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WASHINGTON UNIVERSITY IN ST. LOUIS

Division of Biology and Biomedical Sciences

Molecular Cell Biology

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Functional Significance of Glycoprotein Clearance by the Asialoglycoprotein Receptor and the Mannose/GalNAc-4-SO_4 Receptor

by

Lindsay Michelle Steirer Taylor

A dissertation presented to the
Graduate School of Arts and Sciences
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

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Saint Louis, MO

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ABSTRACT OF THE DISSERTATION

Functional Significance of Glycoprotein Clearance by the Asialoglycoprotein Receptor and the Mannose/GalNAc-4-SO_4 Receptor

By

Lindsay Michelle Steirer Taylor

Doctor of Philosophy in Molecular Cell Biology

Washington University in St. Louis, 2009

Professor Jacques U. Baenziger, Chairperson

Glycosylation plays an important role in many biological functions. Two highly abundant, carbohydrate-specific, endocytic receptors reside in parenchymal and endothelial cells of the liver. Our lab has shown that the asialoglycoprotein receptor (ASGR) is capable of clearing glycoproteins bearing terminal Siaα2,6GalNAc as well as ones bearing terminal Gal/ GalNAc and that the Mannose/GalNAc-4-SO_4 receptor (MR) is capable of clearing glycoproteins bearing terminal GalNAc-4-SO_4. I am taking a genetic approach identifying endogenous ligands for the ASGR and the MR *in vivo* and establishing the biologic significance of clearing these glycoproteins from the blood. A number of glycosylated hormones such as luteinizing hormone (LH), thyroid stimulating hormone, and the prolactin like proteins bear structures that would be recognized by either the ASGR or the MR and clearance would potentially help regulate their concentrations following release into the blood.

I have obtained ASGR-/-, MR-/-, and ASGR-/-MR-/- mice. I am using mass spectrometric methods to identify glycoproteins that are elevated in the blood of these

mice. Glycoproteins bearing Siaα2,6Gal are elevated in ASGR-/- mice suggesting that glycoproteins with Siaα2,6Gal rather than terminal Gal or GalNAc are cleared by the ASGR. Many are acute phase proteins and we propose that the ASGR helps regulate their relative concentrations *in vivo* and enhances their increase during the acute phase response. LH bears terminal GalNAc-4-SO_4 and the half life of LH is increased in MR-/- mice indicating that the MR does account for LH clearance in vivo. ASGR-/- mice also have elevated LH, but the half life is not increased indicating an alternative mechanism of elevating LH in ASGR-/-, likely through a protein bearing Siaα2,6Gal/GalNAc. Ablation of both the MR and ASGR results in mice that are fertile, but unable to induce parturition. This suggests clearance of proteins bearing Siaα2,6Gal/GalNAc and GalNAc-4-SO_4 is critical for appropriate plasma protein levels changes associated with parturition. Clearance by the ASGR and MR may contribute to regulating the concentrations of a range of glycoproteins including acute phase proteins and hormones.

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Steirer LM, Park EI, Townsend RR, and Baenziger JU. "Plasma concentrations of glycoproteins terminating with Siaα2,6Gal are regulated by the asialoglycoprotein
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Chapter 1

Introduction

Glycosylation is essential for a variety of biological processes including protein folding [1], proteolytic stability [2], and clearance rate [3]. Most proteins are glycosylated [4, 5]. Here, we focus on the significance of glycosylation in clearance of glycoproteins circulating in the blood. Clearance of glycoproteins is based on the specific recognition of the protein's oligosaccharide structure by carbohydrate specific receptors. We propose two receptors, the asialoglycoprotein receptor (ASGR) and the mannose/GalNAc-4-SO_4 receptor (MR) regulate plasma protein levels of proteins bearing specific carbohydrates.

The ASGR and MR are known to bind and clear glycoproteins bearing unique N-linked oligosaccharides terminating with Siaα2,6GalNAcβ1,4GlcNAcβ1,2Manα (Siaα2,6GalNAc) [6],and SO_4-4GalNAcβ1,4GlcNAcβ1,2Manα (GalNAc-4-SO_4) (Figure 1), respectively [7-10]. Only a few proteins bearing these unique structures have been identified, but the proteins that have been identified play a major role in reproduction. The synthesis of the oligosaccharide structures is regulated hormonally and developmentally [11, 12] . However, the significance of clearing circulating glycoproteins bearing these structures is unknown in terms of regulating circulating levels and glycoprotein turnover. There is evidence the ASGR and MR are involved during two significant events, the acute phase response and sexual reproduction. In this thesis, we set out to understand the importance of two highly abundant receptors found in the liver, the ASGR and MR, in regulating the circulating concentrations of proteins bearing Siaα2,6Gal and GalNAc-4-SO_4 in unperturbed animals and during the acute phase response and sexual reproduction.

The Asialoglycoprotein Receptor and Mannose/GalNAc-4-SO_4 Receptor. There are two highly abundant receptors found in the liver, the ASGR and the MR, known to bind and clear glycoproteins bearing two unique oligosaccharides, Siaα2,6GalNAc and GalNAc-4-SO_4, respectively. Both receptors were identified based on their ability to bind other structures, terminal galacose (Gal) and mannose (Man), but our lab has established they are capable of binding these two additional structures. Both receptors have been studied extensively to understand their carbohydrate specificity and function; however the precise function of both receptors remains unknown.

Asialoglycoprotein Receptor

The ASGR was first identified in the early 1970s by Ashwell and Morell [13]. Early studies determined that while native ceruloplasmin, a copper binding protein modified with sialic acid (Sia), circulates in plasma for days, removal of the Sia and exposure of the penultimate structure, Gal, results in ceruloplasmin that circulates in plasma for minutes. Therefore, the terminal structure on ceruloplasmin determines the circulatory half life. While observing clearance of ceruloplasmin, it was found to accumulate in the parenchymal cells of the liver [14] and binding is to the plasma membrane of the liver [15]. For maximal binding to occur, the pH needs to be 7.8-8.5 and there is an absolute requirement of calcium [15]. No more than two Gal residues on a single protein are necessary for rapid removal from circulation [13], and the affinity of different ligands for the receptor depends on the position [16] and number of carbohydrate chains [17].

Clearance studies in which exogenous ligands are radiolabeled and injected into an animal are useful for determining circulatory half-life. When the ASGR was

originally identified, clearance studies were conducted using radiolabeled native ceruloplasmin, which bears Sia, and asialoceruloplasmin, which bears terminal Gal. It was found that within 15 minutes of injection, 90% of native ceruloplasmin remained in the blood whereas less than 10% of asialoceruloplasmin remained in the blood. The majority of the radioactivity was found in the liver [14]. This suggests the terminal structure present on oligosaccharides terminal the circulatory half-life of proteins in the blood.

The ASGR has been isolated from a number of hepatic organelles, including the golgi, microsomes, and lysosomes [18]. This in combination with the topological distribution of the receptor, in which the receptor is found on the luminal membrane surface of the golgi complex [19] suggest it undergoes a recycling mechanism. This was later confirmed and studied extensively by Gueze et al [20]. The ligand and receptor are internalized together by endocytosis [21] in coated pits and vesicles [22]. The ASGR is an abundant receptor with as many as 500,000 surface receptors per cell [23] but there may be twice as many receptors present including internalized receptors[24].

It was presumed for nearly 30 years that the ASGR binds asialolglycoproteins; however, in this time, proteins bearing terminal Gal were never identified. To identify endogenous ligands and attempt to elucidate the function of the ASGR, two knockout mouse models were produced, one for each subunit. In the mouse, the ASGR is composed of two subunits, mouse hepatic lectins 1 and 2 (MHL1/2), and both are required for expression of a functional receptor [25-28] Studies have been conducted on each of the subunits to elucidate their roles [29, 30]. In 1994, a genetic ablation model for mice lacking one of the two receptor subunits, the subunit that does not bind, MHL2

was generated [31]. Surprisingly, it was found that there was no accumulation of asialoglycoproteins in the blood. Several years later, the MHL1 subunit was knocked out in mice, and again, there was no accumulation of asialoglycoproteins in the blood [32]. Interestingly, in the absence of the ASGR, no proteins were found to accumulate in the blood. Ablation of either receptor should result in a nonfunctional receptor and any proteins that utilize the receptor should accumulate in the plasma. If the true ligands for the ASGR are proteins bearing terminal Gal/GalNAc, proteins bearing these structures should have accumulated in the plasma. The absence of elevated proteins, specifically bearing terminal Gal/GalNAc begged the question of what is the true function of the ASGR.

To date, no endogenous ligands had been identified for the ASGR. While studying a protein specific GalNAc-transferase, we identified a group of proteins, prolactin-like proteins (PLPs) bearing a unique oligosaccharide terminating with Siaα2,6GalNAc [12]. Surprisingly, neoglycoconjugates bearing this structure were cleared by the ASGR, and proteins bearing Siaα2,6GalNAc were identified as the first potential endogenous ligands for the ASGR. We found the optimal pH and Ca^{2+} dependence are similar, suggesting it coordinates with the same calcium as Gal/GalNAc. Additionally we found the ASGR is partially inhibited by 20mM Sia, suggesting Sia may also bind HL1 of the ASGR, just as Gal/GalNAc do [33]. Using bacterially expressed CRDs of HL1 we studied binding of sialylated glycoproteins [6]. The binding of three neoglycoconjugates bearing Siaα2,6GalNAc, Siaα2,6Gal, and Siaα2,3Gal was compared to the binding of Gal. We found binding activity for Siaα2,6Gal/GalNAc, but Siaα2,3Gal. Biantennerary core-fucosylated N-glycan (BiF)10-[^{125}I]BSA, bearing

terminal Gal, SiaGGnM-[^{125}I]BSA, bearing Siaα2,6GalNAc, and BiF1226-[^{125}I]BSA bearing Siaα2,6Gal, bound with a K_ds of 0.45 μM, 1.26 μM and 3.6 μM respectively. There was an 8-16 fold reduction in the binding of Siaα2,6Gal compared to terminal Gal, but binding of Siaα2,6Gal is detectable and highly specific.

Utilizing the MHL2 knockout (ASGR-/-) model, we examined clearance rates of Gal and Siaα2,6Gal/GalNAc. We found clearance was greatly reduced in ASGR-/- mice for both Gal and Siaα2,6GalNAc. The majority of plasma proteins bear Sia linked to the C3 or C6 hydroxyl of Gal. There was no specific clearance mechanism known for either Siaα2,3Gal or Siaα2,6Gal but the clearance of proteins bearing each structure has been examined in the plasma. Clearance of Siaα2,3Gal and Siaα2,6Gal is difficult to detect because the half-life is long and a large number of proteins bearing these structures exist in circulation that neoglycoconjugates would compete with, but it is clear Siaα2,6Gal has a shorter half-life that Siaα2,3Gal, suggesting there is a specific clearance mechanism for Siaα2,6Gal, but not Siaα2,3Gal [34].

The ASGR has been implicated in an inflammatory stimulus known as the acute phase response (APR). During the APR, protein concentrations of acute phase proteins (APPs) change dramatically. The cause of the elevation is known to be synthesis, but the ASGR may also be involved. The ASGR shuts down within hours following exposure to cytokines. Within the first few hours, the decrease in binding is due to the inactivation of the receptor by phosphorylation, and hours later, there is decreased synthesis of the receptor[35]. Sialic acid terminating proteins that have been implicated in the APR begin to increase within 3-6 hours. In animals with a functioning ASGR, any immediate

increase in APPs may be due to decreased removal from the blood by inactive ASGR rather than increased synthesis by the liver.

The ASGR has also been implicated in pregnancy. It was found that the binding activity of the ASGR begins to elevate at day 10 of pregnancy and elevates at much as three fold immediately prior to parturition. Additionally, there is enhanced endocytosis of the ASGR[36]. There may be a drastic change in proteins produced by the mother and fetus during pregnancy that the ASGR is responsible for clearing.

Most recently, Grewal et al [37] showed elevations of vonWillebrand factor (vWF) and reduced bleeding time in mice lacking MHL1. Additionally, they found platelets are eliminated after they are desialylated by a sialidase produced by *Streptococcus pneumoniae.* These two observations suggest a function for the ASGR. Interestingly, they did not find the same phenotype in mice ablated for MHL2. We and others have shown MHL1 and MHL2 are both required for binding and clearance of asialolglycoproteins.

Clearance by carbohydrate specific receptors may be an essential mechanism for plasma glycoprotein turnover. Rates of turnover are hard to determine for proteins bearing Siaα2,6Gal is because the half life is extremely long. Additionally, the methods currently used inject exogenous ligands, which would compete with endogenous ligands for binding and clearance. In this thesis, I use an alternative method in which two dimensional difference gel electrophoresis and lectin blotting is used to identify proteins bearing Siaα2,6Gal as endogenous ligands for the ASGR.

Mannose/GalNAc-4-SO4 Receptor

In addition to the ASGR, there is a second highly abundant receptor found in the liver. Following the identification of the ASGR, the Mannose Receptor (Man-R) was identified in the late 1970s in alveolar macrophages as a protein that binds to Man, GlcNAc, and fucose [38, 39]. Many years later, this receptor was implicated in clearance of an alternative unique structure GalNAc-4-SO_4. Similar to the ASGR, the receptor requires a pH above 5.0 for binding, but binding of GalNAc-4-SO_4 is not dependent on a divalent cation [40].

Clearance of a specific protein by the MR, luteinizing hormone (LH) bearing GalNAc-4-SO_4, was compared to that of desulfated LH, bearing terminal GalNAc , and recombinant sialylated LH to determine the role of its glycosylation. The circulatory half-life was determined in order to establish the significance of the GalNAc-4-SO_4 on bovine LH (bLH). We found the circulatory half-life of native LH is cleared 4-5 times faster than recombinant LH bearing sialylated oligosaccharides, 7.3%/min compared to 1.7%/min. The desulfated bLH is rapidly cleared by the liver, presumably by the ASGR. The clearance of native bLH is approximately 7.3%/min while desulfated LH clears at 35%/min. We concluded the GalNAc-4-SO_4 protects the bLH from this rapid clearance[40].

Our lab subsequently purified the GalNAc-4-SO_4 receptor from the liver [41]. The receptor, termed the S4GGnM-receptor (S4GGnM-R), was similar to the MR in amino terminal sequence, peptide maps, peptide sequences, and molecular weight [42-45]. The main difference was the lung form of the receptor (Man-R) bound Man and the liver form of the receptor (S4GGnM-R) bound GalNAc-4-SO_4. Remarkably, Man-R

cDNA expressed in CHO cells binds both Man and GalNAc-4-SO_4, confirming the Man-R and the S4GGnM-R are encoded by the same gene. Further analysis of these two receptors determined they were actually the same receptor, and the receptor was renamed the Mannose/GalNAc-4-SO4 Receptor (MR) [46].

The MR has different ligand specificities suggesting it is used for multiple functions. We postulated there must be structural features of the MR that differentiate the MR in the liver and the MR in the lung. We found that the receptor exists in a monomeric form while binding mannose and a dimeric form while binding GalNAc-4-SO_4. Posttranslational modification in macrophages and hepatic endothelial cells likely account for the different specificities of the two forms of the receptor [47].

The MR is comprised of a cysteine-rich (cys-rich) domain, fibronectin type II repeats, carbohydrate recognition domains (CRDs), transmembrane domain, and a cytoplasmic tail [44, 45]. Binding of glycoproteins bearing Man is to CRDs 4-8 and multiple Man termini are optimal for effective ligands [48, 49]. Previously, the CRDs were the only region of the MR to bind proteins. Using deletion mutations, we determined an alternative region is responsible for binding to GalNAc-4-SO_4, the cys-rich region [50]. Crystal structure analysis determined the Cys-rich region forms a Ricin-type β-trefoil structure and binds GalNAc-4-SO_4 [51].

The MR is found in hepatic endothelial cells and Kupffer cells, is highly abundant in the liver with up to 600,000 receptors per cell, and rapidly internalizes glycoproteins for degradation in the lysosome [40]. Extensive research has been conducted on binding of LH to the MR. The binding affinity for LH is $1.7x10^{-7}$ M and circulating LH levels are in the range of $0.1x10^{-9}$-$1.0x10^{-9}$ M [47]. Although circulating LH levels fall below the

K_d for binding to the MR, the abundance of the MR on the cell surface provide sufficient capacity to bind LH.

We determined the MR clears LH based on the GalNAc-4-SO_4 oligosaccharide, but this does not prove the MR mediates clearance of proteins bearing GalNAc-4-SO_4 *in vivo.* In order to accomplish this, we needed genetic evidence. Our lab and Michel Nussenzweig's lab have produced mice with genetically ablated MR [52, 53]. In our model, we found clearance of bLH, which bears GalNAc-4-SO_4, is slowed in mice heterozygous for the MR [53]. Nussenzweig's model showed decreased clearance of a number of neoglycoconjugates including GalNAc-4-SO_4-BSA and Man-BSA. However, clearance of porcine LH clearance is not prolonged [52]. Enormous amounts, 50 μg of porcine LH, potentially saturated the MR and porcine LH may bear oligosaccharides terminating with Siaα2,6Gal/GalNAc, which would be cleared by the ASGR, accounting for this discrepancy.

Previous experiments have determined the MR binds and clears GalNAc-4-SO_4 found on several proteins including glycoprotein hormones. However, little information about clearance of endogenous ligands is known and the function of the MR needs to be elucidated. Investigation of significance of the MR in regulating plasma protein concentrations *in vivo* is key to understanding its function. In this thesis, we provide evidence that the MR regulates endogenous LH and elevated LH leads to multiple physiologic changes.

Proteins recognized by the ASGR and MR are modified by a protein-specific GalNAc-transferase and an α2,6sialyltransferase or GalNAc-4-sulfotransferase.

There are three structures that are focused on in this thesis: Siaα2,6Gal/GalNAc and GalNAc-4-SO_4. Little is known about the clearance of Siaα2,6Gal, but it is a common N-linked structures found on many plasma proteins. The Sia may be linked to Gal in one of two linkages, either α2,6 or α2,3. To synthesize this structure, there are two separate enzymes; the first enzyme, β1,4galactosyltransferase adds Gal to GlcNAc [54] and the second enzyme α2,6sialyltransferase adds Sia to Gal [55]. The second less common structure found on a few plasma proteins terminates with Siaα2,6GalNAc. Siaα2,6GalNAc binds and is cleared by the ASGR [6]. This structure also utilizes the α2,6sialyltransferase, following modification by another unique enzymes β1,4GalNAc-transferase (βGT). The third structure, GalNAc-4-SO_4 is also a unique structure, and is recognized by the MR. The structure GalNAc-4-SO_4 utilizes β1,4GalNAc-transferase [56, 57], and those structures are further modified by GalNAc-4-sulfotransferase. Addition of GalNAc and sulfate is done on a protein specific bases. These transferases are found in multiple locations, but most importantly, they are found in the anterior pituitary and the placenta, where the glycoprotein hormones bearing these structures are synthesized. These enzymes are conserved from fish to man, suggesting oligosaccharides modified by these enzymes are significant[58].

Regulation of glycoproteins bearing GalNAc-4-SO_4 and Siaα2,6GalNAc are during sexual reproduction. Essential reproductive hormones are regulated by an endocrine system known as the hypothalamic-pituitary-gonadal axis (Figure 2). Luteinizing

hormone, synthesized and released from the anterior pituitary stimulates its receptor on the gonads, inducing production of steroid hormones including testosterone, progesterone, and estrogen. The steroid hormones in turn feedback to the hypothalamus and pituitary to control synthesis and release of LH from the pituitary.

The LH receptor (LHR) is a G-protein coupled receptor with seven transmembrane domains. Steroiogenic actions of LH are mainly through cAMP-mediated events in the gonads [59]. In order for maximal stimulation of the LHR on the gonads, the LHR must be episodically stimulated. Episodic stimulation by pulsatile release of LH from the pituitary and possibly clearance by the MR may be necessary to maintain LHR levels and major increases in LH can cause downregulation of the LHR and desensitization to the hormonal signal [59, 60].

Rapid clearance of LH may be essential for maximal stimulation of the LHR. Our lab has determined LH bears the unique oligosaccharide structure GalNAc-4-SO4 and is rapidly cleared by the MR. The oligosaccharides on a number of species' LH have GalNAc and sulfate, and we determined GalNAc-4-SO_4 is present on glycoprotein hormones of all vertebrates [58]. Bovine LH and ovine LH both bear predominantly sulfated oligosaccharides, but human LH bears both sulfated and sialylated oligosaccharides [8, 9, 58]. This suggests LH may be cleared by both the MR and the ASGR.

LH is comprised of two subunits, a common alpha subunit utilized by other proteins including follicle-stimulating hormone (FSH) and thyroid stimulating hormone (TSH) and a unique beta subunit. The alpha subunit is identical for each of the proteins [61, 62], but the oligosaccharide modifications found on the beta subunits differ. The

alpha subunit of the glycoprotein hormones has two N-linked glycosylation sites and the beta subunit has one or two N-linked glycosylation sites. LH specifically may bear none, one, or two sulfated structures. Although it is unknown what percentage of the N-linked glycosylation sites bear Siaα2,6GalNAc, recently we found evidence that LH may bear Siaα2,6GalNAc in addition to GalNAc-4-SO_4.

Our lab has produced a genetic model knocking out the G4ST1 (G4ST1-/-) that alters the clearance of LH by the MR. We found that in G4ST1-/- mice, terminal GalNAc is modified with Sia. Clearance of LH is slightly longer; however, LH is still specifically cleared. Circulating LH levels are elevated causing elevated estrogen and testosterone. Female have enlarged uteri, presumably caused by the elevated estrogen. Additionally, females are very fertile producing 50% more pups than Wt females. These results suggest rapid clearance of LH is significant for regulation of the HPG axis [63].

During pregnancy, proteins bearing Siaα2,6GalNAc have been identified in the placenta. There is expression of βGT in the placenta, but G4STs are not found in the placenta, leading us to identify proteins bearing Siaα2,6GalNAc. Prolactin-like proteins (PLPs) were identified in the placenta bearing the unique structure Siaα2,6GalNAc [12]. The significance of the structure and function of the oligosaccharide on the PLPs is not clear; however, clearance of the PLPs by the ASGR likely plays a key role in pregnancy.

The role of the MR needs to be elucidated. Circulatory half-life may be important in maintaining appropriate LH levels and regulating the HPG axis. Further studies with MR-/- mice and ASGR-/- mice are crucial for understanding the importance of regulating LH levels. In this thesis, we use MR-/- and ASGR-/- mice to elucidate the role of these receptors in regulating the HPG axis. Additionally, steroid hormones are key during

pregnancy, but how these receptors are involved in pregnancy remains unknown. We provide evidence that receptor message levels are regulated throughout pregnancy, suggesting they play a role in pregnancy.

Regulation of plasma protein concentrations by the Asialolglycoprotein Receptor and Mannose/GalNAc-4-SO4 Receptor is essential. This thesis focuses on determining the biological significance of protein level regulation of proteins bearing Siaα2,6Gal/GalNAc and GalNAc-4-SO_4 by the ASGR and the MR. Both receptors are highly abundant in the liver, but their precise function still needs to be elucidated. The ASGR binds proteins bearing Siaα2,6Gal/GalNAc and we can observe clearance of neoglycoconjugates bearing Siaα2,6GalNAc. Mice lacking the ASGR have decreased clearance of Siaα2,6GalNAc. Clearance of Siaα2,6Gal is slow and this structure is found on a large number of proteins, causing competition and therefore cannot be observed in the liver by traditional clearance studies.

For many years, it has been presumed the ASGR clears circulating glycoproteins bearing Gal; however, proteins bearing terminal Gal have not been identified. We have evidence that the ASGR binds to glycoproteins bearing Siaα2,6Gal and we now propose the main glycoproteins cleared by the ASGR bear Siaα2,6Gal. Mice with ablated MHL2 have previously been studied to identify proteins elevated in the plasma, but proteins bearing terminal Gal/GalNAc or Siaα2,6Gal/GalNAc were not identified. New methods in which proteins are fluorescently labeled and separated by two dimensional difference gel electrophoresis, then visualized together are used in this thesis to identify proteins that are elevated in the plasma of ASGR-/- mice. In Chapter 2, I identify proteins that are

elevated in ASGR-/- plasma bearing Siaα2,6Gal. The proteins are highly abundant proteins, including haptoglobin and SAP, and many are involved in the APR.

In the plasma of the ASGR knockouts, proteins are significantly elevated and the majority of those proteins are APPs. Furthermore, ASGR has been previously implicated in the elevation of those specific proteins during APR. In Chapter 3, I demonstrate the ASGR does play a role in elevating proteins during the APR. We identified multiple proteins, including two of the proteins previously identified in unperturbed ASGR-/- mice. In ASGR-/- mice, the fold change is not as dramatic compared to Wt but nonetheless, the overall protein levels do change. We propose that in addition to increased synthesis of APPs by the liver, decreased clearance by the ASGR assists in elevating APPs.

Glycoprotein hormones bear two unique structures, Siaα2,6GalNAc cleared by the ASGR and GalNAc-4-SO_4 cleared by the MR. In the absence of one or both of these receptors, we would expect LH to be elevated because the half-life should be increased and downstream targets of LH, such as testosterone, should be elevated. In Chapter 4, I determine the endogenous half-life of LH in ASGR-/-, MR-/- and MR-/-ASGR-/-. The half life of LH is longer in MR-/- and MR-/-ASGR-/- mice leading to elevated LH levels. ASGR-/- mice also have elevated LH, but there clearance rate is not decreased, suggesting there is an alternative mechanism elevating LH in these animals. Mice with elevated LH have additional physiologic changes associated with elevated LH, including elevated testosterone and enlarged seminal vesicles.

In Chapter 5, I determine the significance of the ASGR and the MR in the estrus cycle, during pregnancy, and at the time of parturition. As previously determined,

ablation of the ASGR and MR results in elevated steroid hormone levels. Steroid hormones, and specifically progesterone play a major role in pregnancy and parturition. We show that MR-/-ASGR-/- mice are fertile, but are unable to induce parturition. The message levels of the MR and ASGR drastically change throughout pregnancy, suggesting they play a major role in pregnancy and parturition.

Chapter 6 summarizes the work of my thesis and suggests potential roles of the ASGR and MR in physiological events including the APR and pregnancy. Additionally, I discuss future important studies including determining the regulation of the ASGR and MR during physiological events such as the APR and pregnancy.

