TALES OF METABOLIC REGULATION: ADIPONECTIN IN THE COW AND SEL1L IN THE MOUSE

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TALES OF METABOLIC REGULATION: ADIPONECTIN IN THE COW AND SEL1L IN THE MOUSE

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The first part of this dissertation assessed factors regulating the insulin sensitizing hormone adiponectin in dairy cows. Plasma adiponectin is reduced during the transition from late pregnancy (LP) to early lactation (EL) in parallel with rapid changes in the plasma concentration of metabolic hormones [i.e. insulin, leptin, and growth hormone (GH)] and non-esterified fatty acids (NEFA). The first study of this dissertation showed that none of these factors contribute to the regulation of plasma adiponectin. The second study tested the possibility that altered endoplasmic reticulum (ER) chaperone expression is involved. This was motivated by the following observations: 1) Plasma adiponectin variation in transition dairy cows occurs in absence of changes in adiponectin mRNA. 2) In rodents, the master regulator of ER homeostasis x-box binding protein 1 (Xbp1) regulates adiponectin production by increasing ER chaperone expression. We showed that Xbp1 expression decreased from LP to EL in the dairy cow. Using a primary bovine adipocyte system, overexpression of Xbp1 had no effect on adiponectin, despite increasing expression of several ER chaperone proteins. The factors regulating plasma adiponectin in the transition dairy cow remain unknown.

The second part of this dissertation addressed the role of sel-1 suppressor of lin-12-like protein (Sel1l) in lipid metabolism. Sel1l is a critical component of the

endoplasmic reticulum associated degradation (ERAD) system required for translocation and degradation of misfolded or aggregated proteins. Adipose-specific deletion of Sel1l in mice has implicated an ERAD-independent role for Sel1l in lipid metabolism. To this end, we generated liver-specific Sel1l knockout mice ($\Delta Sel1l^{Liver}$) to assess whether Sel1l plays a similar role in lipid homeostasis in liver. Lipid metabolism was nearly normal in $\Delta Sel1l^{Liver}$ mice fed chow or high fat diet. $\Delta Sel1l^{Liver}$ mice on an atherogenic diet (AD) showed increased hyperlipidemia, hypercholesterolemia, liver damage, and occasional death compared to wildtype (WT) mice. This phenotype was associated with increased 3-hydroxy-3-methylglutaryl-CoA reductase (Hmgcr) and decreased low-density lipoprotein receptor (Ldlr) in liver of $\Delta Sel1l^{Liver}$ mice. Together, our results reveal a previously unknown role for Sel1l in lipid homeostasis in liver, but further studies are needed to elucidate the mechanism.

BIOGRAPHICAL SKETCH

Christopher Steven Krumm was born in Taegu, South Korea on November 22, 1988 and adopted by Steven and Colleen Krumm. His first inspiration to work in a field related to science came from the film Jurassic Park. The experiences he gained while working for an exotic veterinarian in high school were the primary driver influencing him to pursue a career in veterinary medicine. After graduating in the top 10% of his class, he chose to enroll at Rutgers University. In 2009 he joined the lab of Dr. Wendie S. Cohick to conduct an honors research project entitled "The Role of Insulin-Like Growth Factor Binding Protein-3 in Stress-Induced Apoptosis in Mammary Epithelial Cells". His new found passion for research led him to pursue a PhD degree rather than attend veterinary school. In May 2011, he went on to graduate with a bachelor of science degree in Animal Science and a bachelor of arts degree in Biological Sciences. In August 2011, he began his doctoral studies at Cornell University under the mentorship of Dr. Qiaoming Long to study the role of Sel-1 suppressor of lin-12-like protein (Sel1l) in lipid metabolism. During the summer of 2013, he continued his doctoral studies under the mentorship of Dr. Yves Boisclair to identify factors regulating adiponectin in dairy cattle. After defending his dissertation, he is excited to begin his postdoctoral studies at Weill Cornell Medical College in New York City under the supervision of Dr. Ann-Hwee Lee.

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LIST OF ABBREVIATIONS

AD	Atherogenic diet
AdipoR1	Adiponectin receptor 1
AdipoR2	Adiponectin receptor 2
ALT	Alanine transaminase
AMPK	Adenosine monophosphate-activated protein kinase
APPL1	Amyloid precursor protein-like 1
ATF6α	Activating transcription factor α
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid assay
BSA	Bovine serum albumin
cDNA	Complementary deoxynucleic acid
Chop	DNA-damage inducible transcript 3
CP	Crude protein
Cyp7a1	Cholesterol 7-α hydroxylase
DM	Dry matter
DN	Dominant negative
DsbA-L	Glutathione S-transferase kappa 1
DTT	Dithiothreitol
EL	Early lactation
ER	Endoplasmic reticulum

ERAD	Endoplasmic reticulum-associated degradation
ERdj3	DnaJ heat shock protein family (Hsp40) member B11
ERO1-Lα	Endoplasmic reticulum oxidoreductase 1-like α
Erp44	Endoplasmic reticulum protein 44
Fabp4	Fatty acid binding protein 4
FFA	Free fatty acid
G6P	Glucose-6-phosphate
Gabra1	Gamma-aminobutyric acid (GABA) A receptor subunit alpha-1
GDM	Gestational diabetes mellitus
Gga1	Golgi associated, gamma adaptin ear containing, ARF binding protein 1
GH	Growth hormone
GHR	GH receptor
Grp78	Glucose-regulated protein, 78 kDa
GWAS	Genome wide association study
H&E	Hematoxylin and Eosin
Hdl	High-density lipoprotein
HEK293	Human embryonic kidney 293
HEK293a	Human embryonic kidney 293a

Herp	homocysteine-inducible endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1
HFD	High fat diet
HMG CoA	3-hydroxy-3-methyl-glutaryl-coenzyme A
Hmgcr	3-hydroxy-3-methylglutaryl-Co A reductase
HMW	High molecular weight
Hrd1	Synovial apoptosis inhibitor 1, synoviolin
Hsp90	Heat shock protein 90
Idl	Intermediate-density lipoprotein
IBMX	3-isobutyl-1-methylxanthine
IGF-1	Insulin-like growth factor-1
IM	Intramuscular
IR	Insulin resistance
Ire1α	endoplasmic reticulum to nucleus signaling 1
ITT	Insulin tolerance test
IV	Intravenous
Ldl	Low-density lipoprotein
Ldlr	Low-density lipoprotein receptor
LMW	Low molecular weight
LP	Late pregnancy
LRP	Ldlr-related protein
MAPK	Mitogen-activated protein kinase

MAT	Bone marrow adipose tissue
MMW	Medium molecular weight
NEB	Negative energy balance
NEFA	Non-esterified fatty acid
NE_L	Net energy of lactation
NTCP	solute carrier family 10 (sodium/bile acid cotransporter family), member 1
p38 MAPK	p38 mitogen-activated protein kinase
p58IPK	Protein kinase inhibitor of 58 kDa
p70S6K	Ribosomal protein S6 kinase
PBS	Phosphate-buffered saline
PDI	Protein disulfide isomerase
Pdia6	Protein disulfide isomerase associated 6
PERK	PRKR-like endoplasmic reticulum kinase
PI3K	Phosphoinositide-3-kinase
PMSF	Phenylmethylsulfonyl fluoride
PPARα	Peroxisome proliferator-activated receptor α
PPARγ	Peroxisome proliferator-activated receptor γ
PPRE	PPAR response element
RAMP4	Stress-associated endoplasmic reticulum protein 1
SC	Subcutaneous

scWAT	Subcutaneous white adipose tissue
slc22a7	Solute carrier family 22 (organic anion transporter), member 7
Sel1l	Sel-1 suppressor of lin-12-like protein
Stat5	Signal transducer and activator of transcription-5
SVC	Stromovascular cells
T2D	Type 2 diabetes
TGN	Trans golgi network
TMR	Total mixed ration
TNFα	Tumor necrosis factor α
TZD	Thiazolidinedione
UPR	Unfolded protein response
Vldl	Very low-density lipoprotein
WAT	White adipose tissue
WT	Wild type
Xbp1	X-box binding protein 1
$Xbp1_S$	Spliced variant Xbp1
$Xbp1_T$	Total Xbp1

