INTERACTIONS OF NEU1 WITH ASGR AND LDLR

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Abstract

Development of atherosclerosis, the hardening of the arteries, is dependent on levels of serum cholesterol, which is regulated by the liver via LDL receptors (LDLR). The expression and internalization of LDL receptors depend on several proteins including PCSK9. In fact, previous studies in our laboratory have shown that NEU1 down regulation leads to LDLR hypersialylation which results in its stabilization via reduced interactions with PCSK9. New evidence suggests that NEU1 which de-sialylates LDLR, may affect the ability of another hepatic receptor, the asialoglycoprotein receptor (ASGR), which is comprised of ASGR1 and ASGR2, to interact with LDLR potentially causing its internalization and therefore reduced ability to take up LDL. We investigated how sialidase plays a role in the interaction of ASGR with LDLR. Knockdown and overexpression experiments suggest that NEU1 allows stabilization of LDLR at the cell membrane via ASGR interactions. Treatment of HepG2 cells with monensin which inhibits recycling from the early endosome, unveiled a new truncated ASGR1 isoform potentially lacking its lectin motif. This may be a novel regulatory step in ASGR biosynthesis that warrants further studies. Lysosomal inhibition with chloroquine resulted in concurrent accumulations of NEU1, LDLR and ASGR1, further suggesting these proteins are biosynthetically connected. Our studies revealed a novel isoform of ASGR1 in membrane fractions of HepG2 cell lysates that can associate with NEU1 and LDLR. The impact of NEU1 and ASGR1 on the function and stability of LDLR might lead to new clues for lowering serum cholesterol and reducing atherosclerosis.

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Abbreviations

ApoE	Apolipoprotein W
ARH	Autosomal recessive hypercholesterolemia
ASGP	Asialylated glycoprotein
ASGR	Asialylated glycoprotein receptor
ASGR1	Asialylated glycoprotein receptor 1
ASGR2	Asialylated glycoprotein receptor 2
BSA	Bovine serum albumin
CD36	Cluster of differentiation 36
Dab2	Disabled homolg 2
DANA	2-deoxy-2,3-dehydro-N-acetylneuraminic acid
DAPI	4,6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
DPBS	Dulbecco's phosphate-buffered saline
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HDL	High density lipoprotein
IDOL	Inducible degrader of LDL
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LOF	Loss of function
LSB	Laemmeli sample buffer
M6P	Mannose 6-phosphate
MG132	Carbobenzoxy-Leu-Leu-leucinal
MOI	Multiplicity of infection
NEU1	Neuraminidase 1
OPTI-MEM	Minimal essential medium
PCSK9	Proprotein convertase subtilisin/kexin 9
SDS	Sodium dodecyl sulfate
siRNA	Small inducible RNA
SNARE	Soluble NSF attachment protein receptor
SNX17	Sortin Nexin 17
SRB1	Scavenger receptor class B type 1
TBST	Tris buffered saline Tween 20
TLR4	Toll-like receptor 4
VLDL	Very low density lipoprotein
VWF	Von Willebrand factor

Declaration

All work was performed by Kathryn Fisher.

Chapter 1: Introduction

1.1 Lipoprotein Metabolism

Cholesterol serves two important function in mammals; it is the major component of cellular membranes which determines fluidity and permeability and is a precursor of both bile acids and steroid hormones. Due to its importance, the metabolism and homeostasis of cholesterol is tightly regulated, and an imbalance can lead to detrimental effects and disease, for example atherosclerosis. Cholesterol can be obtained through endogenous biosynthesis from metabolism of acetyl-CoA, as well as from the diet through absorption by enterocytes at the small intestine (Goedeke and Fernandez-Hernando 2012).

The main constituents in cholesterol transport are lipoproteins. These spherical particles are composed of lipids and proteins at their core and an amphiphatic phospholipid surface that allows for their movement through the blood to various tissues. They are responsible for carrying cholesterol between the liver and peripheral tissues within their hydrophobic core (Hoofnagle and Heinecke 2009). Different types of lipoproteins are responsible for carrying different densities of cholesterol. Cholesterol not synthesized by cells and obtained in the diet is packaged into chylomicrons along with triglycerides and transported from the small intestine to the liver to be taken up by hepatocytes (Goedeke and Fernandez-Hernando 2012). From the liver, cholesterol can be delivered to peripheral tissues by very low-density lipoproteins (VLDL), which transport them into the cell to be hydrolyzed by lysosomes into free cholesterol (Goedeke and Fernandez-Hernando 2012, Biggerstaff and Wooten 2004). As cholesterol is removed

from VLDL, its density is lowered, and it becomes a low-density lipoprotein (LDL). Conversely, high density lipoproteins (HDL) will become denser as they take up more cholesterol. These lipoproteins primarily transport cholesterol back to the liver where it can be taken up by receptors (Biggerstaff and Wooten 2004). For this reason, HDL is classically known as "good cholesterol", while LDL is known as "bad cholesterol" as it can lead to excessive delivery of cholesterol to peripheral tissues, as in the case of atherosclerosis (Biggerstaff and Wooten 2004).

Risk factors in the development of atherosclerosis include hypertension, diabetes mellitus, male gender, obesity, smoking, family history, high fat diet, and elevated serum cholesterol levels (Hegele 2009). Elevated LDL and VLDL cholesterol are the main risk factors of this condition and are sufficient even in the absence of other risk factors to trigger atherosclerosis (Glass and Witztum 2001).

1.2 Atherosclerosis

Atherosclerosis is the accumulation of lipid-laden macrophages under the endothelial layer of arteries, leading to chronic inflammation. This is largely driven by elevated serum cholesterol levels (Hegele 2009). This process can lead to the formation of plaques that protrude into the arterial lumen (Glass and Witztum 2001). Plaque formation begins with the formation of fatty streaks due to the accumulation of macrophages and their uptake of low-density lipoproteins (LDL) underneath the endothelium of arterial walls (Glass and Witztum 2001). Once LDL reaches the subendothelial space, its susceptibility to being oxidized is greatly increased. Once

oxidized, LDL will remain in circulation as it is prevented from being bound by low density lipoprotein receptors (LDLR) and taken up into cells (Levitan et al., 2010). The uptake of oxidized LDL by macrophages through scavenger receptors causes the formation of lipid filled foam cells (Glass and Witztum 2001). Macrophages interact with T cells leading to chronic inflammation and the development of atherosclerotic lesions (Glass and Witztum 2001). The subsequent migration, proliferation and secretion of extracellular matrix proteins by smooth muscle cells from the medial arterial wall leads to the formation of fibrous plaques. The rupture of these plaques can result in complications such as stroke and myocardial infarction, of which are the primary cause of death in the developed world (Glass and Witztum 2001). Therefore, understanding the development and progression of this disease, including mechanisms involving lipid metabolism, is of utmost importance.

1.3 LDLR and atherosclerosis

Increased risk of atherosclerosis and cardiovascular disease is associated with increased levels of low-density lipoproteins (LDL). The low-density lipoprotein receptor (LDLR) is responsible for bringing LDL cholesterol into cells. Therefore, levels of circulating LDL is largely determined by LDLR levels. These receptors bind lipoproteins which are subsequently internalized into cells by clathrin-coated vesicles. LDL and LDLR are dissociated when vesicles fuse with early endosomes. LDLR is either recycled to the membrane or degraded by lysosomes, while LDL is sorted into the late endosome and lysosome and will be taken up by the cell (Wijers et al., 2015).

The process of LDLR recycling is beginning to be understood, including the important regulatory proteins. Sorting Nexin 17 (SNX17) has been demonstrated to bind to LDLR and increase the rate that it moves through the endosome, while the number of cell surface receptors remains stable. This suggests that SNX17 plays a role in recycling, but not degradation of LDLR (Stockinger et al., 2002). LDLR recycling is also mediated by Rab proteins. Rab3b has been demonstrated to transport LDLR directly from the Golgi apparatus to the plasma membrane, while Rab13 mediates transport through the recycling endosome. LDLR may also be endocytosed without the binding of LDL, with the help of ARH or Dab2, depending on cell type (Nokes et al., 2008).

Due to the importance of this receptor, many intricate mechanisms for its regulation and degradation are in place. These regulators include proprotein convertase subtilisin/kexin type 9 (PCSK9) and inducible degrader of LDLR (IDOL) (Grefhorst et al., 2008). IDOL acts by ubiquitnation of itself and LDLR present in lipid rafts at the plasma membrane, allowing for internalization mediated by Epsin (Kang et al., 2013). PCSK9 is a protein that preferentially binds LDLR on its epidermal growth factor-like repeat A domain, interfering with a change in conformation that is required for its recycling (Zhang et al., 2007). Once bound, LDLR is internalized and degraded by lysosomes, reducing levels of surface LDLR (Zhang et al., 2007). PCSK9 can also bind LDLR directly after synthesis within the Golgi apparatus (Wijers et al., 2015) While gain of function mutations of PCSK9 have been shown to cause atherosclerosis, loss of function mutations show protection against it (Grefhorst et al., 2008). For this reason, PCSK9 is being heavily researched as a target molecule in cardiovascular disease.

Different approaches for therapeutically inhibiting PCSK9 are being developed and tested in clinical trials, including various monoclonal antibodies (Chaudhary et al., 2017).

The importance of LDLR in disease has been demonstrated in patients with homozygous familial hypercholesterolemia. In this condition, patients lack functional LDL receptors, and this was shown to be responsible for the significant accumulation of LDL in the serum (Goldstein and Brown, 1977). Many studies have examined the effects of deficiency in LDLR on the development of atherosclerosis in mice and noted the marked increase in LDL cholesterol when fed high fat diets, but no difference when fed a chow diet (Ishibashi et al., 1994). More recently, scientists have reported the creation of $LDLR^{-/-}$ Yucatan miniature pigs with targeted disruption of LDLR to provide a large animal model of familial hypercholesterolemia and atherosclerosis (Davis et al., 2014). This model displays considerably elevated LDL levels when fed a standard diet and atherosclerotic lesions resembling human atherosclerosis, with more severe phenotypes when fed a diet high in cholesterol (Davis et al., 2014).

1.4 Sialidase and atherosclerosis

Sialidase (Neu1) is one of four mammalian sialidases (Miyagi and Yamaguchi 2012). It is an enzyme localized to lysosomes and the plasma membrane which cleaves sialic acid residues from glycoproteins (Miyagi and Yamaguchi 2012). Sialic acid residues are negatively charged terminal sugars found on glycoconjugates of proteins and lipids (Chen and Varki 2010, Pshezhestky and Ashmarina 2001). Sialic acid removal is important as it leads to altered chemical properties of proteins, for example their

conformation and presence of functional binding sites, and therefore their interactions and functions in biological processes (Miyagi and Yamaguchi 2012). These processes include cell adhesion, proliferation, differentiation, glycoprotein and glycolipid metabolism, and receptor modification (Monti 2010). Sialidase has been shown to play an important role in mediating cell signaling through surface receptors (Pshezhetsky and Ashmarina 2013). Removal of sialic acid residues by sialidases or degradation of sialoglycoconjugates modulate changes in structure and function over time of cellular receptors (Pshezhetsky and Ashmarina 2013).

Several studies have examined the role of sialic acid in the development of atherosclerosis and noted the increase in serum sialic acid levels in patients with coronary heart disease (Allain et al., 1996, Haq et al., 1993, Knuiman et al., 2004). Studies by Lindberg et al., have shown that concentration of sialic acid in the serum can be used to predict coronary heart disease and mortality from stroke (1991). Sialic acid content of platelets and LDL have shown to be important factors in heart disease as well. Patients were found to have significantly lower sialic acid levels in their platelets, and this was hypothesized to play a role in facilitating platelet aggregation (Mandic et al., 2002). LDL that has been desialylated by Neu1 has been shown to cause lipid accumulation in patients with atherosclerosis, in agreement with the observation that sialic acid content of LDL is much lower in these patients (Orekhov et al., 1992). Studies in fibroblasts and endothelial cells showed that treatment with Neu1 markedly alters the binding, uptake and degradation of LDL (Sprague et al., 1988).

A more recent study investigated how Neu1 may play a role in regulating LDL levels through LDLR. LDLR, an integral membrane glycoprotein, possesses terminal sialic acid residues (Sprague et al., 1988). In hypomorphic Neu1 mice, increased levels and recycling of LDLR in the liver result in decreased cholesterol levels in serum (Yang et al., 2012). Additionally, studies of ApoE deficient mice with hypomorphic Neu1 expression show reduced serum VLDL and LDL as well as reduction in formation of atherosclerotic lesions (White et al., 2018). Treatment of $Apoe^{-/-}$ mice with the sialidase inhibitor DANA also displayed an anti-atherogenic effect (White et al., 2018). The mechanism behind the effect of sialic acid content and LDL levels has yet to be understood, however, one possible mechanism is through the action of ASGR. It is hypothesized that in wild-type mice, the asialylated form of LDLR interacts with ASGR and is internalized by endocytosis (Nioi et al., 2012). In hypomorphic NEU1 mice, LDLR is sialylated and therefore more stable on the hepatocyte surface and less prone to recycling, leading to increased uptake of LDL by hepatocytes and decreased serum cholesterol (Nioi et al., 2016).

1.5 ASGR and atherosclerosis

In addition to Neu1, many other proteins are involved in lipid metabolism. One such protein is the asialoglycoprotein receptor (ASGR). ASGR is an integral membrane receptor and possess two homologous subunits, ASGR1, the major subunit and ASGR2, the minor subunit (Gupta 2012). Each subunit contains four domains, including a cytoplasmic domain, transmembrane domain, extracellular stalk and a carbohydrate recognition domain with disulfide bridges (Fig. 1). As part of the C-type lectin family, it

requires calcium ions for ligand binding (Gupta 2012). ASGR binds and mediates the uptake and degradation of glycoproteins containing a terminal N-acetylgalactosamine or galactose sugar via clathrin coated pits (Gupta 2012). These galactose residues are exposed when terminal sialic acid residues are removed: substrates for ASGR consists of asialylated glycoproteins which are generated by the action of sialidase (Tybjaerg-Hansen 2016). One study by O'Sullivan et al., demonstrated this relationship between sialic acid and ASGR (2016). It was demonstrated that reduction of N-linked sialylation of the von Willebrand factor (VWF) leads to increased clearance of this protein by ASGR (O'Sullivan et al., 2016).

ASGR is expressed on the sinusoidal surface of mammalian hepatocytes and in macrophages (Gupta 2012, Harris et al., 2012). Expression of ASGR vary between hepatocytes and macrophages (Harris et al., 2012). THP1 macrophages express both subunits of ASGR, at levels 4-6 times lower than in HepG2 cells (Harris et al., 2012). This observation proposed the idea that macrophages may serve as a different, and mobile source of ASGR (Harris et al., 2012). Differences in ASGR in the liver versus macrophages could be attributed to splice variants of this protein (Harris et al., 2012, Liu et al., 2010).