Abbreviations

ASGR, asialoglycoprotein receptor; MR, mannose/GalNAc-4-SO_4 receptor; Sia, sialic acid; Gal, galactose; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; Man, mannose; MHL, mouse hepatic lectin; PLP, prolactin-like proteins; CRD, carbohydrate recognition domain; APR, acute phase response; APP, acute phase protein; vWF, vonWillebrand factor; BiF, biantennerary core-fucosylated N-glycan; LH, luteinizing hormone; LHR, LH receptor; CHO, Chinese hamster ovary cells; BSA, bovine serum albumin; HPG, hypothalamic-pituitary-gonadal.

Figures

Figure 1. Three structures are focused on in this thesis, found on the glycoproteins of interest. **Panel A:** Siaα2,6Gal; **Panel B:** Siaα2,6GalNAc: **Panel C:** GalNAc-4-SO_4

Figure 2. Hypothalamic-pituitary-gonadal axis. LH binds to LHR on leydig cells in the testes causing production of testosterone. Testosterone induces spermatogenesis in the sertoli cells of the testes, signals to other target tissues, such as seminal vesicles, and signals to the hypothalamus and pituitary to regulate synthesis and release of LH. LH is released into the blood from the pituitary and once in the blood, it will stimulate the LHR on leydig cells or be cleared from circulation by the MR or AR.

Figure 1.

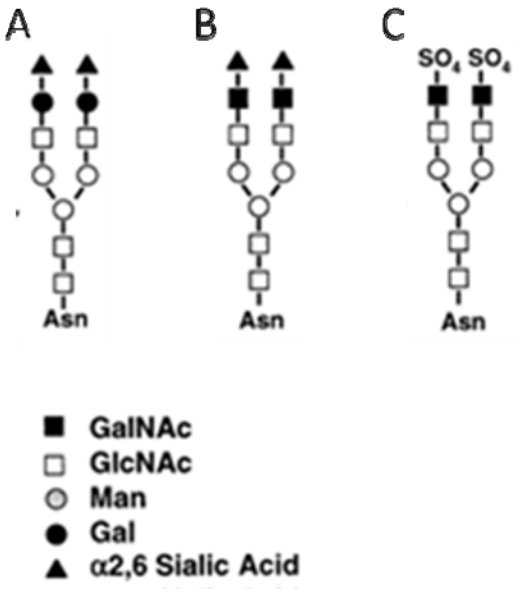
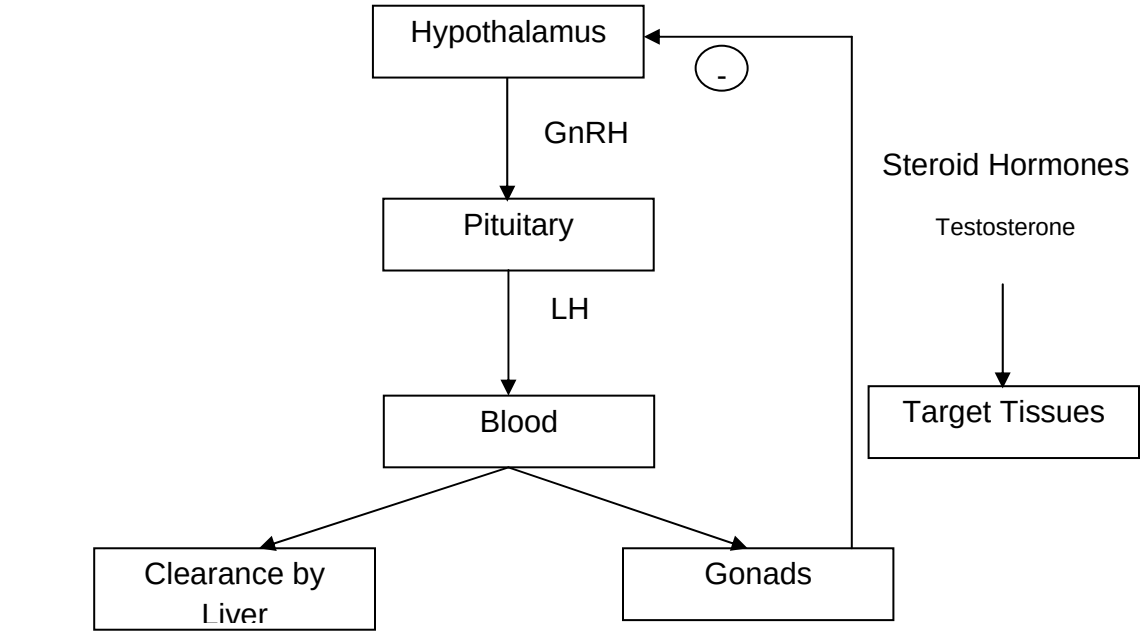


Figure 2.


Hypothalamus
-
GnRH
Steroid Hormones
Testosterone
Pituitary
LH
Blood
Target Tissues
Clearance by Liver
Gonads
Mannose/GalNAc-4-SO4 Receptor(MR)

Chapter 2

The Asialoglycoprotein Receptor Regulates Levels of Plasma Glycoproteins Terminating with Sialic Acid α2,6Galactose

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The Asialoglycoprotein Receptor Regulates Levels of Plasma Glycoproteins Terminating with Sialic Acid α2,6-Galactose*

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The asialoglycoprotein receptor (ASGP-R) is an abundant, carbohydrate-specific, endocytic receptor expressed by parenchymal cells of the liver. We recently demonstrated that the ASGP-R mediates the clearance of glycoproteins bearing Siaα2,6GalNAc as well as those bearing terminal Gal or GalNAc. We now report that glycoproteins such as haptoglobin, serum amyloid protein (SAP), and carboxylesterase that bear oligosaccharides with terminal Siaα2,6Gal are elevated in the plasma of ASGP-R-deficient mice. Because of their abundance in plasma, glycoproteins bearing terminal Siaα2,6Gal will saturate the ASGP-R and compete with each other on the basis of their relative affinities for the ASGP-R and their relative abundance. We propose that the ASGP-R mediates the clearance of glycoproteins that bear oligosaccharides terminating with Siaα2,6Gal and thereby helps maintain the relative concentrations of these glycoproteins in the blood.

The asialoglycoprotein receptor (ASGP-R)[3] was initially identified and characterized by Ashwell and co-workers (1, 2) on the basis of its ability to rapidly remove glycoproteins bearing oligosaccharides terminating with β1,4-linked Gal from the circulation. The ASGP-R has been extensively characterized since its initial discovery; however, its biologic function *in vivo* has remained unclear. This endocytic receptor is highly abundant with 500,000 receptors expressed at the plasma membrane of hepatocytes (3–5) and is rapidly internalized (3, 6). The abundance of the ASGP-R and its rapid rate of internalization in combination with the large number of hepatocytes that are present in the liver, 1.35×10^8/g of liver (7, 8), results in an enormous potential capacity to remove glycoproteins from the circulation. Until recently, mice that have had either subunit of the ASGP-R ablated, subunit 1 ASGP-R1(−/−) or subunit 2 ASGP-R2(−/−), have not been reported to have altered levels of circulating glycoproteins in their blood or to have a physiologic phenotype (9, 10). However, Grewal *et al.* (11) have reported that the ASGP-R plays a role in von Willebrand factor homeostasis and promotes thrombocytopenia during *Steptococcus pneumoniae* sepsis by removing platelets that have had their surface sialic acid removed by the bacterial neuraminidase.

We recently established that glycoproteins bearing Asn-linked oligosaccharides terminating with the sequence Siaα2,6GalNAcβ1,4GlcNAc are recognized by the ASGP-R and rapidly removed from the blood (12, 13). Glycoproteins bearing terminal Siaα2,6GalNAcβ1,4GlcNAc are the first examples of endogenous glycoproteins that can be recognized by the ASGP-R without further modification; *i.e.* removal of terminal Sia. Glycoproteins bearing these structures, for example the prolactin-like proteins (14), glycodelin (15), urokinase (16), and glycoprotein hormones (17), are not highly abundant, suggesting that the ASGP-R recognizes and clears additional more abundant glycoproteins. The most likely candidates are glycoproteins bearing Asn-linked oligosaccharides that terminate with the sequence Siaα2,6Galβ1,4GlcNAc. We have reported that the ASGP-R recognizes these structures with an avidity that is in the micromolar range (13). The avidity of the ASGP-R for structures terminating with Siaα2,6Galβ1,4GlcNAc is predicted to be sufficient to mediate binding and clearance of glycoproteins bearing structures terminating with Siaα2,6Galβ1,4GlcNAc from the blood. This concept is supported by indications that neo-glycoproteins bearing structures terminating with Siaα2,6Galβ1,4GlcNAc are removed from the blood at a faster rate than those bearing Siaα2,3Galβ1,4GlcNAc (18). Slow clearance of glycoproteins bearing Siaα2,6Galβ1,4GlcNAc, however, hampers accurate measurement of their half-lives by injection of radiolabeled ligands.

We now report that multiple glycoproteins bearing oligosaccharides that terminate with Siaα2,6Galβ1,4GlcNAc are elevated in the plasma of ASGP-R-deficient ASGP-R2(−/−) mice as compared with wild-type (Wt) mice. The elevation of multiple glycoproteins bearing terminal Siaα2,6Galβ1,4GlcNAc supports our proposal that the ASGP-R accounts for the clearance of these glycoproteins. This previously undiscerned role of the ASGP-R now allows us to develop a model of how this

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[3] The abbreviations used are: ASGP-R, asialoglycoprotein receptor; ASGP-R1, subunit 1 of the ASGP-R; ASGP-R2, subunit 2 of the ASGP-R; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; Sia, sialic acid; SAP, serum amyloid protein; Wt, wild-type; PBS, phosphate-buffered saline; 2D-DIGE, two-dimensional difference gel electrophoresis; IPG, immobilized pH gradient; SNA-I, *S. nigra I*; RCA-I, *R. communis I*; APP, acute phase protein; PVDF, polyvinylidene difluoride; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; CRP, C-reactive protein; MOPS, 4-morpholinepropanesulfonic acid; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight.

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receptor may contribute to the regulation of the concentration of many different glycoproteins in the blood.

MATERIALS AND METHODS

Mice—ASGP-R2(−/−) mice obtained from the Jackson Laboratories were kindly provided by David Ginsburg, University of Michigan, Dept. of Internal Medicine and Human Genetics. The mice were backcrossed with C57Bl/J6 for five generations. C57Bl/J6 were used as Wt controls.

Plasma Preparation—Blood was collected from the inferior vena cava of Wt and ASGP-R2(−/−) mice using a 1-ml syringe and a 23-gauge needle treated with EDTA to prevent coagulation. The blood was diluted 1:1 with 20 mM PO_4, pH 7.4, 150 mM NaCl (PBS) containing 20 mM EDTA. Leukocytes and erythrocytes were separated from the plasma by sedimentation for 10 min at 0.5 × *g*. The protein concentration of the plasma was determined using a modified Lowry method (PlusOne 2D-Quant Kit, GE Healthcare).

Cyanine Dye Labeling—Plasma samples from Wt and ASGP-R2(−/−) mice containing 25 μg of protein were separately diluted 1:10 in 30 mM Tris-HCl, pH 8.5, 7 M urea, 2 M thiourea, and 4% CHAPS (w/v). The diluted samples from Wt and ASGP-R2(−/−) mice were labeled with 200 pmol of either 3-(4-carboxymethyl)phenylmethyl-3′-ethyloxacarbocyanine halide *N*-hydroxysuccinimidyl ester (Cy2), or 1-(5-carboxypentyl)-1′-methylindodicarbocyanine halide *N*-hydroxysuccinimidyl ester (Cy5), respectively, in the dark for 30 min at 4 °C in a total volume of 20 μl. Excess *N*-hydroxysuccinimidyl esters were consumed by adding 1 μl of 10 mM lysine and incubating for 10 min at 4 °C in the dark. The labeled Wt and ASGP-R2(−/−) samples were combined and brought to a final volume of 450 μl with 400 μl of 7 M urea, 2 M thiourea, 4% CHAPS, 10% isopropanol, 5% glycerol (w/v), 5.4 μl Destreak Rehydration Solution (GE Healthcare), and 2.25 μl of ampholyte pH 3–11 (GE Healthcare).

Two-dimensional Differential In-gel Electrophoresis (2D-DIGE)—Isoelectric focusing of the combined, labeled samples was conducted using 24-cm 3–10 NL-immobilized pH gradient (IPG) strips for 6500 Volt-h using an IPGPhor (GE Healthcare). After focusing, the IPG strips were incubated in 10 ml of 50 mM Tris, pH 8.8, 6 M urea, 30% glycerol, and 2% SDS containing 50 mg dithiothreitol. The reduced proteins were alkylated by incubation in the same buffer containing 600 mg of iodoacetamide. Electrophoretic separation by SDS-PAGE was performed by layering the IPG strips onto 10% polyacrylamide gels. The Cy2- and Cy5-labeled images were acquired using a Typhoon Imager (GE Healthcare, Cy2: Blue 488 nm laser, 520 BP40 filter; Cy5: Red 633 nm laser, 670BP30 filter). Relative quantification of specific gel spots was determined using the DIA and BVA modules of the DeCyder software.

Protein Identification of Gel Spots by Mass Spectrometry—Specific gel spots were selected using the Decyder Software and excised from the gel using an x,y robot (ProPic, Genomic Solutions, Ann Arbor, MI). The gel pieces were trypsin-digested. Mass spectrometric analysis was performed on the tryptic peptides as previously described (19) using either a MALDI-TOF/TOF instrument (ABI4700) or a nano LC-MS using a QSTAR-L mass spectrometer.

Lectin Blots—Plasma proteins, 75 μg, from Wt and ASGP-R2(−/−) mice were labeled with Cy5 and individually separated by two-dimensional gel electrophoresis as described above on 12–16% polyacrylamide gels and electrophoretically transferred to Immobilon-FL PVDF membranes. The membranes were each incubated in 125 ml of horseradish peroxidase-conjugated *Sambucus nigra I* (SNA-1) lectin (EY Labs) diluted 1:1000 in 20 mM Tris, 500 mM NaCl, 0.05% Tween 20, pH 7.5 containing 1% (w/v) bovine serum albumin (Sigma A-7030) for 45 m. The membranes were washed and developed by incubation in 30 ml of ECL plus (GE Healthcare) working solution at 25 °C for 5 m. Digital images were generated by scanning the blots with Typhoon Imager (HRP: 457 nm laser 520BP40 filter; Cy5: 633 nm laser, 670BP40 filter).

Neuraminidase Treatment, Wisteria Floribunda (WFA)-Agarose Affinity Chromatography, and Western Blotting—Plasma proteins, 400 μg, from Wt and ASGP-R2(−/−) mice was digested with neuraminidase from *Arthrobacter ureafaciens* (Roche Applied Science) at 37 °C overnight. WFA-agarose (EY Laboratories) was prepared for incubation with each sample by washing 50 μl, with 2.5 ml of PBS. Each sample of neuraminidase-treated plasma was applied to WFA-agarose in a Handee mini-spin column (Pierce) and incubated overnight at 4 °C. Unbound proteins were collected by sedimenting the WFA-agarose (200 × *g* for 1 min) in the spin column. The columns were washed three times with 200 μl of PBS, and each wash fraction was collected by centrifugation. Bound proteins were eluted with 50 mM GalNAc and analyzed by Western blot following separation by SDS-PAGE using 4–12% Bis-Tris gels. Glycoproteins bearing terminal β1,4-linked GalNAc were identified using the monoclonal antibody SMLDN1:1 that is specific for this structure (20) at a dilution of 1:1,000,000 followed by horseradish peroxidase-conjugated goat anti-mouse IgM at a dilution of 1:500,000. Glycoproteins recognized by SMLDN1:1 were excised from a gel, trypsin-digested, and analyzed by mass spectrometry.

Quantitative Western Blotting—The relative quantities of multiple plasma proteins, haptoglobin, SAP, hemopexin, transferrin, and C-reactive protein (CRP) were determined. Plasma proteins from individual Wt and ASGP-R2(−/−) mice were separated by SDS-PAGE and electrophoretically transferred to Immobilon-FL PVDF membranes (Millipore). For hemopexin detection, 1 μg of plasma proteins was loaded onto a 4–12% Bis-Tris gel. For haptoglobin, transferrin, and CRP detection, 10 μg of plasma proteins was loaded onto 4–12% Bis-Tris gels. For SAP detection, 100 μg of plasma proteins was loaded onto 10% Bis-Tris gels (Invitrogen). The plasma was separated using MOPS Running Buffer for all gels (Invitrogen). Following electrophoretic transfer membranes were incubated in 0.1% casein (Hammersten grade, BDH Chemicals) for 1 h to block nonspecific binding during incubation with antibodies. The relative quantity of each protein in each sample was determined by incubating the membranes overnight at 4 °C with the appropriate antibody in 25 ml of PBS. The dilutions were: rabbit anti-mouse haptoglobin (Life Diagnostics) 1:20,000, rabbit anti-rat SAP 1:20,000 rabbit anti-rat hemopexin (Life Diagnostics) 1:2,500, rabbit anti-rat transferrin 1:10,000, and rabbit anti-rat CRP 1:10,000. The membranes were washed three times for 10

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min at 25 °C with 0.01% Tween 20 in PBS and incubated for 1 h at room temperature with IReDye800 labeled goat anti-rabbit IgG (Rockland). After washing three times for 10 min at 25 °C with 0.01% Tween 20 in PBS, a LICOR Odyssey was used to scan and quantitate the relative amount of protein in each fraction.

Sambucus Nigra I (SNA-I)-Agarose and Ricinus Communis I (RCA-I)-Agarose Affinity Chromatography—SNA-I-agarose and RCA-I-agarose (EY Laboratories) were prepared for incubation with each sample by washing 50 μl with 2.5 ml of PBS. Plasma proteins, 25 μg in 50 μl of PBS, from Wt or ASGP-R2(−/−) mice were incubated overnight at 4 °C with 50 μl SNA-I or RCA-I-agarose in Handee mini-spin columns (Pierce). Unbound proteins were collected by centrifugation (200 × *g* for 1 min). The columns were washed three times with 200 μl PBS and each wash fraction was collected by centrifugation. The bound proteins were eluted from the column using 45 μl of NuPAGE LDS sample buffer and 5 μl of NuPAGE sample reducing agent (Invitrogen) and boiling for 5 min. The amount of haptoglobin in each fraction was determined by Western blot analysis as described above.

RESULTS

Multiple Plasma Proteins Are Elevated in the Plasma of ASGP-R2(−/−) Mice as Compared with Wt Mice—The levels of ASGP-R1 are markedly reduced in ASGP-R2(−/−) mice (21), and clearance of glycoproteins bearing terminal β1,4-linked Gal is abolished (13, 21). In addition, membranes prepared from ASGP-R2(−/−) mice do not exhibit detectable binding of glycoproteins such as orosomucoid that have been treated with neuraminidase to expose terminal Gal (not shown). Thus, even though the minor subunit of the ASGP-R has been ablated in ASGP-R2(−/−) mice, the mice are functionally deficient in ASGP-R activity in the liver.

The vast majority of proteins found in plasma are glycosylated. Many of these plasma glycoproteins bear one or more *N*-linked oligosaccharides that have branches that terminate with either Siaα2,6Gal or Siaα2,3Gal. If, as we have proposed (13), oligosaccharides terminating with Siaα2,6Gal are recognized by the ASGP-R and cleared from the blood at a more rapid rate than those terminating with Siaα2,3Gal, glycoproteins bearing oligosaccharides terminating with Siaα2,6Gal should be elevated in the plasma of ASGP-R2(−/−) mice. We have used fluorescence 2D-DIGE to address this issue. Plasma was isolated from Wt and ASGP-R2(−/−) mice, and equal amounts of plasma protein were fluorescently labeled with Cy2 and Cy5, respectively. The Cy2- and Cy5-labeled samples were combined and then separated into components by two-dimensional electrophoresis. The gel was scanned using the excitation and emission wavelengths specific for Cy2 or Cy5, and the false colored images were digitally combined to produce the composite image illustrated in Fig. 1.

As many as 160 individual spots were elevated >3-fold in the plasma of ASGP-R2(−/−) mice as compared with Wt mice. Seven of the most prominent groups of spots that were elevated are circled and designated 1–7 in Fig. 1. The presence of multiple spots differing in charge but not size is characteristic of glycoproteins. Tryptic peptides from individual spots within the regions marked 1–7 were identified by tandem mass spectrometry as summarized in Table 1. All of the spots identified are glycoproteins. Several are associated with the acute phase response including SAP, haptoglobin, serine protease inhibitor clade A, inter-α-trypsin inhibitor, and α1-B glycoprotein. Carboxylesterase and the IgM heavy chain are also glycoproteins but are not considered acute phase proteins. The majority of spots are not elevated in ASGP-R2(−/−) mice as indicated by the features colored *yellow* in Fig. 1. Thus, only specific plasma proteins are elevated in ASGP-R2(−/−) mice. DeCyder Software was used to determine the relative fold-change for each of the spots that were identified as summarized in Table 1. Individual spots were elevated between 2.8 (inter-α trypsin inhibitor) and 33-fold (SAP) in ASGP-R2(−/−) mice as compared with Wt mice (Table 1).

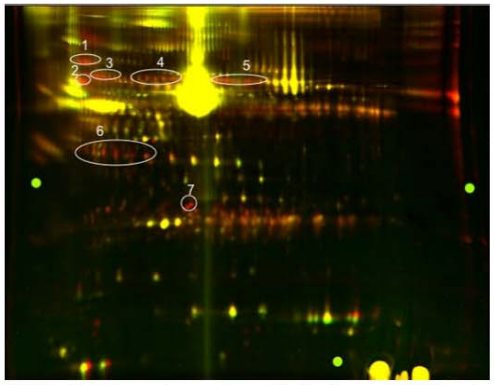

FIGURE 1. **Multiple proteins are elevated in the plasma of ASGP-R2(−/−) mice.** Equal amounts of plasma protein, 25 μg, from Wt and ASGP-R2(−/−) mice were labeled with Cy2 and Cy5, respectively, as described under "Materials and Methods." The cyanine dye-labeled samples were subsequently combined and separated by 2D-DIGE. The images were acquired with a Typhoon Imager and combined to generate the false colored image shown. Wt Cy2-labeled proteins are colored *green* and ASGP-R2(−/−) Cy5-labeled proteins are colored *red*. *Spots*, designated 1–7, that are *red* represent plasma proteins that are elevated in ASGP-R2(−/−) mice and were subsequently identified by MALDI-TOF/TOF following trypsin digestion. The majority of proteins are colored *yellow* indicating they are present in equal amounts in plasma from Wt and ASGP-R2(−/−) mice. DeCyder Software was used to quantitate the relative levels of plasma proteins in these images as summarized in Table 1.

Plasma Glycoproteins Bearing Terminal Siaα2,6Gal Are Elevated in the Plasma of ASGP-R2(−/−) Mice—The results obtained by 2D-DIGE suggest that the ASGP-R regulates the concentration of a select group of plasma proteins. Based on the ability of the ASGP-R to bind glycoproteins bearing structures that terminate in Siaα2,6Gal (13), we predicted that the glycoproteins that are elevated in the plasma of ASGP-R2(−/−) mice will be enriched for structures that terminate with Siaα2,6Gal.

The lectin SNA-I binds saccharides terminating with Siaα2,6Gal/GalNAc but not those terminating with Siaα2,3Gal (22, 23). Cyanine dye-labeled plasma proteins from Wt and ASGP-R2(−/−) mice were separately subjected to two-dimensional gel electrophoresis and electrophoretically transferred to PVDF membranes. The relative locations and levels of spots containing terminal Siaα2,6Gal/GalNAc were determined by

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TABLE 1

Identification of proteins increased in plasma from ASGP-R2(−/−) mice

Spots identified as being elevated in the plasma of ASGP-R2(−/−) mice, indicated by the numbers 1–7 in Fig. 1, were identified by tandem mass spectrometry of tryptic fragments. The identification and fold increase in level of plasma proteins from ASGP-R2(−/−) as compared to Wt mice are indicated for individual spots in each group. APP indicates acute phase proteins; i.e. protein that increase during the acute phase response.

Protein ID	Location	GI number	Fold change (−/−)/Wt	Function
Serine protease Inhibitor Clade A Member 1A	1	6678079	6.934.48	Endopeptidase inhibitor, APP
Carboxylesterase	2	2921308	3.739.987.35	Hydrolyze and inactivate toxins
α1-B glycoprotein	3	63680085	8.92	Not known, APP
Inter-α trypsin inhibitor Heavy chain 4	4	12836422	3.972.78	Inhibits trypsin and plasmin, APP
IgM	5	52382	3.163.67	Antibody
Haptoglobin	6	2144486	8.954.14	Bind hemoglobin to prevent oxidative damage, APP
SAP	7	54045	33.3820.11	Controls chromatin degradation, APP

Western blot using SNA-I-HRP as shown in Fig. 2. Both the level and number of spots that react with SNA-I are increased in the plasma from ASGP-R2(−/−) mice. Furthermore, at least 6 of the 7 groups of spots identified in Fig. 1 (labeled 1–4, 6–7) and Table 1 as representing elevated plasma glycoproteins correspond to the spots that react with SNA-I. Because the proteins were cyanine dye-labeled, we could confirm the correspondence between the SNA-I reactive spots and the labeled spots.

SNA-I will bind to structures terminating with Siaα2,6GalNAc as well those terminating with Siaα2,6Gal; however, the only glycoproteins in plasma known to bear Siaα2,6GalNAc in rodents are prolactin-like proteins (PLP) in the rat (14) and glycoprotein hormones such as luteinizing hormone (LH) in the mouse (24). The former are only present in plasma during pregnancy (14) and both are present in small quantities. We determined that the predominant, if not exclusive, structure detected by SNA-I in the plasma of Wt and ASGP-R2(−/−) mice is Siaα2,6Gal. Plasma from Wt and ASGP-R2(−/−) mice was treated with neuraminidase to remove sialic acid and expose underlying Gal or GalNAc. The neuraminidase-digested plasma was then incubated with WFA-agarose, which binds oligosaccharides bearing terminal β1,4-linked GalNAc but not those bearing terminal β1,4-linked Gal (25). Glycoproteins bound to WFA-agarose were eluted analyzed by Western blot using the SMLDN1:1 mAb, a monoclonal antibody specific for terminal β1,4-linked GalNAc (20). There was no evidence of any glycoprotein in either the bound or unbound fraction containing terminal GalNAc (not shown). Carbonic anhydrase-VI, which bears oligosaccharides terminating with Siaα2,6GalNAc (26), was used as a positive control. Thus, the glycoproteins in plasma that react with SNA-I bear oligosaccharides that terminate with Siaα2,6Gal.