CHAPTER 1

INTRODUCTION

Part I: Adiponectin

The productivity and profitability of the modern dairy cow is largely dependent on the efficiency of adaptation to the metabolic challenges associated with the transition period between late pregnancy (LP) and early lactation (EL). At the onset of lactation in high-yielding dairy cows, energy requirements rise nearly 4-fold in order to meet the demands for milk synthesis [1]. Feed intake alone is not sufficient to meet the sudden increased nutritional demands of the mammary gland in EL, resulting in major deficits in virtually all major classes of organic nutrients including the lactose precursor glucose [1]. Dairy cows cope with this nutritional insufficiency through a growing insulin resistance (IR) in the transition period [1]. IR facilitates the use of glucose by the mammary gland after parturition, [1]. However, excessive IR due to over-nutrition during the dry period and/or excessive fatness at parturition [2,3] can lead to the development of an array of metabolic diseases. For example, excessive plasma non-esterified fatty acids (NEFA) has been associated with inefficient utilization of lipid and protein reserves, loss of milk production, and increased susceptibility to several metabolic diseases (e.g. ketosis, metritis, mastitis) [4–6]. Mechanisms regulating IR in transition dairy cows remain ill-defined. Growth hormone (GH) may be involved, due to its ability to suppress insulin-stimulated glucose uptake in adipose tissue and skeletal muscle [1]. One hormone, however, that has not been extensively studied in the context of IR regulation in transition dairy cows is adiponectin. Adiponectin may be relevant to the regulation of the IR state in dairy cows, because it was previously implicated as an insulin sensitizer in rodents and humans [7,8]. Moreover, the plasma concentration of adiponectin drops in

parallel with the rise of IR in transition dairy cows [9–11]. Factors regulating the periparturient drop of plasma adiponectin in dairy cows are unknown.

In the first part of this thesis, I will review the literature pertaining to adiponectin function and focus primarily on factors regulating its production and secretion from white adipose tissue (WAT). I will then report on experiments examining the role of metabolic factors and hormones in regulating plasma adiponectin in transition dairy cows (Chapter 3). Second, I will report on experiments used to determine whether variation of endoplasmic reticulum (ER) chaperones could explain changes in adiponectin production. ER chaperones may be important, because adiponectin must be assembled into oligomers in the ER before secretion (Chapter 4).

Part II: Sel1

Hepatocytes carry out a myriad of functions involving the ER including triglyceride synthesis, lipoprotein assembly, and plasma protein synthesis and secretion [12]. For example, approximately 75% of all proteins destined for secretion are synthesized within the ER [13]. Preserving ER homeostasis is critical for maintaining the biosynthetic capacity of highly secretory cells such as the hepatocyte.

Imbalances in these pathways due to high demand for protein synthesis and secretion can enhance protein misfolding and impair ER-folding machinery function. These disruptions of ER homeostasis lead to the accumulation of improperly folded or aggregated proteins and the development of ER stress [14,15]. Adaptive mechanisms including the endoplasmic reticulum associated degradation (ERAD) system are activated

in order to cope with increased ER stress [16]. ERAD is a highly conserved system that aims to promote cell survival by eliminating aggregated and misfolded proteins [15,17,18].

Sel-1 suppressor of lin-12-like protein (Sel1l) is an 89-kDa transmembrane protein that nucleates the ERAD complex required for translocation of misfolded or aggregated proteins from the ER to the cytosol for degradation [19]. Sel1l expression is induced by ER-stress inducing agents such as tunicamycin and thapsigargin in human embryonic kidney 293 cells (HEK293) [20]. Targeted disruption of Sel1l in transgenic mice leads to embryonic lethality and increased ER stress [21]. These studies provide evidence of the critical role of Sel1l in maintaining ER homeostasis. Adipose tissue deletion of Sel1l in mice has demonstrated a pivotal role of Se1l in maintaining ER function and accumulation of triglyceride [22]. However, studies assessing the role of Sel1l in lipid homeostasis in secretory organs such as the liver are nonexistent.

In the second part of this thesis, I will report on the effect of liver-specific Sel1l ablation on lipid metabolism (Chapter 5).

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CHAPTER 2:

LITERATURE REVIEW

I. STRUCTURE

Four independent groups identified a common 30-kDa secretory protein highly expressed in adipose tissue and named it Acrp30, AdipoQ, apM1 or GBP28. Acrp30 and AdipoQ were cloned using mouse complementary deoxynucleic acid (cDNA) through differential display before and after differentiation of 3T3-L1 and 3T3-F442 adipocyte cells, respectively [1,2]. apM1 was isolated from a human adipose tissue cDNA library, while GBP28 was purified from human plasma [3,4]. The generic name adiponectin was eventually accepted for this 30 kDa protein [5]. Adiponectin was found to share sequence homology to the complement factor C1q family of proteins and structural homology with tumor necrosis factor α (TNFα), another adipokine highly secreted by adipocytes and implicated in the induction of insulin resistance (IR) [6]. Adiponectin consists of an amino-terminal signal sequence, a hypervariable region that is highly divergent between species, a collagenous domain, and a globular domain [7,8].

Adiponectin circulates at around 10-50 μg/mL or ~ 0.01% of total plasma proteins [9,10]. Unlike most other circulating adipokines, adiponectin circulates as homomultimers of 3, 6, or 18-24 units respectively known as low molecular weight (LMW ~ 67 kDa), medium molecular weight (MMW ~ 120 kDa), and high molecular weight (HMW > 300 kDa) adiponectin [11,12]. Freeze-etch electron microscopy of adiponectin monomers revealed a ball and stick-like structure, while analysis of HMW adiponectin by dynamic light scattering and transmission electron microscopy revealed an asymmetric bouquet-like structure [13,14]. Experiments utilizing purified complexes demonstrated that adiponectin oligomers do not interconvert once secreted in plasma [12].

The molecular weight distribution of plasma adiponectin varies by species and gender. Plasma adiponectin circulates in a 2:1 ratio of HMW and MMW oligomers in female mice, while it exists exclusively as the HMW oligomer in chickens and cattle [15–17]. Moreover, the number of monomers within the HMW adiponectin complex is exactly 18 in mice and cattle, but ranges from 18-30 monomers in humans [14,17–19]. Sexual dimorphism has also been reported for plasma adiponectin. Total adiponectin is more abundant in females than males in humans and mice [20–22]. Moreover, HMW adiponectin accounts for 50% of total plasma adiponectin in women, whereas it is equally distributed among all complexes in men [15].

Formation of the LMW complex is driven by hydrophobic interactions between globular domains of individual monomers [6]. Within LMW adiponectin, the conserved cysteine residue in the collagenous domain is used to covalently link 2 of the 3 monomers [6,12]. The cysteine residue in the remaining monomer interacts with the free cysteine residue from another LMW complex, leading to the formation of MMW and HMW adiponectin [23]. Substitution of the cysteine residue to alanine impaired formation of MMW and HMW adiponectin oligomers, but had no impact on LMW formation [13].

II. POST-TRANSLATIONAL MODIFICATIONS

Many studies have reported changes in plasma adiponectin in absence of mRNA variation. This suggests that post-translational modifications play a role in determining both the absolute level of plasma adiponectin as well as the adiponectin complex distribution [17,24]. Indeed, adiponectin oligomer assembly and multimerization requires

extensive post-translational modifications within the endoplasmic reticulum (ER) before secretion [25].

Hydroxylation & glycosylation

Wang et al made the initial discovery that adiponectin was highly hydroxylated and subsequently glycosylated on four proline residues (Pro^{39}, Pro^{42}, Pro^{48}, and Pro^{86}) and on five lysine residues (Lys^{28}, Lys^{60}, Lys^{63}, Lys^{72}, and Lys^{96}) in bovine adiponectin and on several lysine residues (Lys^{68}, Lys^{71}, Lys^{80}, and Lys^{104}) in mouse adiponectin [23,26]. These proline and lysine residues are conserved in human adiponectin [18,26]. Additional proline residues undergoing hydroxylation have been identified in human adiponectin (Pro^{71}, Pro^{76}, and Pro^{95}) [18]. Substituting the conserved lysine residues with alanine ablated hydroxylation and subsequent glycosylation, and led to impaired intracellular assembly and secretion of adiponectin in Cos-7 cells [23]. Similar conclusions were reached using prolyl- and lysylhydroxylase inhibitors in human embryonic kidney 293 (HEK293) cells [18]. These studies implicate hydroxylation and glycosylation as post-translational modifications important for the proper assembly of adiponectin complexes in the adipocyte.

ER chaperone proteins

Proper assembly and secretion of oligomers such as adiponectin require ER chaperone proteins [27–31]. Treatment of 3T3-L1 adipocytes with the reducing agent 2-mercaptoethanol stimulated adiponectin secretion [28]. Consistent with this finding,

replacement of the cysteine residue required for disulfide bond formation between adiponectin units exacerbated LMW adiponectin secretion in HEK293 cells [12].