Previously, ASGR was hypothesized to be responsible for clearance of plasma lipoproteins, but plasma lipoprotein levels were observed to be unaffected in ASGR knockout mice along with circulating desialylated glycoprotein levels (Tozawa et al., 2001). However, knockout mice were unable to clear exogenous sialylated glycoprotein, indicating that ASGR is not essential under normal conditions (Tozawa et al., 2001). On

the contrary, ASGR has been shown to play a role in the metabolism of both LDL particles and lipoprotein (a), a subtype of LDL (Windler et al., 1991, Hrzenjak et al., 2003). Windler demonstrated that ligands known to be specific for ASGR (ASGPs) were able to compete with the binding of LDL and chylomicron remnants and that lipoproteins were able to inhibit ASGPs from binding (1991). Furthermore, when an antibody against ASGR was applied to HepG2 cells, association of remnants and LDL decreased (Windler et al., 1991). Lipoprotein (a) was shown to be catabolized at a much faster rate in wild type mice compared to ASGR knockout mice (Windler et al., 1991).

A very important study that revealed evidence towards a physiologic function for ASGR was a recent genetic study of an Icelandic population. The study by Nioi et al., demonstrated that a loss of function of the primary subunit of ASGR, ASGR1, has shown to have a strong link with reduction in non-HDL cholesterol levels in plasma and reduced risk of coronary artery disease (2016). A deletion of 12 base pairs located in intron 4 of ASGR1 was identified as having a causal association with this phenotype; a 34 percent reduced risk of atherosclerosis and 9 percent reduction of non-HDL cholesterol (Nioi et al., 2016). Interestingly, this reduction was much larger than that observed with other genetic variants, however its effect on cholesterol levels was smaller, indicating that this deletion may protect against the disease through other independent mechanisms, for instance reduced inflammation (Nioi et al., 2016, Tybjaerg-Hansen 2016). One possibility could be through the sialylation state of chemokines and their receptors which can influence migration of inflammatory cells to atherosclerotic lesions (Nioi et al., 2016).

The findings of this study in combination with that of the hypomorphic Neu1 mice leads to the question: does sialylation play a role in the interaction between LDLR and ASGR, and could it be used as a target for neutralizing ASGR (Nioi et al., 2016).

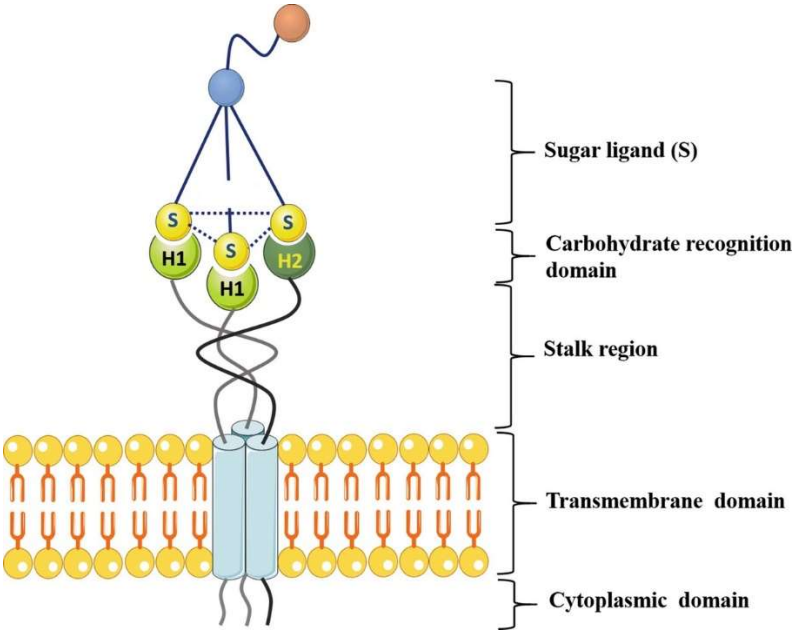


Figure 1. ASGR protein structure. Each receptor is comprised of two major subunits (ASGR1/ H1) and one minor subunit (ASGR2/ H2). ASGR1 is synthesized as a type II transmembrane glycoprotein containing a cytoplasmic domain, transmembrane domain, and an extracellular domain. The extracellular domain contains a stalk region that contributes to oligomerization and a Ca2+ dependent C-type lectin domain that recognizes and binds carbohydrates. (Modified from Das et al. 2019).

1.6 Chemical Inhibitors

An important tool in the study of cell function, dynamics and signaling is the use of chemical inhibitors. Inhibitors of endocytosis interfere with the invagination of the plasma membrane into endosomes which modulates the uptake of ligands and receptors such as LDLR and ASGR. Previous studies have begun to analyze the dynamics of these proteins by way of inhibitors which prevent endosomal maturation for example chloroquine and monensin. Chloroquine is a weak base which can raise the pH of cellular compartments, which was originally employed as a medication for use in the treatment of malaria (Mauthe et al.,2018, Al-Bari 2015). While it was previously understood that chloroquine substantially lowers lysosomal acidity and therefore its degradative activity, recent studies have shown its action is through blockage of autophagosome-lysosome fusion and impairing receptor-mediated endocytosis (Mauthe et al., 2018). As a result of reduced endocytic transfer, degradation of both ligand (EGF) and receptors (EGFR) proteins was impaired (Mauthe et al.,2018).

1.7 Inhibitors effects on ASGR and LDLR

Studies looking at the effects of chloroquine on ASGR in HepG2 cells have observed reduction in surface ASGR through pulse chase experiments, attributing this loss to impaired ASGR recycling rather than the effect of chloroquine on the receptor directly (Schwartz et al., 1984). When looking at chloroquine's effect on uptake of asialylated glycoproteins, there is an inhibition of dissociation of ligands from their receptor, resulting in failure to be degraded by lysosomes and their accumulation in

endocytic vesicles (Harford et al., 1983). This study also noted a similar response to the inhibitor monensin (Harford et al., 1983).

Monensin is a polyether antibiotic which prevents acidification of endosomes (Dhaliwal, 2013). Studies have demonstrated its variety of effects on membrane dynamics, including the disruption of membrane vesicle transport from the Golgi to the membrane, interference with endocytosis related events and altered introduction of membrane and secretory proteins to the cell surface (Tartakoff, 1983). When looking at the effects of monensin on ASGR, studies in Huh7 hepatocytes showed a fifty percent reduction in surface ASGR expression with monensin treatment (Stockert et al., 1995).

Effects of monensin and chloroquine have also been studied regarding cholesterol metabolism. Both inhibitors were shown to inhibit the recycling of LDLR to the plasma membrane in primary mouse hepatocytes without altering total cell expression, however this same effect was not seen by chloroquine when looking at SRB1 surface expression (Minahk et al., 2008).

1.8 Rationale and Objectives

Many factors have been identified thus far to play an important role in the development of atherosclerosis. Recently, the notion that ASGR plays a role in atherosclerosis and lipid metabolism was suggested by Nioi et al., (2016). Loss of function variants of ASGR1 were demonstrated to be associated with a decrease in non-HDL cholesterol levels and an even greater reduction in risk for cardiovascular disease.

Additionally, it has been shown that in PCSK9 knockout hepatocyte cell lines, in which LDLR levels are greatly increased, there is a concurrent significant increase in ASGR1 levels (Lebeau et al., unpublished data). This demonstrates that there is a correlation between increased LDLR levels and increased ASGR1 levels. Finally, in hypomorphic Neu1 mice, increased levels and recycling of LDLR in the liver result in decreased cholesterol levels in serum (Yang et al., 2012). The mechanism behind the effect of sialic acid content on LDLR and LDL levels has yet to be understood, however one possible mechanism is through the action of ASGR. With these lines of evidence, it is apparent that Neu1, LDLR and ASGR are all related in regulating cholesterol levels and ultimately in risk for cardiovascular disease. The following aims will attempt to better understand the mechanisms by which these proteins interact and regulate one another.

Objective 1

To examine the hypothesis that Neu1 actively regulates ASGR and LDLR levels, we observed the effects of overexpression of Neu1 in a human hepatoma cell line, HepG2. The liver is the main known source of ASGR protein expression and is known to be highly expressed in the HepG2 cell line. Additionally, it plays an important role in cholesterol metabolism and uptake of LDL and with significant impact on the development of atherosclerosis. The effect of Neu1 overexpression on the expression of LDLR and ASGR1 was assessed through Western blot.

Objective 2

To investigate the hypothesis that knockdown of ASGR1 destabilizes LDLR expression, ASGR1 was knocked down in HepG2 cells. The effect of ASGR1 knockdown on the expression of LDLR and ASGR1 was assessed through siRNA transfections followed by Western blot analysis.

Objective 3

To elucidate how NEU1, ASGR1 and LDLR are associated with one another, we next utilized chemical inhibitors of secretory and endocytic pathways and observed how these proteins responded. We hypothesized if these proteins are working in conjunction, they would display similar dynamics in response to these chemicals. HepG2 cells were treated with monensin or chloroquine for increasing periods of time and protein expression was assessed through Western blotting techniques. The effects of inhibitors on the expression and localization of ASGR was visualized using immunocytochemistry. The chemical inhibitors monensin and chloroquine are well characterized and have been used extensively in the literature. They are known to affect the secretory and endosomal pathways in which the proteins of interest may interact and were therefore selected to monitor response of the proteins to endosomal inhibition.

Objective 4

Previous studies have shown that NEU1 is responsible for modulating receptor activity in various pathways through sialic acid removal, and in some cases has been demonstrated to have this effect through a physical association, for example in conjunction with CD36 (Kawecki et al., 2019).To analyze whether the association of ASGR1, LDLR and NEU1 was through a physical interaction, HepG2 cell lysates were analyzed using immunoprecipitation in the presence or absence of monensin and MG132, to prevent proteasomal degradation. Cells were fractionated into membrane and soluble proteins to determine the location of potential interactions

Chapter 2: Methods

2.1 Cell culture

HepG2 liver hepatocellular carcinoma cells were cultured with Dulbecco's modified eagle medium (DMEM) (Gibco, 11965092) containing 10% Fetal bovine serum (FBS) (Gibco, A3160602), 1% penicillin/streptomycin/L-glutamine (Invitrogen, 15140122), and 0.1% Amphotencin B (Invitrogen, 15290018). Cells were kept in a 37°C incubator with 5%CO_2 and 21% O_2. Cells were passaged with 1X DPBS (Gibco, 14190144) and 0.05% Trypsin-EDTA (Gibco, 2530054).

2.2 siRNA Transfections

HepG2 cells were transfected with small interference RNAs (siRNAs) targeting ASGR1 (Ambion, s224109) or a scramble siRNA (Ambion, 4611) as a negative control. The transfection reagent Lipofectamine 3000 (Invitrogen, L3000001) was incubated with siRNAS for 15 minutes at room temperature. After removal of culture media from cells, OPTI-MEM media was added (Gibco, 31985088) along with the siRNA and lipofectamine solution. Plates were incubated for 24 hours, media was removed and replaced with DMEM before another 24 hours of incubation.

2.3 Adenoviral infections

An adenovirus expressing normal human sialidase was generated as per Pattison et al., 2004 using a pDC316 shuttle plasmid and an adenoviral genomic plasmid. HepG2 cells were transduced with the adenovirus at MOI values of 0.1, 10 and 100 or a control

empty plasmid (pFG) and incubated for 72 hours. Cells were then lysed and analyzed by Western blot.

2.4 Protein assay

To determine total protein concentration of HepG2 cells lysates, the DC Protein Assay (Bio-Rad, 5000111) was used. Bovine Serum Albumin (BSA) (NEB, 9998) standards and samples were loaded in duplicate in a 96-well microplate. After addition of protein assay reagents, the microplate was incubated at room temperature for 15 minutes. Absorbance values to determine total protein concentration were determined using a microplate reader (SpectraMax Plus 384).

2.5 Membrane extraction

Membrane protein extraction from HepG2 cells was performed using the Mem-PER Plus Kit (Thermo Scientific, 89842Y). The adherent mammalian cells protocol was followed. Cells were resuspending in the growth media using a cell scraper followed by centrifugation at 300g for 5 minutes. The cell pellet was washed twice with 3mL and 1.5mL of cell wash solution and centrifuged for 5 minutes at 300g. Protease inhibitors (Thermo Scientific, 1862209) were added to the permeabilization and solubilization buffers. To the cell pellet, 0.75mL of permeabilization buffer was added followed by briefly vortexing to homogenize and incubation for 10 minutes at 4°C with rotation. Permeabilized cells were centrifuged for 15 minutes at 16000g at 4°C. The supernatant containing cytosolic protein was transferred to a fresh tube. The cell pellet was solubilized by resuspension in 0.5mL of solubilization buffer and a 30 minute incubation

with rotation at 4°C. After centrifuging at 16000g for 15 minutes at 4°C, the supernatant containing membrane and membrane-associated proteins was added to a new tube and proceeded to immunoprecipitation.

2.6 Immunoprecipitation

HepG2 cells were washed with cold 1X DPBS (Gibco, 14190144) and lysed in RIPA lysis buffer (50mM Tris-HCl pH 8, 150mM NaCl, 0.5% sodium deoxycholate, 1% NP40, and 0.1% SDS) with protease inhibitors (Thermo Scientific, 1862209) and EDTA (0.5M) if not already solubilized from membrane extraction. Proteins of interest were immunoprecipitated with target-specific antibodies conjugated to Dynabeads Protein G (Life Technologies, 10004D). Antibodies were each diluted in 200µL DPBS with 0.02% Tween-20 at varying concentrations (1-5µg) and added to 20µL of the Dynabeads. To allow the antibody to bind to the beads, tubes were incubated with rotation for 1 hour at 4°C. Samples were then added to the Dynabeads-Antibody complex and incubated overnight with rotation at 4°C. Magnetic beads were collected on a magnetic and washed 3 times with 200µL wash buffer (DPBS, 0.02% Tween-20), separating the beads on the magnet and removing the supernatant between each wash. Magnetic beads were then resuspending in 100µL wash buffer and transferred to a clean bead to avoid coelution of protein bound to the tube wall. After separation on the magnet and removal of the supernatant, 20µL of loading buffer (1X LSB) was used to resuspend the beads. Samples were heated for 10 minutes at 70°C followed by separation of the sample from the beads on the magnetic rack. Samples were then analysed by Western blot.