Taken together, the results indicate that glycoproteins bearing oligosaccharides terminating with Siaα2,6Gal represent the major fraction of glycoproteins that are increased in the plasma of ASGP-R2(−/−) mice.

Haptoglobin and SAP Are Elevated in Plasma from ASGP-R2(−/−) Mice—Spots identified on two-dimensional gels represent individual isoforms of glycoproteins. It is possible that individual isoforms are elevated at the expense of other isoforms, but that the overall level of a given plasma glycoprotein is not increased in ASGP-R2(−/−) mice. Western blot analysis using antibodies specific for haptoglobin and SAP was used to determine if the total level of these two glycoproteins was elevated in the plasma of ASGP-R2(−/−) mice.

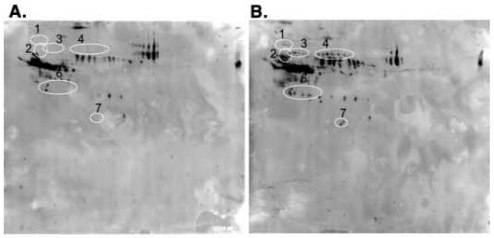

FIGURE 2. **Glycoproteins bearing terminal Siaα2,6Galβ1,4GlcNAc are elevated in the plasma of ASGP-R2(−/−) mice.** Equal amounts of Cy5-labeled plasma proteins, 75 μg from Wt (*panel A*) and ASGP-R2(−/−) (*panel B*) mice, respectively, were separately subjected to 2D-GE and electrophoretically transferred to Immobilon-FL PVDF membranes. The membranes were then probed with HRP-SNA-I, which recognizes structures terminating with Siaα2,6Galβ1,4GlcNAc. All spots except one that were identified with an increase in ASGP-R2(−/−) mice corresponded to glycoproteins that are recognized by SNA-I and show increased reactivity in plasma from ASGP-R2(−/−) mice. Compare circled regions in *panels A* and *B*.

Plasma obtained from 7 Wt and 11 ASGP-R2(−/−) mice was examined as summarized in Fig. 3. Haptoglobin increased 3–5-fold in ASGP-R2(−/−) plasma ($p = 0.0112$) when compared with Wt (Fig. 3*A*). SAP increased 2–3-fold ($p = 0.0146$) when compared with Wt (Fig. 3*B*). These represent highly significant increases for these two major plasma glycoproteins.

Hemopexin, transferrin, and CRP provide examples of plasma proteins that were not elevated in ASGP-R2(−/−) mice when examined by 2D-DIGE. Quantitation was performed by Western blot using antibodies specific for hemopexin, transferrin, and CRP respectively. In agreement with the 2D-DIGE analysis, neither hemopexin (Fig. 3*D*) or transferrin (Fig. 3*E*), which are glycoproteins, nor CRP (Fig. 3*C*), which is not glycosylated, were elevated. The absence of an increase in CRP levels in ASGP-R2(−/−) mice indicates that the increased levels of Siaα2,6Gal-bearing glycoproteins in ASGP-R2(−/−) mice is not due to induction of the acute phase response.

Haptoglobin from Both Wt and ASGP-R2(−/−) Mice Is Modified with Siaα2,6Gal—Haptoglobin is a major plasma glycoprotein known to bear two *N*-linked oligosaccharides that are extensively modified with terminal Siaα2,6Gal (27). The SNA-I lectin blot in Fig. 2 shows that the isoforms of haptoglobin that are elevated in plasma from ASGP-R2(−/−) mice bear terminal Siaα2,6Gal; but do not establish that these are the major isoforms of haptoglobin.

Plasma, 25 μg, from Wt and ASGP-R2(−/−) mice was incubated overnight at 4 °C with SNA-I immobilized on agarose

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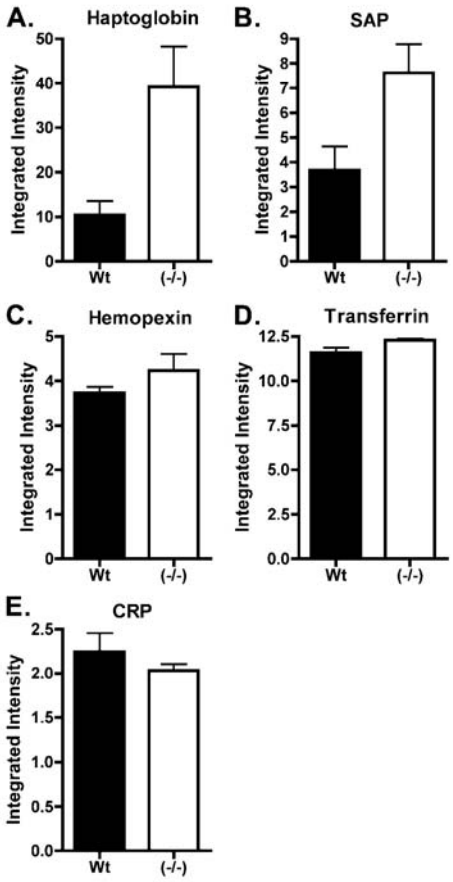

FIGURE 3. **Levels of haptoglobin and SAP but not hemopexin, transferrin, or CRP are elevated in the plasma of ASGP-R2(−/−) mice.** Equal amounts of plasma protein per sample, 1 μg for hemopexin, 10 μg for haptoglobin, transferrin, and CRP or 100 μg for SAP detection, from 7 Wt and 11 ASGP-R2(−/−) mice were separated by SDS-PAGE and electrophoretically transferred to Immobilon-FL PVDF membranes. Western blots were performed to quantitate the relative amount of: (*panel A*) haptoglobin, (*panel B*) SAP, (*panel C*) hemopexin, (*panel D*) transferrin, and (*panel E*) CRP in each sample. Haptoglobin is elevated 4-fold, $p = 0.01$, and SAP 2-fold, $p = 0.01$ in plasma from ASGP-R2(−/−) mice. Hemopexin, transferrin, and CRP did not differ significantly in levels in Wt and ASGP-R2(−/−) mice.

(Fig. 4*A*). After washing to remove unbound proteins, bound glycoproteins were eluted from the column by boiling in SDS. Less than 10% of the haptoglobin remained in the unbound fraction following incubation with SNA-I agarose and >90% of the bound haptoglobin was present in the fraction eluted with SDS (Fig. 4*A*). In contrast, when Wt or ASGP-R2(−/−) plasma was incubated with immobilized RCA-I, which binds oligosaccharides with terminal Gal (28), the major fraction of haptoglobin was present in the unbound fraction and the first wash fraction. The differences in the amount of haptoglobin in the

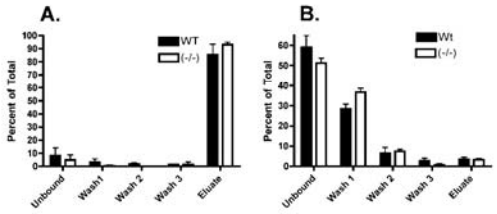

FIGURE 4. **Haptoglobin-bearing oligosaccharides that terminate with Siaα2,6Gal predominate in the plasma of Wt and ASGP-R2(−/−).** Plasma, 25 μg, from Wt and ASGP-R2(−/−) mice was incubated with SNA-I-agarose (*panel A*) or RCA-I-agarose (*panel B*). Equal aliquots of the supernatant containing unbound haptoglobin, each of three wash fractions, and material eluted by warming the agarose in SDS-PAGE-loading buffer was subjected to SDS-PAGE and electrophoretic transfer to Immobilon-F PVDF membranes. The amount of haptoglobin in each fraction was determined by Western blotting using rabbit anti-mouse haptoglobin. The amount of haptoglobin in each fraction is shown as a percent of the total.

Unbound and Wash1 fractions from Wt and ASGP-R2(−/−) mice does not reach statistical significance. The amount of haptoglobin present in the bound fraction did not differ, 3% in plasma from Wt mice *versus* 4% in plasma of ASGP-R2(−/−) (Fig. 4*B*). Thus, the major, if not exclusive, form of haptoglobin in both Wt and ASGP-R2(−/−) mice bears oligosaccharides terminating with Siaα2,6Gal whereas haptoglobin bearing terminal Gal is a minor fraction in Wt and ASGP-R2(−/−) mice. Furthermore, the amount of haptoglobin bearing terminal Gal does not increase significantly in ASGP-R2(−/−) mice as compared with Wt mice. Thus, the increase in haptoglobin in ASGP-R2(−/−) mice represents a reduced clearance rate for glycoproteins bearing oligosaccharides that terminate with Siaα2,6Gal.

DISCUSSION

Our observation that multiple glycoproteins bearing terminal Siaα2,6Gal are elevated in the plasma of ASGP-R2(−/−) mice indicates that the ASGP-R serves to control the concentration of glycoproteins bearing structures terminating with Siaα2,6Gal by clearing them from the blood. The ASGP-R, an abundant carbohydrate-specific, endocytic receptor that is expressed by parenchymal cells in the liver (1), is well suited for this purpose. It is among the most abundant receptors known in mammals with as many as 500,000 asialoglycoprotein binding sites expressed at the plasma membrane of hepatocytes (3–5). The receptor is rapidly endocytosed and recycled back to the cell surface. The ASGP-R is capable of internalizing 0.1 pmol of ligand per minute per 10^6 parenchymal cells (3, 29). The 1.5 g of liver in the mouse is capable of removing 1.2 nmol of ligand per h from the blood if it is saturated with ligand! This is the equivalent of clearing 110 μg of haptoglobin dimer with a molecular weight of 90,000 per hour. The normal concentration of haptoglobin, one of the most abundant glycoproteins present in plasma, is 600–2700 μg/ml or 7.0–30 μM. If the ASGP-R binds haptoglobin with a K_d of 3.6 μM, the K_d that we obtained for binding bovine serum albumin chemically modified with oligosaccharides terminating with Siaα2,6Gal (13), the receptor would be saturated at the concentrations of haptoglobin

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seen *in vivo*. As a result, if haptoglobin were the only glycoprotein recognized the ASGP-R would remove 110 μg of haptoglobin from the blood per h or between 2 and 9% of the haptoglobin present in the entire circulation of the mouse every h.

However, haptoglobin is not the only glycoprotein that bears oligosaccharides that terminate with Siaα2,6Gal, nor is it the only glycoprotein that is elevated in the plasma of ASGP-R2(−/−) mice. The glycoproteins listed in Table 1 as well as a number of other glycoproteins also bear oligosaccharides terminating with Siaα2,6Gal and will compete with haptoglobin as well as each other for binding to the ASGP-R. The ASGP-R will, therefore, be saturated with respect to binding ligands that bear terminal Siaα2,6Gal. As a result the fraction of ASGP-R binding sites that are occupied by individual glycoproteins will reflect their relative affinity and concentration relative to the other glycoproteins in plasma that are recognized. For example, if haptoglobin is bound with a K_d of 3.0 μM and SAP with a K_d of 15 μM and both are present in plasma at the same concentration, 80% of the ASGP-R will be occupied with haptoglobin and 20% with SAP. Under this condition haptoglobin will be cleared at a 4-fold greater rate than SAP.

The affinity of individual plasma glycoproteins that bear oligosaccharides that terminate with Siaα2,6Gal for the ASGP-R will reflect the number and location of these structures. The concentrations of plasma glycoproteins will reflect their rates of synthesis and their rates of removal by mechanisms related to their function such as the binding of free hemoglobin by haptoglobin as well as clearance by the ASGP-R. We propose that the ASGP-R acts much like a "buffer" *in vivo*. If the concentration of a plasma glycoprotein increases due to increased synthesis, it will occupy a greater fraction of the ASGP-R and be cleared at a more rapid rate. If the concentration of a plasma glycoprotein decreases due to consumption, for example in the case of haptoglobin by increased hemolysis, a smaller fraction of the ASGP-R will be occupied by that glycoprotein and it will be cleared at a slower rate.

We postulate that the ASGP-R serves to help regulate the relative concentrations of a wide range of plasma glycoproteins that all bear terminal Siaα2,6Gal. Because the total amount of these glycoproteins is more than sufficient to saturate the binding sites on the hepatocytes, each glycoprotein will occupy a fraction of the receptor proportionate to the concentration and affinity of that glycoprotein relative to all the other glycoproteins in the plasma. Should the concentration of an individual glycoprotein increase or decrease relative to all the others due to some perturbation, the ASGP-R will serve to return the glycoprotein to its same relative concentration after the perturbation has ceased by increasing or decreasing its rate of clearance. Such a function may well explain why so many different glycoproteins in plasma bear oligosaccharides that terminate with Siaα2,6Gal. An attractive feature of such a mechanism is that it would also serve to return glycoproteins to hepatocytes where their amino acids and sugars can be recycled following degradation in lysosomes. The rapid clearance of glycoproteins bearing terminal Siaα2,6GalNAc, Gal, or GalNAc will continue because glycoproteins bearing these structures will be bound with affinities that are 100-fold stronger than those for glycoproteins bearing structures terminating with Siaα2,6Gal. The rapid clearance of glycoproteins bearing terminal Siaα2,6GalNAc, Gal, or GalNAc may reflect additional functions that require rapid clearance by the ASGP-R. For example, the reported removal of platelets from the blood by the ASGP-R during *S. pneumoniae* sepsis due to the action of the bacterial neuraminidase may comprise such a distinct function (11).

The results we have presented indicate that the ASGP-R does indeed mediate the clearance of a wide range of glycoproteins that bear terminal Siaα2,6Gal. Ablation of the ASGP-R results in significant increases in the plasma concentrations of these glycoproteins. We propose that an important function of the ASGP-R is to contribute to the regulation of the relative concentrations of plasma glycoproteins bearing terminal Siaα2,6Gal.

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Chapter 3

The ASGR regulates plasma protein concentrations during the acute phase response

Introduction

Following an inflammatory stimulus, changes in physiologic functions occur within hours. The acute phase response (APR) is an inflammatory stimulus, such as an infection or trauma[64], and is induced by the rapid release of cytokines, such as TNF-α, interleukin-1, and interleukin-6[65]. This process results in concentration changes of a number of proteins, including C-reactive protein, serum amyloid A (SAA), haptoglobin, and hemopexin [64, 66-69]. Increased synthesis of these glycoproteins plays a major role in elevating their circulating concentrations [70]. Additionally, multiple plasma proteins that are involved in the APR terminate with Siaα2,6Gal/GalNAc [71]and changes in structure and ASGR binding activity suggest the ASGR may decrease clearance in order to increase plasma protein concentrations.

During the APR, the expression level of the ASGR decreases in HepG2 cells. The ASGR shuts down within hours following exposure to cytokines, specifically IL-2. Following 4 hours of incubation with IL-2, 50% of the binding activity remains, and following 24 hours of incubation, only 20% of the binding activity remains. Within the first few hours, the decrease in binding is due to the inactivation of the receptor by phosphorylation[35]. There is decreased synthesis of the receptor also, but this does not occur until hours later. Sialic acid terminating proteins that have been implicated in the APR begin to increase within 3-6 hours, long before the synthesis of receptor is decreased. In animals with a functioning ASGR, any immediate increase in APPs may be due to decreased removal from the blood by inactive ASGR rather than decreased synthesis of the ASGR in addition to increased synthesis of APPs by the liver. If this is

the case, ASGR-/- animals may have increased concentrations of essential APPs much earlier than normal animals.

Dramatic increases in plasma proteins terminating with sialic acid (Sia), the structure that has been implicated in our studies, have also been implicated in the APR. Almost all of the APPs are glycoproteins, and recent studies have suggested that there is a change in expression of sialyltransferases, including ST6GalI, which is the sialyltransferase that adds terminal sialic acid (Sia) to Gal to form Siaα2,6Gal, and Sia terminating glycoproteins, including haptoglobin β-chain and hemopexin during the APR[72]. Essentially all Gal residues become modified with Sia[73]. If the APPs are modified to terminate with Sia, then the ASGR may have a critical role in their clearance from the blood during the APR.

Many of the proteins elevated in ASGR-/- mice are APPs, including haptoglobin, hemopexin and SAP. The ASGR-/- mice have elevated APPs, but a major APP, C-reactive protein, is not elevated, indicating these unperturbed animals are not undergoing an APR [71]. Here, we show that when ASGR-/- mice undergo an APR, the APPs further increase. Specific isoforms, mainly acidic isoforms are highly elevated compared to Wt. However, the total protein levels of two proteins, haptoglobin and hemopexin do not have as dramatic a fold change in ASGR-/- compared to Wt between times 0 and 24 hrs. This suggests clearance by the ASGR in addition to increased synthesis of APPs by the liver is required to elevate APPs bearing Siaα2,6Gal.

Methods

Cecal ligation and puncture (CLP) – Male mice were anesthetized with halothane and one treatment group underwent laparotomy and CLP as previously described [74] and the other treatment group, sham, underwent laparotomy but no CLP. Animals were sacrificed at 24 hrs and plasma was collected.

Plasma Preparation—Blood was collected from the inferior vena cava of Wt and ASGR-/- mice at 0 hrs and 24 hrs after an APR using a 1-ml syringe and a 23-gauge needle treated with EDTA to prevent coagulation. The blood was diluted 1:1 with 20 mM PO_4, pH 7.4, 150 mM NaCl (PBS) containing 20 mM EDTA. Leukocytes and erythrocytes were separated from the plasma by sedimentation for 10 min at 0.5 x *g*. The protein concentration of the plasma was determined using a modified Lowry method (PlusOne 2D-Quant Kit, GE Healthcare).

Cyanine Dye Labeling—Plasma samples from ASGR-/- sham, ASGR-/- CLP and Wt CLP mice containing 25 µg of protein were separately diluted 1:10 in 30 mM Tris-HCl, pH 8.5, 7 M urea, 2 M thiourea, and 4% CHAPS (w/v). The diluted samples from ASGR-/- sham, ASGR-/- CLP and Wt CLP mice were labeled with 200 pmol of 3-(4-carboxymethyl)phenylmethyl-3'-ethyloxacarbocyanine halide *N*-hydroxysuccinimidyl ester (Cy2),

1-(5-carboxypentyl)-10-propylindocarbocyanine halide *N*-hydroxysuccinimidyl ester (Cy3) or 1-(5-scarboxypentyl)-1'-methylindodicarbocyanine halide *N*-hydroxysuccinimidyl ester (Cy5), respectively, in the dark for 30 min at 4 °C in a total volume of 20 µl. Excess *N*-hydroxysuccinimidyl esters were consumed by adding 1 µl of 10 mM lysine and incubating for 10 min at 4 °C in the dark. The three labeled samples

were combined and brought to a final volume of 450 µl with 400 µl of 7 M urea, 2 M thiourea, 4% CHAPS, 10% isopropanol, 5% glycerol (w/v), 5.4 µl Destreak Rehydration Solution (GE Healthcare), and 2.25 µl of ampholyte pH 3–11 (GE Healthcare).

Two-dimensional Differential In-gel Electrophoresis (2D-DIGE)—Isoelectric focusing of the combined, labeled samples was conducted using 24-cm 3–10 NL-immobilized pH gradient (IPG) strips for 6500 Volt-h using an IPGPhor (GE Healthcare). After focusing, the IPG strips were incubated in 10 ml of 50 mM Tris, pH 8.8, 6 M urea, 30% glycerol, and 2% SDS containing 50 mg dithiothreitol. The reduced proteins were alkylated by incubation in the same buffer containing 600 mg of iodoacetamide. Electrophoretic separation by SDS-PAGE was performed by layering the IPG strips onto 10% polyacrylamide gels. The Cy2-, Cy3- and Cy5-labeled images were acquired using a Typhoon Imager (GE Healthcare). Relative quantification of specific gel spots was determined using the DIA and BVA modules of the DeCyder software.

Protein Identification of Gel Spots by Mass Spectrometry—Specific gel spots were selected using the Decyder Software and excised from the gel using an x,y robot (ProPic, Genomic Solutions, Ann Arbor, MI). The gel pieces were trypsin-digested. Mass spectrometric analysis was performed on the tryptic peptides as previously described using either a MALDI-TOF/TOF instrument (ABI4700) or a nano LC-MS using a QSTAR-L mass spectrometer.

Lipopolysaccaride(LPS) Injection – To determine the optimal amount of LPS to induce, multiple concentrations of LPS were given to Wt mice. The concentrations given are: saline (no LPS), 5µg/kg, 50 µg/kg, 500 µg/kg, and 5 mg/kg. The LPS was diluted with

saline and injected intraperitonially. The optimal dose was determined to be 500 µg/kg and this is the dosage that was used.

Quantitative Western Blotting—The relative quantities of two plasma proteins, haptoglobin and hemopexin were determined. Plasma proteins from individual Wt and ASGR-/- mice at 0 hrs and 24 hrs after LPS was injected were separated by SDS-PAGE and electrophoretically transferred to Immobilon-FL PVDF membranes (Millipore). For hemopexin detection, 1 µg of plasma proteins was loaded onto a 4–12% Bis-Tris gel. For haptoglobin, 10 µg of plasma proteins was loaded onto 4–12% Bis-Tris gels. The plasma was separated using MOPS Running Buffer for all gels (Invitrogen). Following electrophoretic transfer membranes were incubated in 0.1% casein (Hammersten grade, BDH Chemicals) for 1 h to block nonspecific binding during incubation with antibodies. The relative quantity of each protein in each sample was determined by incubating the membranes overnight at 4 °C with the appropriate antibody in 25 ml of PBS. The dilutions were: rabbit anti-mouse haptoglobin (Life Diagnostics) 1:20,000 and rabbit anti-rat hemopexin (Life Diagnostics) 1:2,500. The membranes were washed three times for 10 min at 25 °C with 0.01% Tween 20 in PBS and incubated for 1 h at room temperature with IReDye800 labeled goat anti-rabbit IgG (Rockland). After washing three times for 10 min at 25 °C with 0.01% Tween 20 in PBS, a LICOR Odyssey was used to scan and quantitate the relative amount of protein in each fraction.

Results

Multiple proteins are elevated in ASGR-/- during APR. In unperturbed ASGR-/- plasma**,** multiple APPs are elevated; however, ASGR-/- mice do not have elevated CRP and therefore, are not undergoing an APR [71]. Once an APR is induced in ASGR-/- mice, we expect the APPs to further increase in concentration compared to unperturbed ASGR-/- mice. To visualize changes in plasma protein concentrations, we used 2D-DIGE to separate the cyanine-labeled proteins into individual components and scanned the gel with a Typhoon Imager. In the false-colored image, elevated ASGR-/- sham proteins appear green, elevated ASGR-/- CLP proteins appear blue, and elevated Wt CLP proteins appear red. Proteins with equal amounts in all samples, such as albumin, appear white.

Multiple proteins that are elevated in unperturbed ASGR-/- further elevate in ASGR-/- CLP plasma (Figure 1). Tryptic peptides from multiple proteins were identified by tandem mass spectrometry. The identities of these proteins, labeled 1-7 are listed in Table 1. The column ASGR-/- CLP/ASGR-/- sham contains data showing the elevation of proteins during the APR compared to the control group. The column labeled ASGR-/- CLP/Wt CLP compares the elevation of ASGR-/- APR to Wt APR animals. Proteins do elevate during the APR in ASGR-/- mice compared to the sham. We identified a total of seven elevated proteins by mass spectrometry: ceruloplasmin, inter-alpha trypsin inhibitor heavy chain 4, hemopexin, alpha-1-acid glycoprotein, haptoglobin, SAP and complement 3. Two of the proteins previously identified in unperturbed ASGR-/- plasma, haptoglobin and SAP further elevate in ASGR-/- CLP plasma. Haptoglobin and SAP elevate 2.22 and 3.99-fold respectively, in ASGR-/- CLP mice compare to ASGR-/- sham mice. Hemopexin is not significantly elevated in unperturbed ASGR-/- plasma.

Haptoglobin, SAP, and hemopexin elevate significantly in ASGR-/- plasma compared to Wt CLP plasma. Haptoglobin elevated 7.34-fold, SAP elevated 4.88-fold and hemopexin elevated 3.75-fold in ASGR-/- CLP plasma, compared to Wt CLP plasma**.**

While the 2D-DIGE technique is useful for initial identification of proteins that are elevated in the plasma, the 2D gel only gives us information about specific isoforms. Many of the isoforms identified are elevated, but other isoforms in the group are not elevated. Additional methods are necessary to establish whether total protein concentrations increase. In addition, only three samples can be run on a 2D-DIGE gel at a time so the ultimate comparison of Wt sham, ASGR-/- sham, Wt CLP, and ASGR-/- CLP cannot be conducted. Using an alternative method to induce an APR, we have compared haptoglobin and hemopexin levels before an APR and 24 hrs after the induction of an APR.

Haptoglobin and hemopexin are elevated in ASGR-/- during the APR. LPS, found in the outer membrane of Gram-negative bacteria, acts as an endotoxin and induces an APR. Several dilutions of LPS were used to determine the optimal dose of LPS to induce an acute phase response. The concentrations of LPS injected intraperitonially into our mice are saline with no LPS, 5 μg/kg, 50 μg/kg, 500 μg/kg, and 5 mg/kg (Figure 2). Quantification of haptoglobin was used to determine whether an APR was initiated, and we found that 500 μg/kg and 5 mg/kg both caused a dramatic increase in haptoglobin within 24 hrs. For our studies, we used 500 μg/kg to induce an APR in our mice.

Based on the 2D-DIGE gel, haptoglobin and hemopexin are both significantly elevated in ASGR-/- CLP plasma compared to Wt CLP plasma. We further investigated these two proteins using quantitative western blotting. Plasma was collected from Wt

and ASGR-/- mice, termed 0 hrs, the mice were injected with LPS, and 24 hrs following the injection, plasma was collected and analyzed.