Endoplasmic reticulum protein 44 (Erp44) and Endoplasmic reticulum oxidoreductase 1-like α (ERO1-Lα) are two ER resident chaperone proteins induced during adipogenesis in 3T3-L1 adipocytes [28,29]. Both have been shown to regulate the retention and secretion of adiponectin complexes through direct interaction with the highly conserved Cys^{39} residue [28,29]. For example, Erp44 forms a mixed disulfide bond with the Cys^{39} residue of improperly assembled MMW and HMW adiponectin, thus preventing their secretion through thiol-mediated retention [28]. Overexpressing Erp44 expression decreased adiponectin secretion in HEK293 cells and 3T3-L1 adipocytes [28]. In contrast, silencing Erp44 increased adiponectin secretion in 3T3-L1 adipocytes [28]. ERO1-Lα is an ER membrane-associated oxidoreductase that utilizes the oxidizing power of oxygen to generate disulfide bonds, which can then be transferred to substrates such as adiponectin through intermediate proteins [e.g. protein disulfide isomerase (PDI)] [32,33]. Overexpressing ERO1-Lα expression increased adiponectin secretion from HEK293 cells and 3T3-L1 adipocytes [28]. In contrast, silencing ERO1-Lα decreased adiponectin secretion [29].

It has been suggested that the high affinity between ERO1-Lα and Erp44 could reduce the binding of adiponectin complexes by Erp44, thereby favoring adiponectin secretion [34]. Co-expression of ERO1-Lα and Erp44 increased adiponectin secretion in HEK293 cells [28]. Thus, Erp44 and ERO1-Lα control the maturation and abundance of adiponectin oligomer complexes secreted from the adipocyte.

Glutathione S-transferase kappa 1 (DsbA-L) is a disulfide isomerase identified as an adiponectin-interactive protein through a yeast two hybrid screen [30]. While DsbA-L is expressed in a wide array of mouse tissues including liver, lungs, kidney, heart, and pancreas, highest expression is in adipose tissue [30]. Overexpression of DsbA-L in 3T3-L1 adipocytes increased production and secretion of HMW adiponectin, whereas RNAi-mediated suppression of DsbA-L reduced secretion of both total and HMW adiponectin into conditioned media [30]. Mutating a conserved Ser^{16} residue in DsbA-L led to decreased HMW adiponectin secretion into the media in Chinese hamster ovary cells overexpressing the insulin receptor [30]. Direct evidence of a mechanistic role of DsbA-L in adiponectin multimerization was obtained in genetically engineered mice [35]. DsbA-L overexpression in mouse adipose tissue increased HMW adiponectin in adipose tissue and plasma [35]. In contrast, global DsbA-L knockout mice showed reduced plasma adiponectin [36]. Confocal immunofluorescence studies showed that DsbA-L localized within the ER and mitochondria [37]. Preventing DsbA-L translocation to the ER ablated its ability to increase adiponectin multimerization [37]. DsbA-L therefore is an ER-associated protein involved in the assembly of adiponectin complexes.

Trafficking of properly assembled adiponectin oligomer complexes through the trans golgi network (TGN) is dependent on golgi associated, gamma adaptin ear containing, ARF binding protein 1 (Gga1) - coated vesicles [31]. Gga1 is a member of a family of ubiquitously expressed adaptor Gga coat proteins required for the proper sorting and trafficking of cargo proteins and vesicles through the TGN [38,39]. Confocal microscopy and transmission electron microscopy results indicated that adiponectin co-

localized with Gga1 in the Golgi and TGN [31]. No direct interaction between adiponectin and Gga1 was observed in the golgi and TGN. Silencing Gga1 in 3T3-L1 adipocytes suppressed adiponectin secretion, whereas over-expression of Gga1 had the opposite effect. Similar results were obtained when wildtype (WT) or mutant Gga1 were co-expressed with adiponectin in HEK293 cells [31]. Collectively, this study shows that adiponectin secretion from the adipocyte is dependent on Gga1 trafficking.

III. ADIPONECTIN SIGNALING

Two adiponectin receptors were identified through screening of a cDNA library derived from human skeletal muscle. These receptors are known as adiponectin receptor 1 (AdipoR1) and adiponectin receptor 2 (AdipoR2) and have molecular weights of 42 and 35 kDa respectively [40]. Mouse AdipoR1 and AdipoR2 share 66.7% sequence homology and are conserved from yeast to humans [40]. AdipoR1 and AdipoR2 were identified as integral membrane proteins featuring 7 transmembrane domains, but exhibiting inverse topology to G-protein coupled receptor proteins i.e. C-terminus external and N-terminus internal to the cell membrane [40–42]. While AdipoR1 and AdipoR2 are ubiquitously expressed, AdipoR1 is most abundantly expressed in skeletal muscle, while AdipoR2 is most abundantly expressed in the liver [40].

AdipoR1 signaling is primarily mediated through adenosine monophosphate-activated protein kinase (AMPK), whereas AdipoR2 signaling is mediated through activation of peroxisome proliferator-activated receptor α (PPARα) by p38 mitogen-activated protein kinase (p38 MAPK) [43,44]. Recombinant adiponectin induced

phosphorylation of AMPK and p38 MAPK in C2C12 myocytes and hepatocytes. Signaling events were abrogated by dominant negative (DN)-AMPK or an inhibitor of p38 MAPK in C2C12 myocytes [40]. Adenovirus encoding AdipoR1 or AdipoR2 increased phosphorylation of AMPK or PPARα in liver tissue of ob/ob mice respectively [45]. Moreover, AdipoR1 knockout (ΔAdipoR1) mice exhibited decreased AMPK signaling with no effect on PPARα signaling in liver tissue, while AdipoR2 knockout (ΔAdipoR2) mice exhibited the reciprocal results [45]. Mice harboring knockouts of both receptors (ΔAdipoR1; ΔAdipoR2) displayed defects in both AMPK and PPARα signaling in liver tissue [45].

Use of the cytoplasmic domain of AdipoR1 as bait in a yeast two-hybrid assay led to the identification of amyloid precursor protein-like 1 (APPL1) as a receptor interacting protein. APPL1 is a pleckstrin homology domain protein expressed in several mouse tissues including brain, heart, fat muscle, skeletal muscle, spleen, kidney, and pancreas [46]. APPL1 co-localized with adiponectin as shown through confocal microscopy in C2C12 myocytes [46]. In addition, the C-terminus and N-terminus of AdipoR1 directly interacted with adiponectin and APPL, respectively [46]. RNAi-mediated suppression of APPL1 decreased adiponectin signaling, whereas overexpression induced the opposite effect [46].

IV. FACTORS REGULATING ADIPONECTIN PRODUCTION

Obesity and energy overconsumption

Excessive energy intake and adiposity are two factors associated with reductions in adiponectin mRNA in adipose tissue and plasma adiponectin [47,48]. Plasma adiponectin

levels were significantly lower in obese subjects and animals when compared to controls [2,5]. Reciprocally, weight loss following gastric bypass surgery caused a significant rise in adipose adiponectin mRNA and plasma adiponectin [49–51]. Obese diabetic patients that underwent weight reduction were also characterized by increased plasma adiponectin levels [52]. In contrast, one study reported that reduced adiposity resulting from exercise did not lead to an increase in plasma adiponectin [53].

Qiao et al studied the effects of either a normal chow diet or high fat diet (HFD) on adiponectin in the mouse [54]. Plasma adiponectin did not differ when diets were fed on an isocaloric basis. On the other hand, adiponectin mRNA and plasma levels were elevated upon caloric restriction, irrespective of diets. Time-dependent rises in mRNA and plasma adiponectin levels were observed in calorie-restricted ob/ob mice when compared to WT controls [54]. Moreover, only the HMW adiponectin isoform increased in abundance upon caloric restriction [55]. Overall, these data suggest that energy intake, but not dietary fat regulates production and secretion of HMW plasma adiponectin.

Exercise

Acute exercise has been associated with improved insulin action [56]. Reports pertaining to acute exercise and adiponectin have produced inconsistent results. Acute exercise had no effect on plasma adiponectin in healthy and obese individuals in some studies, whereas it increased plasma adiponectin levels in other studies [57–62]. Effects of acute exercise on adiponectin oligomers have also been contradictory [63,64]. These inconsistencies could be due to several factors such as the overall health of subjects

(trained vs. not trained; lean vs. obese), type of exercise (cycling, running, rowing), measurement time points, and insufficient time required for changes in hormonal adaptations.