2.7 Western blot analysis

Western blotting was used for protein analysis of HepG2 cell lysates. If not already lysed, cells were rinsed with cold 1X DPBS (Gibco, 14190144) and lysed in RIPA lysis buffer (50mM Tris-HCl pH 8, 150mM NaCl, 0.5% sodium deoxycholate, 1% NP40, and 0.01% sodium dodecyl sulfate) with protease inhibitors (Thermo Scientific, 1862209) and EDTA (0.5M). Samples were boiled in 6X Laemmli Sample Buffer (0.5M Tris pH 6.8, 1.2% SDS, 50% Glycerol, 600mM DTT and 0.3% Bromophenol Blue) at 100°C for 10 minutes. Equal protein amounts of each sample were separated by SDS-PAGE using 5-10% protein gels (0.5M-1.5M Tris pH 8.8, 40% Polyacrylamide and 1% APS) for 120 minutes at 100V at room temperature and transferred onto nitrocellulose membrane for 1 hour at 100V at 4°C. Membranes were blocked with 5% non-fat dry milk (Carnation) in Tris- buffered saline with 0.1% Tween-20 (TBST), incubated with primary antibody in 5% milk TBST overnight at 4°C. Primary antibody was removed and membranes were washed with TBST 4 times for 10 minutes, followed by incubation with horseradish peroxidase conjugated secondary antibody in 5% milk TBST for 1 hour at room temperature. Membraned were washed 4 times with TBST for 10 minutes before incubation with Amersham ECL Western Blotting Detection Regents (GE Healthcare, RPN2106). Blotted membranes were exposed using Amersham Hyperfilm ECL film (GE Healthcare, 28906839) for various time increments and developed. If blots were to be analyzed for other proteins of interest, membranes were rinsed with TBST and stripped with stripping buffer (Glycine, SDS, Tween-20, pH 2.2) twice for 10 minutes before re-blotting.

2.8 Western blot quantification

Densitometry was measured through the gel analysis feature in ImageJ (v1.52a, NIH, USA) using scans of films in JPEG format. Values were normalized to respective GAPDH or β-actin measurements.

2.9 Immunocytochemistry

HepG2 cells were plated onto 12mm circular glass coverslips (Fisher Scientific, 1254580) nested in a 24-well plate and cultured for 24 hours before treatment. After treatment, cells were washed in cold DPBS two times for 1 minute, followed by fixation in 3.5% formaldehyde (Caldeon, 3500-1) in DPBS for 45 minutes at room temperature. Next, 0.5% Triton-X 100 in DPBS was used to permeabilize cells for 30 minutes. Cells were blocked with 10% Goat serum in DPBS for 1 hour before incubation with primary antibody cocktail. Primary antibody was diluted 1:200 in 10% Goat serum in DPBS and left on cells overnight at 4°C. Cells were washed 6 times for 5 minutes at room temperature using 0.05% Tween 20 in DPBS. A cocktail of corresponding secondary antibodies was diluted in 10% goat serum at a 1:500 dilution and added to cells for 1 hour at room temperature and washed 6 more times for 5 minutes, as above. Finally, glass coverslips were rinsed with double distilled H_2O mounted onto microscope slides using ProLong Gold antifade reagent with DAPI (Invitrogen, P36941) to visualize nuclei. Slides were left at room temperature for 1 hour to dry followed by storage at -20°C.

2.10 Fluorescence Microscopy

Immunofluorescence slides were imaged using a fluorescent microscope (Zeiss, Axiovert200) under the 40x magnification lens. Images were captured using the Leica AxioVision software (V4.8.2.0) using multidimensional acquisition to view the double or triple labeling.

2.11 Antibodies

The following primary antibodies were used in Western blot and immunofluoresence analysis: anti-NEU1 (mouse anti-NEU1, 1:100, Santa Cruz, sc-166824), anti-ASGR1 (rabbit anti-ASGR1, 1:1000, Proteintech 117391AP, anti-hLDLR (goat anti-hLDLR, 1:1000, R&D Systems AF2148), and anti-GAPDH (goat anti-GAPDH, 1:1000, R&D Systems AF5718). The respective secondary antibodies used were anti-mouse IgG HRP (1:2000, Cell Signaling 7076S), anti-rabbit IgG HRP (1:2000, Cell Signaling 7074S) and donkey anti-goat IgG HRP (1:2000, Invitrogen A15999) for Western blot experiments. For immunofluorescence experiments Alexa Fluor 488 anti-mouse IgG (Invitrogen, A11029) and Alexa Fluor 594 anti-rabbit IgG (Invitrogen, A11037) were used.

2.12 Statistical Analysis

For comparisons of data sets involving two groups, t-tests were used at $P<0.05$ to determine a significant difference between means. For comparison of data sets with three or more groups, One-way ANOVA was used at $P<0.05$ to determine significant differences between means. Dunnett's multiple comparison test was used to determine

which groups were significantly different. Statistical analysis was performed using GraphPad Prism 6 (V6.01).

Chapter 3: Results

3.1 NEU1 overexpression in HepG2

To examine the hypothesis that NEU1 actively regulates LDLR levels and in turn may affect ASGR levels, the effect of NEU1 overexpression on LDLR and ASGR1 expression was assessed. HepG2 cells, a human hepatocyte cell line, was transduced with an adenovirus expressing his-NEU1 at various multiplicity of infections (MOI) and incubated for 24 hours. Protein expression of NEU1, LDLR and ASGR were analyzed using Western blotting (Fig. 2). Experiments demonstrated that overexpression of NEU1 in HepG2 cells was correlated with increased levels of ASGR1 and LDLR protein levels. Expression of His-NEU1 is significantly present at a MOI of 10, to a lesser extent at 100 MOI, but not at 0.1 MOI (Fig. 2D). In HepG2 cells significantly overexpressing his-NEU1, a significantly increased expression of both LDLR and ASGR1 is observed (1B, C). However, in cells expressing little or no his-NEU1, LDLR and ASGR1 protein expression is comparable to control cells or those transduced with a control empty virus (pFG).

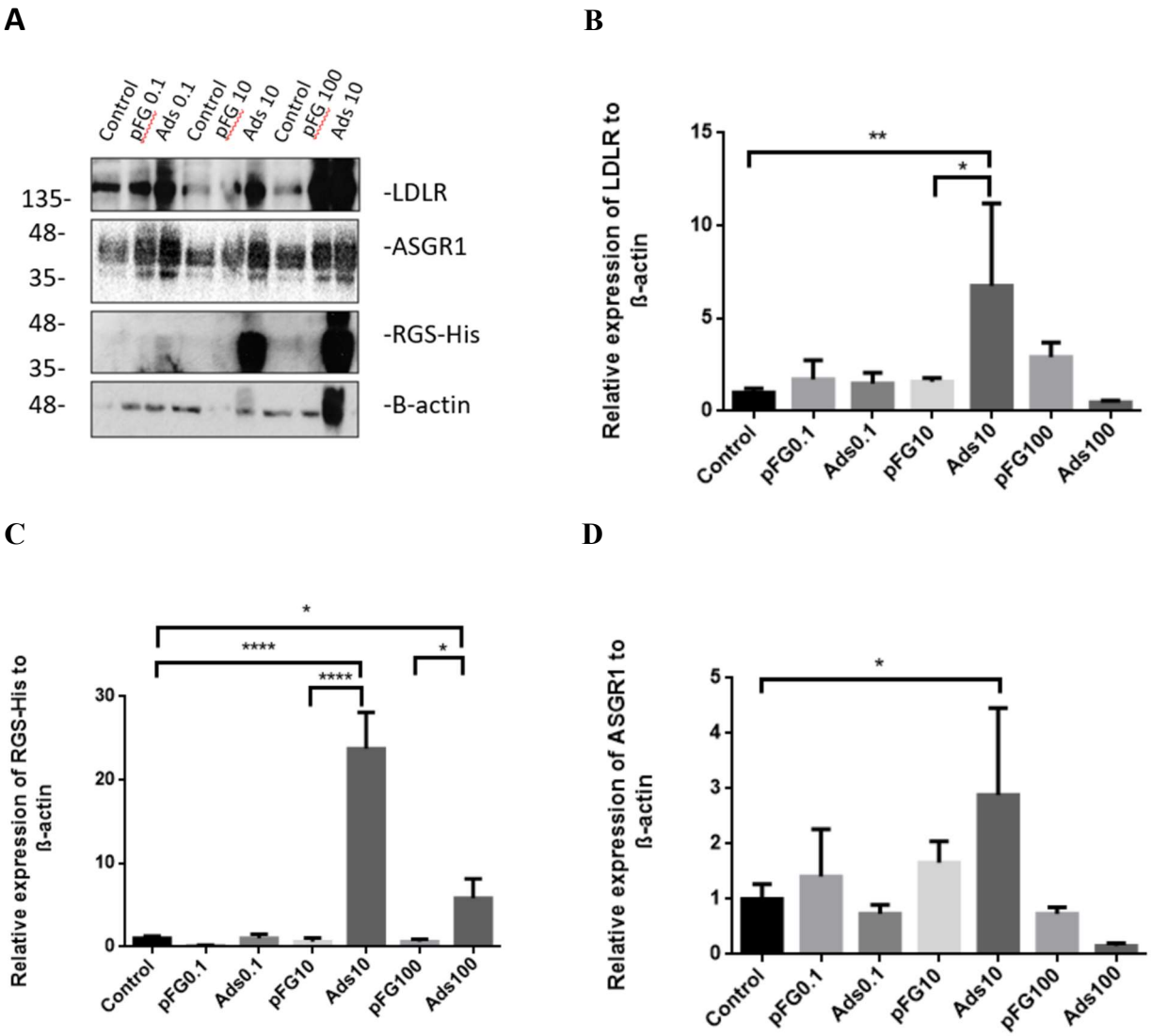


Figure 2. NEU1 over-expression effect on LDLR and ASGR1 protein levels. Transduced HepG2 cells were analyzed by Western blot. Cells were treated with increasing concentrations of a control virus (pFG) and a his-NEU1 expressing Adenovirus (Ads) for 72 hours. A) Cell lysates were subjected to SDS-PAGE (10%) and membranes were probed with anti-LDLR, anti-ASGR1, anti-RGS-His and anti-β-actin as a loading control. Densitometry of immunoblot bands was measured using ImageJ and normalized to the loading control. Expression of LDLR and ASGR1 are significantly increased with NEU1 overexpression at 10 MOI (B,C,D). E) Expression of His-NEU1 is significantly present at 10 MOI of the NEU1 adenovirus. The results indicate that the expression of LDLR and ASGR1 are affected by overexpression of NEU1. Representative blots for n=3 for each group. One-way ANOVA, Dunnett's multiple comparison test *P < 0.05, **P < 0.01.

3.2 Effect of ASGR1 Knockdown on NEU1 and LDLR

To further examine if the expression levels of NEU1 and LDLR is dependent on ASGR1 expression, the effect of knocking down ASGR1 on NEU1 expression in HepG2 cells was assessed. Cells were transfected with scrambled siRNA or siRNA specific to ASGR1 for 24 hours and protein expression was analyzed by Western blot (Fig. 3) Results showed a significant decrease in ASGR1 levels in cells treated with ASGR1 siRNA (Fig. 3B). When compared to control cells and those treated with scrambled siRNA, cells with ASGR1 knocked down had a significant decrease in LDLR expression as seen in densitometry analysis (Fig. 3C). However, NEU1 levels appear to be unaffected by knockdown of ASGR1 (Fig. 3D).

3.3 Effects of Monensin on ASGR1, LDLR and Neu1 expression and secretion

To elucidate if NEU1, ASGR1 and LDLR are associated with one another, we next utilized chemical inhibitors of secretory and endocytic pathways and observed how these proteins responded. We hypothesized if these proteins associate at early stages of synthesis , they would display a similar expression pattern in response to these inhibitors of secretion . To investigate this hypothesis, HepG2 cells were treated with monensin, an early endosomal proton transport inhibitor that prevents endosome maturation. Monensin treatment inhibits recycling from the early endosome and is expected to cause the accumulation of ASGR1 and LDLR. Cells were exposed to the treatment for 0, 6, 12 or 24 hours. In addition to cell lysates, the media of each sample was collected to asses protein secretion. Protein levels were analyzed and quantified using Western blot and

densitometry (Fig. 4). At 24 hours of exposure to monensin, protein levels of LDLR were increased by 3 fold when compared to the control (Fig. 4). With increasing exposure to monensin, a gradual decrease in levels of full length ASGR1 was observed, however a lower molecular weight form of ASGR1 accumulated by 24 hours of treatment (Fig. 4C). Expression of NEU1 was increased not only within the cell but was shown to be secreted and detected in the media in increasing amounts with increasing exposure to monensin.

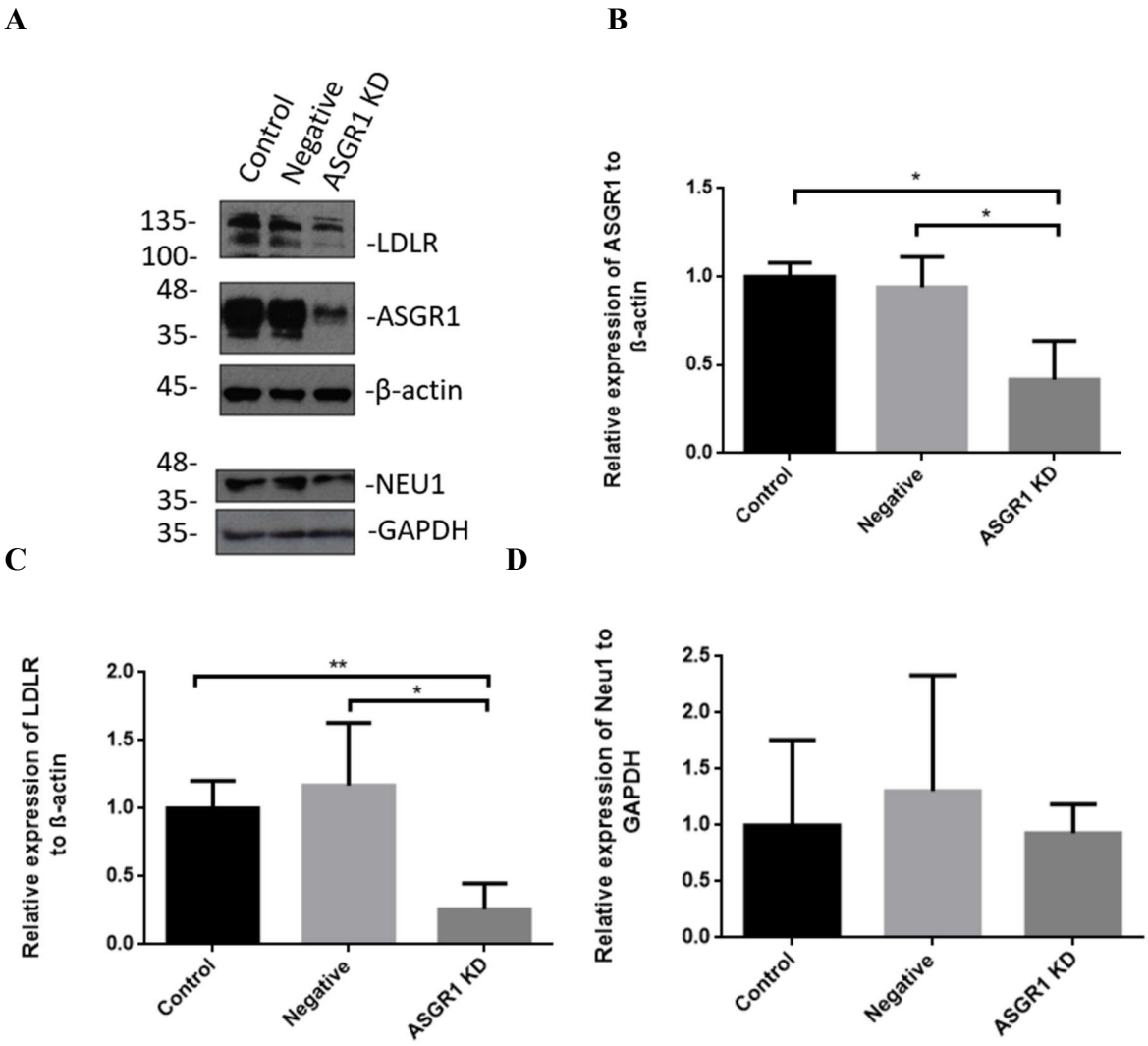


Figure 3. Effect of ASGR1 knockdown on LDLR and NEU1 protein expression. Western blot analysis of LDLR, ASGR1 and NEU1 expression in transfected HepG2 cells. Cells were treated with scrambled siRNA (Negative) or ASGR1 siRNA (ASGR1 KD) for 24 hours. Cell lysates were subjected to SDS-PAGE (10%). Membranes were probed with anti-LDLR, anti-ASGR1, anti-NEU1 and anti- β-actin or anti-GAPDH as a loading control. Band intensities for LDLR (B), ASGR1 (C) and NEU1 (D) were measured by ImageJ software and normalized. Reduction of ASGR1 expression by siRNA knockdown (C) leads to decreased levels of LDLR (B). This indicates these proteins could be associated with one another. Representative blots for n=3 for each group. Unpaired t-test *$P < 0.05$, **$P < 0.01$.