Similar to what we had seen previously, before LPS was injected, haptoglobin was elevated 3.4-fold. After the APR was induced with LPS, haptoglobin increased 21.3-fold in Wt plasma, but only 7.54-fold in ASGR-/- plasma. The relative quantities of haptoglobin in Wt and ASGR-/- mice were not significantly different at 24 hrs. Hemopexin was slightly increased in ASGR-/- mice before injection. Following the LPS injection, hemopexin elevated 2.67-fold in Wt plasma, but only 1.35-fold in ASGR-/- plasma. The relative quantities of hemopexin were not significantly different at 24 hrs either. Haptoglobin and hemopexin in Wt mice elevates more significantly that in ASGR-/- plasma, and we propose this increase is due to decreased clearance.

Discussion

During the APR, there is an increase in a large number of proteins, many of which are glycoproteins. There are two types of APPs, positive APPs those that increase at least 25% during the APR and negative APPs, those that decrease at least 25% during the APR [75]. We have previously shown in ASGR-/- plasma, many proteins are elevated, and these proteins are mainly positive APPs [71]. Based on the identity of these proteins, we would expect them to further elevate during the APR. By 2D-DIGE, we have shown multiple proteins elevate during the APR and two of the proteins that are elevated in unperturbed ASGR-/- plasma, haptoglobin and SAP, further increase in ASGR-/- mice undergoing an APR.

Many of the proteins elevated in ASGR-/- plasma are APPs, and since these proteins are already elevated in ASGR-/- plasma, when these animals undergo an APR, these proteins will further elevate due to increased synthesis. However, if increased synthesis and decreased clearance by the ASGR both account for the elevation of these proteins, we should not see as dramatic of an increase in ASGR-/- CLP plasma. Using LPS to induce an APR, we have found that there is an elevation of haptoglobin and hemopexin in Wt and ASGR-/- plasma, but the increase is not as dramatic as in ASGR-/- plasma. Haptoglobin increases 21-fold in Wt plasma, and it only increases 7-fold in ASGR-/-. Similarly with hemopexin, in Wt plasma during the APR, hemopexin elevates 3-fold and in ASGR-/- plasma, hemopexin does not elevate in ASGR-/- plasma. This strongly suggests that in addition to elevated synthesis, decreased clearance accounts for the striking increase in APPs.

We have previously shown the ASGR clears glycoproteins bearing Siaα2,6Gal [71]. Many of the proteins found to be elevated during the APR bear Siaα2,6Gal. Treichel et al [35] previously showed in HepG2 cells, the ASGR becomes inactive within hours of exposure to IL-2 and ultimately is down regulated, suggesting the ASGR may play a role in regulating APP concentrations during the APR. Additionally, it has been shown the transferase that adds Sia to Gal, α2,6-sialyltransferase is up regulated and nearly all terminal Gal is capped with Sia [72, 73]. Twenty-four hours following the induction of an APR, the concentration of proteins in Wt and ASGR plasma are similar, despite the dramatic differences seen in unperturbed animals. This suggests in the Wt mice, the function of the ASGR has changed, becoming more similar to ASGR-/- mice. Combined, this information strongly suggests during the APR, decreased clearance of glycoproteins by the ASGR aids in elevating the concentration of proteins bearing Siaα2,6Gal.

We postulate that in addition to increased synthesis, decreased clearance by the ASGR during the APR elevates the APPs. Based on previous literature, the ASGR responds within the first few hours of an APR by phosphoylating the ASGR, rendering it inactive, and multiple hours later, synthesis of the receptor is decreased. Both of these events would result in decreased clearance by the ASGR. Future experiments include determining the binding activity of the ASGR in unperturbed animals and throughout the APR to elucidate the precise function of the ASGR throughout the APR.

Abbreviations

APR, acute phase response; APP, acute phase protein; ASGR, asialoglycoprotein receptor; Sia, sialic acid; Gal, galactose; GalNAc, N-acetylgalactosamine; CLP, cecal ligation and puncture

Tables

Table 1. Identification of proteins increased in plasma from ASGR–/– CLP mice.

Spots identified as being elevated in the plasma of ASGR–/– CLP mice, indicated by the numbers 1–7 in Fig. 1, were identified by tandem mass spectrometry of tryptic fragments. The identification and fold increase in level of plasma proteins from ASGR–/– CLP as compared to ASGR-/- and Wt CLP mice are indicated for individual spots in each group.

Protein	Location	GI Number	Fold change (-/-)CLP/ (-/-)sham	Fold change (-/-)CLP/Wt CLP
Ceruloplasmin	**1**	**38614350**	**2.72**	**1.09**
inter-alpha trypsin inhibitor heavy chain 4	**2**	**38614350**	**3.18** **1.41** **4.41**	**3.09** **2.65** **1.11**
Hemopexin	**3**	**22022646**	**1.29**	**3.75**
alpha-1-acid glycoprotein	**4**	**109548**	**1.88**	**4.13**
Haptoglobin	**5**	**2144486**	**2.22**	**7.34**
SAP	**6**	**54045**	**3.99**	**4.88**
Complement C3	**7**		**3.17**	**3.02**

Figures

Figure 1. Multiple proteins are elevated in ASGR-/- during the APR. Equal amounts of plasma from Wt CLP, ASGP-/- CLP, and ASGR-/- CLP were labeled with Cy2, Cy3, and Cy5, respectively. The cyanine-dye labeled samples were processed, separated by 2D-DIGE, and protein spots were identified by mass spectrometry as previously described. Proteins appearing in green are highest in the ASGR-/- sham, proteins in blue are highest in ASGR-/- CLP, and proteins in red are highest in Wt CLP. Proteins that appear white are equal in all three samples. The protein identifications, labeled 1-7 can be found in Table 2. The protein spots identified include two proteins identified in unperturbed ASGR-/- mice, haptoglobin and SAP, as well as five additional proteins, ceruloplasmin, inter-alpha trypsin inhibitor heavy chain 4, hemopexin, alpha-1-acid glycoprotein and component C3.

Figure 2. Induction of APR with LPS. Four different concentrations of LPS, 5 μg/kg, 50 μg/kg, 500 μg/kg, and 5 mg/kg were injected intraperitonially into Wt mice to determine the optimal concentration of LPS to inject to induce an APR. The relative quantity of haptoglobin was used to determine whether an APR was induced. An APR was induced with all 4 doses; The 500 μg/kg and 5 mg/kg doses caused over a 20-fold increase in haptoglobin. The dosage chosen for further experiments was 500 μg/kg**.**

Figure 3. Haptoglobin and hemopexin elevated in ASGR-/- during the APR, but not as dramatically as Wt. Plasma was collected at time 0, 500 μg/kg of LPS was injected intraperitonially into Wt and ASGR-/- mice, and the animals were sacrificed 24 hours later and blood was collected. Quantitative western blotting was conducted on the plasma, and the integrated intensity was used to determine relative amounts of

haptoglobin and hemopexin. Haptoglobin is elevated 3.4-fold in the 0 hr ASGR-/- plasma. At 24 hours, haptoglobin is elevated 7.54-fold in ASGR-/- plasma and 21.3-fold in Wt plasma ($p=0.0002$). Hemopexin is slightly elevated at 0 hrs in ASGR-/- plasma, but does not further elevate after 24 hrs in ASGR-/-. Hemopexin elevates 2.67-fold in Wt plasma.

Figure 1.

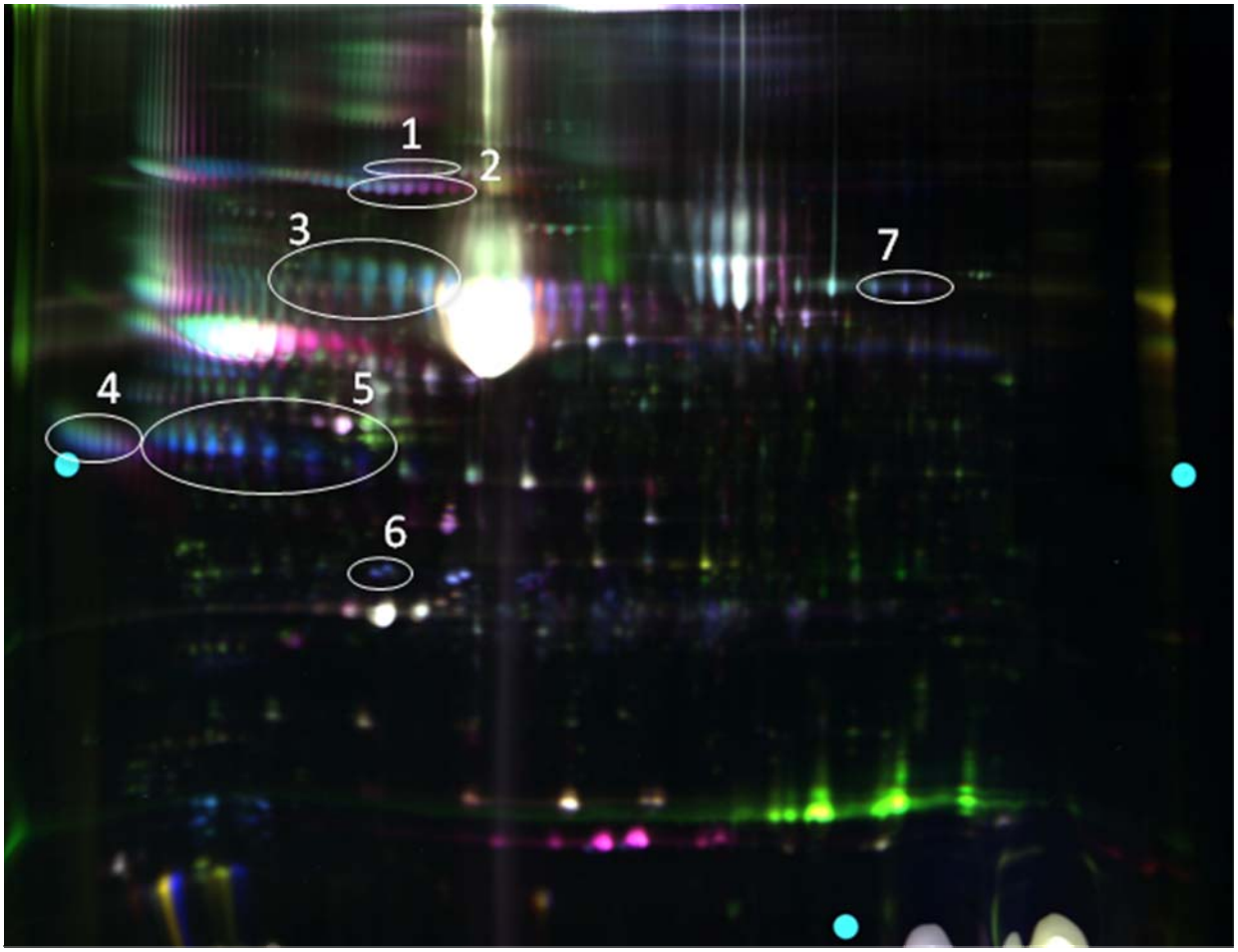
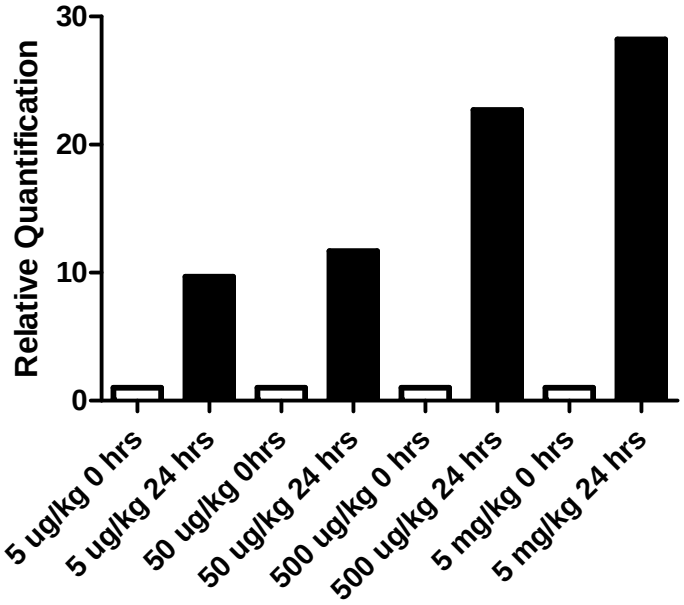


Figure 2.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20
10
0
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5 ug/kg 24 hrs
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50 ug/kg 24 hrs
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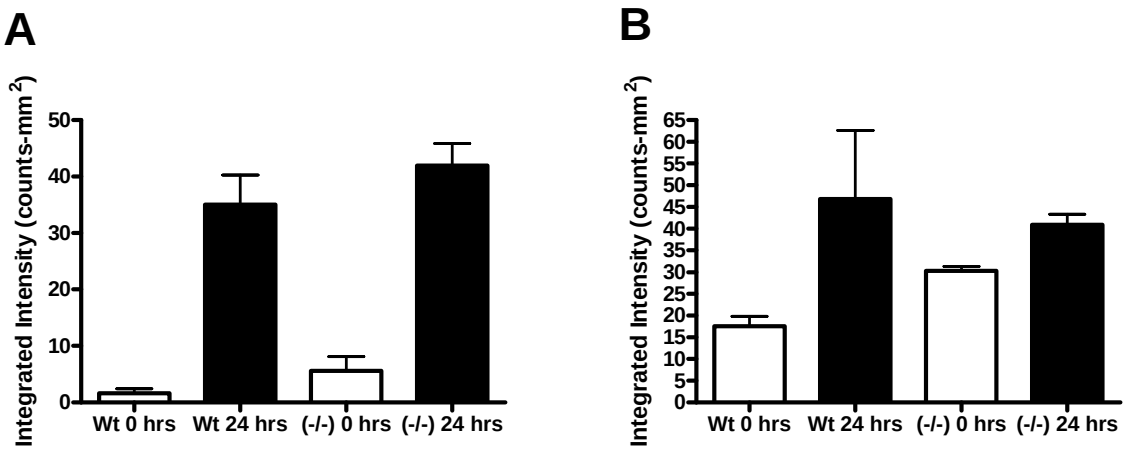
500 ug/kg 24 hrs
5 mg/kg 0 hrs
5 mg/kg 24 hrs

Figure 3.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40
30
20
10
0
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Wt 24 hrs
(-/-) 0 hrs
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B
Integrated Intensity (counts-mm^2)
65
60
55
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45
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Wt 0 hrs
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(-/-) 0 hrs
(-/-) 24 hrs

Chapter 4

The mannose/GalNAc-4-SO_4 receptor and asialoglycoprotein receptor regulate steroid hormone levels

Introduction

Luteinizing hormone (LH) bears unique N-linked oligosaccharides that terminate with the sequence SO_4-4-GalNAcβ1,4GlcNAc (GalNAc-4-SO_4) [7-10]. Two β1,4N-acetylgalactosaminyltransferases (βGT3 and βGT4) that may account for the protein-specific modification of LH and other glycoproteins bearing this modification have recently been cloned [76-78] and found to have the expected protein specificity [79]. Two GalNAc-4-sulfotransferases, GalNAc-4-ST1 and GalNAc-4-ST2, have also been cloned [80-82] and can selectively modify the terminal β1,4-linked GalNAc on N-linked oligosaccharides [83]. We recently demonstrated that following ablation of GalNAc-4-ST1, the predominant form of GalNAc-4-ST in the pituitary, sulfation of LH oligosaccharides is abolished and there is a marked increase in GalNAc bearing α2,6-linked sialic acid (Sia) on LH. The half-live for endogenous LH in the blood increases from 7.2 min in Wt mice to 10.1 min in GalNAc-4-ST1-/- mice. Furthermore, there are significant elevations in levels of LH, estrogen, and testosterone in the GalNAc-4-ST1-/- mice demonstrating that terminal glycosylation determines the half-life and as a result the potency of LH *in vivo* [63].

We demonstrated that the Cysteine-rich domain of the Mannose/GalNAc-4-SO_4-Receptor (MR), a ricin-type beta-trefoil, binds terminal GalNAc-4-SO_4 on oligosaccharides such as those found on LH [50] and that the asialoglycoprotein receptor (ASGR) binds oligosaccharides terminating Siaα2,6GalNAcβ1,4GlcNAc (Siaα2,6GalNAc) [33]. Lee et.al. [52] reported that ablation of MR did not increase the half life of radiolabeled porcine LH even though the half life of the neoglycoprotein S4GGnM-BSA was increased in MR-/- mice. In contrast, Mi et al. [53] reported a slight

increase in the half life of bovine LH and reduced implantation in heterozygous MR mice. Thus, while MR and the ASGR are able to bind oligosaccharides terminating with GalNAc-4-SO_4 and Siaα2,6GalNAc, respectively, their role in clearing endogenous LH remains to be definitively established.

We recently established a method to examine the time course for clearance of endogenous LH in mice and have used this method to determine if MR and/or ASGR determine the half life for endogenous LH. The clearance of endogenous LH was examined in MR-/-, ASGR-/-, and MR-/-ASGR-/-. In addition, hormone levels and mRNA levels for various genes were compared in mice with one or both receptors ablated to determine if there is a physiologic impact. The MR does indeed account for the clearance of endogenous LH in the mouse. Furthermore, changes in hormone levels and steady state levels of mRNAs indicate that both the MR and ASGR contribute to regulation of LH levels *in vivo*.

Methods

Serum Preparation and tissue collection – Serum was collected by terminal cardiac puncture. Mice were anesthetized with ketamine (87mg/kg) and xylazine (13 mg/kg), and blood was collected and allowed to clot for 30 min in serum separator tubes (BD). The serum was separated by centrifugation for 30 min at room temperature. Serum was stored at -80^{o}C. Tissues, including pituitary and testes were collected from the same mice and stored at -80^{o}C for further use.

Hormone Assays – Quantification of LH, FSH, and testosterone was conducted at University of Virginia Ligand Core. An immunoradiometric assay (IRMA) is used to quantify LH and a radioimmunoassay (RIA) to quantify FSH and testosterone.

Clearance of endogenous LH – Clearance of endogenous LH in castrated male mice was carried out as described previously [53]. Mice were anesthetized with ketamine and xylazine. A baseline sample of blood, 75 µl, was drawn, then 10 µg of Acyline (Woods Assay, Inc.), was injected into the retro-orbital sinus. Additional samples of 75 µl were collected at 5 and 10 min, and 150 µl was collected at 20 min. At 30 min, the mouse was bled by cardiac puncture. The mice were kept warm on a heating pad for the duration of the experiment, and after each blood draw, 200 µl of warm saline was injected intraperitoneally.

Castration – Mice were anesthetized with ketamine and xylanine. The scrotum was shaved and cleaned with isopropanol and povidone. A 2 inch midline incision was made in the scrotum, the tunica was pierced, and the testis was pushed out with gentle pressure. The spermatic artery was cauterized and cut to remove the testis. The epididymis, deferential vessels, and ductus were placed back in the tunica. Several drops

of lidocaine were placed in the incision, and the incision was closed with 6-0 Ethilon sutures.

RNA Isolation – RNA was isolated from individual pituitaries using MagMAX-96 Total RNA Isolation Kit (Ambion). Each pituitary was incubated in 100 µl Lysis/Binding Solution for 2 h at room temperature, and then homogenized using a 20-gauge needle and 1 ml syringe. Homogenized samples were transferred to a 96-well Processing Plate, 60 µl of 100% isopropanol was added, and the plate was shaken for 1 min on an orbital shaker. Bead mix containing RNA binding beads and Lysis/Binding Enhancer, 20 µl, was added to each sample and shaken for 5 min. The RNA binding beads were captured on the magnetic stand for 3 min and the supernatant was aspirated and discarded. The beads were washed once with 150 µl Wash I and once with150 µl Wash II. Nucleic acids were released from the binding beads with 50 µl diluted TURBODNase to degrade genomic DNA. RNA was rebound to the beads using 100 µl RNA Rebinding Solution. The beads were captured and the supernatant was aspirated and discarded. The beads were then washed twice with 150 µl Wash II, and the beads were dried. Finally, 30 µl of Elution Buffer was added to each sample and shaken vigorously. The supernatant containing the RNA was collected for further use.

For isolation of RNA from testes, tissue was homogenized in 1 ml TRIZOL Reagent. The homogenized samples were incubated for 5 min at room temperature to dissociate nucleoprotein complexes. Chloroform, 0.7 ml, was added and the mixture was shaken vigorously for 15 sec then incubated at room temperature for 3 min. Phase separation was completed by centrifuging at 13,000 x g for 15 min at 4^{o}C, and the aqueous phase was collected. The RNA was precipitated with 0.5 ml isopropanol,

incubated on ice for 15 min, and centrifuged at maximum speed for 30 min. RNA cleanup was done using RNeasy Mini Kit (Qiagen) exactly as indicated in the manufacturer's protocol. Briefly, the precipitated RNA was dissolved in 100 µl RNase-free water, ethanol was added, and the mixture was put onto an RNeasy Mini spin column. The column was washed and RNA eluted with 100 µl RNase-free water. Quantification of RNA from both pituitary and testes was determined using a Nanodrop (Thermo Scientific).

Quantitative RT-PCR (qPCR) – Template cDNA was generated using Omniscript Reverse Transcription kit (Qiagen). On ice, template RNA was thawed and fresh master mix was prepared. For the master mix, 1 µl 10x Buffer RT, 1 µl dNTP Mix, 1 µl Oligo-dT primer, 0.5 µl RNase inhibitor and 0.5 µl Omniscript Reverse Transcriptase were mixed together. To the master mix, 1 µg template RNA was added, and the final volume was brought to 10 µl with RNase-free water. The mixture was incubated at 37^{o}C for 1 h, then diluted 5-fold, to a final volume of 50 µl. The qPCR reaction was set up in triplicate for each sample with 2 µl diluted cDNA, 1 µM primers, 10 µl 2x Sybr Green Mix, and water to a final volume of 20 µl. The primer sequences used listed in Table 1. The qPCR was run on an ABI 7500 using standard conditions. The data was imported into Prism and analyzed.

Results

The MR mediates the rapid clearance of secreted LH from the blood.

The detailed structures of the N-linked oligosaccharides on murine (m) LH have not to date been examined. We have, however, shown that LH from the pituitary of wild type (Wt) mice contains significant amounts of terminal GalNAc-4-SO_4 and is the major glycoprotein in the pituitary bearing such structures [9, 63]. Furthermore, we determined that there is little terminal GalNAc on mLH and relatively little Siaα2,6GalNAc suggesting that, as in other mammalian species, N-linked oligosaccharides terminating with GalNAc-4-SO_4 predominate on mLH. Male mice were castrated in order to elevate the circulating levels of LH. Blood was withdrawn at time 0 and at 5, 10, 20, and 30 min after injection of Acyline, a GnRH antagonist that acutely blocks secretion of LH. The amount of mLH remaining in the blood at each time point was determined by IRMA. Examples of clearance curves obtained for Wt, MR-/-, ASGR-/-, and MR-/-ASGR-/- mice are shown in Figure 1, Panels A-D.

The half life for clearance of endogenous mLH increases from 8.4 min to 15.6 min in MR-/- mice as compared to Wt mice (Fig.1 Panels B and E). In contrast ablation of the ASGR does not change the half life of mLH as compared to Wt mice (Fig.1 Panels B and E). The increase in the half life of mLH in MR-/- mice indicates that MR does indeed account for the rapid clearance of LH from the blood following GnRH stimulated secretion. Since there is no increase in the half life of mLH in ASGR-/- mice, the ASGR must not account for much, if any of the clearance of LH in Wt mice. In addition, the vast majority of LH must bear multiple oligosaccharides that terminate with one of more GalNAc-4-SO_4 moieties. Mice that have had both receptors ablated, MR-/-ASGR-/-

mice (Fig 1.D), also remove LH from the circulation at a slower rate than Wt mice. The mean half life of 11.6 min is significantly longer than in Wt mice, p=0.042, but not significantly different from the half life seen in MR-/- mice. The half life obtained for LH clearance in MR-/-ASGR-/- mice also supports the conclusion that recognition of terminal GalNAc-4-SO_4 on LH by MR-/- is the predominant if not exclusive basis for the rapid clearance of LH from the blood.

Circulating LH is elevated in MR-/-, ASGR-/-, and MR-/-ASGR-/- mice.

GalNAc-4-ST1-/- mice modify the GalNAc termini on LH N-linked oligosaccharides with α2,6-linked Sia rather than SO_4 [63]. LH bearing terminal Siaα2,6GalNAc is likely cleared from the circulation by the ASGR but at a slower rate than LH bearing GalNAc-4-SO_4. As a result the half life increases from 7.2 min in Wt to 10.1 min in GalNAc-4-ST1-/- mice. Levels of LH and testosterone are increased in GalNAc-4-ST1-/- mice and they have enlarged seminal vesicles. We examined MR-/-, ASGR-/-, and MR-/-ASGR-/- mice for changes in LH, FSH, testosterone, and seminal vesicle size (Fig. 2).

LH levels (Fig. 2A) are significantly elevated 3.5-fold in MR-/-, 2.5-fold in ASGR-/-, and 2.9-fold in MR-/-ASGR-/- mice compared with Wt mice, $p<0.005$ for all three genotypes. In contrast, there were no significant differences in FSH levels (Fig. 2B) in any of the genotypes. There is a corresponding increase in testosterone levels in all three genotypes (Fig. 2C), 2.9-fold in MR-/-, 4.7-fold in ASGR-/-, and 3.4-fold in MR-/-ASGR-/- mice as compared to Wt mice. The size of the seminal vesicles, which is determined by testosterone levels, is increased in all three genotypes (Fig. 2D).

As with the GalNAc-4-ST1-/- mice, a reduced rate of LH clearance is associated with an increase in LH and testosterone in MR-/- and MR-/-ASGR-/- mice. The increase in LH and testosterone in the ASGR-/- mice is unexpected since the rate of LH clearance is not altered as compared to Wt. Regulation of LH and testosterone levels *in vivo* is complex and involves the hypothalamus, pituitary, and gonad; i.e. the HPG axis [84]. GnRH produced by the hypothalamus stimulates the production and release of LH by the pituitary and the released LH stimulates production of testosterone by the testis. Testosterone in turn regulates the production and release of GnRH. It is possible that the ASGR mediates the clearance of glycoproteins, other than LH itself, that modulate the production of and release of LH by gonadotrophs in the pituitary. The increased levels of LH and testosterone in MR-/-, ASGR-/-, and MR-/-ASGR-/- mice indicates that the HPG axis cannot fully compensate for the changes that result from reduced rates of clearance by these receptors. We examined steady state mRNA levels for a number of proteins in the pituitary and testis in order to determine how the HPG axis is responding to the altered rates of clearance in the receptor deficient mice.