Chronic exercise has also been associated with improved insulin action [65]. Inconsistent results have also been reported between chronic exercise and plasma adiponectin. Chronic exercise increased plasma adiponectin in obese, type 2 diabetic (T2D) individuals [66]. Moreover, one study observed a significant increase in the ratio of HMW/total adiponectin upon chronic exercise in healthy and obese individuals [67]. On the other hand, chronic exercise had no effects on plasma adiponectin in healthy or obese individuals in other studies, even when leading to a reduction in adiposity [53,67–69]. In conclusion, it is still not clear whether acute or chronic exercise play a role in the regulation of plasma adiponectin.

Metabolic hormones

Insulin

Several studies have demonstrated that insulin directly alters adiponectin expression or secretion [70–72]. Insulin treatment of cultured human adipose tissue or of the adipocyte cell lines T37i and 3T3-L1 increased adiponectin mRNA [71–73]. Inhibition of the insulin-signaling pathways such as phosphoinositide-3-kinase (PI3K), mitogen-activated protein kinase (MAPK), or ribosomal protein S6 kinase (p70S6K) reduced adiponectin mRNA expression and secretion in 3T3-L1 adipocytes [72,74].

For unclear reasons, opposite results were obtained by another group in the same cell line (i.e. 3T3-L1 adipocyte). Insulin treatment of 3T3-L1 adipocytes suppressed adiponectin mRNA expression and secretion into the media [75]. Furthermore, the inhibitory actions of insulin on adiponectin secretion could be rescued by either treatment with pharmacological inhibitors of PI3K, MAPK, or p70S6K or removal of insulin from the media in 3T3-L1 adipocytes [75]. This latter results agrees with most of the *in vivo* data. Injection of human insulin in mice caused a significant decrease in plasma levels of HMW, but not LMW or MMW adiponectin [12]. Transgenic mice with a deletion of the insulin receptor in adipose tissue showed increased plasma adiponectin levels [76]. Moreover, human subjects with a loss of function mutation in the insulin receptor have increased plasma adiponectin [77] . Hyperinsulinemia induced a significant drop in plasma adiponectin in human subjects undergoing a hyperinsulinemic-euglycemic clamp [78]. A significant drop in plasma HMW, but not LMW adiponectin were also observed in diabetic and non-diabetic subjects under hyperinsulinemic conditions [79].

Growth hormone (GH)

GH is considered an upstream regulator of adiponectin signaling as it has been known to antagonize insulin action [80]. Treatment of cultured human adipose tissue with GH suppressed adiponectin secretion [81]. Injection of GH or of itjs mediator, insulin-like growth factor-I (IGF-I), decreased plasma adiponectin in mice [82]. Consistent with these data, mice lacking the growth hormone receptor (ΔGHR) have increased plasma adiponectin, despite exhibiting greater fatness [83]. On the other hand, the opposite effect

was seen in transgenic mice overexpressing GH (Tg-GH) [81,84]. Finally, the repressive effects of exogenous GH therapy on the HMW adiponectin oligomer were observed in both mice and human children [82,85]. Together, these studies suggest that GH reduces plasma adiponectin levels.

Leptin

Some studies have suggested that leptin regulates adiponectin production and secretion. Human adipocytes treated with recombinant leptin expressed higher levels of adiponectin mRNA and produced more adiponectin; opposite results were observed when a leptin antagonist was used [86]. *E*x *vivo* treatment of human adipose tissue with leptin led to increased adiponectin mRNA and protein [86].

Other reports suggest that leptin had no effect on adiponectin secretion. Healthy and obese human subjects treated with human leptin experienced no significant changes in plasma adiponectin [87]. Plasma adiponectin was increased in obese leptin deficient humans subjects treated with human leptin [88]. However, the latter is likely caused by the dramatic loss of adiposity during leptin therapy. Rats treated with recombinant leptin had no change in adiponectin mRNA in adipose tissue [89]. In conclusion, *in vitro* data support a positive role for leptin in regulating adiponectin production, but this conclusion is not supported *in vivo*.

V. EVIDENCE IMPLICATING ADIPONECTIN IN THE REGULATION OF INSULIN ACTION

Clinical evidence in humans and mice

Plasma adiponectin is frequently reduced in human subjects experiencing conditions of diseases associated with IR. For example, plasma adiponectin is decreased in obese individuals when compared to lean controls [5]. Plasma adiponectin was also reduced in conditions associated with insulin insensitivity such as T2D. This negative association between plasma adiponectin and insulin action has been observed across ethnic groups including the Japanese, African Americans, Chileans, and Asian Indians [52,90–95]. Similar trends were observed in other animals including mice, monkeys, marmots, and dolphins [2,96–99].

Rodent models were used to investigate whether reduced plasma adiponectin was a mere consequence of obesity, IR, and T2D or if it contributed to the pathologic defects of these conditions. For example, mice lacking the leptin receptor (db/db) and mice overexpressing the agouti gene (KKA^{y}) suffer from low adiponectin and IR. Infusion of adiponectin restored plasma adiponectin to levels comparable to WT mice and reversed IR [100]. In a second independent experiment, IR was induced in heterozygous peroxisome proliferator-activated receptor γ (PPARγ) knockout mice by reducing PPARγ action with a PPARγ antagonist. Adiponectin infusion in these mice reduced plasma free fatty acid (FFA) and triglyceride levels in skeletal muscle and liver [100]. Together, these studies provided evidence that adiponectin could exhibit anti-diabetic and hypolipidemic effects.

Freubis et al induced metabolic syndrome in WT mice by feeding a HFD. Administration of globular adiponectin to these mice caused a significant drop in body weight and plasma triglyceride, FFA, and glucose [101]. Similarly, Yamauchi et al showed that globular adiponectin induced AMPK signaling in skeletal muscle in mice and C2C12 myocytes [102]. This effect was not seen in C2C12 myocytes overexpressing a dominant negative version of AMPK. Together, these data suggest that globular adiponectin improves insulin action in skeletal muscle by activating the AMPK signaling pathway.

Berg et al treated diabetic mouse models with full-length adiponectin produced in mammalian cells. This treatment led to improved systemic insulin sensitivity as shown by decreased plasma glucose levels [103]. Using the insulin clamp technique, they showed that adiponectin inhibited endogenous glucose production, but had no effect on peripheral glucose uptake [104]. Consistent with these data, adiponectin decreased expression of enzymes involved in gluconeogenesis in liver, which included glucose-6-phosphatase and phosphoenolpyruvate carboxykinase [104]. Collectively, these results suggest that plasma adiponectin increases hepatic insulin sensitivity, leading to reduced glucose production [102–104].

Genetic evidence

Genetically engineered mouse models were developed to assess the functional role of adiponectin *in vivo*. The ability of insulin to inhibit hepatic glucose production was impaired in adiponectin knockout (ΔAdiponectin) mice, whereas no effects were observed on peripheral glucose disposal [105]. Transgenic adiponectin overexpression (Tg-

Adiponectin) mice displayed decreased hepatic glucose production, plasma glucose and insulin levels, and increased phosphorylation of AMPK in liver [106]. More recently, Kim et al created an ob/ob mouse strain overexpressing adiponectin in adipose tissue (Tg-Adiponectin; ob/ob) [107]. In response to a bolus infusion of 2-deoxyglucose, these mice experienced a reduction in hepatic glucose output, resulting in normalization of plasma glucose levels, whereas this effect was not seen in ob/ob mice. The Tg-Adiponectin; ob/ob mice also exhibited significant improvements in hepatic insulin sensitivity as shown by increased phosphorylated Akt [107]. Together, these studies point to the liver as a primary target tissue responsible for full-length adiponectin-mediated alterations in insulin sensitivity.

Genetic evidence has also been obtained in humans. The adiponectin gene is located on chromosome 3q27, close to loci associated with metabolic diseases including T2D [108,109]. Several mutations within the adiponectin gene have been linked to hypoadiponectinemia, IR, and T2D. SNP276 within intron 2 (G vs. T) was associated with decreased plasma adiponectin and increased IR, obesity, and T2D in Japanese, American, and German subjects [110–112]. SNPs 11377 and 11391 in the promoter region of the adiponectin gene were associated with hypoadiponectinemia and T2D in French Caucasians [109]. Two missense mutations (I164T and R112C) disrupted assembly of LMW adiponectin and adiponectin secretion in human subjects with the metabolic syndrome [19,113]. Similarly, G84R and G90S mutations were responsible for inhibition of the assembly and secretion of the HMW adiponectin [19]. These results show that insufficient

production and secretion of adiponectin oligomers are associated with several metabolic diseases, including T2D.