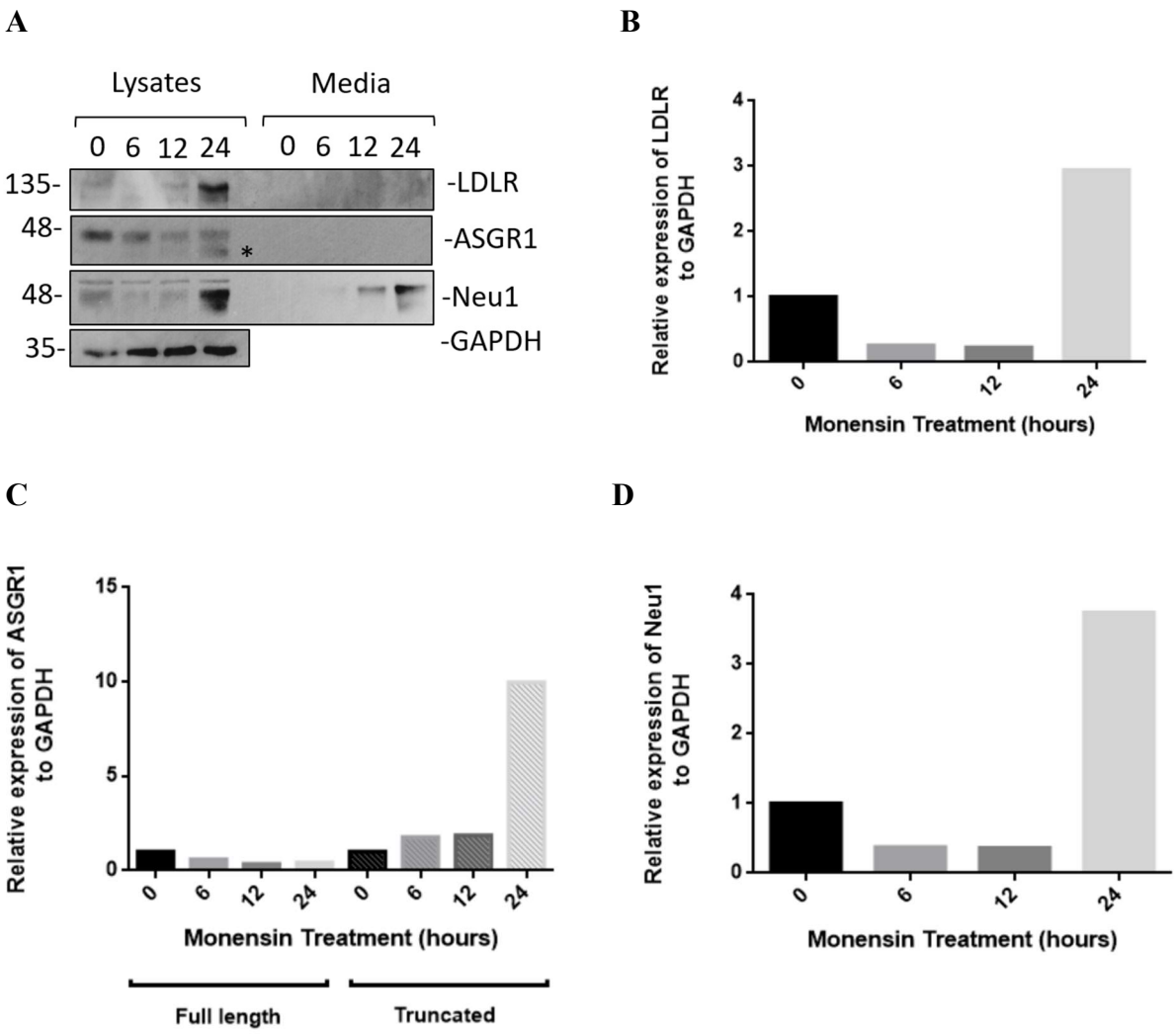
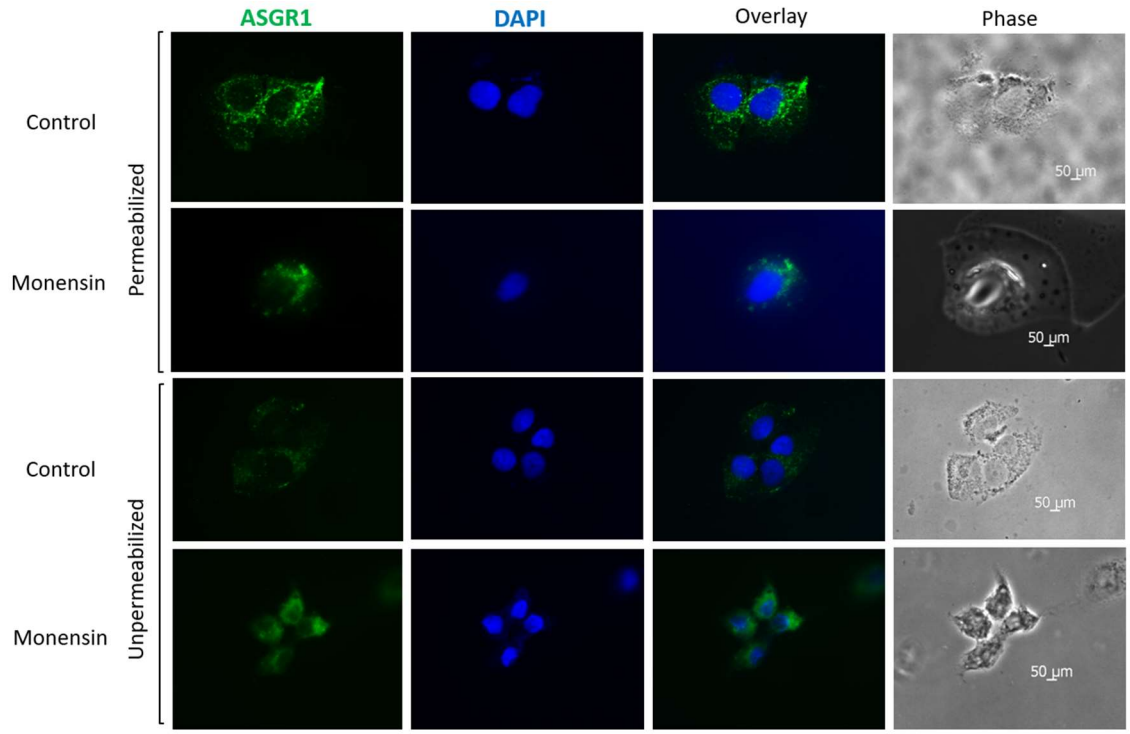


Figure 4. Expression of NEU1, ASGR1 and LDLR protein expression and secretion in HepG2 cells with increased exposure to monensin treatment. A) HepG2 cell lysates were separated by SDS-PAGE (10%) after monensin treatment of 0, 6, 12 or 24 hours (3µM)(Lanes 1-4). Cell media was collected and concentrated using protein concentration columns for each respective treatment (Lanes 5-8). Blotting was performed using anti-LDLR, anti-ASGR1, anti-NEU1 antibodies and anti-GAPDH as a loading control. Densitometry of immunoblot bands for LDLR/GAPDH (B), ASGR1/GAPDH (C) and NEU1/GAPDH (D) demonstrate accumulation of LDLR and NEU1 inside cells with monensin treatment while secretion of NEU1 appears to be increased by 24 hours treatment. In addition, a decrease in the expression level of mature ASGR1 and accumulation of a truncated ASGR1 isoform (*) was apparent after 24h of monensin treatment. Representative blots for n=2 for each group

3.4 Monensin mediated effects of ASGR1 expression and distribution

To further understand how ASGR1 respond to monensin treatment which inhibits recycling from the early endosome, their intracellular localization was analyzed through immunocytochemistry after 24 hours of treatment (Fig. 5). Cells were either permeabilized with Triton-X to observe total cell ASGR1 or left unpermeabilized to assess cell surface ASGR1. With exposure to monensin, ASGR1 appeared to accumulate within cells and staining was more clustered when compared to punctate perinuclear staining in the control (Fig. 5A). When compared to the punctate staining in unpermeabilized control samples, cells treated with monensin had a more diffuse and even staining pattern. Staining intensity was quantified by rating 100 cells from each treatment group on a scale from low to high intensity staining (Fig. 6). For staining of the total HepG2 cell, untreated cells (UP) were most frequently rated at an intensity of 3, and when compared to those exposed to monensin (MP), significantly more cells were rated at an intensity of 4, along with significantly less cells rated at an intensity of 1 (Fig. 5B). Untreated cells stained with anti-ASGR1 on the cell surface (UU) had a median rating of 2, while those treated with monensin had significantly more cells rated at a 1 or a 2. These results indicate that ASGR1 is accumulating inside the cell and decreased on the cell surface in response to monensin treatment suggesting active recycling of the ASGR1 protein.

A


ASGR1
DAPI
Overlay
Phase
Control
Monensin
Permeabilized
Control
Monensin
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50 µm
50 µm
50 µm
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B

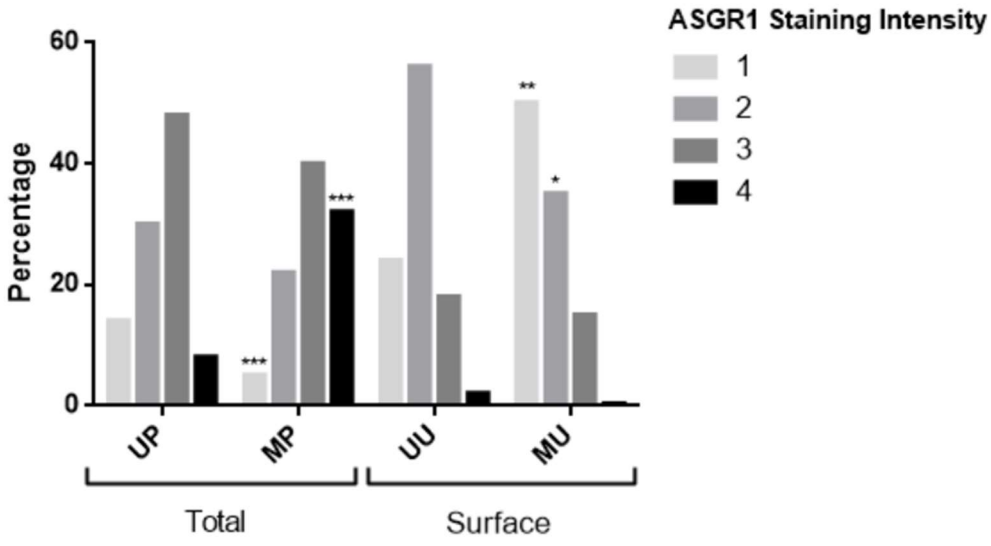

ASGR1 Staining Intensity
1
2
3
4
Percentage
60
40
20
0
UP
MP
UU
MU
Total
Surface
***
***
**
*

Figure 5. Cell surface and total ASGR localization and expression in response to monensin treatment A) Localization of ASGR1 protein in HepG2 cells by immunocytochemistry. HepG2 cells were grown on glass coverslips, and permeabilized with Triton-X or left unpermeabilized to stain the cell surface. Cells were stained with anti-ASGR1 antibody and nuclear counterstain DAPI after 24 hour monensin treatment (3μM). Permeabilized control cells showed punctate perinuclear staining compared to a more intense clustered perinuclear staining with monensin treatment. ASGR1 on the cell surface of control cells was weaker with membranous punctations. With monensin treatment staining was more even and diffuse B) Quantification of ASGR1 staining intensity in HepG2 cells. n=100 cells were rated on a 1-4 scale from low to high intensity. Increased ASGR1 staining inside cells and decreased cell surface ASGR1 after 24 hour monensin treatment. Monensin treatment appears to impair transport of ASGR1 to the cell surface as it remains trapped inside the cell. Chi-square goodness of fit test, all groups compared to untreated control cells. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

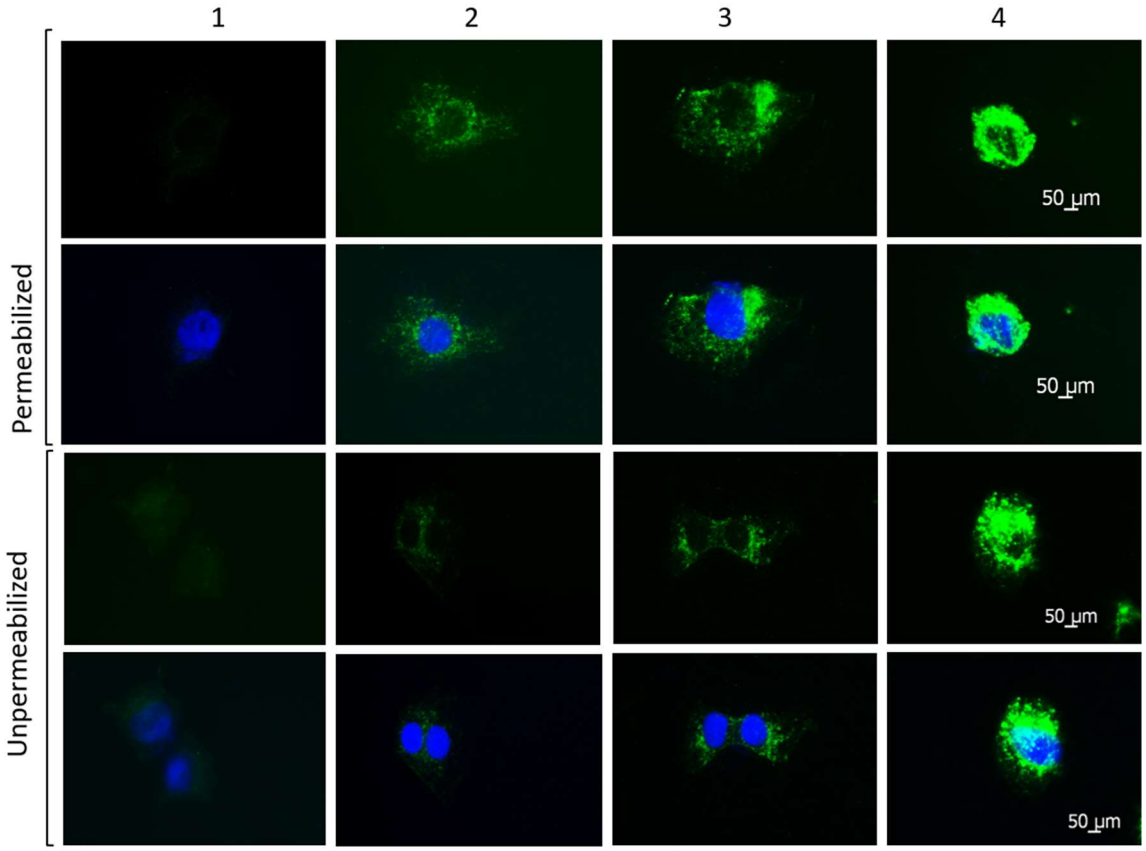


Figure 6. Representative images of ASGR1 staining intensity in permeabilized and unpermeabilized HepG2 cells, ranging from low (1) to high (4). HepG2 cells were grown on glass coverslips, and were either permeabilized with Triton-X or left unpermeabilized to stain the cell surface. Cells were stained with anti-ASGR1 antibody and nuclear counterstain DAPI.