Glycoprotein hormone and glycosyltransferase expression in the pituitary.

We examined the steady state mRNA levels for LH, FSH, and TSH beta subunits in individual pituitaries for all three genotypes using quantitative RT-PCR. LHβ levels are elevated 1.3 fold in MR-/- mice and 2.0 fold in ASGR-/- mice (Fig.3A). The increased levels of LHβ mRNA in the ASGR-/- mice is striking since neither FSHβ nor TSHβ differ from Wt mice in MR-/- or ASGR-/- mice (Fig. 3B and C). The 1.3 fold increase in LHβ in MR-/- mice suggests that the reduced rate of clearance rather than increased synthesis accounts for the elevated LH in the blood whereas the 2.0 fold

increase in LHβ in ASGR-/- mice suggests that increased synthesis accounts for the elevated levels of circulating LH.

Steady state levels of mRNA for βGT3, βGT4, GalNAc-4-ST1, and α2,6SiaylT were determined for the same genotype (Fig. 4). GalNAc-4-ST1 (Panel A), and α2,6SiaylT (Panel B) increased 1.6 and nearly 2.0 fold, respectively, in MR-/- and ASGR-/- mice. In contrast, βGT3 and βGT4 message levels decreased 10-20% in the same mice (Panel C and D). The changes in GalNAc-4-ST1, and α2,6SiaylT suggest that they are regulated in parallel to LHβ in these mice but that this is not the case for either βGT3 or βGT4 message. In contrast to the increases in LHβ message seen in MR-/- and ASGR-/- mice, LHβ message is reduced in MR-/-ASGR-/- mice (Fig. 3). Similarly the message levels for both GalNAc-4-ST1 and α2,6SiaylT are lower in MR-/-ASGR-/- mice (Fig.4 A and B). TSH also bears N-linked oligosaccharides that terminate with GalNAc-4-SO_4 [8, 9] and TSHβ message is decreased in MR-/-ASGR-/- mice (Fig.3C). Thus, altering clearance by ablation of both receptors also affects TSH expression in these mice.

Gene expression in the testis of MR-/-, ASGR-/-, and MR-/-ASGR-/- mice.

LH serves to regulate the production of testosterone in the testis of male mice. The expression of a number of genes in the testis is regulated by LH including the LH receptor (LHR) [85], steroidogenic acute regulatory (StAR) protein [86], activin and inhibin [87, 88]. Testosterone and activin [89] in turn are known to regulate the expression of LHβ. Since LH and testosterone are elevated in all three receptor

knockouts we compared the expression these genes in the testis of MR-/-, ASGR-/- and MR-/-ASGR-/- mice (Fig. 5).

Despite the elevated levels of LH and testosterone in MR-/- and ASGR-/- mice, expression of LHR, StAR, 20α-HSD, inhibin, and activin are not significantly changed. In marked contrast, the expression of each of these genes is increased in the MR-/-ASGR-/- mice with 20α-HSD being elevated 12.5-fold and activin and inhibin being elevated 4.6 and 5.4-fold, respectively (Fig.5). Loss of clearance by both the MR and the ASGR has a different impact on gene expression in both the testis and the pituitary than loss of clearance by either receptor individually suggesting that both receptors contribute to the regulation of the HPG axis *in vivo*.

Discussion

LH was the first of the pituitary glycoprotein hormones shown to bear unique N-linked oligosaccharides terminating with SO_4-4-GalNAcβ1,4GlcNAc [7-10]. We determined that oligosaccharides terminating with GalNAc-4-SO_4 are recognized by the ricin-type beta-trefoil Cysteine rich domain that is located at amino terminus of the MR and hypothesized that the MR accounts for the rapid clearance of LH from the blood following its stimulated secretion and that rapid clearance is critical for the function of LH *in vivo* [50]. We have taken a genetic approach to determine if the MR does indeed account for the rapid clearance of LH from the blood and if rapid clearance plays a role in LH function *in vivo*. Terminal GalNAc on LH N-linked oligosaccharides synthesized by mice that have had GalNAc-4-ST1 ablated is not modified with SO_4 but is instead modified with α2,6-linked Sia. LH bearing Siaα2,6GalNAc rather than terminal GalNAc-4-SO_4 is recognized by the ASGR rather than the MR and is cleared from the blood at a slower rate following secretion, half live of 10.1 vs 7.2 min [63]. Circulating levels of LH, testosterone, and estrogen are increased in GalNAc-4-ST1-/- mice. Tissues that are sensitive to testosterone and estrogen, seminal vesicles in males and uteri in females, are enlarged in GalNAc-4-ST1-/- mice and sexual maturation occurs at an earlier age. The GalNAc-4-ST1-/- mice demonstrate that the structure of the N-linked carbohydrates on LH determines which receptors mediates LH clearance and as a consequence the amount of estrogen and testosterone produced in response to the LH.

The half life of endogenous LH in MR-/- mice increased from 8.4 min to 15.6 min demonstrating that the MR indeed accounts for the rapid clearance of LH from the blood and that the vast majority, if not all, of the LH in Wt mice is modified with

terminal GalNAc-4-SO_4 on at least two N-linked oligosaccharides. Earlier conclusions that the half life of LH was not altered in MR-/- mice were based on the injection of exogenous, radiolabeled porcine LH [52] and may not accurately reflect the half life of the endogenous hormone for a number of reasons. The amount of radiolabeled LH injected would have resulted in micromolar concentrations of LH in the mouse potentially saturating the MR. In any case, the half life of 15.6 min in MR-/- is consistent with clearance of the hormone by renal filtration. The absence of any change in the half life of endogenous LH in ASGR-/- mice indicates that there is not sufficient terminal GalNAc or Siaα2,6GalNAc on endogenous LH for the ASGR to contribute to the clearance of LH. Male MR-/- mice, like GalNAc-4-ST1-/- mice [63], have elevated levels of LH and testosterone. In addition their seminal vesicles, which are responsive to testosterone, are larger than those of Wt males. The increase in LH and testosterone levels is significant and indicates that the HPG axis cannot fully compensate for the changes resulting from a prolonged LH half life.

The half life of endogenous LH in ASGR-/- mice is not increased; however, like MR-/- mice the levels of LH and testosterone are elevated. A further indication that the increased levels of LH and testosterone are physiologically significant comes from the increased size of the seminal vesicles. Since the half life of LH is not altered in ASGR-/- mice, the increased levels of LH and testosterone indicate that the half life of another component of the HPG axis that influences LH levels is likely altered and may be modified with Siaα2,6GalNAc. The half life of endogenous LH in MR-/-ASGR-/- mice, like that in MR-/- mice, is prolonged and the levels of LH and testosterone are increased. Regulation of the HPG axis is complex but serves to maintain very precise levels of

hormone in the blood. LH and testosterone levels are not only increased in MR-/-, ASGR-/-, and MR-/-ASGR-/- mice but also are more variable. This suggests that in the absence of clearance by either the MR, ASGR, or both receptors it is more difficult to maintain hormone levels at the precise levels required. Furthermore, the differences in expression of mRNAs in the pituitary and testis in the MR-/-, ASGR-/-, and MR-/-ASGR-/- mice indicate that the manner in which LH and testosterone levels are being maintained in the different receptor knockouts is not the same and likely reflect the HPG axis attempting to compensate for the changes produced by altering clearance rates.

Our current studies confirm that N-linked structures terminating with GalNAc-4-SO_4 predominate on murine LH and demonstrate that the MR mediates the rapid clearance of LH from the blood. Furthermore, clearance by the MR is an important component of HPG axis regulation and maintenance of LH and testosterone levels *in vivo*. Our studies also implicate the ASGR in the maintenance of LH and testosterone levels *in vivo*; however, further studies will be required to identify the glycoproteins recognized by the ASGR.

Abbreviations

MR, mannose/GalNAc-4-SO4 receptor; ASGR, asialoglycoprotein receptor; bGT, b1,4 N-acetylgalactosyaminyltransferase; GalNAc, N-acetylgalactosamine; ST, sulfotransferase; Sia, sialic acid; Wt, wild-type; IRMA, immunoradiometric assay; RIA, radioimmunoassay; GnRH, gonadotropin releasing hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; TSH, thyroid stimulating hormone; LHR, LH receptor; StAR, steroidogenic acute regulatory protein; 20α-HSD, 20 alpha hydroxysteroid dehydrogenase; HPG, hypothalamic-pituitary-gonadal

Tables.

Table 1. qPCR primers used for LHβ, FSHβ, TSHβ, LHR, StAR, 20αHSD, Activin, Inhibin reactions

Primer	Sequence
LH□F	**GTCAACGCAACTCTGGCCGCAGAGAA**
LH□R	**GCACACTGGCTGAGGCACAGGAGGCAA**
FSH□F	**GCTTTCCCCAGAAGAGACAGCTGA**
FSH□R	**CTACTGAGATGGTGATGTTGGTCAA**
TSH□F	**GGAGAGAGTGTGCCTACTGCCTGA**
TSH□R	**CCTGAGAGAGTGCATATTTGGGAA**
LHRF	**ACATAACCACCATACCAGGG**
LHRR	**ACTGTGCATCTTCTCCAGG**
StARF	**ATCACTCATGAGCTGGCTGCG**
StARR	**GTGAGTTTAGTCTTGGAGGG**
20□HSDF	**ACTTCCCATCGTCCAGAGTTGG**
20□HSDR	**AGCTCATTCCCTGGCTTCAGAG**
ActivinF	**ACGGGTATGTGGAGATAGAGG**
ActivinR	**AGTGCAGTGTCTTCCTGGC**
InhibinF	**ACAGGACCTCTGAACCAGAG**
InhibinR	**AGTGAAGAGGCCTTCCTCAG**

Figures.

Figure 1. Clearance of endogenous LH is slower in MR-/- and MR-/-ASGR-/- mice. Mice were castrated to maximize LH five days prior to clearance studies. Plasma was collected retroorbitally prior to and following a 10 μg injection of acyline at times 0, 5, 10, 20, and 30 min. Plasma LH concentrations were determined by immunoradiometric assay and analyzed using Prism in (A) Wt, (B) MR-/-, (C) ASGR-/-, and (D) MR-/-ASGR-/-. The half-life of LH (E) is nearly doubled, 8.4 min compared to 15.6 min ($p=0.0228$), in MR-/- mice. The half-life of LH is also prolonged in MR-/-ASGR-/- mice, 11.6 min ($p=0.0419$); however, not as significantly as in MR-/- mice.

Figure 2. Levels of LH and testosterone, but not FSH are elevated in MR-/-, ASGR-/- and MR-/-ASGR-/-. Plasma was collected from adult male Wt, MR-/-, ASGR-/-, and MR-/-ASGR-/- mice. Circulating LH levels (A) were determined using an IRMA and analyzed using a parametric t-test. LH levels are significantly elevated in all three genotypes compared to Wt ($p<0.005$). Testosterone levels (B) were determined by RIA and analyzes using a non-parametric t-test, Mann-Whitney since testosterone does not follow a normal distribution. Testosterone is significantly elevated in all three knockout genotypes compared to Wt ($p<0.05$). FSH levels (C) were determined by RIA, and there is no significant difference between Wt and any of the knockout genotypes. Seminal vesicles (D) are a target of testosterone and are enlarged significantly in all three genotypes, but most significantly enlarged in MR-/-ASGR-/-mice.

Figure 3. The expression of LHβ, FSHβ, and TSHβ decreases in the pituitary of MR-/-ASGR-/- mice. Relative mRNA transcript levels of LHβ, FSHβ, and TSHβ were determined using qPCR. Pituitaries from Wt, MR-/-, ASGR-/-, and MR-/-ASGR-/- mice

were collected and the mRNA from each knockout was compared to Wt using an ABI 7500 PCR system. The expression levels were normalized to 18s transcript levels. LHβ expression (A) doubles in ASGR-/- mice ($p<0.0001$), but declines 1.7 fold in MR-/-ASGR-/- mice ($p=0.0059$) FSHβ expression (B) remains unchanged in MR-/- and ASGR-/- mice, but declines 1.7 fold in MR-/-ASGR-/- ($p=0.0129$). TSHβ mRNA expression (C) declines 3.5 fold in MR-/-ASGR-/- ($p<0.0001$).

Figure 4. Alterated transcription of GalNAc-transferases 3 and 4, GalNAc-4-sulfotransferase, and α2,6-sialyltransferase. Individual pituitaries were isolated and the relative mRNA levels in Wt, MR-/-, ASGR-/- and MR-/-ASGR-/- compared to Wt were determined using qPCR. The expression of GalNAc-transferase 3 (A) declines 1.6-fold in MR-/- and ASGR-/- mice ($p=0.0102$, 0.0165) and the expression of GalNAc-transferase 4 (B) declines 1.44-fold in MR-/- and MR-/-ASGR-/- ($p<0.01$). The expression of GalNAc-4-sulfotransferase significantly increases in MR-/- ($p=0.0014$) and ASGR-/- ($p<0.0001$). The expression of α2,6-sialyltransferase changes similar to that of LHβ. There is a significant increase in the MR-/- and ASGR-/- pituitaries ($p=0.0023$, 0.0002) and a 4-fold decrease in the MR-/-ASGR-/- pituitaries ($p<0.0001$).

Figure 5. Transcription of LHR, StAR, 20αHSD, Activin and Inhibin increases in the testes of MR-/-ASGR-/-. Testes were collected and processed individually to isolate mRNA from each testis. Relative mRNA transcript levels of LHR, StAR, 20αHSD, activin, and inhibin were determined using qPCR. In MR-/-ASGR-/- testes, LHR expression (A) elevates 1.9 fold ($p=0.0051$), StAR expression (B) elevates 2.2 fold ($p=0.0002$), and 20αHSD expression (C) elevates 13 fold ($p<0.0001$), activin expression (D) elevates 4.2 fold ($p=0.0008$), inhibin expression (E) elevates 5.7 fold ($p=0.0021$).

Figure 1.

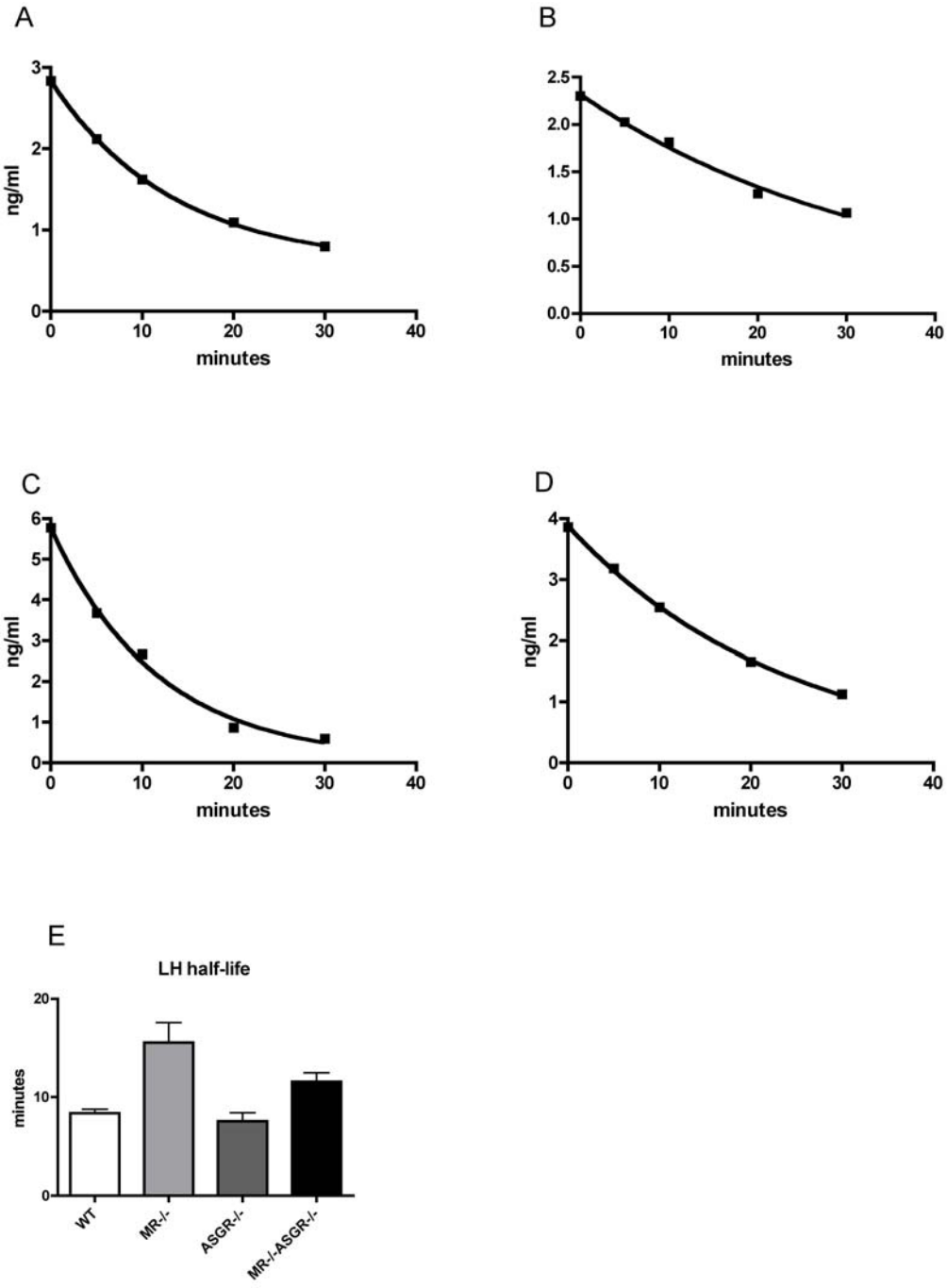
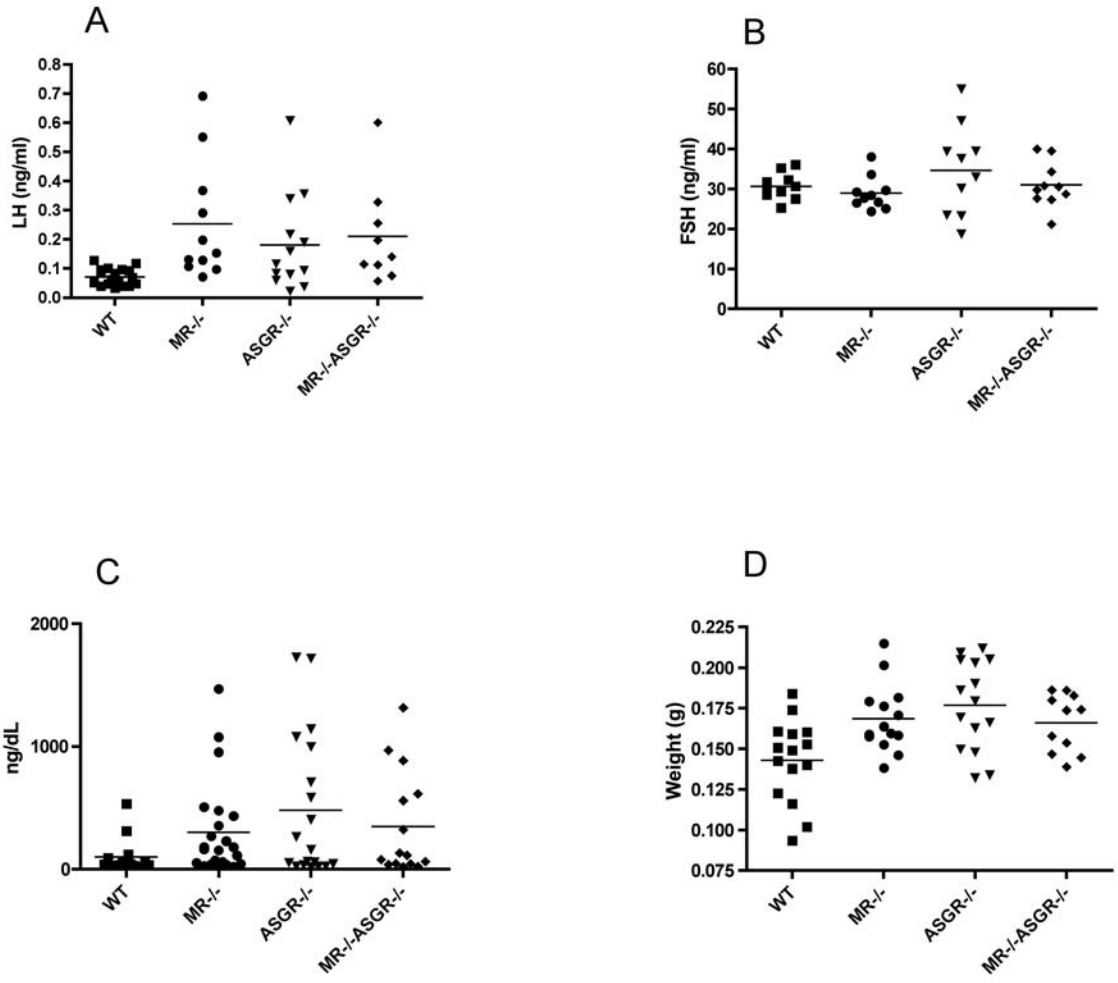

A
ng/ml
minutes
B
minutes
C
ng/ml
minutes
D
ng/ml
minutes
E
LH half-life
minutes
WT
MR-/-
ASGR-/-
MR-/-ASGR-/-

Figure 2.


A
LH (ng/ml)
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0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
WT
MR-/-
ASGR-/-
MR-/-ASGR-/-
B
FSH (ng/ml)
60
50
40
30
20
10
0
WT
MR-/-
ASGR-/-
MR-/-ASGR-/-
C
ng/dL
2000
1000
0
WT
MR-/-
ASGR-/-
MR-/-ASGR-/-
D
Weight (g)
0.225
0.200
0.175
0.150
0.125
0.100
0.075
WT
MR-/-
ASGR-/-
MR-/-ASGR-/-

Figure 3.

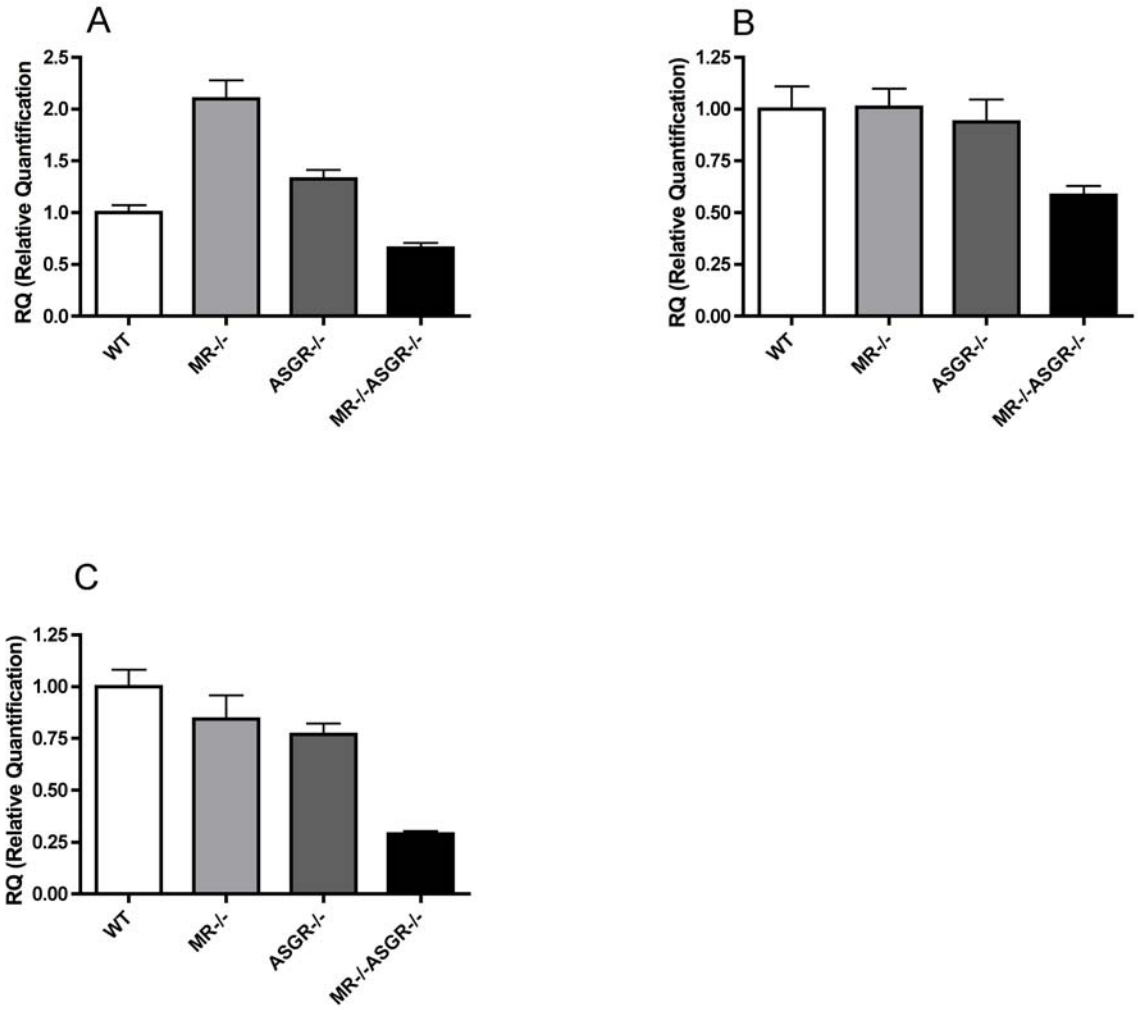

A
RQ (Relative Quantification)
2.5
2.0
1.5
1.0
0.5
0.0
WT
MR-/-
ASGR-/-
MR-/-ASGR-/-
B
RQ (Relative Quantification)
1.25
1.00
0.75
0.50
0.25
0.00
WT
MR-/-
ASGR-/-
MR-/-ASGR-/-
C
RQ (Relative Quantification)
1.25
1.00
0.75
0.50
0.25
0.00
WT
MR-/-
ASGR-/-
MR-/-ASGR-/-

Figure 4.