HMW adiponectin

Additional evidence implicating adiponectin as an insulin sensitizer came from studies investigating whether all adiponectin isoforms were equally potent. Plasma abundance of HMW adiponectin was correlated with indices of insulin action, but total adiponectin was not [114]. It was later found that increased changes in the ratio of plasma HMW/total adiponectin were positively correlated with increased insulin sensitivity in patients of T2D and gestational diabetes mellitus (GDM) [114].

Functional assays and genetically engineered animal models were developed to determine whether plasma HMW adiponectin was causally involved in regulating insulin action. Administration of HMW, but not LMW adiponectin induced a dose-dependent drop in plasma glucose levels in ΔAdiponectin mice, indicating a stimulation of insulin sensitivity [114]. To a similar extent, acute injection of HMW adiponectin in mice triggered the activation of downstream hepatic adiponectin signaling, while chronic infusion led to alleviation of hyperglycemia and IR in db/db mice [23]. In agreement with these *in vivo* data, an adiponectin variant unable to assemble into HMW isoforms was less effective than WT adiponectin in alleviating the hyperglycemia, hypertriglyceridemia, and IR of db/db mice [23]. Together, these data implicate HMW adiponectin as the most potent oligomer capable of modulating insulin action.

Pharmacological evidence

A final piece of evidence relating adiponectin to insulin action comes from studies of thiazolidinedione (TZD), a class of drugs known to act on PPARγ. They are used as therapeutics to treat IR and the metabolic syndrome. TZDs improve insulin action, through mechanisms that include suppression of hepatic gluconeogenesis and stimulation of peripheral glucose disposal [115–117]. Use of TZDs led to partial correction of hyperglycemia and hyperinsulinemia in humans and db/db mice [2,118–120]. All of these actions occurred in parallel with a rise in plasma adiponectin, more specifically the HMW oligomer [9,100,114,121]. Thus, these data suggest the possibility that adiponectin mediates the insulin-sensitizing effects of TZDs.

A number of studies have focused on the molecular mechanisms accounting for positive effects of TZDs on adiponectin mRNA. A significant rise in adiponectin mRNA was reported upon treatment with TZDs [10,121]. Consistent with transcriptional regulation, a PPARγ response element (PPRE) was identified within the human and mouse adiponectin promoters [70,122]. PPARγ was shown to specifically bind to the PPRE in the human adiponectin promoter [122]. Both human or mouse adiponectin promoters were activated in HEK293 cells co-transfected with a PPARγ expression-vector [70,122]. TZD treatment further stimulated human and mouse adiponectin promoter activity, and these effects were lost when the PPRE was mutated [70,122]. Together, these studies suggest that the positive effects of TZD on plasma adiponectin involve a stimulation of adiponectin transcription.

Functional evidence for the regulatory role of PPARγ signaling on adiponectin expression and secretion was observed using genetically engineered mouse models. Transgenic mice with a targeted deletion of PPARγ in adipose tissue ($\Delta PPAR\gamma^{Adipose}$) exhibited substantially decreased plasma adiponectin levels [123]. Moreover, TZD treatment had no effect on insulin sensitivity in the adipose tissue of these mice. Therefore, this study suggested that TZDs target adipose PPARγ to increase adiponectin production and secretion [123].

A series of experiments focused on whether TZDs mediate insulin sensitivity in an adiponectin dependent or independent manner. First, murine models of obesity including db/db and KKAy mice displayed significantly lower plasma adiponectin levels than WT mice and suffer from IR [100]. Second, TZD treatment of ob/ob or db/db mice increased plasma adiponectin levels and improved insulin action [100,124]. Third, overexpression of adiponectin in ob/ob mice improved insulin action [105,107]. Fourth, TZD-mediated improvements in insulin action were diminished in ob/ob mice lacking adiponectin [105]. Collectively, these findings show that TZD-induced improvements in insulin sensitivity and glucose tolerance are mediated in an adiponectin-dependent manner.

In many studies however, TZD increased plasma adiponectin in absence of increased mRNA, suggesting that post-translational effects are also involved [125,126]. In this context, TZD has been shown to activate the transcription of the genes encoding DsbA-L and Erp44, two ER protein involved in the assembly of adiponectin oligomers. Both PPARγ agonists and PPARγ overexpression increased mRNA expression of DsbA-

L and Erp44 in HEK293 cells and 3T3-L1 adipocytes, whereas silencing PPARγ with genetic or pharmacological approaches led to the opposite effect [125,126]. Moreover, TZD treatment of HEK293 cells overexpressing PPARγ led to increased production of HMW adiponectin, but not MMW and LMW adiponectin, suggesting that this effect was through increased expression of ER-associated proteins [125,126].

VI. PHYSIOLOGICAL STATE; PREGNANCY TO LACTATION

IR is a naturally occurring maternal adaptation of pregnancy that ensures adequate glucose availability to the developing fetus [127]. A number of studies have focused on the possibility that reduced adiponectin could contribute to the onset of IR during pregnancy. Variation in plasma adiponectin levels during different stages of pregnancy has been reported in rodents and human subjects. Plasma adiponectin significantly dropped during late pregnancy (LP) in mice [128]. On the other hand, a significant drop in plasma adiponectin starting from the first week of pregnancy and ending during lactation was observed in mice [20]. In women, one group reported a significant drop in plasma adiponectin from the 1st to the 3rd trimester of pregnancy [129]. Plasma adiponectin levels during lactation were comparable to 3rd trimester levels in women [129]. Mazaki-Tovi et al, however, reported that plasma adiponectin was significantly lower in lactation relative to pregnancy in women [130]. On the other hand, other studies found no significant changes in plasma adiponectin during human pregnancy [131].

GDM is a disorder associated with IR during human pregnancy. A relationship was identified between low plasma adiponectin levels and increased risk of GDM [132,133]. A number of studies observed decreased plasma adiponectin in pregnant women with GDM when compared to pregnant women without GDM [132–138]. Furthermore, plasma adiponectin decreased in all three trimesters of pregnancy as well as at parturition in human subjects with GDM [132,133,135,138]. In agreement with these results, decreased adiponectin was observed at parturition in women with GDM relative to healthy controls [136].

VII. THE TRANSITION PERIOD IN DAIRY COWS AS AN INSULIN-RESISTANT STATE

Metabolic challenges during the transition period

The productivity and profitability of the modern dairy cow is largely dependent on adaptations to the metabolic challenges associated with the transition from LP to early lactation (EL). These adaptations have been studied during the transition period extending from 3 weeks before to 3 weeks after parturition [139]. This period is dominated by parturition and the substantial increase in nutritional demands associated with milk synthesis [139]. As the dominant precursor for lactose synthesis, glucose demand by the lactating mammary gland requires up to 80% of whole body glucose supply during EL [80]. Within a few days of parturition, total energy requirements increase by 4-fold [127,140]. Voluntary feed intake fails to increase sufficiently to cover these additional needs of the mammary gland during EL, leading to a state of negative energy balance (NEB) [140]. Tight

regulation of the mobilization of endogenous lipid reserves is critical for a successful transition from LP to EL in the dairy cow. When excessive, dairy cows are more susceptible to health disorders, including ketosis, fatty liver, displaced abomasum, retained placenta, and ultimately decreased milk yield [141–144]. Overfeeding during the dry period and/or excessive fatness at parturition can lead to excessive mobilization of lipid reserves and an exaggerated increase in plasma non-esterified fatty acid (NEFA) levels [145–148].

IR as an adaptive and maladaptive mechanism

Mobilization of endogenous reserves is regulated through changes in plasma hormones and/or the ability of tissues to respond to these hormones [149]. For example, increased adipose tissue responsiveness to catecholamines leads to increased lipolysis [150]. Induction of an IR state in EL attenuates insulin action (i.e. stimulation of lipogenesis, inhibition of lipolysis, and inhibition of proteolysis) [151]. As a result, FFA and glycerol are mobilized and provide additional precursors for adenosine triphosphate (ATP) generation (FFA) and hepatic gluconeogenesis (glycerol) [80,152]. IR also favors glucose uptake by the mammary gland, because its uptake is insulin-independent [153]. In addition, hypoinsulinemia in EL leads to decreased insulin-mediated glucose uptake in skeletal muscle and adipose tissue [80,152].

Several factors have been shown to promote IR in the periparturient period. Increased plasma NEFA, as a consequence of excessive fat mobilization, has been linked to IR in dairy cows. Increased plasma NEFA and decreased insulin-stimulated glucose

uptake were observed in non-pregnant, non-lactating dairy cows fasted for 4 days [154]. In agreement, non-pregnant, non-lactating dairy cows infused with triglyceride exhibited increased plasma NEFA and decreased insulin action [155].

