generating GlcNH$_3^+$, and S-nitrosoylation (SNO) of conserved cysteines in the core protein by endogenously formed nitric oxide (NO) in a copper-dependent redox reaction (Figure 1B and C) initiates this processing (Mani et al., 2000; Ding et al., 2002). Later, presumably in late endosomes (Fivaz et al., 2002), NO is released from SNO and probably converted to nitrosoy (HNO) completing the copper redox cycle (Figure 1D). HNO then catalyzes deaminative cleavage at the GlcNH$_3^+$ residues of HS in Gpc-1 generating HS oligosaccharides with reducing terminal anhydromannose (anMan) residues. These oligosaccharides eventually separate from the truncated Gpc-1. Some of the oligosaccharides are transported...
HNO-catalyzed, copper-dependent deaminative cleavage of HS at the unknown endogenous reducing agent triggers a nonenzymatic, possibly NO- and copper-dependent processing of recycling Gpc-1 (Fivaz 2005), possibly because cholesterol does not interact with internal vesicles.

When cells with arrested deaminative autoprocessing of Gpc-1 receive ascorbate, there is formation of nitrotyrosine. This can be diminished by inhibition of heparanase with suramin.

Results

Defective or depressed NO-dependent, deaminative Gpc-1 HS autoprocessing in NPC1 fibroblasts and U18666A-treated normal fibroblasts

When Gpc-1 recycles through cells, it is processed by enzymatic (heparanase) as well as nonenzymatic (NO-catalyzed) de-glycinations. These constitute two competing degradative processes (Figure 2). Whereas heparanase is expected to degrade HS on both SNO-free and SNO-containing Gpc-1, deaminative de-glycination can only take place on Gpc-1-SNO (Fransson et al., 2004). As shown in Figure 3A and B, deaminative processing of Gpc-1 was active in growing human fetal lung fibroblasts (HFL-1) as indicated by the formation of anMan-containing HS oligosaccharides (Figure 3B), which was determined by an mAb raised against deaminative degradation products of heparin (Pejler et al., 1988). In contrast, formation of such oligosaccharides in growing NPC1 fibroblasts was reduced to ~10% of the level in normal fibroblasts (cf. Figure 3B and D, insets), although the expression of Gpc-1 appeared unaffected (cf. Figure 3A and C).

NPC1 cells might fail to generate high amounts of anMan-containing HS oligosaccharides either because there are few NO-sensitive sites in Gpc-1 HS or because there is defective NO release from SNO groups in Gpc-1 or because Gpc-1 is poorly S-nitrosylated. Exogenous addition of ascorbate to cells can trigger deaminative cleavage of HS in SNO-containing Gpc-1 (Fransson et al., 2004). Moreover, by inhibition of heparanase degradation of HS by suramin, which does not interfere with NO-dependent degradation (Cheng et al., 2002), more Gpc-1 HS will be available for deaminative cleavage (Figure 2). Accordingly, when NPC1 fibroblasts were exposed to ascorbate, formation of anMan-containing HS degradation products increased (Figure 3E and F), especially when heparanase was inhibited by suramin (Figure 3F). Then, the amount of oligosaccharides generated by ascorbate increased 3-fold compared with untreated cells (cf. Figure 3D and F, insets) and reached ~50% of the level in normal fibroblasts (cf. Figure 3B and F, insets).

![Fig. 1. Schematic model of cholesterol and Gpc-1 trafficking via an endosomal recycling route.](image-url)
The cationic amphiphile U18666A causes arrest of cholesterol recycling, yielding a cellular phenotype similar to that of NPC cells. We therefore examined whether NO-dependent Gpc-1 autoprocessing could be abrogated by treating normal fibroblasts with U18666A. Although treatment with 3 μg/mL of U18666A for 8 h was sufficient to cause cholesterol accumulation in HFL-1 cells as determined by staining with filipin (result not shown), inhibition of HS degradation required treatment with 10 μg/mL of U18666A for 16 h (Figure 3H), presumably because growing fibroblasts have an exceptionally active NO-dependent HS degradation (Figure 3B; Cappai et al., 2005). Oxidation of Cu(I) to Cu(II) may be required to generate HNO from NO released from SNO or to provide electrons to trigger a direct reaction between –SNO and GlcNH₃⁺. Heparanase catalyzes hydrolytic endo-β-glucuronidic cleavage of HS in both SNO-containing and SNO-free Gpc-1 (bottom left). This process is suppressed by suramin. In the event of extensive enzymatic de-glycanation of Gpc-1 before deaminative cleavage (bottom right), tyrosine nitration (NO₂) may take place.