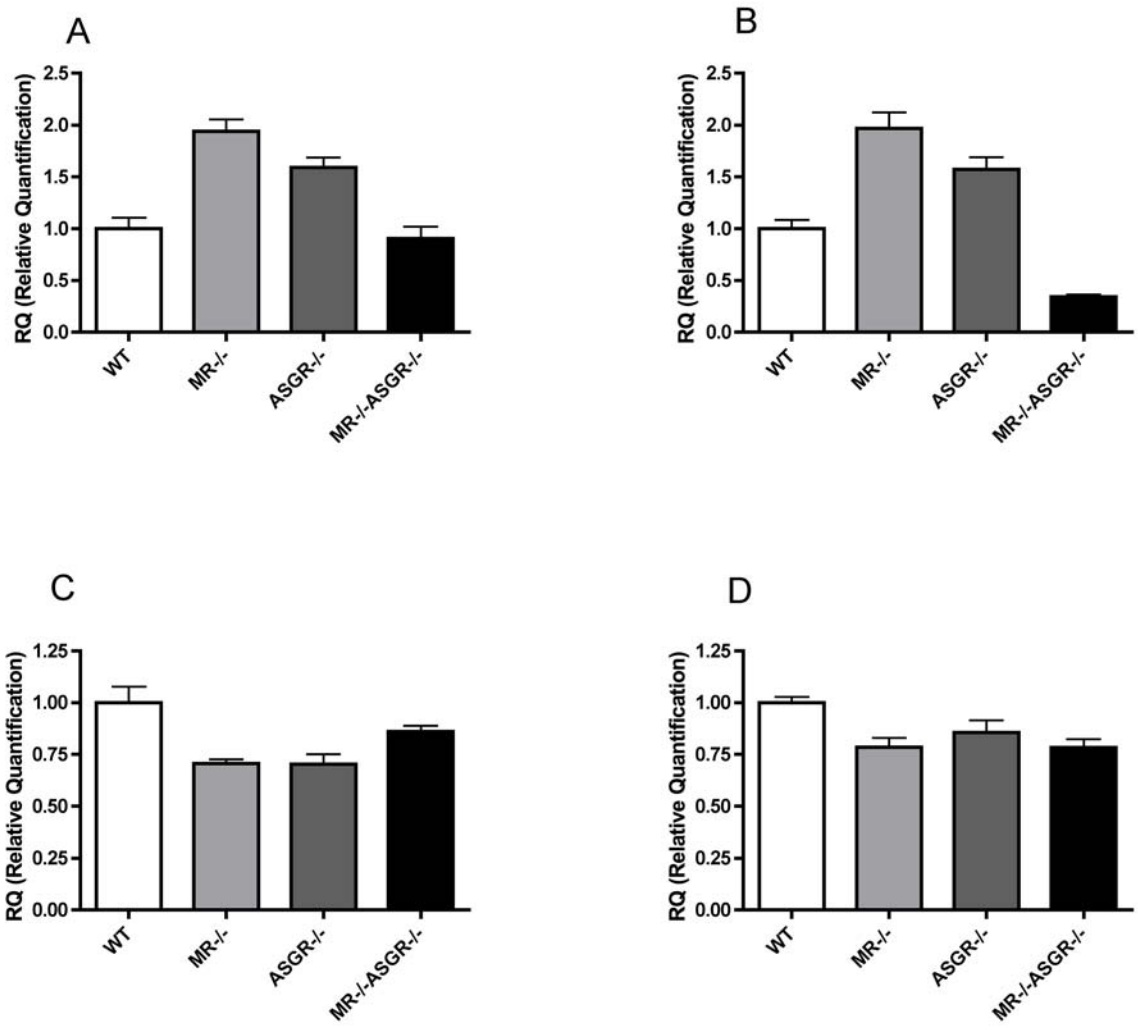
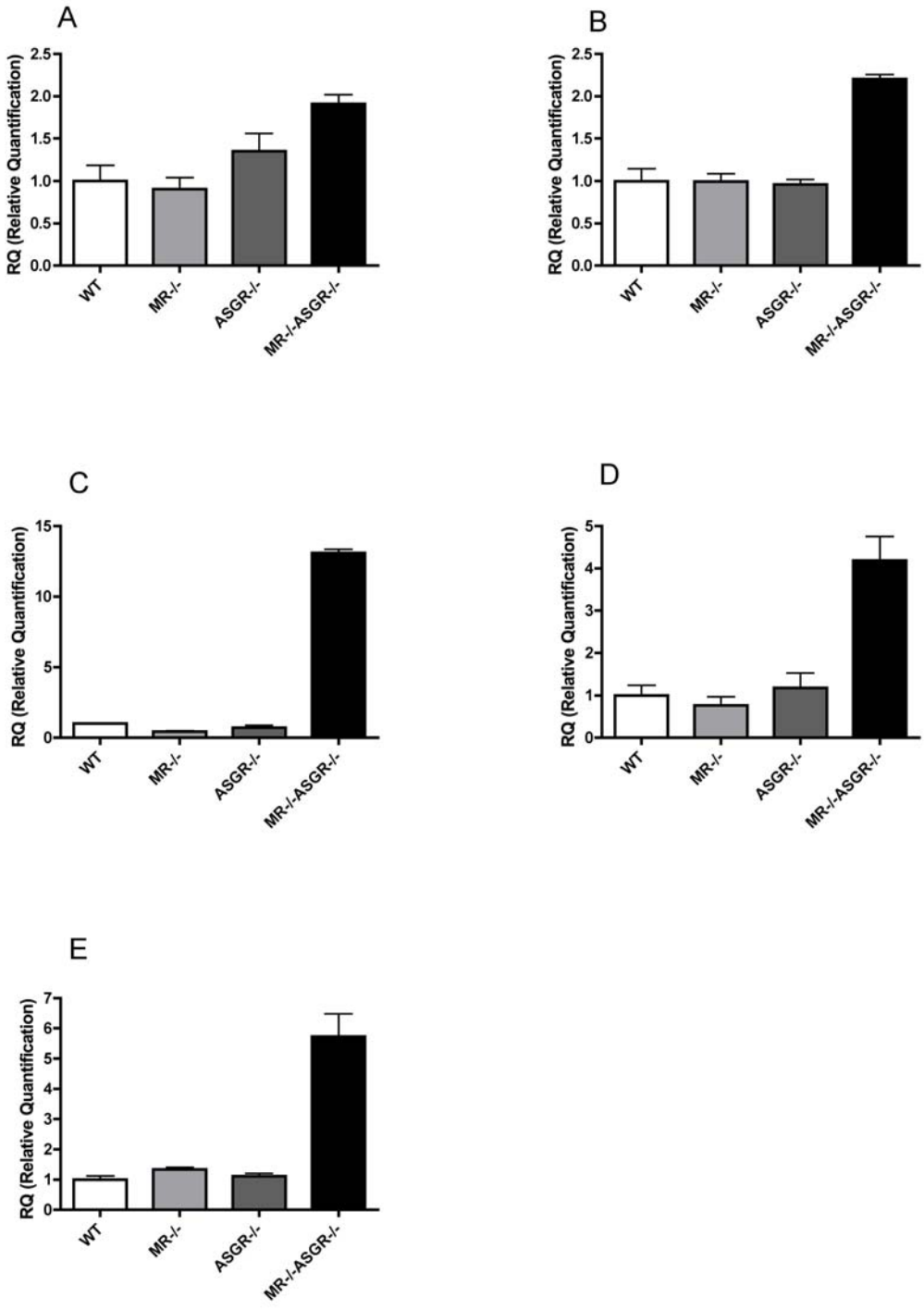

A
RQ (Relative Quantification)
2.5
2.0
1.5
1.0
0.5
0.0
WT
MR-/-
ASGR-/-
MR-/-ASGR-/-
B
RQ (Relative Quantification)
2.5
2.0
1.5
1.0
0.5
0.0
WT
MR-/-
ASGR-/-
MR-/-ASGR-/-
C
RQ (Relative Quantification)
1.25
1.00
0.75
0.50
0.25
0.00
WT
MR-/-
ASGR-/-
MR-/-ASGR-/-
D
RQ (Relative Quantification)
1.25
1.00
0.75
0.50
0.25
0.00
WT
MR-/-
ASGR-/-
MR-/-ASGR-/-

Figure 5.


A
RQ (Relative Quantification)
2.5
2.0
1.5
1.0
0.5
0.0
WT
MR-/-
ASGR-/-
MR-/-ASGR-/-
B
RQ (Relative Quantification)
2.5
2.0
1.5
1.0
0.5
0.0
WT
MR-/-
ASGR-/-
MR-/-ASGR-/-
C
RQ (Relative Quantification)
15
10
5
0
WT
MR-/-
ASGR-/-
MR-/-ASGR-/-
D
RQ (Relative Quantification)
5
4
3
2
1
0
WT
MR-/-
ASGR-/-
MR-/-ASGR-/-
E
RQ (Relative Quantification)
7
6
5
4
3
2
1
0
WT
MR-/-
ASGR-/-
MR-/-ASGR-/-

Chapter 5

Regulation of the mannose/GalNAc-4-SO_4 receptor and asialoglycoprotein receptor during pregnancy

Introduction

A number of glycosylated hormones bear N-linked carbohydrate structures that are selectively modified with β1,4-linked N-acetylgalactosamine (GalNAc) [90]. The terminal GalNAc on luteinizing hormone (LH) is further modified with SO_4 to produce the unique terminal sequence SO_4-4-GalNAcβ1,4GlcNAcβ1,2Man (GalNAc-4-SO_4) [7-10, 80-82]. Other glycoproteins such as glycodelin in humans and certain prolactin like proteins (PLP) in rodents that are produced during pregnancy are modified with terminal α2,6-linked sialic acid (Sia) to generate another unique terminal sequence on their N-linked oligosaccharides, Siaα2,6GalNAcβ1,4GlcNAcβ1,2Man (Siaα2,6GalNac) [12, 91]. Glycoproteins bearing terminal GalNAc-4-SO_4 or Siaα2,6GalNAc are recognized by two highly abundant receptors located in endothelial cells [40] and parenchymal cells [14] of the liver, respectively. Glycoproteins bearing oligosaccharides terminating with GalNAc-4-SO_4 are bound by the ricin-type beta-trefoil domain of the Mannose/GalNAc-4-SO_4-receptor (MR) [51] whereas glycoproteins bearing oligosaccharides terminating with Siaα2,6GalNAc are bound by the asialoglycoprotein-receptor (ASGR) [6]and in both cases are rapidly internalized. As a consequence, glycoproteins bearing either of these structures have relatively short half-lives when injected into the blood[33, 40].

We recently reported [63] that ablation of the sulfotransferase that mediates sulfate addition to the β1,4-linked GalNAc on LH, GalNAc-4-ST1, results in LH that terminates with Siaα2,6GalNAc rather than GalNAc-4-SO_4. LH bearing Siaα2,6GalNAc is cleared more slowly than LH bearing GalNAc-4-SO_4. As a consequence circulating levels of LH are elevated as are levels of testosterone in male mice and estrogen in

female mice. Thus, the amount of testosterone or estrogen produced in response to LH can be modulated by altering the carbohydrate structure on the hormone *in vivo*. In the current study we have taken a genetic approach to determine the role of the MR and the ASGR in reproduction in female mice. We have examined male mice deficient for the MR (MR-/-), the ASGR (ASGR-/-), and both the MR and the ASGR (MR-/-ASGR-/-). The half-life of LH is increased from 8.4 m to 15.6 m in MR-/- indicating that the MR does indeed account for the rapid clearance of LH *in vivo*. Furthermore, the levels of LH and testosterone are elevated in the MR-/- males. The clearance rate for LH is unchanged in ASGR-/- male mice; however, ASGR-/- mice also have elevated levels of LH and testosterone suggesting that other glycoproteins that are cleared by the ASGR may have an impact on LH levels *in vivo* even though the half life for LH is unchanged.

We have now examined MR-/-, ASGR-/-, and MR-/-ASGR-/- female mice to determine what role the MR and ASGR play in reproduction. Our results indicate that the expression of both receptors is highly regulated during the course of pregnancy. In addition, in the absence of both receptors pregnant mice are not able to initiate parturition most likely due to an inability to modulate the levels of progesterone.

Methods

Ovulatory cycle – The esturs or ovulatory cycle in mice consists of four stages that can be differentiated by vaginal smears. The stage of estrus cycle was determined daily by vaginal smear for individual mice over 20 consecutive days. The smears were stained with methylene blue. The four stages are defined by the major cell type present: predominantly nucleated epithelial and some cornified epithelial cells in proestrus, only cornified epithelial cells in estrus, cornified epithelial cells and leukocytes in metestrus, and predominantly leukocytes in diestrus**.**

Serum preparation and Tissue collection - Serum was collected by terminal cardiac puncture. Mice were anesthetized with ketamine (87mg/kg) and xylazine (13 mg/kg), and blood was collected and allowed to clot for 30 min in serum separator tubes (BD). The serum was separated from cells and clot by centrifugation for 30 min at room temperature and stored at -80^{o}C until use. Tissues, including pituitary, ovaries and liver were collected from the same mice and stored at -80^{o}C for further use.

RNA Isolation – For isolation of RNA from liver, tissue was homogenized in 1 ml TRIZOL Reagent. The homogenized samples were incubated for 5 min at room temperature to dissociate nucleoprotein complexes. Chloroform, 0.7 ml, was added and the mixture was shaken vigorously for 15 sec then incubated at room temperature for 3 min. Phase separation was completed by centrifuging at 13,000 x g for 15 min at 4^{o}C, and the aqueous phase was collected. The RNA was precipitated with 0.5 ml isopropanol, incubated on ice for 15 min, and centrifuged at maximum speed for 30 min. RNA cleanup was done using RNeasy Mini Kit (Qiagen) exactly as indicated in the manufacturer's protocol. Briefly, the precipitated RNA was dissolved in 100 µl RNase-

free water, ethanol was added, and the mixture was put onto an RNeasy Mini spin column. The column wass washed and RNA eluted with 100 µl RNase-free water. Quantification of RNA from both pituitary and testes was determined using a Nanodrop (Thermo Scientific).

Quantitative RT-PCR (qPCR) – Template cDNA was generated using Omniscript Reverse Transcription kit (Qiagen). On ice, template RNA was thawed and fresh master mix was prepared. For the master mix, 1 µl 10x Buffer RT, 1 µl dNTP Mix, 1 µl Oligo-dT primer, 0.5 µl RNase inhibitor and 0.5 µl Omniscript Reverse Transcriptase were mixed together. To the master mix, 1 µg template RNA was added, and the final volume was brought to 10 µl with RNase-free water. The mixture was incubated at 37^{o}C for 1 h, then diluted 5-fold, to a final volume of 50 µl. The qPCR reaction was set up in triplicate for each sample with 2 µl diluted cDNA, 1 µM primers, 10 µl 2x Sybr Green Mix, and water to a final volume of 20 µl. The primer sequences used are listed in Table 1. The qPCR was run on an ABI 7500 using standard conditions. The data was imported into Prism and analyzed.

Vasectomy - Mice were anesthetized with ketamine and xylanine. The scrotum was shaved and cleaned with isopropanol and povidone. A 2 cm midline incision was made in the scrotum, the tunica was pierced, and vas deferens was cut. Several drops of lidocaine were placed in the incision, and the incision was closed with 6-0 Ethilon suture. The mice were bred with Wt females to verify the vasectomized mice were sterile.

Pseudopregnancy – Female mice were mated with vasectomized males. The day a vaginal plug was seen was considered day one of pseudopregnancy. Four days following identification of the vaginal plug, one group of females was injected with oil

into the uterine horn. The second group of females was manipulated in the same manner but not oil was injected. Nine days following identification of the vaginal plug, the two groups of females were sacrificed. The oil injected females were divided into two groups, those that had decidua and those that did not. Liver was collected for analysis.

Progesterone injections – Progesterone, 100 μg was injected into Wt females for 6 days. After six days, the females were sacrificed and the livers were collected for analysis.

Progesterone assay – Progesterone levels in serum were analyzed by a solid-^{125}I assay according to the manufacturer's protocol (Siemens).

Results

Comparison of the estrus cycle and hormone sensitive tissues in female Wt, MR-/-, ASGR-/-, and MR-/-ASGR-/- mice. We previously reported that the MR accounts for the rapid clearance of LH from the blood following its stimulated release from the pituitary. In contrast, the ASGR does not appear to contribute to clearance of LH in mice; however, both MR-/- and ASGR-/- male mice have elevated circulating levels of LH and testosterone suggesting that the MR and the ASGR both contribute to the regulation of LH and testosterone levels *in vivo*. We therefore examined female MR-/-, ASGR-/-, and MR-/-ASGR-/- mice for evidence of alterations in the regulation of LH and estrogen levels.

All three genotypes progress through the stages of the estrus cycle (Fig.1) and are fertile. The number of cycles occurring over a period of 20 consecutive days for 10 individual mice of each genotype was determined (Fig1.E). The average length of the estrus cycle in Wt and MR-/- was 5.3 days. In contrast the ASGR-/- and the MR-/-ASGR-/- mice have average estrus cycle times of 6.9 and 6.5 days, both of which are significantly longer than those of Wt mice. Unlike Wt mice, cornified epithelial cells were typically present even during diestrus in MR-/-, ASGR-/-, and MR-/-ASGR-/- mice.

Other than at the time of ovulation circulating levels of estrogen fall below the levels of detection with currently available immunoassays. This was true for all three genotypes, indicating that unlike male mice, female receptor knockout mice do not have markedly elevated levels of estrogen. The uterus is sensitive to estrogen levels. In GalNAc-4-ST1-/- mice the uterus was 2.3 fold larger than in Wt mice. The uteri from Wt, MR-/-, and ASGR-/- mice did not differ in their average weight, whereas the uteri

from MR-/-ASGR-/- mice weighed on average 0.044 g compared to 0.079 g for Wt mice (Fig.2). Therefore, neither in MR-/-, ASGR-/-, nor MR-/-ASGR-/- female mice show evidence of increased estrogen levels as compared to Wt mice.

Hormonal Regulation of the MR and ASGR in mice during pregnancy. The ASGR and the MR mediate the rapid clearance of glycoproteins bearing N-linked oligosaccharides with terminal Siaα2,6GalNAcβ1,4GlcNAc [33] and SO_4-4-GalNAcβ1,4GlcNAc [40], respectively. It has been reported that levels of the ASGR are increased late in pregnancy in rodents [36]. A number of the prolactin-like proteins (PLP) produced by the placenta during pregnancy in rodents are selectively modified with terminal Siaα2,6GalNAcβ1,4GlcNAc [12] and represent the first ligands described for the ASGR that would not require further modification to be recognized by this receptor. Since the MR and ASGR may regulate the circulating levels of a number of different hormones that are critical for the maintenance of pregnancy we examined the expression of message for these receptors at different stages during pregnancy by qPCR (Fig.3).

Steady state levels of mRNA for the MR and for both subunits of the ASGR increase 3-6 fold by day 3.5 of pregnancy (Fig.3). Levels of all three mRNAs continue to increase until day 12.5. After day 12.5 message levels begin to decline but are still elevated at post natal day 2. The increase in the steady state levels of message for the MR and the ASGR is remarkable since both are highly abundant receptors in endothelial and parenchymal cells of the liver, respectively [23, 40].

Since implantation occurs on day 4 in the mouse [53], the elevated levels of MR, ASGR-1, and ASGR-2 message on day 3.5 suggest that implantation is not essential for

at least the initial increase in message levels. This was confirmed by mating female mice with vasectomized male mice to induce pseudopregnancy. Four days after a vaginal plug was observed oil was injected in the uterus of one group of female mice and another group was manipulated but not injected. On day 9 the mice were sacrificed and those injected with oil were divided into those that had or had not developed decidua. Livers were collected from each group and message levels were determined (Fig.4). The message levels for the MR, ASGR-1, and ASGR-2 are increased at least 3 fold in pseudopregnant mice regardless of the presence or absence of decidual tissue. Thus neither implantation nor decidua formation are essential for at least the initial increase in steady state message levels for the MR and the ASGR.

The rise and fall in message levels for the MR and the ASGR most closely parallels the rise and fall of progesterone that is seen in the pregnant and pseudopregnant mouse. We examined the livers of female mice given progesterone for 6 days and found that the MR, ASGR-1, and ASGR-2 message was increased at least 3-fold (Fig.5). Our results indicate that progesterone modulates the steady state levels of message for the MR and the ASGR during pregnancy. Increasing the expression of these highly abundant endocytic receptors has the potential to have an impact on the clearance of a large number of different glycosylated hormones and other glycoproteins during pregnancy.

MR-/-ASGR-/- mice do not proceed through parturition normally. The rise and fall in MR and ASGR message and activity levels in the liver over the course of pregnancy suggested they play a role in some aspect of pregnancy. Even though the estrus cycle is longer in ASGR-/- and MR-/-ASGR-/- mice than in Wt or MR-/- mice, all three receptor genotypes are fertile. However, pregnant MR-/-ASGR-/- mice, unlike

pregnant Wt, MR-/-, and ASGR-/- mice, do not initiate parturition by day 19.5. Instead, the pregnancy continues for 2-4 additional days when the pregnant mother and the fetuses die while attempting to deliver larger than normal pups. We compared weights for fetuses in Wt, MR-/-, ASGR-/-, and MR-/-ASGR-/- mice on day 18.5 (Fig.6). The weights for the three genotypes do not differ significantly from those of Wt mice at day 18.5. Fetuses taken from pregnant MR-/-ASGR-/- mice on day 21 or 22 weigh 1.7 g as compared to 1.3 g for 18.5 day fetuses indicating that the fetuses continue to develop after day 19.5 and are not dying *in utero*.

The initiation of parturition at the appropriate time is a complex process requiring multiple precisely controlled events to occur [92]. Levels of $PGF_{2\alpha}$ and estrogen increase while levels of progesterone decrease. The inability of MR-/-ASGR-/- mice to initiate parturition by day 19.5 is similar to the phenotype observed in mice that have had genes ablated that regulate the levels of $PGF_{2\alpha}$ and progesterone [93-97]. RU486 is a progesterone receptor antagonist that can induce parturition in mice that are not able to reduce their progesterone levels by day 19 [98]. Administration of RU486 to pregnant MR-/-ASGR-/- mice the evening of day 18 or 19 induces parturition by the next morning with the delivery of live pups. The ability of RU486 to induce parturition in MR-/-ASGR-/- mice suggests that some aspect of the pathway that regulates progesterone production late in pregnancy is not functioning in a normal fashion in MR-/-ASGR-/- mice.

Late in pregnancy levels of the prostaglandin $PGF_{2\alpha}$ rise. The $PGF_{2\alpha}$ down regulates the production of activated Stat5 via the prolactin receptor [99]. The reduced levels of activated Stat5 in combination with $PGF_{2\alpha}$ allow for increased transcription of

20α-hydroxysteroid dehyrogenase (20αHSD) [100, 101]. 20αHSD in turn converts progesterone into an inactive metabolite thus reducing the levels of circulating progesterone and initiating parturition. In addition, estrogen levels rise as progesterone levels fall. $PGF_{2\alpha}$ reaches the same level in MR-/-ASGR-/- and Wt mice (Fig.7A) and message levels are similar for 20αHSD (Fig.7B) at day 18.5. Plasma estrogen and progesterone levels were also compared for Wt, MR-/-, ASGR-/-, and MR-/-ASGR-/- on day 18.5 (Fig.7C and D). Estrogen levels are similar in Wt and ASGR-/- mice whereas they are decreased in both MR-/- and MR-/-ASGR-/-. Levels of progesterone are similar or less than Wt in ASGR-/- and MR-/-ASGR-/- mice but increased in MR-/- mice. Thus, in the absence of the MR it appears pregnant mice have difficulty increasing their production of estrogen prior to parturition whereas only MR-/- mice are not able to reduce their progesterone levels to those of Wt mice.

Discussion

The steroid hormones progesterone, estrogen, testosterone, and their derivatives have an impact on a wide range of gender specific processes that require precise regulation of their concentrations. Regulation of steroid hormone production is accomplished by a complex feedback system referred to as the hypothalamic-pituitary-gonadal (HPG) axis[84]. LH, synthesized by gonadotrophs in the anterior pituitary, is stored in dense core granules and released upon stimulation by gonadotropin releasing hormone (GnRH) produced by the hypothalamus. LH activates the LH receptor in the gonad resulting in increased production of testosterone in males and progesterone and estrogen in females. Testosterone and estrogen in turn reduce GnRH production by the hypothalamus.

LH is remarkable because it bears N-linked oligosaccharides that terminate with the unique sequence SO_4-4-GalNAcβ1,4GlcNAc[7-10, 84]. LH bearing terminal GalNAc-4-SO_4 is rapidly removed from the blood by the highly abundant MR expressed at the surface of hepatic endothelial cells and Kupffer cells[40]. Mice that have had the sulfotransferase that mediates sulfate addition to the GalNAc on LH ablated, GalNAc-4-ST1, produce a form of LH bearing N-linked oligosaccharides that terminated with Siaα2,6GalNAc rather than GalNAc-4-SO_4 [63]. LH bearing Siaα2,6GalNAc is removed from the blood by another receptor located in parenchymal cells of the liver, the ASGR. LH bearing Siaα2,6GalNAc is removed from the blood at a slower rate than LH bearing than GalNAc-4-SO_4. As a result the half life of LH in the blood increases from 7.4 m in Wt mice to 10.1 m in GalNAc-4-ST1-/- mice. This results in increased levels of LH and increased testosterone in male mice and increased estrogen in female mice.

Sexual development is precocious in both sexes and the mice are fertile. The changes observed in the GalNAc-4-ST1-/- mice indicate that the carbohydrate structures on LH play a role in determining the amount of steroid hormone that is ultimately produced in both sexes.