Inflammation has been associated with IR [156]. Inflammatory cytokines such as TNF-α are known to cause impaired insulin action in cattle [157]. Whether variation in immune responses during the transition period promote IR in the periparturient period is not completely understood [158]. Increased inflammation has been linked to IR in EL [159].

GH has been known to promote IR in dairy cows [160]. It does this by suppressing insulin-stimulated glucose uptake in adipose tissue and skeletal muscle and by decreasing the ability of insulin to inhibit hepatic gluconeogenesis [127,160]. The antagonistic effects of GH on insulin action, however, have not been demonstrated during the transition period in dairy cows [161,162].

Additional factors are likely involved in modulating insulin action in the transition dairy cow. Adiponectin is another hormone shown to modulate the effects of insulin in other species. It's regulation and actions in transition dairy cows, however, has not been studied extensively.

VIII. ADIPONECTIN DURING THE TRANSITION PERIOD IN DAIRY COWS

Investigation of the adiponectin system in dairy cows has been primarily focused on the transition period.

Reports of plasma adiponectin regulation during the transition period have been relatively consistent. Plasma adiponectin varied, with highest levels during LP, a

maximal reduction around parturition, and a gradual rise to LP levels during the first few weeks of EL [17,163–167]. Furthermore, adiponectin exists nearly exclusively as the HMW isoform in dairy cows [17]. Inconsistent evidence currently exists on whether adiponectin mRNA is regulated during the transition period. A significant drop in adiponectin mRNA levels from LP to EL was found in one transition cow study [168], whereas other studies found no such effect [17,169,170]. In particular, Saremi et al reported that adiponectin mRNA did not change over the first few weeks of lactation when measured in subcutaneous (tail-head, withers, or sternum) or visceral (omental, mesenteric, and retroperitoneal) adipose tissue in primiparous dairy cows [170]. On the other hand, adiponectin protein abundance in these cows was increased over the first few weeks of lactation in both subcutaneous and visceral (retroperitoneal, mesenterial, and omental) adipose tissue [163,164].

Collectively, these studies illustrate effects of the transition period on adiponectin mRNA and protein, but do not specifically address the mechanism accounting for these changes. This dissertation will therefore aim to identify potential potent factors involved in the production and secretion of adiponectin in the modern dairy cow.

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CHAPTER 3

REDUCED PLASMA ADIPONECTIN IN TRANSITION DAIRY COWS IS NOT CAUSED BY CHANGING PLASMA LEPTIN, INSULIN, GROWTH HORMONE, OR NEFA[1]

[1]Krumm C.S., Giesy S.L., Caixeta L.S., Butler W.R., Sauerwein H., Kim J.W., and Boisclair Y.R. (2016) Reduced plasma adiponectin in transition dairy cows is not caused by changing plasma leptin, insulin, growth hormone, or NEFA. *In preparation*.

INTRODUCTION

Adiponectin is a 30-kDa protein hormone synthesized exclusively by white adipose tissue (WAT) [1–3]. Adiponectin circulates as a homomultimer consisting of low molecular weight (LMW), medium molecular weight (MMW), and high molecular weight (HMW) complexes containing 3, 6, or 18 or more adiponectin monomers [4]. Adiponectin signals through two membrane bound receptors known as adiponectin receptor 1 (AdipoR1) and adiponectin receptor 2 (AdipoR2) found in most tissues including liver, muscle, and adipose tissue [5,6]. Many lines of evidence suggest that adiponectin is an insulin sensitizer. First, decreased plasma adiponectin is observed in conditions and diseases characterized by insulin resistance (IR) such as obesity and type 2 diabetes [7,8]. This inverse relation between IR and plasma adiponectin has been found not only in humas, but also in various animal models ranging from non-human primates to dolphins [7,9–12]. Second, administration of recombinant adiponectin improved insulin action in various mouse models of IR (diet-induced obese, lipoatrophic, db/db, and KKA^{y}) [13]. Third, mutations leading to adiponectin loss of function promote IR in both mice and humans [14,15], whereas adiponectin overexpression in IR ob/ob mice is sufficient to normalize their excessive circulating levels of glucose and insulin [16].

We and others have demonstrated that the plasma concentration of adiponectin varies in a quadratic manner in transition dairy cows, with the highest levels in late pregnancy (LP), a nadir on the day of parturition, and a progressive return to LP values over the first few weeks of lactation [17,18]. This adiponectin profile occurs in parallel with the development of IR and the onset of negative energy balance (NEB), and coincides

with rapid reduction in plasma insulin and leptin and reciprocal changes in plasma growth hormone (GH) and non-esterified fatty acids (NEFA) [17,19,20]. There is evidence in rodents and humans that each of these factors regulate adiponectin production [21–23], but whether they contribute to variation in plasma adiponectin in transition dairy cows remains unknown.

The objectives of the present study were first to determine whether the onset of NEB around parturition contributes to reduced plasma adiponectin, and second whether sudden changes in the plasma concentrations of leptin, insulin, GH, or NEFA regulate plasma adiponectin. Our results demonstrate that plasma adiponectin is sensitive to changes in energy balance in the immediate post-periparturient period, but rule out leptin, insulin, GH, or NEFA as factors contributing to reduced plasma adiponectin in transition dairy cows.

MATERIALS & METHODS

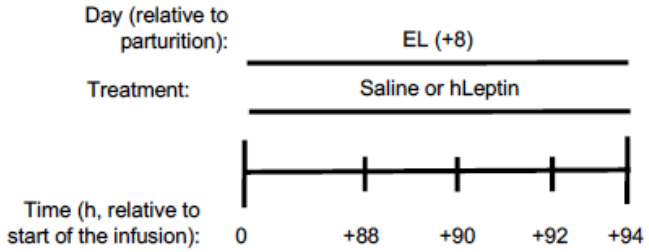
Animals and Design

Plasma samples from five previous experiments designed to identify effects of single metabolic or hormonal factors (energy balance, leptin, insulin, GH, and plasma NEFA) were used [19,24–26]. All experiments were performed in multiparous Holstein cows at Cornell University and were approved by the Cornell Institutional Animal Care and Use Committee. Procedures common to all experiments included housing in individual stalls and blood collection from chronic intrajugular catheters. Blood was processed to obtain plasma following the addition of sodium heparin (15 IU/mL), and centrifugation. Unless otherwise mentioned, cows were fed unlimited amounts of total mixed rations (TMR) using automatic feeders and milked daily at 0600 and 1800 h after parturition. Experiments and associated specific procedures were as follows.

Effect of the periparturient period and leptin

The first study was used to compare assays in their ability to detect changes in plasma adiponectin between LP and early lactation (EL) and to assess the effects of leptin therapy in EL [25]. Ten cows were fed unlimited amounts of TMR formulated for each physiological stage (1.5 Mcal of net energy of lactation (NE_L) and 140 g crude protein (CP) per kg dry matter (DM) in LP and 1.5 Mcal NE_L and 180 g CP per kg DM in EL). Plasma adiponectin was analyzed on 4 blood samples collected at 2 h intervals between 0800 and 1400 h in LP (day -29 ± 2, relative to parturition day 0) and again in EL (day +8). After completing blood sampling on day +8, cows were randomly assigned to

receive a continuous intrajugular infusion of saline (Control) or human leptin (hLeptin, 61 μg/kg body weight/day; Lilly; Indianapolis, IN) for 96 consecutive hours. Plasma adiponectin was measured on 4 samples taken at 2 h intervals between 88 and 94 h of infusion.



Multiparous dairy cows in early lactation (EL) were randomly assigned to a constant intravenous infusion of saline (Control) or human Leptin (hLeptin) for 96 consecutive hours. Plasma samples were collected as shown during the infusion period.