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Fig. 2. The competing enzymatic and nonenzymatic HS degradations. When SNO-containing Gpc-1 (top left) is exposed to a reducing agent, either an endogenous electron donor or an exogenously supplied ascorbate, Gpc-1-bound HS (thick bars) is deaminatively cleaved at the GlcNH₃⁺ units, generating HS fragments (top right). The exact reaction mechanism has not been fully elucidated. However, S-nitrosylation of Gpc-1 is inhibited by Cu(II) chelators, and the ascorbate-induced deaminative cleavage at the GlcNH₃⁺ resides in HS of Gpc-1 is inhibited by Cu(I) chelators indicating that a copper redox cycle is involved (Ding et al., 2002). The reactive NO species may be HNO or there may be a direct reaction between the dipole-ion pair >Cys-S-N=O...H₂N-Glc<, leading to the formation of >Cys-S + H₂O²⁺ + N≡N-Glc< and then sugar ring contraction and cleavage of the glucosaminidic bond (Horton and Philips, 1973). Oxidation of Cu(I) to Cu(II) may be required to generate HNO from NO released from SNO or to provide electrons to trigger a direct reaction between –SNO and GlcNH₃⁺. Heparanase catalyzes hydrolytic endo-β-glucuronidic cleavage of HS in both SNO-containing and SNO-free Gpc-1 (bottom left). This process is suppressed by suramin. In the event of extensive enzymatic de-glycanation of Gpc-1 before deaminative cleavage (bottom right), tyrosine nitration (NO₂) may take place.

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Further experiments showed that the cationic amphiphile imipramine (80 μM for 18 h) did not inhibit formation of anMan-containing degradation products, although it generated accumulation of cholesterol (results not shown). Estrone (3 μg/mL for 16 h), which is not cationic but has a C-17 keto group similar to that of U18666A, caused neither...
cholesterol accumulation nor inhibition of NO-dependent HS degradation (results not shown). Depletion of total cellular cholesterol by treatment with 10 mM methyl-β-cyclo-dextrin for 2 h did not affect the deaminative HS degradation (result not shown).

We then tested whether inhibition of deaminative HS degradation by U18666A in a neuronal cell-line could be overcome by exogenous addition of ascorbate.

**Ascorbate restores NO-dependent Gpc-1 autoprocessing in U18666A-treated N2a cells**

N2a neuroblastoma cells generated constitutively anMan-containing HS oligosaccharides. The staining appeared as a punctate pattern in the periphery of the cytoplasm (Figure 4A and E). Staining for such oligosaccharides was reduced by −40% after treatment with 3 μg/mL of U18666A (Figure 4B and F). Subsequent treatment with ascorbate restored oligosaccharide formation in some of the cells (Figure 4C and G). If heparanase degradation of HS is inhibited by suramin, more HS should be available for NO-dependent deaminative cleavage (Figure 2). Hence, treatment with both suramin and U18666A, followed by triggering of deaminative cleavage by ascorbate, increased the level of anMan-containing HS oligosaccharides in all of the cells, by −2.5-fold compared with U18666A-treated cells and by −1.5-fold compared with untreated cells (cf. Figure 4H with Figure 4F and Figure 4H with Figure 4E, respectively). These results indicated that heparanase-catalyzed degradation of HS was unaffected by U18666A. The oligosaccharide staining generated by exposure to ascorbate also assumed a punctate pattern but was more widely distributed in the cytoplasm.