We have examined the impact of ablating the MR and/or the ASGR in male mice. The half-life of endogenous LH increases from 8.4 m to 15.6 m in MR-/- mice indicating that endogenous LH is cleared by the MR and that endogenous LH is modified with terminal GalNAc-4-SO_4. The half life of endogenous LH bearing GalNAc-4-SO_4 is considerably longer in MR-/- mice than was seen for LH bearing Siaα2,6GalNAc in GalNAc-4-ST1-/- mice. The half life of LH is also prolonged in MR-/-ASGR-/- mice but not in ASGR-/- indicating that the ASGR does not contribute significantly to the clearance of endogenous LH. Remarkably LH and testosterone levels are increased in MR-/-, ASGR-/-, and MR-/-ASGR-/- mice. While the elevated levels of LH in MR-/- can be attributed to the increased half life, the increase in LH levels in ASGR-/- mice is likely to reflect a change in the clearance rate for a glycoprotein recognized by the ASGR. Take together, the changes seen in males in MR-/-, ASGR-/-, and MR-/-ASGR-/- mice indicate that the regulation of LH and steroid hormone levels may be more complex than anticipated and involve both of the highly abundant carbohydrate-specific receptor systems.

The changes we observe in female MR-/-, ASGR-/-, and MR-/-ASGR-/- mice also indicate that both the MR and the ASGR are involved in regulating steroid hormone levels *in vivo*. The low levels of LH and estrogen seen in female mice make it difficult to obtain accurate measurements for comparison among the receptor genotypes. This

difficulty is compounded by the cyclic variation associated with the different stages of the estrus cycle. The uterus is sensitive to estrogen levels and can be used as an indicator of relative estrogen levels *in vivo*. In contrast to GalNAc-4-ST1-/- mice that have uteri with a mean weight that is 2.3 fold greater than in Wt mice [63], the weight of the uterus is not increased in MR-/- or ASGR-/- mice and is reduced to half the weight of Wt mice in MR-/-ASGR-/- mice. Thus, in contrast to male MR-/-, ASGR-/- and MR-/-ASGR-/- mice that have elevated levels of LH and testosterone the female MR-/-, ASGR-/- and MR-/-ASGR-/- mice have normal or low levels of estrogen. The mice are nonetheless fertile and able to proceed normally with pregnancy until the time of parturition.

Expression of the MR and both subunits of the ASGR is highly regulated during pregnancy. The steady state levels of message for both receptors are increased 3-5 fold by the time of implantation on day 4 and increase to a maximum on day 12.5 after which both begin to decline. The increase in message does not require implantation or decidua formation and largely parallels the levels of progesterone. Progesterone increases MR and ASGR message expression in non-pregnant mice and RU486 inhibits the increase in message level in pseudopregnant mice. Thus, progesterone appears to be a major regulator of MR and ASGR expression during pregnancy. Even though MR-/-ASGR-/- mice proceed through pregnancy without obvious difficulty they do not enter parturition and deliver the morning of day 19. The initiation of parturition is a complex process. In the mouse PGF2α begins several days before parturition while progesterone begins to fall. The rapid fall in progesterone is a result of the action of 20αhydroxysteroid dehyrdrogenase (20αHSD) that converts progesterone into an inactive metabolite. Transcription of 20αHSD is suppressed in the ovary during pregnancy by the action of

Stat5a. The levels of activated Stat5 are maintained by activation of the PRL receptor by PRL and PLPs produced in the placenta and decidua of the mouse [102, 103]. Increased PGF2α down regulates the PRL receptor and reduces the levels of activated Stat5 resulting in increased transcription of 20αHSD. This series of events results in the fall in progesterone that is required to initiate parturition.

The inability of MR-/-ASGR-/- mice to initiate parturition and the ability of RU486 to overcome this block suggests that these mice are not able to regulate progesterone levels appropriately near the end of pregnancy. However, we have examined PGF2α, progesterone, and 20αHSD message levels in MR-/-ASGR-/- mice and found them to be similar to Wt mice. We have examined progesterone and estrogen levels in MR-/-, ASGR-/-, and MR-/-ASGR-/- mice at day 18.5 and determined progesterone is high in MR-/- mice and estrogen is low is MR-/- and MR-/-ASGR-/- mice. These differences indicate that the ablating of either or both of these receptors compromises that ability to control steroid hormone levels late in pregnancy.

We previously determined that the N-linked oligosaccharides on a number of the PLPs produced by the placenta in rodents are modified with terminal Siaα2,6GalNAc [12] and may represent the first examples of endogenous ligands for the ASGR since they do not require further modification to be recognized; i.e. removal of the terminal Sia. It is likely that one or more of the PLPs produced during pregnancy are elevated in the blood of pregnant mice and may account for inappropriate activation of a prolactin receptor. Likewise the inability to clear LH and other glycoproteins bearing terminal GalNAc-4-SO_4 may prevent the episodic rise and fall in circulating LH levels that is

thought to be required for maximal activation of the LH receptor. Thus, estrogen my not reach levels that are required for critical functions at the time of parturition.

The initiation of parturition requires multiple precisely timed events to occur. Further studies will be required to determine which combination of events is compromised to the extent of preventing parturition in MR-/-ASGR-/- mice. However, the current studies demonstrate that the MR and the ASGR both play roles in regulating steroid hormone production by determining the half lives and circulating levels of key glycoproteins. The changes in expression of the MR and the ASGR that occur during pregnancy and appear to reflect the action of progesterone may ultimately explain a number of the changes associated with pregnancy as well as gender specific differences.

Abbreviations

LH, luteinizing hormone; PLP, prolactin-like protein; PRL, prolactin; Sia, sialic acid; GalNAc, N-acetylgalactosamine; Wt, wild type; MR, mannose/GalNAc-4-SO_4 receptor; ASGR, asialoglycoprotein receptor; ST1, GalNAc-4-sulfotransferase 1; $PGF_{2\alpha}$, Prostaglandin F2 alpha; 20αHSD, 20 alpha hydroxysteroid dehydrogenase; HPG, hypothalamic-pituitary-gonadal; GnRH, gonadotropin releasing hormone.

Tables.

Table 1. qPCR primers for ASGR1, ASGR2, and MR reactions.

Primer	**Sequence**
ASGR1F	GCCTTGGACTGAAGCTGACAA
ASGR1R	GGTCAGTTAGGCCAATCCAA
ASGR2F	GGGTGGATGGAACTGATTA
ASGR2R	GGCAGAAGTTGTCATTCCAA
MRF	GGGTCAGGCTTCTCTGGAA
MRR	GGTTCAACACGGTATGACAGA

Figures.

Figure 1. The ovulatory cycle of Wt, MR-/-, ASGR-/-, and MR-/-ASGR-/-. Vaginal smears were collected daily for 20 days in 10 mice of each genotype to observe the ovulatory cycle of Wt, MR-/-, ASGR-/-, and MR-/-ASGR-/- mice. The length of the estrus cycle was determined based on the average number of cycles each mouse underwent. The length of the estrus cycle is 5.3 days in Wt mice. It is 1.6 days longer in ASGR-/- mice, taking 6.9 days ($p=0.0018$) and 1.2 days longer, 6.5 days ($p=0.0025$) in MR-/-ASGR-/- mice. MR-/- mice had a normal length cycle.

Figure 2. The uterus is small in MR-/-ASGR-/- mice. Uteri were collected randomly from Wt, MR-/-, ASGR-/-, and MR-/-ASGR-/- mice. MR-/-AR-/- have small, thin uteri as compared to Wt, averaging 0.04401 g compared to 0.07905 g ($p=0.0153$).

Figure 3. MR and ASGR message levels change throughout pregnancy. Livers were collected from pregnant Wt, MR-/-, AR-/-, and MR-/-AR-/- mice at days 0, 3, 6, 9, 12, 15, 18 days of pregnancy and postnatal day 2. Livers were processed and the message levels for (A) HL1 and (B) HL2 of the AR and the (C) MR were determined using qPCR. We found the message for HL1 and HL2 of the AR and the MR elevate the first 12 days of pregnancy and then slowly decline until they reach nearly baseline at postnatal day 2 of pregnancy.

Figure 4. MR and ASGR message levels elevate during pseudopregnancy. Wt females were mated with Wt males and day 1 of pregnancy was documented after seeing a vaginal plug. The females were separated into two groups; group one females were unmanipulated and group two females had oil injected into their uterine horn at day 4 of pregnancy. At day 9 of pregnancy, females were sacrificed and their livers were

collected for analysis. Message levels for (A) HL1 and (B) HL2 of the ASGR and (C) the MR were determined. Both groups of females have up to 6-fold elevated MR and AR expression at day 9 ($p<0.005$).

Figure 5. Progesterone injections increase MR and ASGR message levels. Progesterone, 100 μg, was injected into Wt female mice for 6 days. After 6 days, the animals were sacrificed and the MR and ASGR message levels were determined by qPCR. The message of (A) ASGR HL1 was elevated 3 fold ($p=0.0053$), (B) ASGR HL2 was elevated 4 fold ($p=0.0037$), and (C) MR was elevated 7 fold ($p=0.0028$).

Figure 6. Fetuses continue to grow after failed parturition in MR-/-AR-/- mice. Females that did not induce parturition at day 19 were sacrificed at day 21-22 and the weight of the fetuses was determined. MR-/-ASGR-/- fetuses weigh 1.7g at day 21-22 as compared to 1.3 g at day 18.5 ($p<0.0001$).

Figure 7. PGF2α, 20αHSD, estrogen and progesterone levels during pregnancy in MR-/-ASGR-/- mice. Circulating plasma $PGF_{2\alpha}$, 20αHSD, estrogen and progesterone levels were determined in Wt, MR-/-, AR-/-, and MR-/-AR-/- females. We found all animals have normal $PGR_{2\alpha}$ and 20αHSD levels at day 18.5, immediately before parturition is initiated. AR-/- female mice have normal estrogen and progesterone levels at day 18.5 of pregnancy. MR-/- females have elevated progesterone and decreased estrogen at day 18.5, although these values are not significant**.** MR-/-AR-/- mice have normal progesterone levels and decreased estrogen levels at day 18.5 of pregnancy

Figure 1.

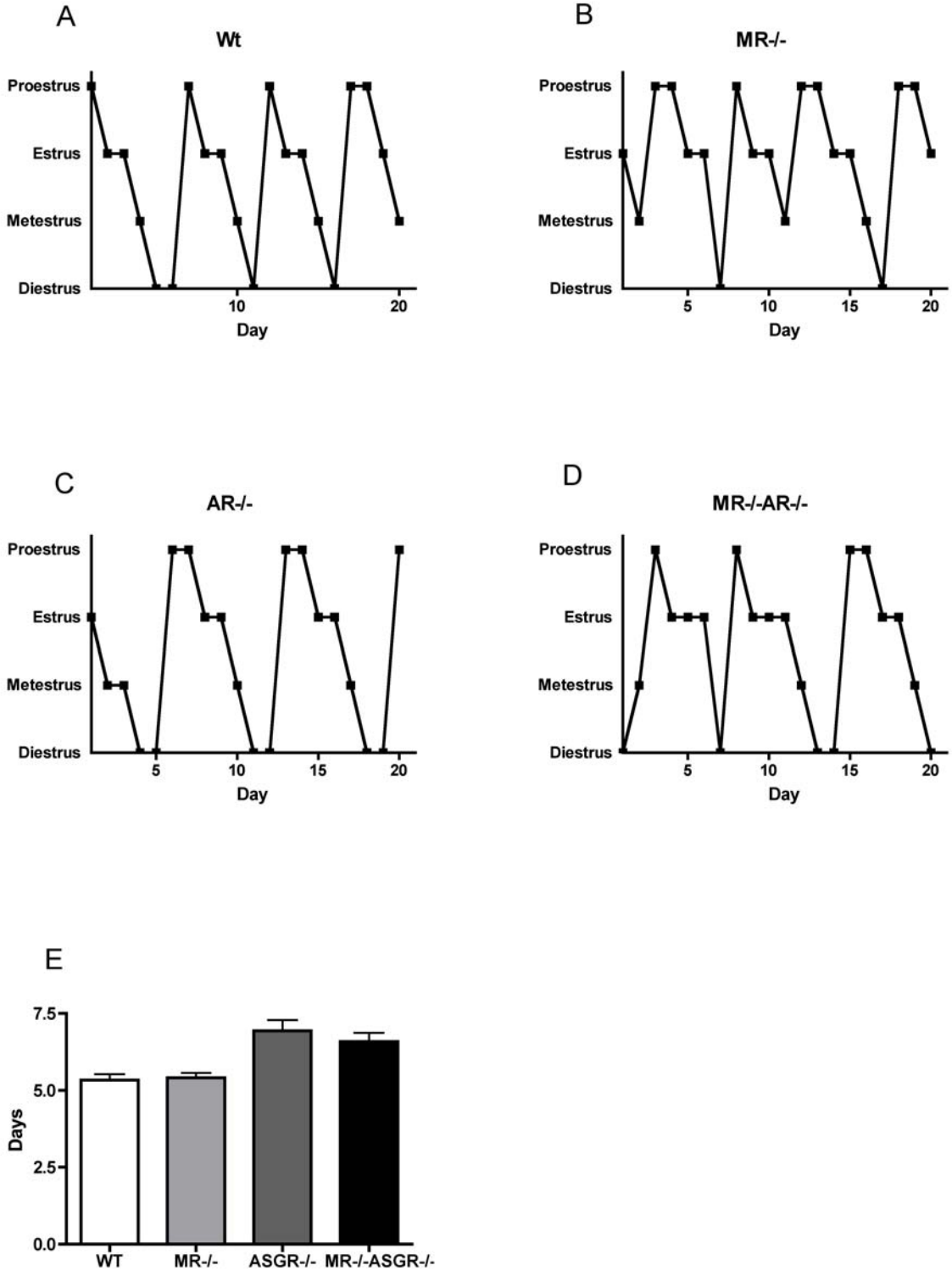

A
Wt
Proestrus
Estrus
Metestrus
Diestrus
10
20
Day
B
MR-/-
Proestrus
Estrus
Metestrus
Diestrus
5
10
15
20
Day
C
AR-/-
Proestrus
Estrus
Metestrus
Diestrus
5
10
15
20
Day
D
MR-/-AR-/-
Proestrus
Estrus
Metestrus
Diestrus
5
10
15
20
Day
E
7.5
5.0
2.5
0.0
Days
WT
MR-/-
ASGR-/-
MR-/-ASGR-/-

Figure 2.

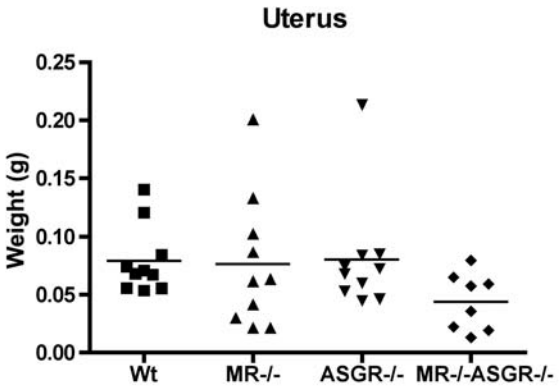


Figure 3.

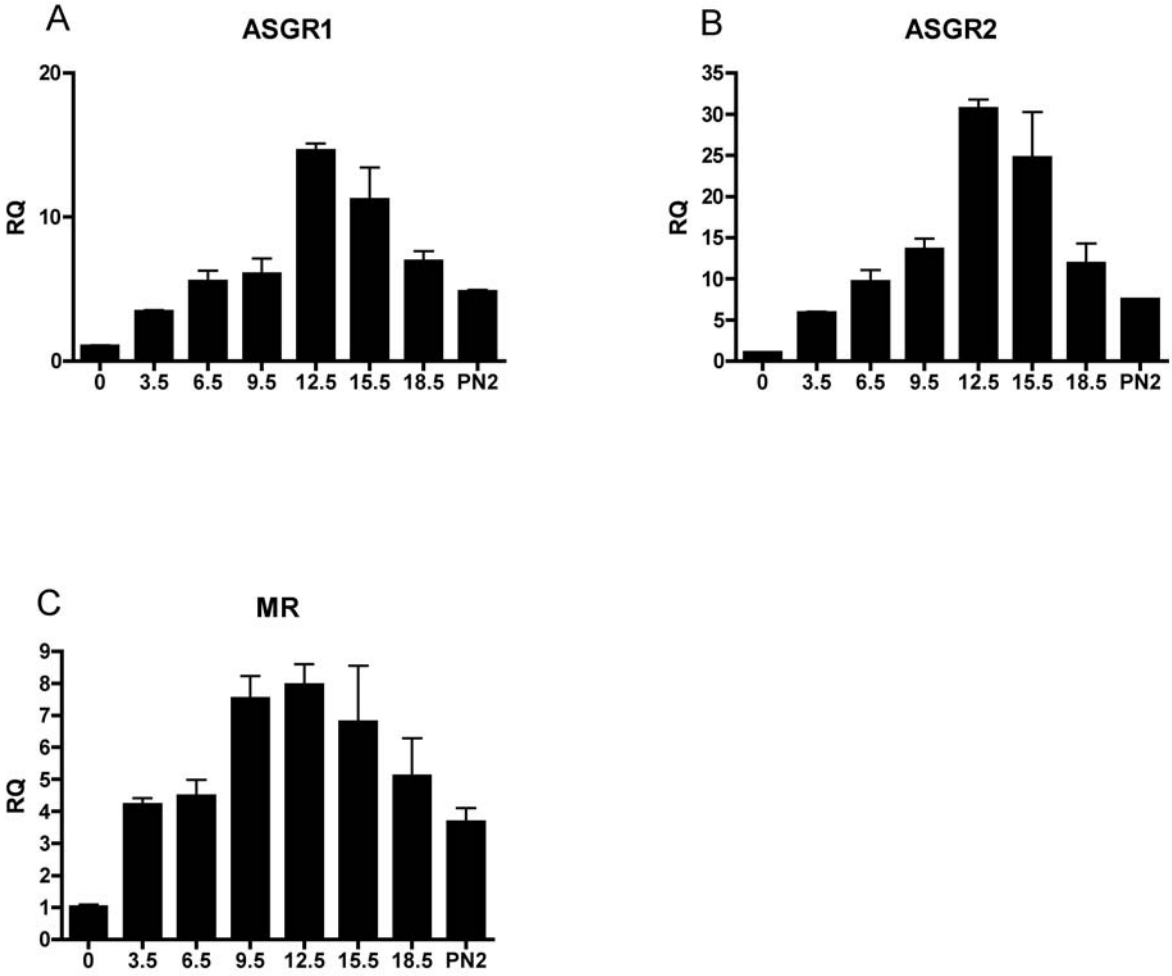
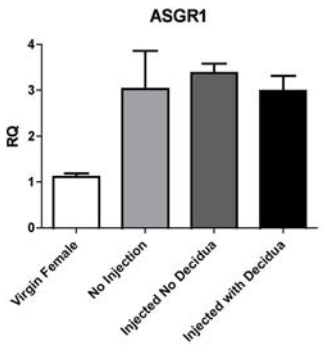
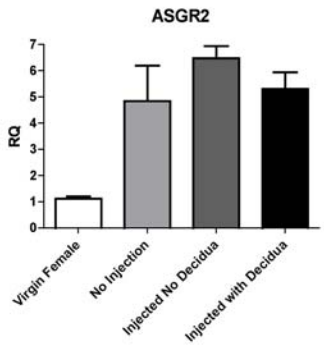

A
ASGR1
RQ
20
10
0
0 3.5 6.5 9.5 12.5 15.5 18.5 PN2
B
ASGR2
RQ
35
30
25
20
15
10
5
0
0 3.5 6.5 9.5 12.5 15.5 18.5 PN2
C
MR
RQ
9
8
7
6
5
4
3
2
1
0
0 3.5 6.5 9.5 12.5 15.5 18.5 PN2

Figure 4.


ASGR1
RQ
0
1
2
3
4
Virgin Female
No Injection
Injected No Decidua
Injected with Decidua


ASGR2
RQ
0
1
2
3
4
5
6
7
Virgin Female
No Injection
Injected No Decidua
Injected with Decidua

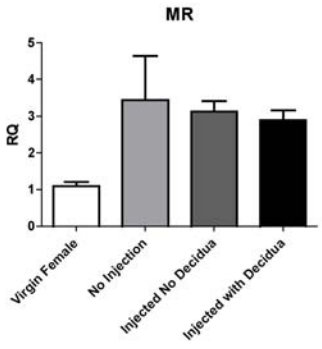

MR
RQ
0
1
2
3
4
5
Virgin Female
No Injection
Injected No Decidua
Injected with Decidua

Figure 5.

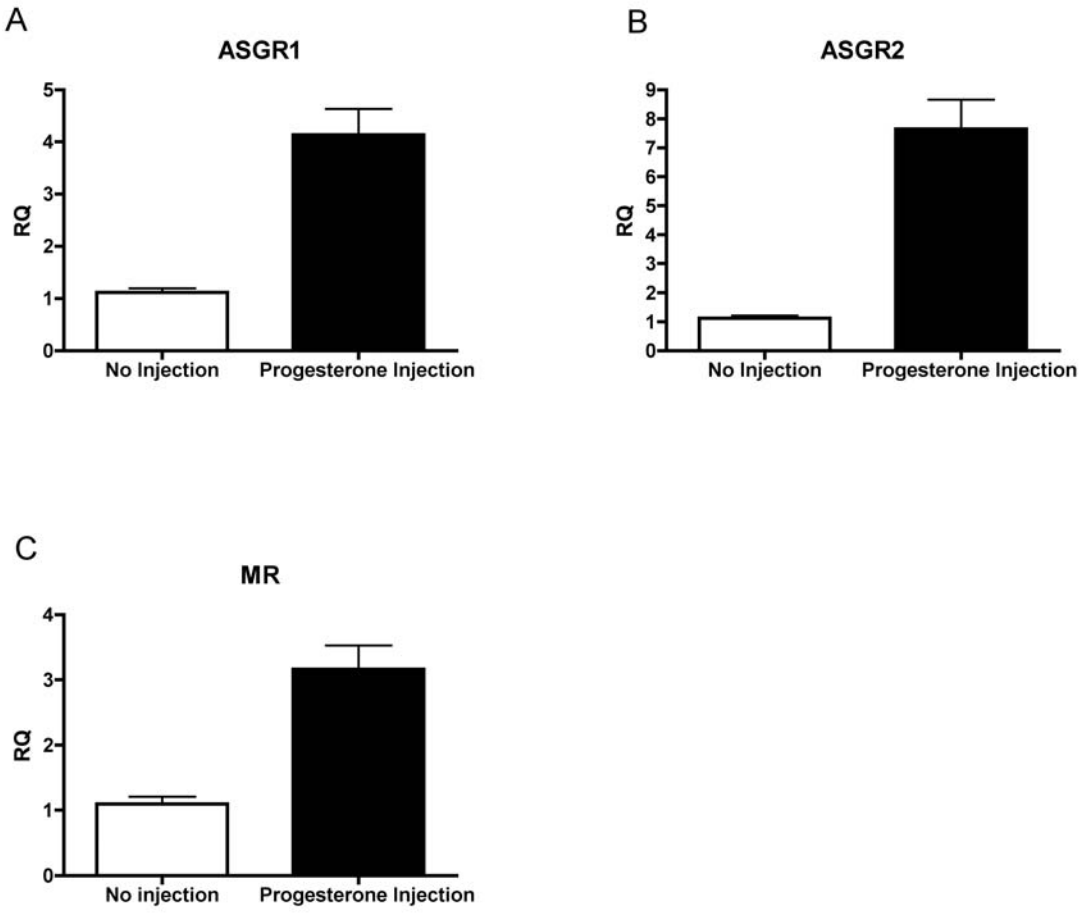

A
ASGR1
RQ
0
1
2
3
4
5
No Injection
Progesterone Injection
B
ASGR2
RQ
0
1
2
3
4
5
6
7
8
9
No Injection
Progesterone Injection
C
MR
RQ
0
1
2
3
4
No injection
Progesterone Injection

Figure 6.

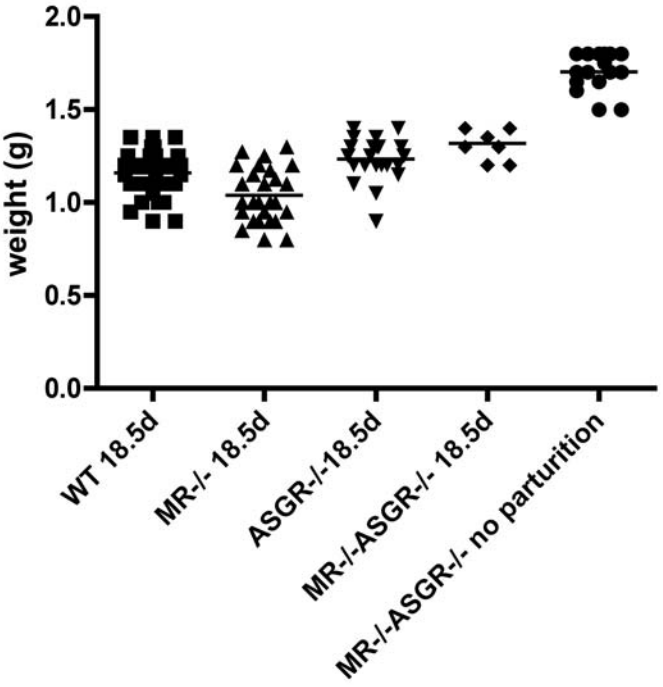
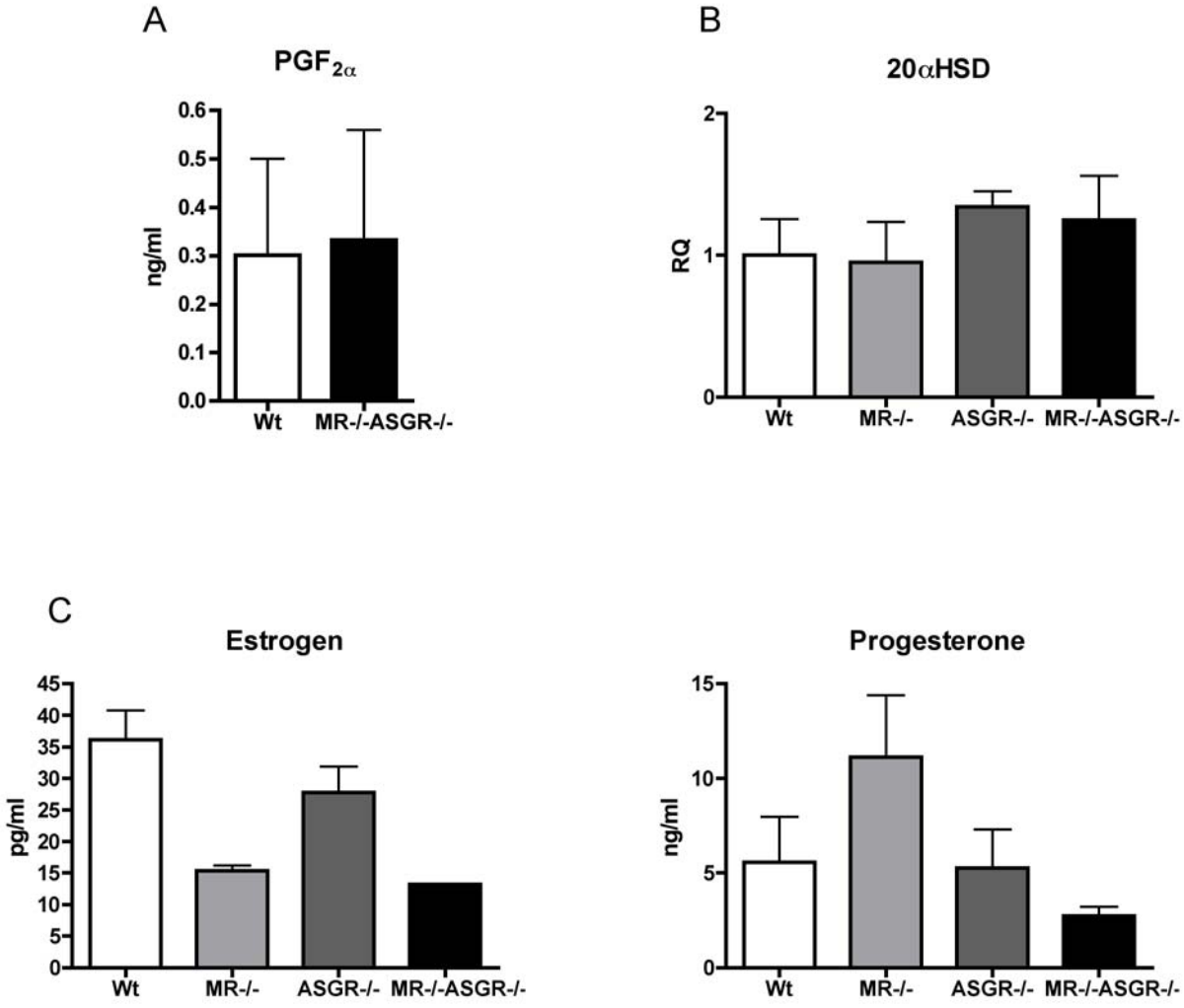

2.0
1.5
1.0
0.5
0.0
weight (g)
WT 18.5d
MR-/- 18.5d
ASGR-/-18.5d
MR-/-ASGR-/- 18.5d
MR-/-ASGR-/- no parturition

Figure 7.