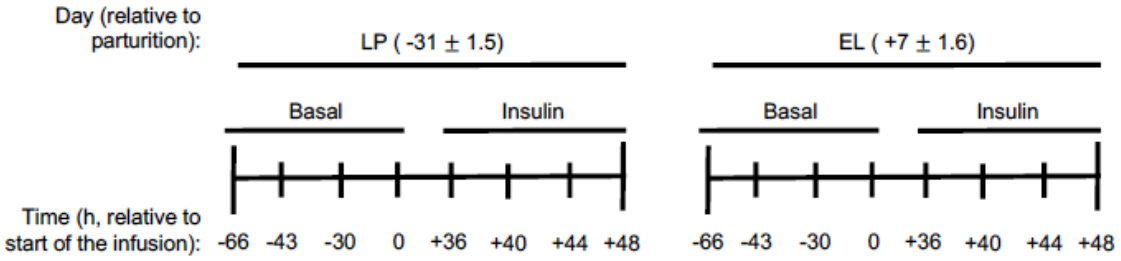
Energy balance after parturition

This study was used to evaluate the effect of positive energy balance in EL on the recovery of plasma adiponectin [19]. Between parturition and day +32 of lactation, cows were offered a low energy TMR (1.52 Mcal NE_L and 189 g CP per kg DM) and milked thrice daily at 0900, 1600, and 2300 h (Lactating, n = 7) or offered a high energy TMR (1.70 Mcal NE_L and 188 g CP per kg DM) and never milked (Non-lactating, n = 7). Lactating cows were fed *ad libitum* during the first week postpartum and thereafter limited to amounts consumed on day +7 (13.6 ± 1.2 kg/day); non-lactating cows were fed *ad libitum* at all times. A body condition score (Thin = 1, Fat = 5) was assigned to each cow on week 1 and 4 by two independent individuals using the system developed by

Wildman et al [27]. Plasma variables including adiponectin were analyzed on 4 blood samples collected every other day between day +5 and +11 and again between day +26 and +32.

Effect of Insulin

This study was used to assess the effect of chronic hyperinsulinemia in LP and in EL on plasma adiponectin [24]. Six cows were fed unlimited amounts of appropriate TMR during LP (1.56 Mcal NE_L and 140 g CP per kg DM) and EL (1.58 Mcal NE_L and 198 g CP per kg DM). They were subjected to the hyperinsulinemic-euglycemic clamp procedure starting on day -31 ±1.5 of LP and day +7 ± 1.6 of EL. The clamp procedure involved a 66 h period of basal blood sampling followed by infusion of bovine insulin at the rate of 1 μg•kg body $weight^{-1}$•h^{-1} for 96 h in LP and 48 h in EL. During insulin infusion, plasma glucose levels were monitored every hour and maintained to the concentration of blood glucose observed during the basal period by varying the rate of infusion of glucose solution (50% wt/vol dextrose solution) [24]. Plasma adiponectin was determined on samples collected during the basal period (-66, -43, -30, and 0 h, relative to initiation of insulin infusion) and on 4 plasma samples collected at 4 h intervals between 36 and 48 h and 84 and 96 h of hyperinsulinemia during LP, and between 36 and 48 h of hyperinsulinemia during EL.

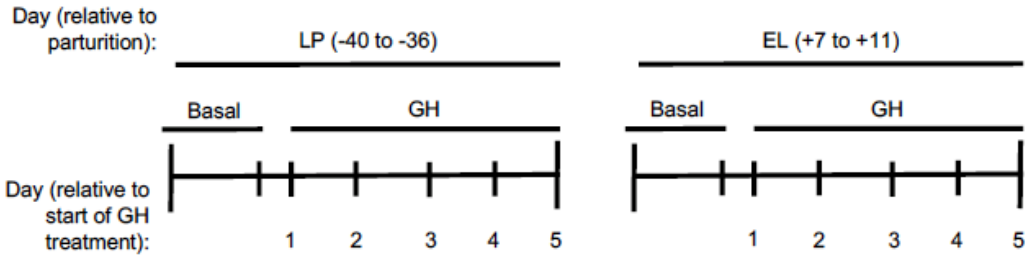


Multiparous dairy cows during late pregnancy (LP) and early lactation (EL) were studied under basal condition (Basal) and during euglycemic-hyperinsulinemia (Insulin). Plasma samples were collected as shown during the basal period and during euglycemic-hyperinsulinemia.

Effect of GH

This study was used to assess the effect of chronic GH treatment in LP and EL on plasma adiponectin [26]. Fourteen cows were offered unlimited amounts of TMR appropriate for each physiological stage (1.52 Mcal of NE_L and 142 g of CP per kg DM in LP and 1.72 Mcal of NE_L and 179 g of CP per kg DM in EL) over a 5-day period (n = 7 per physiological stage). They were studied either in LP (day -40 to -36, relative to parturition on day 0) or EL (day +7 to +11). On the first day of each period, basal blood samples were collected hourly over a 3 h period, followed by biopsy of adipose tissue from the tail-head subcutaneous depot. Immediately after, cows received an intrajugular bolus of GH (20 μg rbST per kg BW; Monsanto Co; St. Louis, MO) and a second adipose tissue biopsy was taken 15 min later. GH treatment was continued over the next 4 days via intramuscular (IM) injections of 40 mg/day of rbST. The rbST dose and length of treatment were based on previous studies that showed effective GH bioactivity in dairy cows during LP and EL [28,29]. Blood samples were collected at hourly intervals between 1000 and 1300 h on the last day of treatment. Blood samples collected during the basal

sampling and on day 5 of GH treatment were analyzed for plasma insulin-like growth factor–I (IGF-I) and adiponectin.

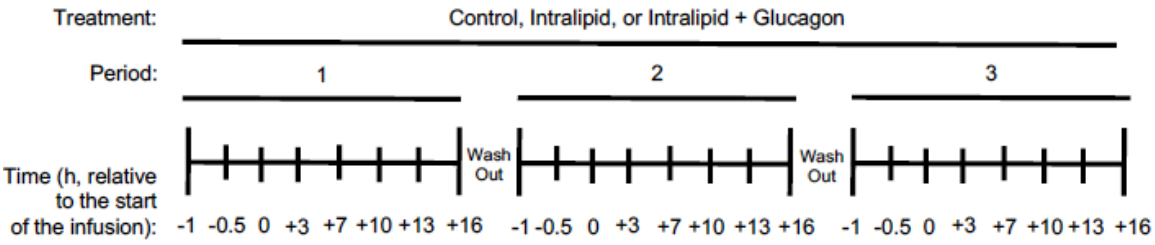


Multiparous dairy cows were studied in late pregnancy (LP) and early lactation (EL) over a 5-day period. On the first day of the study period, basal blood samples were collected hourly over a 3 h period. Adipose tissue was then collected before and 15 min after an intrajugular GH bolus (20 µg rbST/kg BW). On days 2 to 5, cows received daily injections of GH (40 mg rbST/day). Plasma samples collected during the basal period (Basal) and on day 5 of GH treatment (GH).

Effect of NEFA

This study was used to assess the effects of increased plasma NEFA on plasma adiponectin. Six non-pregnant, non-lactating dairy cows were fed a single TMR (1.54 Mcal NE_L and 143 g CP per kg DM) in amounts covering 120% of energy requirements (NRC 2001) throughout the entire experiment. They were randomly assigned to two – 3 x 3 latin squares with experimental periods of 17 h separated by 3-day intervals. Each experimental period included basal blood sampling over 1 h followed by a 16 h period of treatment. Treatments consisted of various combinations of subcutaneous (SC) injections of saline or bovine glucagon (Eli Lilly; Indianapolis, IN) and intravenous (IV) infusion of saline or 20% intralipid solution (Frasenius, Kabi; Deeriled, IL). These treatments were: 1) IV infusion and SC injections of saline (Control); 2) IV infusion of intralipid and SC injection of saline (intralipid); 3) IV infusion of intralipid and (SC) injection of glucagon

(intralipid + glucagon). Intralipid was infused at the rate of 100 mL/hr for 16 consecutive hours and glucagon was administered at the dose of 5 mg at 0 and 8 h relative to the start of treatment. The glucagon dose and time between injections was chosen based on a previous study that maintained elevated glucagon concentrations over a 14-day experimental period in dairy cows [30]. Blood samples obtained during the basal period (-1 and -0.5 h relative to treatment at 0 h) and during treatment (0, +3, +7, +10, +13, and +16 h) were analyzed for plasma NEFA and adiponectin.



Non-pregnant, non-lactating dairy cows were treated with combinations of intravenous (IV) infusion of saline or intralipid 20% and subcutaneous (SC) injections of saline or glucagon. Treatments were IV infusion and SC injections of saline (Control), IV infusion of intralipid and SC injections of saline (intralipid), or IV infusion of intralipid and SC injection of glucagon (intralipid + glucagon). Treatment periods were separated by 3-day wash out periods. Plasma samples were collected as shown during each treatment period.