**Nitration of tyrosine upon exposure to U18666A and ascorbate**

When deaminative cleavage of HS in Gpc-1 is depressed, as in NPC1 fibroblasts and in U18666A-treated cells, Gpc-1 continues to be de-glycanated by heparanase. Hence, an SNO-rich Gpc-1 with truncated HS chains should be obtained (Figure 2, bottom left). When this Gpc-1 is subsequently exposed to a reducing agent, such as ascorbate, the deaminative cleavage process should be initiated. However, the capacity to deaminate may exceed the number of remaining cleavage sites in the HS stubs (Figure 2, bottom right). NO radicals, generated in excess, can then react with molecular oxygen/superoxide anion, forming peroxynitrite (ONOO⁻) or nitrogen dioxide radical (·NO₂), both of which can lead to nitration of tyrosine residues in proteins (Ischiropoulos, 2003). As this is believed to contribute to neurodegeneration, we tested whether nitrotyrosine could be detected in N2a cells after the arrest of NO-dependent HS degradation followed by restoration using ascorbate.

N2a cells that had been treated with 1 mM ascorbate for 1 h displayed no significant increase in nitrotyrosine formation over the low level detected in untreated cells (Figure 5A). Treatment with U18666A to inhibit NO-dependent Gpc-1 processing followed by treatment with ascorbate for 10 min did not significantly affect the nitrotyrosine content that remained undetectable by confocal microscopy (Figure 5A and B). After treatment of U18666A pre-exposed cells with Fig. 4. Restoration of NO-dependent deaminative Gpc-1 autoprocessing in U18666A-treated N2a cells by ascorbate. The confocal laser immunofluorescence microscopy images (A–D) were obtained after staining for anMan-containing oligosaccharides (AM). Quantification of anMan-containing HS degradation products was obtained by flow cytometry (E–H). Cells were either left untreated (A, E) or treated with 3 μg/mL of U18666A for 16 h (B, F) or treated with U18666A for 16 h and then with 1 mM ascorbate for 1 h (C, G) or treated with 0.2 mM suramin for 8 h, then with both suramin and U18666A for 16 h and then with ascorbate for 1 h (D, H). Results of flow cytometry measurements were obtained after staining for anMan-containing oligosaccharides: ***p < 0.001; *p < 0.1. Cells were fixed and permeabilized in acetone/H₂O₂/methanol for confocal microscopy and in 2% (w/v) paraformaldehyde, 0.1% (w/v) Triton X-100 for flow cytometry. Scale bar, 20 μm.
Ascorbate for 1 h, there was a 4- to 5-fold increase in nitrotyrosine formation, which was also easily detectable by confocal microscopy (Figure 5C). The nitrotyrosine staining was diffuse but mostly colocalizing with Gpc-1 (Figure 5C, yellow in inset). Pretreatment with suramin to inhibit heparanase reduced nitrotyrosine formation by almost 50% (Figure 5D).

As NO-dependent HS degradation was defective in NPC1 fibroblasts, we also examined nitrotyrosine formation in these cells and in normal fibroblasts after exposure to ascorbate. In normal fibroblasts (HFL-1), a 1-h ascorbate treatment generated insignificant amounts of nitrotyrosine staining (Figure 5E). However, in untreated NPC1 fibroblasts, there was a small but significantly higher level of nitrotyrosine formation generating a weak punctate staining around the cell surface and in the cytoplasm (Figure 5F). When NPC1 fibroblasts were exposed to ascorbate for 1 h, there was an almost 3-fold increase in nitrotyrosine content and an increased punctate staining colocalizing with Gpc-1 (Figure 5G). Pretreatment with suramin to inhibit heparanase reduced the ascorbate-generated nitrotyrosine formation by almost 50% (Figure 5H).