3.5 Effect of Chloroquine on protein expression

In addition to the chemical inhibitor monensin which is an endosomal recycling inhibitor, the dynamics of NEU1, LDLR and ASGR1 were observed in response to chloroquine, an inhibitor of endosomal acidification and lysosomal maturation (does not affect recycling). HepG2 cells were treated with chloroquine for 0, 6, 12 or 24 hours and both cell lysates and media were collected to analyze protein expression. Western blot analysis was used to observe levels of LDLR, ASGR1 and NEU1 (Fig. 7A). After 24 hours exposure to chloroquine, expression of LDLR, ASGR1 and NEU1 were all increased (Fig. 7B-D). Furthermore, NEU1 was detected in the media starting at 6 hours of treatment and increasing over time.

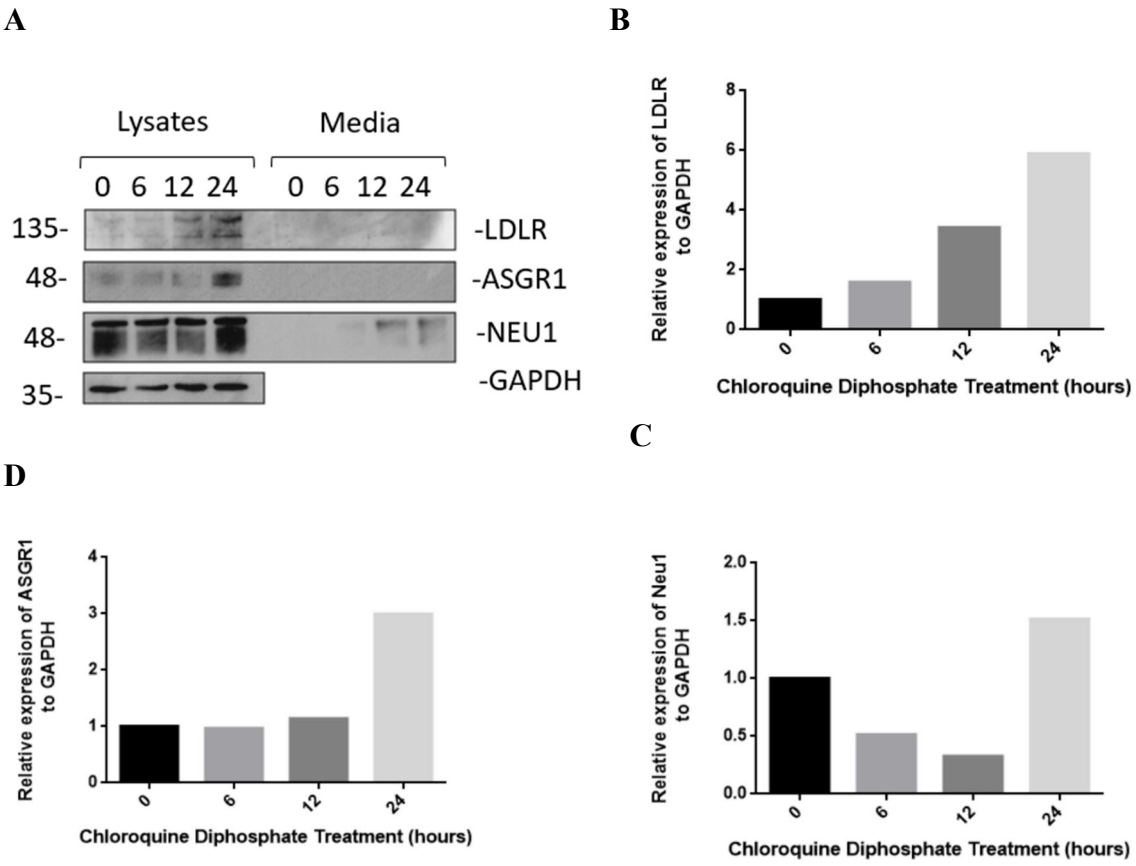
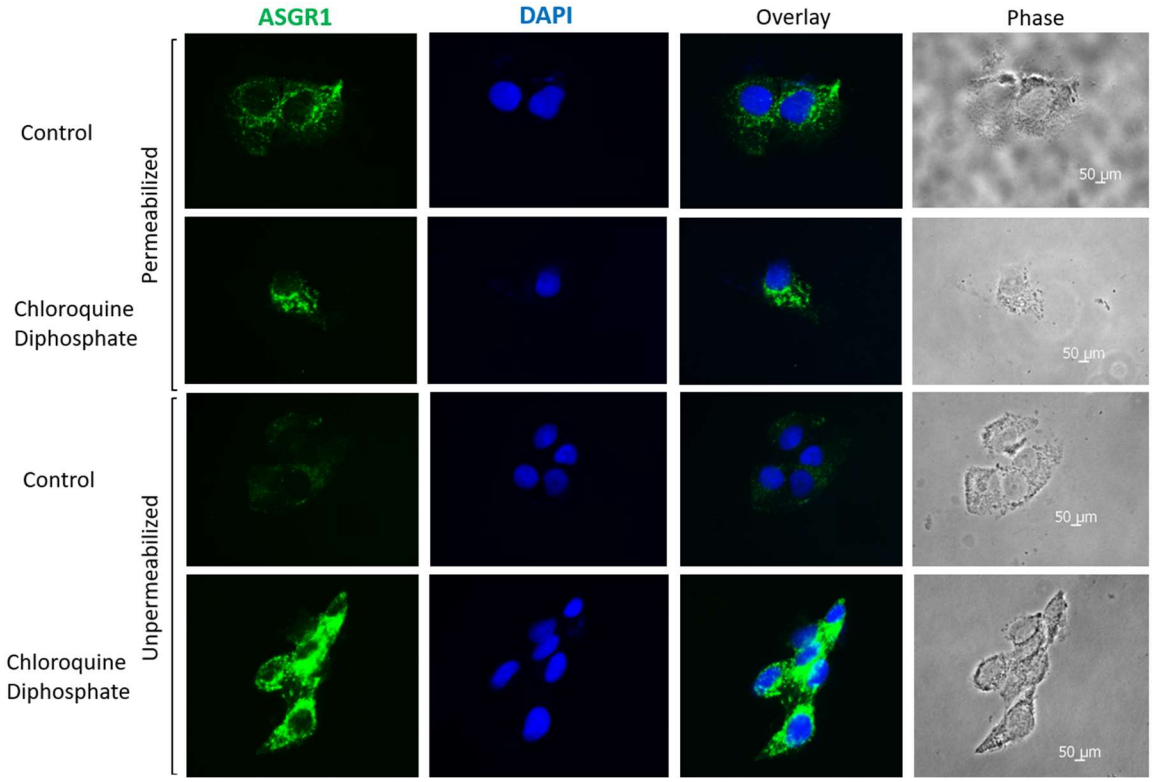


Figure 7. ASGR1, LDLR and NEU1 response to inhibition of lysosomal maturation. A) Western blot analysis of NEU1, ASGR1 and LDLR protein expression and secretion in HepG2 cells with increased exposure to chloroquine treatment. HepG2 cell lysates were separated by SDS-PAGE (10%) after chloroquine treatment of 0, 6, 12 or 24 hours (10μM)(Lanes 1-4). Cell media was collected and concentrated using protein concentration columns for each respective treatment (Lanes 5-8). Blotting was performed using anti-LDLR, anti-ASGR1, anti-NEU1 antibodies and anti-GAPDH as a loading control. Densitometry of immunoblot bands was measured using ImageJ and normalized to GAPDH. Accumulation of LDLR (B), ASGR1 (C) and NEU1 (D) with Chloroquine treatment. Secretion of NEU1 appears to be increased by 24 hours treatment. The results indicate that these proteins are behaving in conjunction and therefore may be associated. Representative blots for n=2 for each group.

3.6 ASGR1 localization and expression altered by chloroquine

The effects of chloroquine were further analyzed using immunocytochemical localization of ASGR1 (Fig. 8). HepG2 cells were cultured and treated with chloroquine for 24 hours. In treated permeabilized cells, both perinuclear and cytoplasmic clusters of staining were seen compared to punctate perinuclear staining in untreated cells. In unpermeabilized cells, a chloroquine induced massive accumulation of ASGR1 on the cell membrane in contrast to a more even punctate pattern in the untreated cells (Fig.8A). Quantification of staining intensity in unpermeabilized cells showed a significant increase of ASGR1 both for total cell and cell surface (Fig. 8B). Cells treated with chloroquine showed a significant increase in cells with a staining intensity rating of 4 and a significant decrease of cells rated at 1 and 2 compared to the control. A very significant increase in cells rated at an intensity of 4 for ASGR1 staining on the cell surface was present with treatment. These results are consistent with the increase in ASGR1 expression observed by Western blot analysis.

A


ASGR1
DAPI
Overlay
Phase
Control
Chloroquine Diphosphate
Permeabilized
Control
Chloroquine Diphosphate
Unpermeabilized
50 µm

B

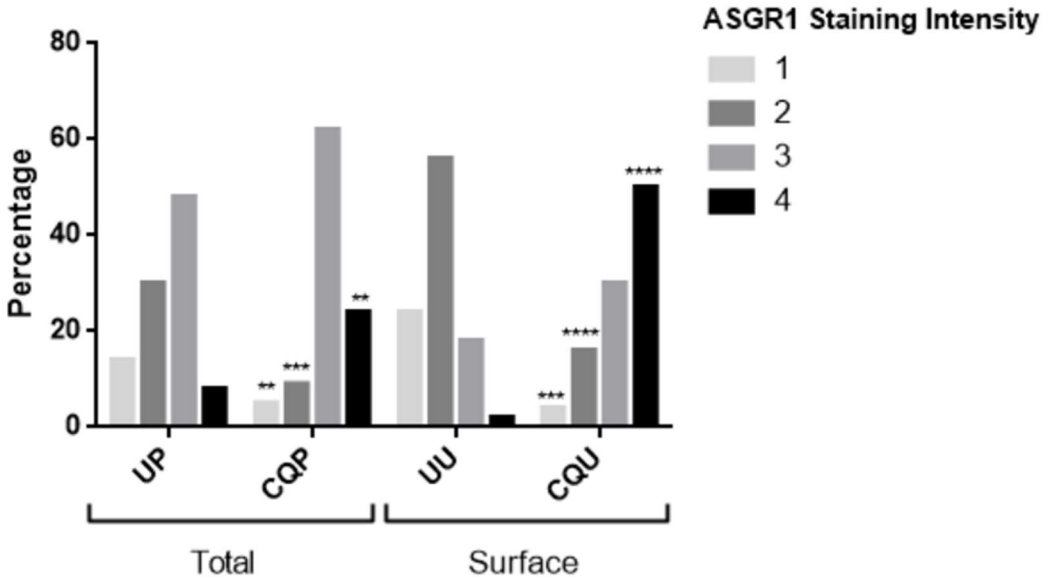

ASGR1 Staining Intensity
1
2
3
4
Percentage
80
60
40
20
0
UP
CQP
UU
CQU
Total
Surface

Figure 8. A) Localization of ASGR1 protein in HepG2 cells by immunocytochemistry after 24h chloroquine treatment. HepG2 cells were grown on glass coverslips, and either permeabilized with Triton-X or left unpermeabilized were stained with anti-ASGR1 antibody and nuclear counterstain DAPI after 24 hours chloroquine treatment (10μM). Permeabilized cells treated with chloroquine showed perinuclear and cytoplasmic clusters of staining compared to more punctate perinuclear staining in the control. On the cell surface massive accumulations of staining were observed compared to much weaker punctate pattern in untreated cells. B) Quantification of ASGR1 staining intensity in HepG2 cells. n=100 cells were rated on a 1-4 scale from low to high intensity. Significant increase of ASGR1 staining inside cells. Chloroquine induced massive accumulation of ASGR1 to membrane. Chi-square goodness of fit test, all groups compared to untreated control cells. **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

3.7 NEU1/LDLR/ASGR1 association analysis

Since previous experiments suggested that NEU1, ASGR1 and LDLR are behaving similarly, we wanted to establish whether this was through a physical association. To analyze if potential associations were occurring at the membrane or in the soluble fraction, HepG2 cells were grown and treated with or without monensin for 24 hours and fractionated into subcellular compartments. Co-immunoprecipitation and western blot allowed the isolation of proteins of interest and potential binding partners. At the cell membrane, no apparent association was seen between NEU1, ASGR1 or LDLR (Fig. 9A). Interestingly, at the membrane both full length and truncated ASGR1 were detected with monensin treatment, as seen in previous experiments on total cell lysates. When analyzing soluble proteins, LDLR is pulled down with NEU1 both with and without monensin (Fig. 9B).

HepG2 cell membrane proteins were further analyzed through immunoprecipitation with the addition of MG132 to both the control and monensin treatment groups (Fig. 10). This was included to prevent degradation of membrane proteins by the proteasome. Again, no detectable association between NEU1, LDLR and ASGR1 was present at the membrane.