A
PGF2α
ng/ml
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Wt
MR-/-ASGR-/-
B
20αHSD
RQ
2
1
0
Wt
MR-/-
ASGR-/-
MR-/-ASGR-/-
C
Estrogen
pg/ml
45
40
35
30
25
20
15
10
5
0
Wt
MR-/-
ASGR-/-
MR-/-ASGR-/-
Progesterone
ng/ml
15
10
5
0
Wt
MR-/-
ASGR-/-
MR-/-ASGR-/-

Chapter 6

General Discussion

In this thesis, I set out to determine how the asialoglycoprotein receptor (ASGR) and mannose/GalNAc-4-SO4 receptor (MR) regulate circulating plasma protein concentrations. In the first experiment, we found a number of proteins bearing Siaα2,6Gal are elevated in mice genetically ablated for the ASGR. Several of the proteins identified are highly abundant proteins in the plasma, including haptoglobin and SAP. We suggest the ASGR plays a regulatory role in maintaining circulating plasma protein levels of proteins bearing Siaα2,6Gal in unperturbed animals. The ASGR and MR bind and clear proteins bearing Siaα2,6Gal/GalNAc and GalNAc-4-SO_4, respectively. In chapters 3-5, we show the ASGR and MR regulate circulating plasma proteins levels involved in regulating the acute phase response (APR), hypothalamic-pituitary-gonadal (HPG) axis, and pregnancy and parturition. We found that acute phase proteins (APPs) bearing Siaα2,6Gal elevated in ASGR-/- mice further elevate in ASGR-/- plasma, but the fold change is not as dramatic as in Wt plasma, suggesting the ASGR plays a major role in elevating these proteins in the APR. Proteins bearing Siaα2,6Gal/GalNAc or GalNAc-4-SO_4, including luteinizing hormone (LH) are regulated by the ASGR and MR for a properly functioning HPG axis. Finally, during pregnancy and parturition, the ASGR and MR elevate and regulate plasma protein concentrations of proteins involved in pregnancy and parturition. MR-/-ASGR-/- mice are unable to induce parturition and this leads to the death of the mother and fetus. We suggest the ASGR and MR play a regulatory role in maintaining plasma protein concentrations of proteins bearing Siaα2,6Gal/GalNAc and GalNAc-4-SO_4 in unperturbed animals and assists in elevating the plasma protein levels following perturbations during the APR and pregnancy.

The ASGR clears glycoproteins bearing Siaα2,6Gal.

The asialolglycoprotein receptor (ASGR) is a highly abundant protein with up to 500,000 receptors per hepatocyte [23]. For nearly 30 years, the function of this receptor was believed to be clearance of glycoproteins bearing Gal/GalNAc terminating oligosaccharides. However, proteins bearing this structure were never identified. The ASGR has been studied extensively with little success elucidating the function until recently. Here, we identify endogenous ligands for the ASGR and begin to understand its role *in vivo.*

Proteins bearing terminal Gal/GalNAc would be cleared so rapidly from the plasma, they would never be found circulating. To identify proteins bearing terminal Gal/GalNAc, two genetic knockout mouse models were generated, one for each subunit of the ASGR , and elevated proteins were sought after [31, 32]. However, no proteins bearing terminal Gal/GalNAc were identified as elevated in the plasma. This finding was interesting since it had been presumed for years that the ASGR clears proteins bearing terminal Gal/GalNAc, and proteins bearing terminal Gal should be elevated in ASGR-/- plasma. There are two possibilities for why these proteins were not found: they are cleared by another mechanism or proteins bearing terminal Gal rarely exist in the plasma.

While studying ligands of a different receptor clearance system, our lab encountered proteins bearing the unique structure Siaα2,6GalNAc in the plasma. Surprising, we found proteins bearing Siaα2,6GalNAc bind to the ASGR. Further studies determined neoglycoconjugates bearing this structure are cleared rapidly and using the genetic mouse models previously generated, we determined clearance was by the ASGR [6, 33]. Few proteins have this oligosaccharide, and the proteins identified,

prolactin-like proteins [12], are only present in the placenta during pregnancy, making them unlikely candidates to fulfill the main function of such an abundant receptor. However, more common structures bearing Sia, Siaα2,6Gal and Siaα2,3Gal exist plentifully in the plasma. Based on our observation that the ASGR can bind proteins unmodified by a neuraminidase, we proposed the ASGR binds and clears proteins bearing on of these more common structures, and the function of the ASGR is to regulate circulating plasma protein levels of proteins bearing Sia.

To test our hypothesis that the ASGR binds and clears proteins bearing Sia, we expressed the CRD of ASGR HL-1 and observed binding. We found that neoglycoconjugates bearing Siaα2,6Gal bind ASGR HL-1 with a lower affinity than terminal Gal but a higher affinity than Siaα2,3Gal, suggesting proteins bearing Siaα2,6Gal bind specifically to ASGR HL-1. Clearance studies comparing Gal and Siaα2,6Gal were conducted [6]; however, proteins bearing Siaα2,6Gal are plentiful in the plasma and we would need to inject enough exogenous ligand to outcompete the endogenous ligands. Addtionally, Siaα2,6Gal has a long half-life, making these experiments difficult. Using traditional clearance studies, it was impossible to verify the ASGR clears proteins bearing Siaα2,6Gal [34].

Recently, Grewal et al [37]studied several elevated proteins in ASGR-/- mice. Using knockout mice ablated for HL-1 and HL-2, they found elevations in vonWillebrand factor (vWF) in HL-1-/- mice, but not HL-2-/- mice. This finding is interesting because we found in HL-2-/- mice, there is no binding activity of

asialoglycoproteins. Nonetheless, they found the HL-1-/- mice had reduced bleeding times and platelet elimination following desialylation by *Streptococcus pneumoniae*.

We used alternative techniques including two dimensional difference gel electrophoresis and lectin blotting to identify endogenous ligands for the ASGR, by identifying elevated proteins in ASGR-/- mice. In chapter 2 of this thesis, we identified multiple proteins that are elevated in ASGR(-/-) plasma, and determined the elevated proteins bear Siaα2,6Gal. The proteins identified are abundant proteins, including haptoglobin and SAP. Specific isoforms are more significantly elevated than others, likely those isoforms bearing more Siaα2,6Gal, but the overall concentration of these proteins is elevated in the plasma. We have begun using a new method involving mass spectrometry to verify proteins that are elevated. The new technique quantifies the frequency of specific peptides seen in the plasma by mass spectrometry, and the proteins that are found most frequently are identified. Using this new technique, we identified a number of proteins elevated in ASGR(-/-) plasma, including haptoglobin. We conclude proteins bearing Siaα2,6Gal are the main ligands for the ASGR.

We have identified endogenous ligands for the ASGR, and determined the ASGR regulates plasma protein concentrations. We must now determine the purpose of rapid clearance by the ASGR. We propose the ASGR in involved in plasma protein turnover and that each protein has a set concentration and to maintain their set point, proteins are synthesized and cleared by their functional receptor and by the maintenance receptor, in this case the ASGR.

To determine the importance of clearance by the ASGR in plasma glycoprotein turnover, we must look at synthesis and degradation rates in Wt and ASGR-/- mice.

Using stable isotopic labeling and the mass spectrometry technique described previously, we can quantify turnover rates of proteins in mice. Briefly, stable isotope is administered to the animal, the stable isotope be incorporated into proteins for a specific amount of time and the ratio of labeled to unlabeled protein allows us to determine the fractional synthesis rate and fractional clearance rate. By determining fractional synthesis clearance rates, we can determine the significance of clearance compared to synthesis by the ASGR increases plasma protein concentrations.

The proteins that were identified as elevated in the plasma are abundant proteins, and could individually saturate the receptor. This suggests there must be a hierarchy of clearance of these proteins. We propose the ASGR regulates these proteins based on their current concentration and their affinity. Experiments determining how proteins compete for the ASGR are necessary to completely understand the function of the ASGR. Experiments using a method such as the Biacore can be conducted to determine the how proteins bind to the ASGR based on their concentration and affinity. The ASGR can be immobilized on a Biacore Chip and binding of proteins with different concentrations and affinities can be determined to elucidate the competition of ligands for the ASGR.

The MR is a second highly abundant endocytic receptor found in the liver [40]. The MR clears glycoprotein hormones bearing GalNAc-4-SO_4; however, it is unknown whether this is the main function of the MR. The MR may also play a functional role in regulating the concentration of proteins bearing GalNAc-4-SO_4 and mannose. It is necessary to determine if the MR has a function similar to that of the ASGR in unperturbed animals for determining the overall function of these two highly abundant receptors.

The ASGR regulates circulating plasma protein levels during the APR

Many of the proteins found elevated in ASGR-/- mice are acute phase proteins (APPs) and therefore, the ASGR may play a major role in the acute phase response. The APR is a response to infection, tissue injury or trauma in which a number of APPs dramatically change in response to inflammation [64]. There are two types of APPs, negative APPs and positive APPs [75]. Negative APPs include normal blood proteins such as albumin and transferrin. Positive acute phase proteins include proteins we identified in ASGR-/- plasma, including haptoglobin and SAP. At the site of the inflammation, a number of proinflammaotry cytokines are release, and these inflammatory cytokines including IL-1, IL-6, and TNF-α stimulate synthesis of APPs in the liver. Synthesis of APPs occurs within hours of the stimulus [70]. The protein changes seen during an APR are drastic and it is difficult to assume these changes are done entirely by increased synthesis. Synthesis may play a major role in increasing the concentration of APPs, but we propose decreased clearance by the ASGR also plays a major role.

It has previously been shown Siaα2,6Gal terminating proteins play a critical role in the APR. Proteins bearing small amounts of terminal Gal may exist in the plasma, but the amount of Gal or the orientation of the oligosaccharide is not sufficient for clearance by the ASGR. In unstressed animals, some Gal remains uncapped. During the APR, nearly 100% of the Gal residues are capped [73]. Additionally, the expression of α2,6sialyltransferase that adds Sia to Gal, ST6GalI elevates during the APR [72]. This suggests the structure Siaα2,6Gal plays a major role in the APR, perhaps by blocking clearance of proteins bearing Siaα2,6Gal.

Studies byTreichel et al [35]have shown the expression levels of the ASGR decrease in HepG2 cells during the APR. The cytokine IL-2 shuts down the ASGR within hours of exposure. Within 4 hours of exposure to IL-2, only 50% of the binding activity remains due to inactivation of the receptor by phosphorylation. Additionally, the message levels of the ASGR are greatly reduced within hours. We propose that down regulation of the ASGR plays a major role of elevating APPs bearing Siaα2,6Gal. If this is the case, in ASGR-/- plasma, we should not see as dramatic a fold change of the APPs because the only mechanism of elevation of proteins in these animals will be synthesis.

Previous experiments suggest that the oligosaccharide structures present on proteins and down regulation of the ASGR are important for an APR. In Chapter 3, we utilized ASGR-/- mice to further our understanding of the function of the ASGR during the APR. Based on Chapter 2, we know APPs are significantly elevated in ASGR-/- mice. We used ASGR-/- mice to determine the significance of protein clearance by the ASGR during the APR. We found that many proteins that are elevated in unperturbed animals further increase during the APR. Following the induction of the APR, 24 hrs later, specific isoforms of proteins including haptoglobin and SAP are significantly elevated. However, the initial levels of these proteins are higher, and the fold change of these proteins is not as significant as the fold change seen in Wt plasma. This suggests that during the APR, increased synthesis and decreased clearance by the ASGR work together to elevate APPs. We propose the ASGR regulates the concentration of proteins during the APR. In the absence of the ASGR, this mechanism for increasing plasma protein concentrations is unavailable, making their fold changes less.

In order to better understand the function of the ASGR in the APR, it must first be determined how the ASGR protein expression changes throughout the APR in Wt mice. The ASGR may be down-regulated to increase plasma protein levels during the APR, then up-regulated to return plasma protein levels to normal levels after the inflammation has been resolved. Determination of protein expression changes throughout the APR will aid in determining its precise function in altering plasma protein levels. Traditional binding studies can be used to determine the role of the ASGR throughout the APR.

Once it has been better established how the ASGR regulates plasma proteins levels throughout the APR, it is important to understand how synthesis and clearance by the ASGR work together to elevate plasma protein levels, and how much each mechanism is involved in changes the levels. Using stable isotopic labeling as previously described, we can assess the significance of decreased clearance compared to elevated synthesis in elevating APP concentrations.

The MR may also play a major role in the regulation of glycoproteins bearing GalNAc-4-SO_4 or mannose during the APR. Lee et al [52]identified four APPs elevated in unperturbed MR(-/-) mice the COOH-terminal propeptide domains of the pro-alpha 1 and 2 chains of type 1 procollagen and the pro-alpha 1 chain of type III procollagen which are elevated during wound healing, and fetuin-B. Additional proteins may be elevated in MR during the APR, they were just undetectable using previous techniques. Determination of proteins elevated in MR-/- plasma compared to Wt plasma in unperturbed animals will give us an idea of whether this receptor is involved in the clearance of APPs. Once we have identified elevated proteins in unperturbed animals, we will identify proteins bearing GalNAc-4-SO_4 and mannose elevated in animals

undergoing an APR. We will also determine whether the MR follows a similar pattern of inactivation and down regulation within hours of the APR.

The HPG axis is regulated by the ASGR and MR

The hypothalamic-pituitary-gonadal (HPG) axis regulates circulating levels of glycoprotein hormones produced in the pituitary and steroid hormones produced in the gonads. A key regulator of the HPG axis is LH, a glycoprotein hormone bearing GalNAc-4-SO_4 [8-10]. LH binds to the LHR on the gonads causing production of steroid hormones. The steroid hormones signal back to the hypothalamus and pituitary signaling for more or less production and release of LH. This closed feedback loop is essential for regulation of the HPG axis and alterations in regulation, specifically high levels of LH often leave animals and humans infertile due to desensitization of the LHR.

Our lab has determined the main oligosaccharide structure on LH is GalNAc-4-SO4, and LH is rapidly cleared by the MR [40]. While we have elucidated LH is cleared by the MR, the impact on circulatory half-life *in vivo* remains unknown. Rapid clearance of LH by the MR has been proposed to be essential for episodic stimulation of the LHR. Changing the half-life may cause chronic stimulation of the LHR leading to infertility.

Previously, Risma et al [104] overexpressed bLH-CTP in mice to produce a model of elevated LH. They found that elevated LH levels leads to chronic anovulation and infertility in females. The ovaries were enlarged with multiple cysts and packed with corpus luteum. Males expressing bLH-CLP were fertile. Additionally, elevated LH levels cause precocious puberty. Chronically elevated LH clearly has an impact on reproduction and we examined the significance of having elevated endogenous LH. By altering the circulatory half life, we may cause changes in the circulatory half life and

altered fertility. To address this issue we have examined the affects of changing the oligosaccharide structure on circulatory half life.

Recently, our lab generated a genetic mouse model lacking the transferase that adds sulfate to GalNAc, GalNAc-4-sulfotransferase (G4ST). We expected LH to bear terminal GalNAc and have a more rapid clearance rate. Surprisingly, we found in the absence of the G4ST, the oligosaccharide is modified with Sia and the final structure is Siaα2,6GalNAc, a structure presumably cleared by the ASGR. We found the circulatory half-life of LH in these animals is longer causing elevated LH; however, they are fertile, suggesting the LHR is still episodically stimulated. There have physiologic changes associated with elevate LH; males have elevated testosterone and females have elevated estrogen, and all mice undergo precocious puberty. Additionally, females have nearly 50% more litters compared to Wt females [63]. Understanding how the MR and ASGR regulate circulating LH levels is critical for understanding the precise role of LH in regulating the HPG axis.

In Chapter 4, we studied the role of the ASGR and MR in regulating LH levels. We found LH levels to be elevated in ASGR and MR plasma; however, the clearance rate of LH was only altered when we knocked out the MR. This suggests the main oligosaccharide structures found on LH are GalNAc-4-SO4 and there is little, if any, Siaα2,6GalNac. Circulating LH levels are elevated in ASGR(-/-) mice, through a mechanism other than clearance. One likely possibility that there is a protein(s) bearing Siaα2,6Gal/GalNAc in the plasma that regulates production or release of LH in the pituitary, and elevation of this protein is causing elevated LH in the plasma. Interestingly, all mice are fertile. LH signals to the gonads for steroid hormone

production, including testosterone and estrogen, and we have shown in male mice, elevated LH causes elevated testosterone and enlarged seminal vesicles. In female mice, the estrus cycle is prolonged in AR-/- and MR-/-AR-/-.

Elevated LH caused by ablation of either the MR or the ASGR does not have any extraordinary affect on the gonads. The testes have similar gene expression of LHR, StAR, 20αHSD, inhibin and activin. Ablation of both receptors, however, has dramatic affects on the gene expression. The expression of all these genes is elevated, suggesting the lack of clearance of LH in combination with the lack of clearance by an unidentified protein cleared by the ASGR results in the increase expression of these genes.

Changing the circulatory half life of LH *in vivo* clearly affects regulation of the HPG axis. By modifying the oligosaccharides structures, we have significantly altered the half life of LH and generated mice that are more fertile. By removing a main mechanism of clearance, the MR , we have significantly altered half life, but these animals are not more fertile. The differences seen are likely due to the stimulation of the LHR. By altering the oligosaccharide structure, the half life is longer, but there is still specific clearance by the ASGR. By removing the MR, clearance may not be specific, but rather through the kidney.

In order to better understand the significance of circulatory half life on LH, we must determine the affects of increased half life on reproduction. Mice with elevated LH, by either overexpressing bLH-CTP or changing the oligosaccharide structure, undergo precocious puberty. Mice lacking the MR, ASGR, or both have elevated LH and should also undergo precocious puberty. Additionally, mice with LH bearing Siaα2,6GalNAc are more fertile. We have not observed an obvious increase in fertility in MR-/- or

ASGR-/- mice, but the frequency of litters is important in understanding the importance of episodic stimulation in these mice and should be documented.

The clearance rate of LH is not altered in ASGR-/- mice, but LH is elevated. There must be a protein bearing Siaα2,6Gal/GalNAc elevated in the plasma of these animals causing elevated LH. The protein is likely coming from the testes. When the testes are intact, there is increased expression of LH in the pituitary. However, once the testes have been removed through castration, the expression of LH is similar to Wt expression. We are in the process of using gene chip analysis to identify genes whose expression is elevated in MR-/-, ASGR-/-, and MR-/-;ASGR-/- testes to identify gene expression elevations in MR-/-, ASGR-/-, and MR-/-ASGR-/- mice. Identification of elevated genes will allow us to more completely understand the HPG axis by identifying additional proteins involved in the regulation of LH**.**

The ASGR and MR are regulated during pregnancy and parturition

For a successful pregnancy in mice, steroid hormone levels must change dramatically. Progesterone levels are high throughout pregnancy [105]and estrogen levels are low. Immediately before parturition, progesterone levels rapidly decline and estrogen levels increase. Regulation of progesterone levels is done through 20αHSD, an enzyme that converts progesterone to its inactive metabolites. During pregnancy, prolactin binds to the prolactin receptor on the ovary causing decreased transcription of 20αHSD [101]. At the time of parturition, $PGF_{2\alpha}$ down regulates the prolactin receptor and up regulates production of 20αHSD, causing the conversion of progesterone to inactive metabolites and therefore decreasing the concentration of progesterone in the

blood [100]. The rapid decline of progesterone is thought to be necessary to induce parturition.

In Chapter 5 of this thesis, we studied the role of the MR and ASGR in reproduction of female mice. We found ASGR-/- and MR-/-ASGR-/- mice have slightly but significantly longer estrus cycles, but all three genotypes are fertile. Interestingly, mice with both receptors ablated undergo what appears to be a normal pregnancy, but they do not induce parturition and ultimately die. We studied the mRNA expression levels of the MR and ASGR throughout pregnancy and found the expression of both receptors changes significantly throughout pregnancy. Additionally, we found through pseudopregnancy, the changes in the receptor expression are driven by the mother, and the presence of deciduas is not necessary.

Our lab is currently conducting several experiments to further understand pregnancy and parturition. First, we are trying to understand the regulation of the MR and ASGR during pregnancy. The expression of MR and ASGR rapidly increases within the first couple days of being pregnant and peaks at day 12.5. Additionally, it has been previously shown binding of the ASGR increases throughout pregnancy, suggesting there are changes in plasma proteins that need to be regulated by the ASGR. The cause of this elevation is unknown. Progesterone elevation and decline follows a similar pattern to that of the receptors, suggesting progesterone may be the cause of the expression changes. Experiments are underway to determine whether progesterone is the cause of the expression changes. Pseudopregnant mice will be injected with RU486, a progesterone antagonist to determine whether progesterone is driving the expression changes.

There is clearly a problem with MR-/-ASGR-/- mice entering parturition. We had hints that the cause of the problem was progesterone, because injection of RU486 results in parturition and the mother and fetuses are saved. However, our preliminary studies suggest progesterone levels in MR-/-ASGR-/- are not different compared to Wt. Additionally, we have determined $PGF_{2\alpha}$ protein levels and 20αHSD mRNA expression levels are normal in MR-/-ARGR-/- mice. There have been suggestions that it is not progesterone alone that induces parturition, but the progesterone:estrogen ratio. We have shown estrogen levels may be low in MR-/-ASGR-/-, but further studies on these levels and the progesterone:estrogen ratio are necessary to completely understand the problem with parturition in these mice.

Finally, delayed clearance of one or more proteins in the MR-/-ASGR-/- plasma must be accounting for the inability to induce parturition. We previously identified prolactin-like proteins bearing Siaα2,6GalNAc that are likely regulated by the ASGR. Additional proteins bearing Siaα2,6GalNAc and GalNAc-4-SO_4 may play a critical role in parturition. Identification of these proteins will be necessary in completely understanding parturition. Using mass spectrometry, we will compare 18.5 day pregnant Wt, MR-/-, ASGR-/-, and MR-/-ASGR-/- plasma to identify elevated proteins in the plasma of MR-/-ASGR-/- plasma.

Conclusions

In this thesis, I have shown clearance of glycoproteins bearing Siaα2,6Gal/GalNAc and GalNAc-4-SO_4 is critical for multiple functions including inducing the acute phase response and parturition. Genetically altered mice lacking the ASGR have elevated levels of multiple plasma proteins including haptoglobin, SAP, and

LH. Haptoglobin and SAP bear the common structure Siaα2,6Gal and their clearance rate is reduced due to loss of the ASGR. The clearance rate of LH is not altered, suggesting there is an unidentified protein bearing Siaα2,6Gal/GalNAc involved in regulating production or release that is regulated by the ASGR.

The MR plays a clear role in determining the circulatory half life of LH in vivo, and the precise circulatory half life is important. We have shown drastic changes in the half life results in drastically different phenotypes. Further experiments to elucidate the role of both the MR and ASGR in reproduction are necessary to further our understanding of the function of these receptors in reproduction.

Abbreviations

ASGR, asialoglycoprotein receptor; MR, mannose/GalNAc-4-SO_4 receptor; Sia, sialic acid; Gal, galactose; GalNAc, N-acetylgalactosamine; APR, acute phase response; APP, acute phase protein; HPG, hypothalamic-pituitary-gonadal; HL, hepatic lectin; CRD, carbohydrate recognition domain; SAP, serum amyloid P; LH, luteinizing hormone; G4ST, GalNAc-4-sulfotransferase; LHR, LH receptor; 20αHSD, 20 alpha hydroxysteroid dehydrogenase; $PFG_{2\alpha}$, Prostaglandin $F_{2\alpha}$; StAR, sterogenic acute regulatory protein; PLP, prolactin-like protein.

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