Fig. 5. Visualization and determination of nitrotyrosine in N2a cells (A–D), in HFL-1 fibroblasts (E), and in NPC1 fibroblasts (F–H) after the treatments indicated in the panels. The confocal laser immunofluorescence microscopy images were obtained after staining for nitrotyrosine throughout and in (C, G) also for Gpc-1 (GPC). Corresponding flow cytometry measurements were made after staining for nitrotyrosine and are shown to the right of the corresponding confocal images. Deaminative cleavage of HS was induced with 1 mM L-ascorbate for 10 min or 1 h, as indicated in the panels. Inhibition of cholesterol transport and NO-dependent processing was obtained by treatment with 3 μg/mL of U18666A for 16 h. Inhibition of heparanase was carried out by exposing cells to 0.2 mM suramin for 24 h. Cells were fixed and permeabilized with 2% (w/v) paraformaldehyde, 0.1% (v/v) Triton X-100 (A–D), or acetone/H2O2/methanol (E–H). Scale bar, 20 μm; NO2-Y, nitrotyrosine; +/-, with or without treatment; vertical connecting lines indicate comparisons; ***p < 0.001.
To show that Gpc-1 was involved in the generation of nitrotyrosine, Gpc-1 expression was silenced using RNAi. The expression level was reduced to ~30% of normal, as estimated by SDS–PAGE and western blotting. When NPC1 fibroblasts were mock-transfected with a vector encoding a scrambled sequence, both Gpc-1 and nitrotyrosine could be detected after treatment with ascorbate (Figure 6A and B), but when the same cells were transfected with a vector expressing human Gpc-1-specific short-interfering RNA (siRNA), expression of Gpc-1 and formation of ascorbate-induced nitrotyrosine were both reduced (Figure 6C and D).

Discussion

During intracellular recycling, Gpc-1 is processed via two independent de-glycanation steps, heparanase-catalyzed endoglycosicid and NO-dependent, deaminative HS degradation, respectively (Figure 2). The deaminative degradation requires prior S-nitrosylation of the Gpc-1 core protein and generation of GlcNH$_3^+$ residues in the HS chains. To initiate deaminative cleavage of HS, the participation of a reducing agent, probably located to late endosomes, is required. Exogenously supplied ascorbate can be taken up by fibroblasts via the sodium ascorbate symport (Corpe et al., 2005) and trigger deaminative cleavage when the endogenous mechanism fails (Fransson et al., 2004).

The present results show that constitutive, NO-dependent, deaminative HS degradation is defective in cells with arrested cholesterol recycling, that is, NPC1 fibroblasts and non-NPC cell lines treated with the cationic amphiphile U18666A. However, the heparanase-catalyzed HS degradation is not affected. Deaminative HS degradation is partially restored by ascorbate in both NPC1 fibroblasts and U18666A-treated non-NPC cells and further augmented when heparanase is simultaneously inhibited by suramin. These findings suggest that Gpc-1 and cholesterol have a common traffic route.

Cholesterol trafficking via multivesicular endosomes is regulated by the NPC1 and NPC2 proteins. It is not clear how NPC1 and NPC2 work in concert to transport cholesterol (Ioannou, 2005). Therefore, the disease pathogenesis remains obscure. One possibility is that a functional NPC1 protein is required for Rab9-mediated vesicular transport from late endosomes to the trans-Golgi network (Seachrist and Ferguson, 2003). Another possibility is that NPC1 is a lipid flippase that collaborates with the obligatory cholesterol transporter NPC2 (Ioannou, 2005). This collaboration could generate the lipid asymmetry required to induce proper membrane curvature. Thereby, fusion of cholesterol- and GPI-anchored protein-containing internal vesicles with the delimiting membrane of late endosomes and the subsequent generation of transport vesicles may be facilitated (Figure 1D). In addition, when NPC1 or NPC2 is defective, cholesterol is not oxidized to 25-hydroxycholesterol (Frolov et al., 2003; Zhang et al., 2004). If GPI-anchored Gpc-1 is transported by the same mechanism, this may explain why the redox-dependent deaminative processing of Gpc-1-HS is also affected in NPC1 fibroblasts.