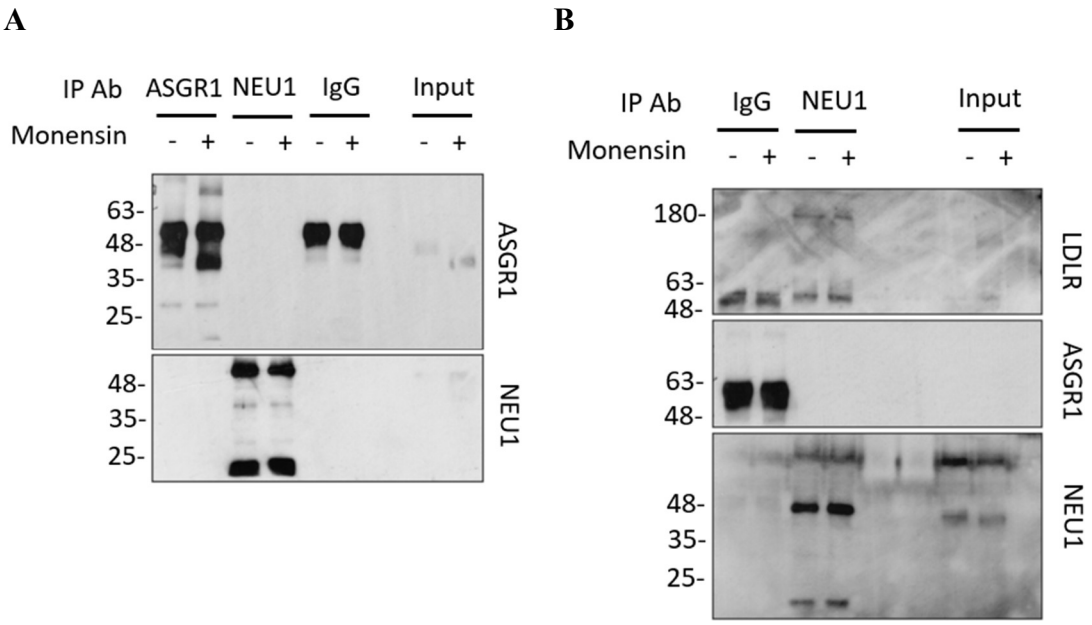


Figure 9. Analysis of protein interactions in HepG2 cells with monensin treatment. Co-immunoprecipitation of membrane (A) and soluble (B) proteins from HepG2 cells with anti-ASGR1, anti-NEU1 or rabbit IgG as a control. Cells were treated with monensin for 24 hours. Lysates were separated by SDS-PAGE (10%) and blotted for anti-ASGR1, anti-NEU1 and anti-LDLR. Monensin-mediated fragmentation of ASGR1 is present at the membrane. While no apparent association of ASGR1 with NEU1 at the cell membrane. LDLR and NEU1 appear to co-precipitate from the cytosol extract.

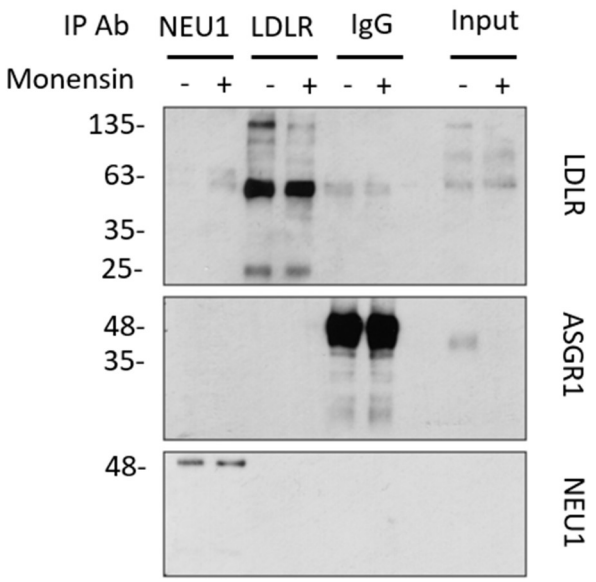


Figure 10. Analysis of protein interactions in HepG2 cells with monensin and MG132 treatment. Membrane proteins from HepG2 cells immunoprecipitated with anti-NEU1, anti-LDLR or rabbit IgG as a control. Cells were treated with or without monensin for 24 hours in the presence of MG132 (1μM). Lysates were separated by SDS-PAGE (10%) and blotted for anti-ASGR1, anti-NEU1 and anti-LDLR. Reduced LDLR expression with monensin treatment at the membrane. No apparent association between NEU1, LDLR and ASGR1 at the membrane.

Chapter 4: Discussion

4.1 NEU1 overexpression in HepG2

This experiment involved the use of three different multiplicity of infection values (MOI) to obtain an optimal expression of his-NEU1 virus in HepG2 cells for NEU1 overexpression. While a MOI of 0.1 appeared to be inadequate to show successful transduction, RGS-His was found to be expressed at a MOI of 10 and 100. At a MOI of 100, cell viability seems to be affected, with expression of both LDLR and ASGR1 reduced to levels below that of the control. At a MOI of 10, western blot analysis of HepG2 cells overexpressing NEU1 revealed this increase is correlated with an increase of both ASGR1 and LDLR protein levels (Fig. 2). These results could indicate that in the presence of NEU1 overexpression, the elevated expression of ASGR1 is a result of a direct interaction of these two proteins. However, co-immunoprecipitation analysis in later experiments suggests that this is not the case (Fig. 9, 10). More likely the overexpression of NEU1 is providing more substrate for ASGR. More NEU1 present in the cell would allow glycoproteins to be desialylated and therefore available for ASGR to bind and degrade. An upregulation of ASGR may be an attempt to offset this additional substrate. No previous studies have looked at the effects of manipulating NEU1 on ASGR. It would be meaningful to assess the expression, activity and localization of NEU1 in B6.SM mice, in which NEU1 expression and activity are significantly reduced (Yang et al., 2012).

In addition to elevated ASGR1, LDLR protein levels simultaneously increased with NEU1 overexpression. This result may indicate that NEU1 is allowing the stabilization of LDLR. Previous studies that have demonstrated NEU1 as an important regulator of receptor-mediated signaling. At the plasma membrane, NEU1 de-sialylates and activates receptors involved in inflammation, immunity, and phagocytosis (Pshezhetsky and Ashmarina 2013). Desialylation of TLR4 by Neu1 has been shown to be essential for the activation of this receptor in macrophages and dendritic cells (Amith et al., 2010). In contrast to our experiments where overexpression of Neu1 leads to stabilization of asialylated LDLR, researchers have suggested that sialylated LDLR is more stable and less prone to recycling, as seen in studies done in hypomorphic NEU1 mice (Yang et al., 2012). It is thought that in its asialylated form, LDLR interacts with ASGR, leading to endocytic recycling (Nioi et al., 2016). Since in our experiment levels of both LDLR and ASGR increase with Neu1 overexpression, an alternate explanation could be that ASGR is allowing stabilization of LDLR at the cell surface (Fig. 11). This hypothesis is further supported when looking at knockdown of ASGR1.

4.2 Effect of ASGR1 Knockdown on NEU1 and LDLR

Knockdown experiments of ASGR1 in hepatoma cells show a subsequent decrease in LDLR expression through western blot analysis, with NEU1 expression unaffected (Fig. 3). This result indicates LDLR is destabilized in the absence of ASGR. This provides further evidence that these proteins are associated with one another. It has

been reported that loss of function (LOF) variants of ASGR1 are associated with decreased LDL cholesterol (Nioi et al., 2016). In the case where ASGR is stabilizing LDLR, perhaps interactions between ASGR and LDLR impair uptake of LDL by LDLR, explaining the observation that patients with LOF ASGR1 would be able to take up more LDL into the liver, decreasing serum cholesterol levels.

Lastly, Neu1 expression is unaltered in HepG2 cells with reduced ASGR1 expression. This has been previously observed in mice lacking the ASGR1 subunit with no difference in Neu1 expression or activity (Yang et al., 2015). While ASGR is dependent on the action of Neu1, by providing substrates through desialylation of glycoproteins, NEU1 expression is not dependent on ASGR.

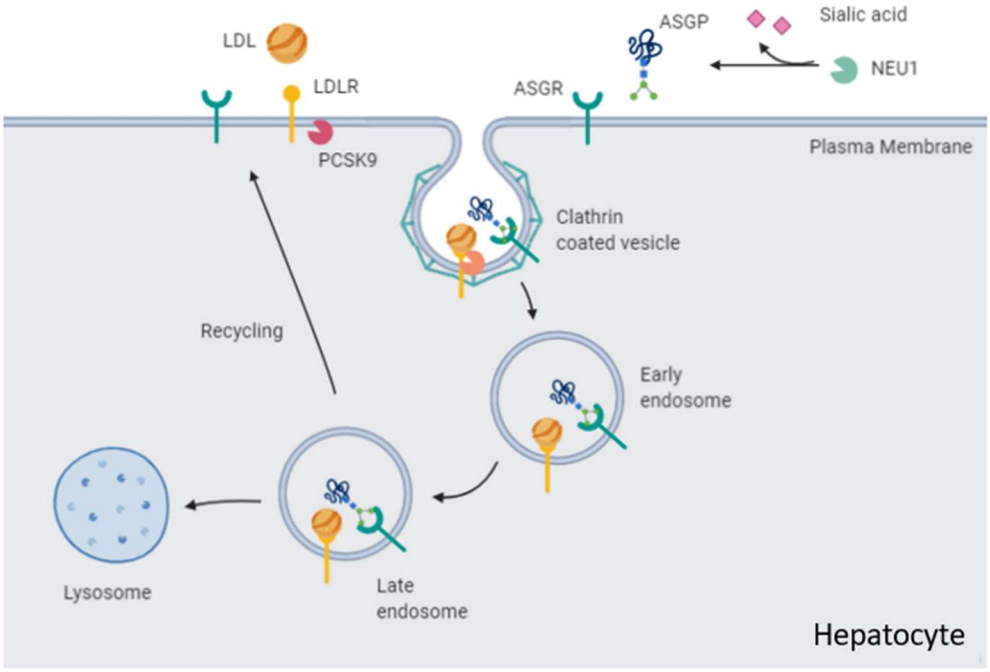


Figure 11. Proposed mechanism for association of ASGR, LDLR and NEU1 in HepG2 cells. NEU1 allows stabilization of ASGR and LDLR on the cell surface. NEU1 provides substrates for ASGR, leading to increased ASGR expression. Presence of ASGR on the cell surface allows stabilization of LDLR.

4.3 Effects of Monensin on ASGR1

The expression of ASGR1 protein was analyzed in response to the inhibitor monensin, which blocks recycling to the membrane. Western blot analysis of total HepG2 cell lysates was analyzed with increasing exposure to monensin (Fig. 4). A gradual decrease in levels of full length ASGR1 was observed with a lower molecular weight isoform of ASGR1 accumulating by 24 hours of treatment. This truncated ASGR1 is also present at the membrane, as seen in immunoprecipitation of HepG2 cell membrane proteins (Fig. 9).

The presence of truncated ASGR1 with treatment reveals that blocking recycling through monensin leaves ASGR1 susceptible to cleavage. The possibility of changes in glycosylation explaining the appearance of truncated ASGR1 is not likely as LDLR which is also a glycosylated protein remained unaffected, therefore serving as a positive control. Experiments involving the inhibitor chloroquine, in which lysosomal degradation is also blocked but recycling to the membrane is not, did not show the accumulation of truncated ASGR1 (Fig. 12). This indicates that this isoform is not a product of being directed to the lysosome, but is associated with recycling.

Quantification of immunolabelling showed increased ASGR1 staining inside cells and decreased cell surface ASGR1 with treatment. This agrees with previous studies in Huh7 hepatocytes which showed a reduction in surface ASGR expression with monensin treatment (Stockert, 1995). These results indicate that monensin treatment traps ASGR1 inside the cell and impairs transport of ASGR1 to the cell surface. Trapping of ASGR1 in

endosomes leaves ASGR1 susceptible to cleavage into the new isoform. Immunoprecipitation of HepG2 cells membrane protein brought down truncated ASGR1, indicating it is a transmembrane and not a soluble protein. Therefore, this is evidence that ASGR1is cleaved at the endosome. Endosomal proteases are known to both activate and inactivate internalized proteins through cleavage (Authier et al., 1996). Two possibilities exist as to which portion of ASGR1 is being cleaved off. In the case where ASGR1 is cleaved at the cytoplasmic tail, this would account for a 4.7 kDa loss in molecular weight. The other more likely possibility is that this ASGR1 isoform is lacking a portion of the lectin binding site in the extracellular domain. This could be validated through isolation and analysis of this ASGR1 isoform. Interestingly, it is possible that the tip of the lectin binding site is cleaved in endosomes before recycling and secretion to prevent binding of glycoproteins extracellularly.

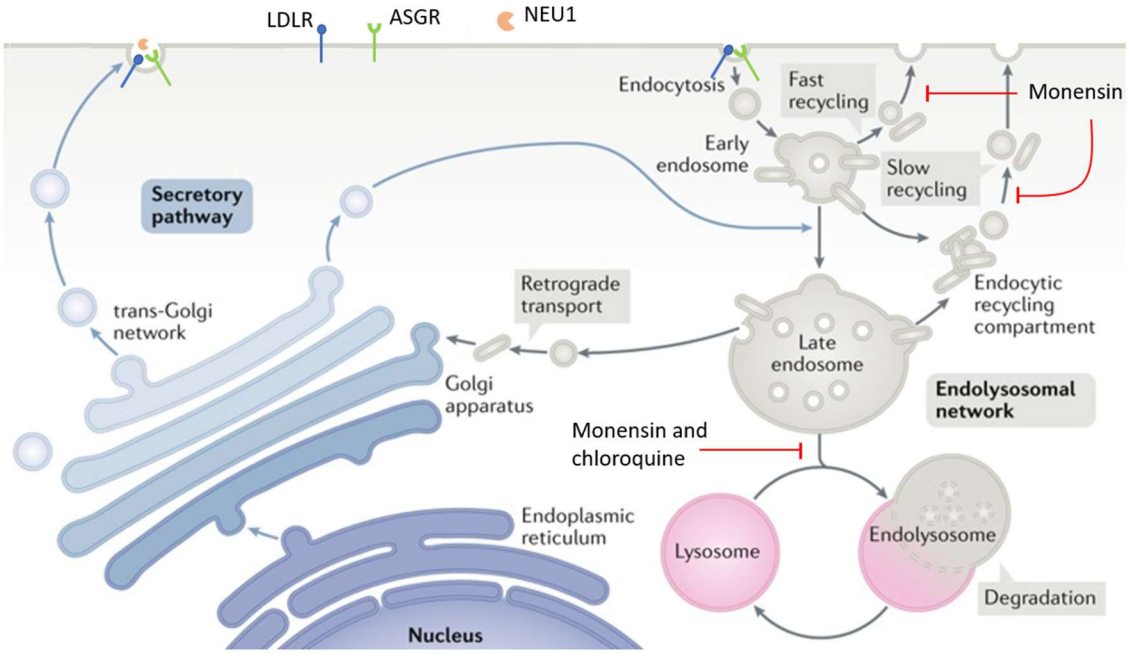


Figure 12. Endolysosomal pathways blocked through monensin and chloroquine. Monensin blocks slow and fast recycling. Both chloroquine and monensin block formation of endolysosome and degradation. (Modified from Cullen and Steinberg, 2018)

4.4 Monensin's effect on LDLR and NEU1

At 24 hours of exposure to monensin, protein levels of LDLR in total HepG2 cell lysates were increased by 3 fold when compared to the control (Fig. 4). Previous work has shown that monensin inhibits recycling of LDLR to the membrane but does not alter total cell expression (Minahk, 2008). Researchers used primary mouse hepatocytes which were treated with monensin for just 1 hour before analysis, whereas in the current study, increased LDLR expression was not evident until 24 hours treatment. Since monensin inhibits recycling to the plasma membrane, this results in the accumulation of LDLR intracellularly.

Expression of NEU1 was increased not only within the cell but was shown to be secreted and detected in the media in increasing amounts with increasing exposure to monensin. Monensin has been previously shown to be responsible for stimulating release of other lysosomal enzymes in macrophages, including β-glucuronidase, β-hexosaminidase and β -galactosidase (Takano et al., 1984). Notably, the secretion of these enzymes required a high concentration of monensin and was proportional to the concentration in this study. Researchers attributed this observation to increased cellular pH. Trafficking of lysosomal enzyme from the Golgi complex to lysosomes is mediated through receptor transport. Interrupting dissociation of receptors and enzymes which requires an acidic environment could lead to mistransport of these enzymes to the membrane (Fig. 13).

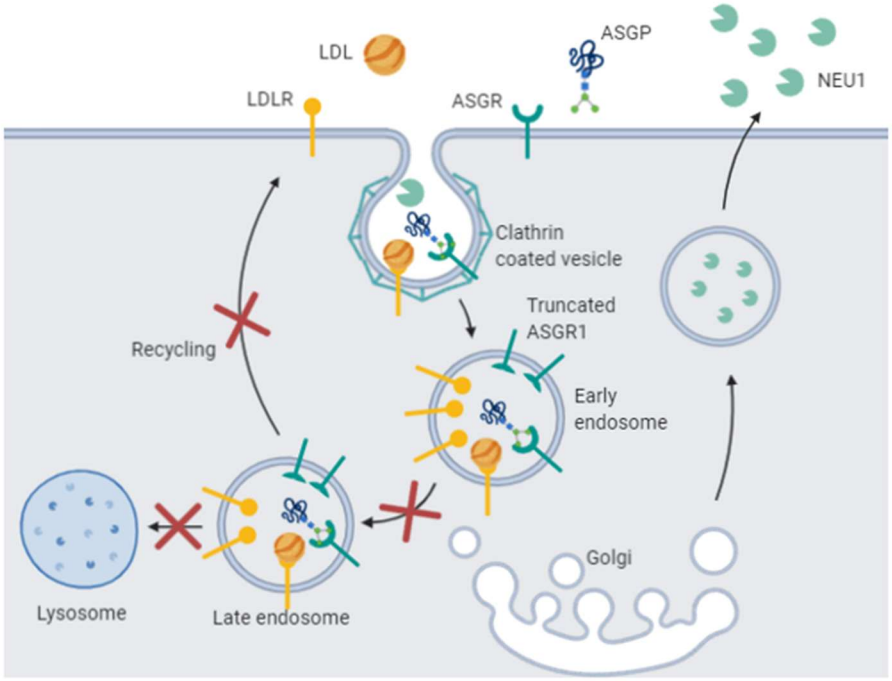


Figure 13. Proposed mechanisms and effect of monensin on ASGR1, LDLR and NEU1. Lower molecular weight ASGR1 is present with monensin treatment. Blocking of recycling may leave ASGR1 susceptible to proteolysis. Trapping of ASGR1 inside the cell and impaired transport to the cell surface result in increased total cell expression of ASGR1 and reduced cell surface ASGR1. Increased levels of LDLR are observed as a result of inhibition of endocytosis and therefore reduced degradation by lysosomes. NEU1 expression and secretion is increased due to interrupted transport to lysosomes and increased trafficking to the membrane.

4.5 Effects of Chloroquine on ASGR1

The consequences of chloroquine treatment on ASGR1 expression was analyzed through Western blot and immunocytochemistry (Fig. 7, 8). After 24 hours exposure to chloroquine, expression of ASGR1 in total HepG2 cell lysates was found to be increased. Immunostaining of ASGR1 showed that while ASGR1 levels were increased for total cell staining, there was also a very significant accumulation of ASGR1 seen on the cell surface.

Chloroquine has been proven to act through both blocking fusion of endosomes and lysosomes in addition to impairing receptor-mediated endocytosis (Steinman et al., 1983, Wang et al., 1993). By blocking these two processes, there will be a reduction of endocytic transfer of ASGR present on the cell surface, and ASGR that is on the path to degradation will fail to be processed and broken down by lysosomes, resulting in accumulations both inside the cell and on the membrane. Previous studies that have looked at the effects of chloroquine on ASGR in HepG2 cells through pulse and chase experiments concluded there is a reduction in surface ASGR due to impaired ASGR recycling (Schwartz et al., 1984). The researchers treated cells with chloroquine for 30 minutes before chasing labelled ASGR over a 2 hour period, in contrast to the 24 hour treatment used in our experiments as well as analyzing ASGR levels as a steady state. Therefore, longer exposures to monensin allow accumulation of ASGR inside and on the cell surface (Fig. 14).

4.6 LDLR and NEU1 response to Chloroquine

Western blot analysis was also used to analyze effects of chloroquine treatment on LDLR and NEU1 expression (Fig. 7). Expression of both LDLR and NEU1 were elevated after 24 hour chloroquine treatment. As a result of blocking lysosomal maturation and endosomal acidification, degradation of these proteins was impaired, resulting in their accumulation in the cell. Previous work has also shown that chloroquine inhibits recycling of LDLR to the membrane without altering total cell expression with 1 hour chloroquine treatment (Minahk 2008). Since chloroquine treatment was only incubated for 1 hour, this demonstrates the accumulation of LDLR may not come to fruition unless exposed to chloroquine for longer. Increased LDLR expression was not evident until 6 hours chloroquine treatment in the present study.

In addition to elevated NEU1 expression, NEU1 was detected in the media at 6, 12 and 24 hours chloroquine treatment, with increasing amounts accumulating over time. Previous work in fibroblasts have noted that chloroquine enhances the secretion of ß-hexosaminidase, possibly by depleting free receptors involved in enzyme transport (Gonzalez-Noriega et al., 1980). This leads to failure of lysosomal enzymes to be segregated into lysosomes and therefore released into the media (Fig. 14). This may also explain the effects of chloroquine on NEU1 secretion through inability to be compartmentalized through M6P receptors for example, which have been shown to have altered distribution in response to chloroquine (Mauthe et al., 2018). Another possibility could be the targeting of NEU1 to the plasma membrane by sorting to major histo-

compatibility complex II-positive vesicles, which merge with the plasma membrane, as seen in THP-1 macrophages (Liang et al., 2006).

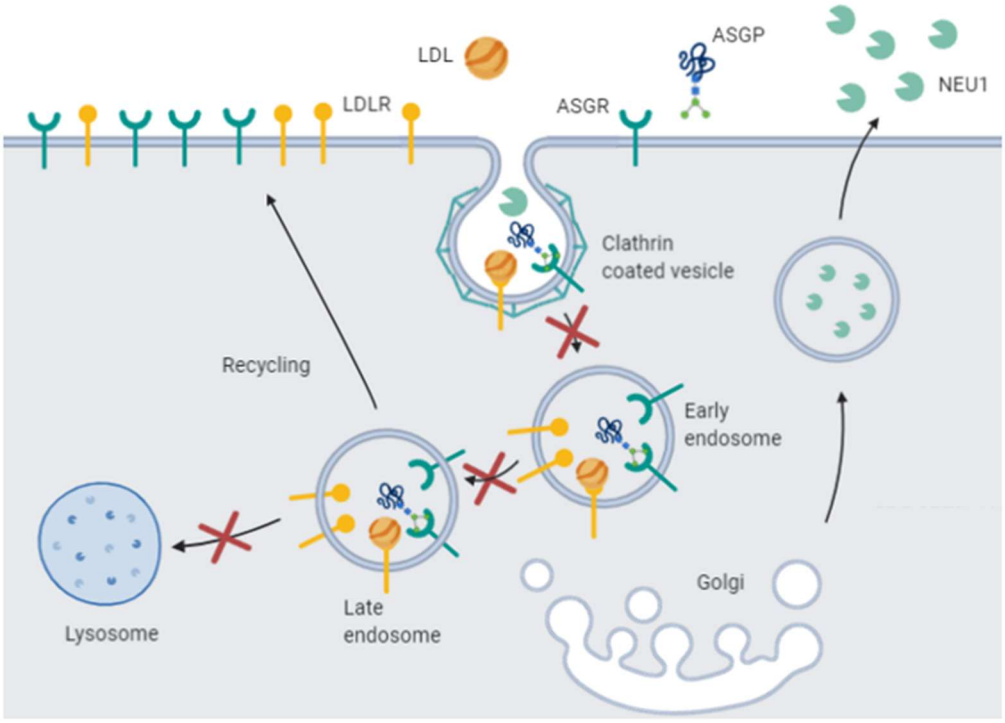


Figure 14. Effect of chloroquine on LDLR, ASGR1 and NEU1 and proposed mechanisms. Chloroquine is known to block endosome-lysosome fusion and impair clathrin-meditated endocytosis. Increased total cell expression of LDLR and ASGR1 as a result of failed degradation by lysosomes, causing accumulation in endocytic vesicles. Increased surface expression of ASGR1 and LDLR expression are observed due to impaired endocytosis. NEU1 accumulation and secretion due to failure of compartmentalization to lysosomes followed by mistransport to the membrane.

4.7 NEU1/LDLR/ASGR1 association analysis

Past and current research indicate that NEU1, ASGR1, and LDLR are somehow associated. Therefore, immunoprecipitation analysis was used to asses whether this interaction was through a physical association by isolating these proteins and their potential binding partners. At the membrane, no apparent association was seen between NEU1, ASGR1, or LDLR. This same result was seen after addition of MG132, indicating that it is not proteasomal degradation of ASGR and LDLR that accounts for the lack of detectable co-precipitation of ASGR1 with LDLR or NEU1. The results indicate that these proteins are not detectably interacting at the HepG2 cell membrane.

A recent study investigated interaction partners of NEU1 in macrophages, identifying interactions between NEU1 and CD36 at the plasma membrane (Kawecki et al., 2019). CD36 is a scavenger receptor and plays a role in many diseases, including atherosclerosis, playing roles in inflammation, foam cell formation and thrombosis. The study also identified many candidates of interaction partners, including proteins localized to the membrane, cytoskeleton and cytoplasm, with some noted to be involved in vesicle trafficking. Analysis of soluble proteins in HepG2 cells showed that LDLR is pulled down with NEU1 both with and without monensin. This indicates that these may be interacting as they co-precipitated. As LDLR is not a soluble protein, this could indicate membrane contamination in the soluble fraction.

Conclusion

Recently, the notion that ASGR plays a role in atherosclerosis and lipid metabolism was suggested, however the mechanisms behind this effect had not been investigated. The current study sought to understand the possibility of NEU1 regulating interactions between ASGR and LDLR. Previous studies in our laboratory has shown that NEU1 down regulation leads to LDLR stabilization via reduced interactions with PCSK9. Our current results suggest that NEU1 over expression allows stabilization of LDLR through interactions with ASGR. This study solidifies ASGR as a novel pathway for the turnover of LDLR and helps understand the link between ASGR and atherosclerosis. To better understand the implications of these results, future studies could address how NEU1 and ASGR play a role in LDL uptake. This could be investigated by assessing the level of LDL uptake by HepG2 cells in the presence or absence of ASGR and the presence or absence of Neu1. HepG2 cell could be treated with fluorescently labelled LDL or unlabelled LDL and cells could be imaged to observe the level of LDL uptake. Additionally, these studies could be extended to an *in vivo* model by assessing LDL uptake in ASGR knockout mice or hypomorphic NEU1 mice.

References

Al-Bari, M. A. A. (2015). Chloroquine analogues in drug discovery: new directions of uses, mechanisms of actions and toxic manifestations from malaria to multifarious diseases. *Journal of Antimicrobial Chemotherapy, 70*(6), 1608-1621. doi: 10.1093/jac/dkv018

Allain, P., Olivier, E., Le Bouil, A., Benoit, C., Geslin, P., & Tadei, A. (1996). [Increase of sialic acid concentration in the plasma of patients with coronary disease]. *Presse Med, 25*(3), 96-98.

Amith, S. R., Jayanth, P., Franchuk, S., Finlay, T., Seyrantepe, V., Beyaert, R., . . . Szewczuk, M. R. (2010). Neu1 desialylation of sialyl alpha-2,3-linked beta-galactosyl residues of TOLL-like receptor 4 is essential for receptor activation and cellular signaling. *Cell Signal, 22*(2), 314-324. doi: 10.1016/j.cellsig.2009.09.038

Authier, F., Posner, B. I., & Bergeron, J. J. (1996). Endosomal proteolysis of internalized proteins. *FEBS Lett, 389*(1), 55-60. doi: 10.1016/0014-5793(96)00368-7

Biggerstaff, K. D., & Wooten, J. S. (2004). Understanding lipoproteins as transporters of cholesterol and other lipids. *Adv Physiol Educ, 28*(1-4), 105-106. doi: 10.1152/advan.00048.2003

Chaudhary, R., Garg, J., Shah, N., & Sumner, A. (2017). PCSK9 inhibitors: A new era of lipid lowering therapy. *World journal of cardiology*, *9*(2), 76–91. doi:10.4330/wjc.v9.i2.76

Chen, X., & Varki, A. (2010). Advances in the biology and chemistry of sialic acids. *ACS Chem Biol, 5*(2), 163-176. doi: 10.1021/cb900266r

Cullen, P.J., Steinberg, F. (2018). To degrade or not to degrade: mechanisms and significance of endocytic recycling. *Nat Rev Mol Cell Biol* 19, 679–696 doi:10.1038/s41580-018-0053-7

Das S., Kudale P., Dandekar P., Devarajan P.V. (2019) Asialoglycoprotein Receptor and Targeting Strategies. In: Devarajan P., Dandekar P., D'Souza A. (eds) Targeted Intracellular Drug Delivery by Receptor Mediated Endocytosis. AAPS Advances in the Pharmaceutical Sciences Series, vol 39. Springer, Cham

Davis, B. T., Wang, X. J., Rohret, J. A., Struzynski, J. T., Merricks, E. P., Bellinger, D. A., . . . Rogers, C. S. (2014). Targeted disruption of LDLR causes hypercholesterolemia and atherosclerosis in Yucatan miniature pigs. *PLoS One, 9*(4), e93457. doi: 10.1371/journal.pone.0093457

Dhaliwal, A. (2013). Activators and Inhibitors in Cell Biology Research. *Materials and Methods, 3*. doi: 10.13070/mm.en.3.185

Dickson, R. B., Willingham, M. C., & Pastan, I. H. (1982). Receptor-mediated endocytosis of alpha 2-macroglobulin: inhibition by ionophores and stimulation by Na+ and HCO3(-). *Ann N Y Acad Sci, 401*, 38-49. doi: 10.1111/j.1749-6632.1982.tb25705.x

Glass, C. K., & Witztum, J. L. (2001). Atherosclerosis. the road ahead. *Cell, 104*(4), 503-516.