U18666A as well as several other cationic amphiphiles is known to interfere with vesicular cholesterol traffic between late endosomes and the Golgi and thereby mimic the cholesterol traffic block and the lack of oxysterol formation characteristic of the NPC diseases. However, inhibition of NO-dependent deaminative processing of Gpc-1-HS in normal fibroblasts required higher concentrations of U18666A than was required to induce cholesterol accumulation. U18666A has a reducible keto group at C-17 that could participate in redox reactions and thereby consume the endogenous reducing power needed to initiate deaminative processing of Gpc-1. Estrone, which also has a C-17 keto group but no cationic group, interferes neither with deaminative HS degradation nor with cholesterol trafficking. Therefore, the cationic head group of U18666A should be important for its localization to late endosomes. Imipramine, which also has a cationic head group and causes cholesterol accumulation, has no reducible group and does not affect deaminative HS degradation.

When NO-dependent, deaminative autoprocessing of Gpc-1 is depressed, as in NPC1 fibroblasts and U18666A-treated normal cells, exogenously supplied ascorbate can partially restore NO-dependent Gpc-1 autoprocessing. The anMan-containing HS oligosaccharides, generated both constitutively and after exposure to ascorbate, appeared to be present largely in vesicular structures, possibly endosomes, of N2a cells. Constitutive formation of oligosaccharides may be restricted to a subset of endosomes. Studies are in progress to determine the precise localization of the HS oligosaccharides.

Unfortunately, restoration of deaminative HS degradation by ascorbate leads to tyrosine nitration. The mechanism...
appears to be as follows. When deaminative cleavage of HS is temporarily inhibited or defective, while the heparanase-catalyzed de-glycanation of Gpc-1 proceeds undisturbed, the HS chains on Gpc-1 should become truncated while the core protein should remain fully S-nitrosylated (Figure 2). Moreover, many of the NO-sensitive GlcN\(\text{H}_2\)\(\text{O}\) units in HS are situated in the HS oligosaccharides that are liberated by heparanase. These segregate from the SNO-containing and HS-truncated Gpc-1 during recycling and will not be cleaved by NO released from Gpc-1-SNO (Fransson et al., 2004). When an excess of NO is released from HS-truncated Gpc-1-SNO, tyrosines in the Gpc-1 core protein as well as in other adjacent proteins can become nitrated, probably by ONOO\⁻\ or \(\cdot\)NO\(\text{2}\) generated from \(\cdot\)NO or HNO. This could be destructive and contribute to neurodegeneration. Although tyrosine nitration was profoundly inhibited by silencing of Gpc-1 expression, it cannot be excluded that other glypicans contribute to the observed effects.

In summary, NO-dependent, deaminative degradation of HS is severely depressed in NPC1 fibroblasts and in U18666A-treated normal fibroblasts and N2a neuroblastoma cells, while heparanase-catalyzed HS degradation is unaffected. Upon exposure to ascorbate, deaminative HS degradation is partially restored, but an excess of reactive nitrogen species generates nitrotyrosines, a potentially destructive process. The present results thus suggest that misprocessing of Gpc-1 in NPC1 cells leads to nitrosative stress and cell damage. As nitrosation is reduced by suramin inhibition of heparanase, this could be a useful treatment in NPC diseases.

Materials and Methods

Materials

HFL-1 and mouse N2a neuroblastoma cells were obtained from ATCC and NPC1 fibroblasts (GM03123) from the Coriell Institute and maintained in minimal essential medium (MEM) supplemented with 10% fetal calf serum. Polyclonal antisera against human Gpc-1, an mAb-recognizing anMan-terminating HS oligosaccharides (Pejler et al., 1998), suitably tagged secondary antibodies, as well as suramin, sodium L-ascorbate, enzymes, pre-packed columns, and other chemicals were generated or obtained as described previously (Cheng et al., 2002; Ding et al., 2002; Belting et al., 2003; Mani et al., 2003, 2004). U18666A was obtained from Biomol (Plymouth Meeting, PA) estrone, filipin, imipramine, and methyl-\(\beta\)-cyclodextrin from Sigma (Stockholm, Sweden), and an mAb to nitrotyrosine from Abcam (Cambridge, UK).

siRNA preparation and transfection

The vector pSilencer 2.0-U6 (Ambion, Austin, TX) containing the sequence GCTGGTCTACTGTGCTCAC (corresponding to nucleotides 977–995 in human Gpc-1) followed by a hairpin sequence TTCAAGAGA, then the reversed complementary Gpc-1 sequence with an additional C in the 5’ end and a stretch of six T for RNA polymerase III termination followed by GGAA in the 3’ end was synthesized by Genscript Corporation (Edison, NJ). Negative control vectors comprising scrambled sequences were also prepared. Transfection was accomplished by using Lipofectamine (Life Technologies, Stockholm, Sweden) according to the description of the manufacturer.

Confocal laser scanning immunofluorescence microscopy

The various procedures including seeding of cells, the use of primary and secondary antibodies, generation of images by sequential scans, and data processing were the same as those used previously (Cheng et al., 2002; Ding et al., 2002; Belting et al., 2003; Mani et al., 2003, 2004) or as recommended by the manufacturers. Cells were fixed and permeabilized either with acetone/H\(\text{2}O\)/methanol or with 2% (w/v) paraformaldehyde, 0.1% (v/v) Triton X-100. Mouse N2a cells were first precoated with 10% anti-mouse immunoglobulin G (IgG) and then exposed to primary antibodies. The second antibody used was either goat anti-mouse total Ig when the primary antibody was monoclonal or goat anti-rabbit IgG when the primary antibody was polyclonal. The second antibodies were tagged with either fluorescein isothiocyanate or Texas Red and appropriately combined for colocalization studies. In the controls, the primary antibody was omitted. Before fixation and confocal microscopy, cells were inspected by light microscopy. Images shown were obtained at a focal plane that was at the center of the cell and of 0.3–0.5 \(\mu\)m thickness. Identical exposure settings were used for image capture. Images were digitized and transferred to Adobe PhotoShop for merging, annotation, and printing. Filipin staining for cholesterol was performed with 50 \(\mu\)g/mL for 2 h. Image capturing of the blue fluorescence was obtained by using a regular fluorescence microscope.

Flow cytometry

Cells were seeded in 24-well plates and grown to near confluence, rinsed with medium, and detached using trypsin [0.5 mL of 0.05% w/v trypsin, 0.5 mL ethylenediaminetetraacetic acid (EDTA) in growth medium for 3–4 min]. Trypsinization was terminated by replacing the trypsin solution with 0.5 mL of medium supplemented with 10% fetal bovine serum (FBS). Cells were recovered by gentle suspension and transferred to tubes, adding 1 volume of phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) (w/v). Cells were then pelleted by centrifugation and resuspended in 0.2 mL of PBS after removal of the supernatant. Cells were fixed for 30 min in 1 mL of PBS containing 4% paraformaldehyde (w/v) whilst initially vortexing. Permeabilization was performed by incubation with 0.2% Triton X-100 in PBS (v/v) for 20 min. Immunostaining of the cells with the mAb specific for anMan-containing HS degradation products as the primary antibody and goat anti-mouse total Ig as the secondary antibody was performed as described for confocal microscopy. In the controls, the primary antibody was omitted. After each step, cells were recovered by centrifugation at 350 \(\times\) \(g\) for 5 min. The cells were finally suspended in PBS containing 1% BSA and analyzed for fluorescence in a fluorescence-assisted cell sorting instrument (Calibur, Becton Dickinson Biosciences, Stockholm, Sweden) operated by Cell-Quest software.
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Conflict of interest statement

None declared.

Abbreviations

anMan, anhydromannose; Gpc-1, glypican-1; GPI, glycosylphosphatidylinositol; HFL-1, human fetal fibroblasts; HNO, nitric oxide; NPC1, Niemann–Pick type C1; NPC2, Niemann–Pick type C2; OONO−, peroxynitrite; PBS, phosphate-buffered saline; sRNA, short-interfering RNA; SNO, S-nitrosylation; U18666A, 3-β-[2-(diethy lamino) ethoxy]androst-5-en-17-one.

References