Goedeke, L., & Fernandez-Hernando, C. (2012). Regulation of cholesterol homeostasis. *Cell Mol Life Sci, 69*(6), 915-930. doi: 10.1007/s00018-011-0857-5

Gonzalez-Noriega, A., Grubb, J. H., Talkad, V., & Sly, W. S. (1980). Chloroquine inhibits lysosomal enzyme pinocytosis and enhances lysosomal enzyme secretion by impairing receptor recycling. *J Cell Biol, 85*(3), 839-852. doi: 10.1083/jcb.85.3.839

Grefhorst, A., McNutt, M. C., Lagace, T. A., & Horton, J. D. (2008). Plasma PCSK9 preferentially reduces liver LDL receptors in mice. *J Lipid Res, 49*(6), 1303-1311. doi: 10.1194/jlr.M800027-JLR200

Gupta, A. (2012). Asialoglycoprotein Receptor and the Macrophage Galactose-Type Lectin *Animal Lectins: Form, Function and Clinical Applications* (pp. 709-724). Vienna: Springer Vienna.

Haq, M., Haq, S., Tutt, P., & Crook, M. (1993). Serum total sialic acid and lipid-associated sialic acid in normal individuals and patients with myocardial infarction, and their relationship to acute phase proteins. *Ann Clin Biochem, 30 (Pt 4)*, 383-386. doi: 10.1177/000456329303000406

Harford, J., Wolkoff, A. W., Ashwell, G., & Klausner, R. D. (1983). Monensin inhibits intracellular dissociation of asialoglycoproteins from their receptor. *J Cell Biol, 96*(6), 1824-1828. doi: 10.1083/jcb.96.6.1824

Harris, R. L., van den Berg, C. W., & Bowen, D. J. (2012). ASGR1 and ASGR2, the Genes that Encode the Asialoglycoprotein Receptor (Ashwell Receptor), Are Expressed in Peripheral Blood Monocytes and Show Interindividual Differences in Transcript Profile. *Mol Biol Int, 2012*, 283974. doi: 10.1155/2012/283974

Hegele, R. A. (2009). Plasma lipoproteins: genetic influences and clinical implications. *Nat Rev Genet, 10*(2), 109-121. doi: 10.1038/nrg2481

Hoofnagle, A. N., & Heinecke, J. W. (2009). Lipoproteomics: using mass spectrometry-based proteomics to explore the assembly, structure, and function of lipoproteins. *J Lipid Res, 50*(10), 1967-1975. doi: 10.1194/jlr.R900015-JLR200

Ishibashi, S., Goldstein, J. L., Brown, M. S., Herz, J., & Burns, D. K. (1994). Massive xanthomatosis and atherosclerosis in cholesterol-fed low density lipoprotein receptor-negative mice. *J Clin Invest, 93*(5), 1885-1893. doi: 10.1172/jci117179

Kang, Y. L., Yochem, J., Bell, L., Sorensen, E. B., Chen, L., & Conner, S. D. (2013). Caenorhabditis elegans reveals a FxNPxY-independent low-density lipoprotein receptor internalization mechanism mediated by epsin1. *Mol Biol Cell, 24*(3), 308-318. doi: 10.1091/mbc.E12-02-0163

Kawecki, C., Bocquet, O., Schmelzer, C. E. H., Heinz, A., Ihling, C., Wahart, A., . . . Maurice, P. (2019). Identification of CD36 as a new interaction partner of membrane NEU1: potential implication in the pro-atherogenic effects of the elastin receptor complex. *76*(4), 791-807. doi: 10.1007/s00018-018-2978-6

Knuiman, M. W., Watts, G. F., & Divitini, M. L. (2004). Is sialic acid an independent risk factor for cardiovascular disease? A 17-year follow-up study in Busselton, Western Australia. *Ann Epidemiol, 14*(9), 627-632. doi: 10.1016/j.annepidem.2003.09.017

Kühnle, N., Dederer, V., & Lemberg, M. K. (2019). Intramembrane proteolysis at a glance: from signalling to protein degradation. *Journal of Cell Science, 132*(16), jcs217745. doi: 10.1242/jcs.217745

L J Goldstein, a., & Brown, S. M. (1977). The Low-Density Lipoprotein Pathway and its Relation to Atherosclerosis. *Annual Review of Biochemistry, 46*(1), 897-930. doi: 10.1146/annurev.bi.46.070177.004341

Levitan, I., Volkov, S., & Subbaiah, P. V. (2010). Oxidized LDL: diversity, patterns of recognition, and pathophysiology. *Antioxid Redox Signal, 13*(1), 39-75. doi: 10.1089/ars.2009.2733

Liang, F., Seyrantepe, V., Landry, K., Ahmad, R., Ahmad, A., Stamatos, N. M., & Pshezhetsky, A. V. (2006). Monocyte Differentiation Up-regulates the Expression of the Lysosomal Sialidase, Neu1, and Triggers Its Targeting to the Plasma Membrane via Major Histocompatibility Complex Class II-positive Compartments. *Journal of Biological Chemistry, 281*(37), 27526-27538. doi: 10.1074/jbc.M605633200

Lindberg, G., Eklund, G. A., Gullberg, B., & Rastam, L. (1991). Serum sialic acid concentration and cardiovascular mortality. *Bmj, 302*(6769), 143-146.

Liu, J., Hu, B., Yang, Y., Ma, Z., Yu, Y., Liu, S., . . . Yang, D. (2010). A new splice variant of the major subunit of human asialoglycoprotein receptor encodes a secreted form in hepatocytes. *PLoS One, 5*(9), e12934. doi: 10.1371/journal.pone.0012934

Mandic, R., Opper, C., Krappe, J., & Wesemann, W. (2002). Platelet sialic acid as a potential pathogenic factor in coronary heart disease. *Thromb Res, 106*(2), 137-141.

Mauthe, M., Orhon, I., Rocchi, C., Zhou, X., Luhr, M., Hijlkema, K.-J., . . . Reggiori, F. (2018). Chloroquine inhibits autophagic flux by decreasing autophagosome-lysosome fusion. *Autophagy, 14*(8), 1435-1455. doi: 10.1080/15548627.2018.1474314

Minahk, C., Kim, K.-W., Nelson, R., Trigatti, B., Lehner, R., & Vance, D. E. (2008). Conversion of Low Density Lipoprotein-associated Phosphatidylcholine to Triacylglycerol by Primary Hepatocytes. *Journal of Biological Chemistry, 283*(10), 6449-6458. doi: 10.1074/jbc.M706995200

Miyagi, T., & Yamaguchi, K. (2012). Mammalian sialidases: physiological and pathological roles in cellular functions. *Glycobiology, 22*(7), 880-896. doi: 10.1093/glycob/cws057

Monti, E., Bonten, E., D'Azzo, A., Bresciani, R., Venerando, B., Borsani, G., . . . Tettamanti, G. (2010). Sialidases in vertebrates: a family of enzymes tailored for several cell functions. *Adv Carbohydr Chem Biochem, 64*, 403-479. doi: 10.1016/s0065-2318(10)64007-3

Nioi, P., Sigurdsson, A., Thorleifsson, G., Helgason, H., Agustsdottir, A. B., Norddahl, G. L., . . . Stefansson, K. (2016). Variant ASGR1 Associated with a Reduced Risk of Coronary Artery Disease. *New England Journal of Medicine, 374*(22), 2131-2141. doi: 10.1056/NEJMoa1508419

Nokes, R. L., Fields, I. C., Collins, R. N., & Folsch, H. (2008). Rab13 regulates membrane trafficking between TGN and recycling endosomes in polarized epithelial cells. *J Cell Biol, 182*(5), 845-853. doi: 10.1083/jcb.200802176

O'Sullivan, J. M., Aguila, S., McRae, E., Ward, S. E., Rawley, O., Fallon, P. G., . . . O'Donnell, J. S. (2016). N-linked glycan truncation causes enhanced clearance of plasma-derived von Willebrand factor. *Journal of Thrombosis and Haemostasis, 14*(12), 2446-2457. doi: 10.1111/jth.13537

Orekhov, A. N., Tertov, V. V., Sobenin, I. A., Smirnov, V. N., Via, D. P., Guevara, J., Jr., . . . Morrisett, J. D. (1992). Sialic acid content of human low density lipoproteins affects their interaction with cell receptors and intracellular lipid accumulation. *J Lipid Res, 33*(6), 805-817.

Pattison, S., Pankarican, M., Rupar, C. A., Graham, F. L., & Igdoura, S. A. (2004). Five novel mutations in the lysosomal sialidase gene (NEU1) in type II sialidosis patients and assessment of their impact on enzyme activity and intracellular targeting using adenovirus-mediated expression. *Human Mutation, 23*(1), 32-39. doi: 10.1002/humu.10278

Pesonen, M., & Kääriäinen, L. (1982). Incomplete complex oligosaccharides in semliki forest virus envelope proteins arrested within the cell in the presence of monensin. *Journal of Molecular Biology, 158*(2), 213-230. doi: https://doi.org/10.1016/0022-2836(82)90430-2

Pshezhetsky, A. V., & Ashmarina, L. I. (2013). Desialylation of surface receptors as a new dimension in cell signaling. *Biochemistry (Mosc), 78*(7), 736-745. doi: 10.1134/s0006297913070067

Schwartz, A. L., Bolognesi, A., & Fridovich, S. E. (1984). Recycling of the asialoglycoprotein receptor and the effect of lysosomotropic amines in hepatoma cells. *J Cell Biol, 98*(2), 732-738. doi: 10.1083/jcb.98.2.732

Sprague, E. A., Moser, M., Edwards, E. H., & Schwartz, C. J. (1988). Stimulation of receptor-mediated low density lipoprotein endocytosis in neuraminidase-treated cultured bovine aortic endothelial cells. *J Cell Physiol, 137*(2), 251-262. doi: 10.1002/jcp.1041370207

Steinman, R. M., Mellman, I. S., Muller, W. A., & Cohn, Z. A. (1983). Endocytosis and the recycling of plasma membrane. *J Cell Biol, 96*(1), 1-27. doi: 10.1083/jcb.96.1.1

Stockert, R. J., Potvin, B., Tao, L., Stanley, P., & Wolkoff, A. W. (1995). Human Hepatoma Cell Mutant Defective in Cell Surface Protein Trafficking. *Journal of Biological Chemistry, 270*(27), 16107-16113. doi: 10.1074/jbc.270.27.16107

Stockinger, W., Sailler, B., Strasser, V., Recheis, B., Fasching, D., Kahr, L., . . . Nimpf, J. (2002). The PX-domain protein SNX17 interacts with members of the LDL receptor family and modulates endocytosis of the LDL receptor. *Embo j, 21*(16), 4259-4267.

Takano, Y., Imai, K., Tanaka, A., Fujimori, K., Yamada, M., & Yamamoto, K. (1984). Stimulation of the Release of Lysosomal and Nonlysosomal Granular Enzymes from Macrophages Treated with Monensin. *Cell Structure and Function, 9*(3), 265-277. doi: 10.1247/csf.9.265

Tartakoff, A. M. (1983). Perturbation of vesicular traffic with the carboxylic ionophore monensin. *Cell, 32*(4), 1026-1028. doi: 10.1016/0092-8674(83)90286-6

Tozawa, R., Ishibashi, S., Osuga, J., Yamamoto, K., Yagyu, H., Ohashi, K., . . . Yamada, N. (2001). Asialoglycoprotein receptor deficiency in mice lacking the major receptor subunit. Its obligate requirement for the stable expression of oligomeric receptor. *J Biol Chem, 276*(16), 12624-12628. doi: 10.1074/jbc.M011063200

Tybjærg-Hansen, A. (2016). The Sialylation Pathway and Coronary Artery Disease. *New England Journal of Medicine, 374*(22), 2169-2171. doi: 10.1056/NEJMe1604773

Wang, L. H., Rothberg, K. G., & Anderson, R. G. (1993). Mis-assembly of clathrin lattices on endosomes reveals a regulatory switch for coated pit formation. *J Cell Biol, 123*(5), 1107-1117. doi: 10.1083/jcb.123.5.1107

Wijers, M., Kuivenhoven, J. A., & van de Sluis, B. (2015). The life cycle of the low-density lipoprotein receptor: insights from cellular and in-vivo studies. *Curr Opin Lipidol, 26*(2), 82-87. doi: 10.1097/mol.0000000000000157

Wileman, T., Boshans, R. L., Schlesinger, P., & Stahl, P. (1984). Monensin inhibits recycling of macrophage mannose-glycoprotein receptors and ligand delivery to lysosomes. *Biochem J, 220*(3), 665-675. doi: 10.1042/bj2200665

Windler, E., Greeve, J., Levkau, B., Kolb-Bachofen, V., Daerr, W., & Greten, H. (1991). The human asialoglycoprotein receptor is a possible binding site for low-density lipoproteins and chylomicron remnants. *Biochem J, 276 (Pt 1)*, 79-87.

Yang, A., Gyulay, G., Mitchell, M., White, E., Trigatti, B. L., & Igdoura, S. A. (2012). Hypomorphic sialidase expression decreases serum cholesterol by downregulation of VLDL production in mice. *J Lipid Res, 53*(12), 2573-2585. doi: 10.1194/jlr.M027300

Yik, J. H. N., Saxena, A., Weigel, J. A., & Weigel, P. H. (2002). Nonpalmitoylated Human Asialoglycoprotein Receptors Recycle Constitutively but Are Defective in Coated Pit-mediated Endocytosis, Dissociation, and Delivery of Ligand to Lysosomes. *Journal of Biological Chemistry, 277*(43), 40844-40852. doi: 10.1074/jbc.M204780200

Zhang, D. W., Lagace, T. A., Garuti, R., Zhao, Z., McDonald, M., Horton, J. D., . . . Hobbs, H. H. (2007). Binding of proprotein convertase subtilisin/kexin type 9 to epidermal growth factor-like repeat A of low density lipoprotein receptor decreases receptor recycling and increases degradation. *J Biol Chem, 282*(25), 18602-18612. doi: 10.1074/jbc.M702027200