Analysis of metabolites and hormones

Plasma NEFA were measured by the acyl-CoA synthetase/oxidase method described previously [31]. Plasma insulin and IGF-I were measured with double-antibody RIA as previously described [31,32]. Plasma leptin was measured with a double-antibody RIA capable of detecting only bovine leptin [19] or hLeptin [25] (Linco Research, Inc.; St. Charles, MI). Effective leptin concentration in hLeptin infused cows was calculated as the sum of bovine leptin and human leptin corrected for its ability to activate the bovine

leptin receptor [25]. Inter-assay and intra-assay coefficients of variation for these assays averaged less than 6% and 8% respectively.

Plasma adiponectin was measured by an indirect competitive ELISA designed to measure bovine adiponectin [18]. The assay was performed exactly as originally described, except for the following modifications. The calibration curve was prepared by a 4-fold serial dilution of the reference serum and ranged from 0.08 to 20 ng/mL. Other modifications included dilution of samples (1:20,000 instead of 1:80,000), volume of diluted samples and standards inputted in the assay (20 μL instead of 50 μL), and dilution of the primary antibody (1:5,000 instead of 1:2,000). The assay was developed with 100 μL of secondary antibody (donkey anti-rabbit HRP [Jackson ImmunoResearch #711-035-152]; 1:15,000 dilution in assay buffer), and 100 μL substrate solution (SureBlue^{TM} TMB Microwell Peroxidase Substrate; KPL; Gaithersburg, MD). The reaction was stopped by adding 100 uL of TMB stop solution (KPL) followed by reading of the optical density at 450 nm with a microplate reader. Inter-assay and intra-assay coefficients of variation for all assays averaged less than 6.4% and 8% respectively. Plasma adiponectin was also measured in a subset of samples by western immunoblotting exactly as described recently [17]. In brief, plasma samples (1.5 μL of 1:10 dilution for plasma) were electrophoresed under reducing conditions on 13% SDS-PAGE gels and electroblotted onto nitrocellulose membranes (Protran, Schleicher & Schuell Bioscience; Keene, NH). The membranes were incubated with bovine adiponectin antiserum (1:1,000 dilution) and developed with a 1:2,000 dilution of IR Dye 800-nm goat anti-rabbit secondary antibody (LI-COR

Bitoechnology; Lincoln, NE). Signals were quantified with the LI-COR Odyssey infrared imaging system using the 800-nm channel.

Western immunoblotting of adipose tissue extract

Bovine adipose tissue was homogenized in 2 mL of lysis buffer [10 mM Tris, pH7.6, 10 mL/L Triton X-100, 1 mM EGTA, 150 mM NaCl, 1 mM Na_3VO_4, 1 mM Na pyrophosphate, 10 mM NaF, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mg/L aprotinin, and 10 mg/L leupeptin]. Homogenates were clarified by centrifugation (10,000 × g for 20 min at 4°C). Protein concentrations of cellular extracts were determined using a bicinchoninic acid assay (BCA) protein assay kit (Thermo Fisher; Waltham, MA). Fixed amounts (55 μg) of protein extract were separated on 10% polyacrylamide gels and transferred onto nitrocellulose membranes (Protran, Schleicher, and Schuell Bioscience; Dassel, Germany). Membranes were immersed in blocking solution (50 mM Tris, pH 7.4, 200 mM NaCl, 1 mL/L Tween 20, 50 g/L nonfat dried skim milk). Membranes were then immunodecorated overnight at 4°C with a 1:4,000 dilution of antibodies against signal transducer and activator of transcription-5 (Stat5; Cell Signaling; Danvers, MA) or a 1:1,000 dilution of antibodies against tyrosine phosphorylated Stat5 (Cell Signaling). Signals were developed with a 1:5,000 dilution of goat anti-rabbit antibody (Thermo Fisher; Waltham, MA) in 5% non-fat dried skim milk and visualized by chemiluminescence exposure to film (Super Signal West Pico Chemiluminescent Substrate, Thermo Fisher). Signals were quantified by densitometry using the NIH 1.6.3 software (National Institutes of Health; Bethesda, MD).

Statistical Analysis

Data were analyzed by a mixed model using the fit model procedure of JMP Pro 11.0 statistical software (SAS Institute; Cary, NC). Data from the periparturient period study were analyzed by a mixed model accounting for physiological stage (LP or EL) as the fixed effect and cow as the random effect. For the leptin infusion portion of the experiment, data were analyzed with the fixed effect of treatment (Control or hLeptin) and cow as the random effect with data obtained on day 8 as a covariate. For the energy balance study, the mixed model accounted for treatment (Lactating *vs*. Non-lactating), time (week 1 *vs.* 4), and their interaction as the fixed effects and animal as the random effect. For the insulin and GH studies, the mixed model accounted for physiological stage (LP *vs.* EL), treatment (either Basal *vs.* Insulin or GH), and their interaction as the fixed effects and animal as the random effect. For the NEFA study, the mixed model accounted for treatment (Saline, Intralipid, and Intralipid + Glucagon), time (0, 3, 7, 10, 13, and 16 h), and their interaction as the fixed effects and animal as the random effect. Values obtained during the basal period (average of -1 and -0.5 h samples) were used as a covariate. Correlations between plasma adiponectin and other variables were performed using the fit model procedure of JMP. Statistical significance was set at $P < 0.05$.

RESULTS

Comparison of adiponectin assays

In previous work, we reported a 30% reduction in plasma adiponectin between day -35 and +7 relative to parturition on day 0 when measured by western immunoblotting [17]. This technique has limited applicability for the analysis of numerous samples across multiple studies. Accordingly, we used another transition cow study to compare a recently developed bovine adiponectin ELISA to our western immunoblotting assay. Energy indicators in that study varied as expected, with energy balance and plasma NEFA averaging 12.1 Mcal NE_L/d and 108 μM in LP and -15.2 Mcal NE_L/d and 386 μM in EL. When measured by ELISA, plasma adiponectin fell from 51 to 36 μg/mL between day -29 of LP and day 8 of EL ($P < 0.05$). Moreover, both assays gave similar relative reductions in plasma adiponectin between LP and EL (Figure 3.1A). The ELISA assay was adopted for all subsequent analysis.

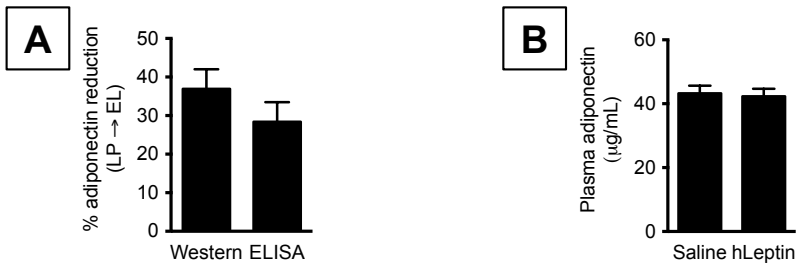


Figure 3.1 Comparison of adiponectin assays and effect of increased plasma leptin in early lactation on plasma adiponectin. Multiparous dairy cows were studied on day -29 ± 2 (relative to parturition on day 0, LP) and again on day +8 of early lactation (EL). **(A)** Plasma samples were collected at 2 h intervals over a 6 h sampling window at both times and analyzed for adiponectin by western immunoblotting (Western) or by a bovine adiponectin ELISA (ELISA). The percent reduction in plasma adiponectin between LP and EL is shown. Each bar represents the mean ± SE of 10 cows. **(B)** Upon completion of the sampling period on day + 8 of EL, dairy cows were randomly assigned to a constant intravenous infusion of saline (Control) or human Leptin (hLeptin) for 96 consecutive hours. Plasma samples were collected between 88 and 94 h of infusion and analyzed for adiponectin concentration with a bovine adiponectin ELISA. Each bar represents the mean ± SE of 5 cows per treatment.

Effect of energy balance immediately after parturition

Next, we asked whether eliminating the energy deficit of EL impacted plasma adiponectin by comparing cows milked thrice daily or never milked. Energy balance and the change in body condition score over the four weeks of the study were -16.8 Mcal NE_L/d and -0.6 units for lactating cows, and +12.1 Mcal NE_L/d and 0.1 unit for non-lactating cows ($P < 0.001$ for both variables). Plasma adiponectin increased by an average of 24% in both groups between week 1 and 4 and was 21% higher across time in non-lactating cows than lactating cows (Figure 3.2A; Treatment and Time, $P < 0.05$ or less). When assessed across treatments, no relation existed between the change in body condition over this period and the change in plasma adiponectin (Figure 3.2B). There were however, modest relations between adiponectin and plasma indicators of energy status, with adiponectin increasing with leptin or insulin, and decreasing with NEFA (Figure 3.2C and results not shown; $P < 0.05$ or less).

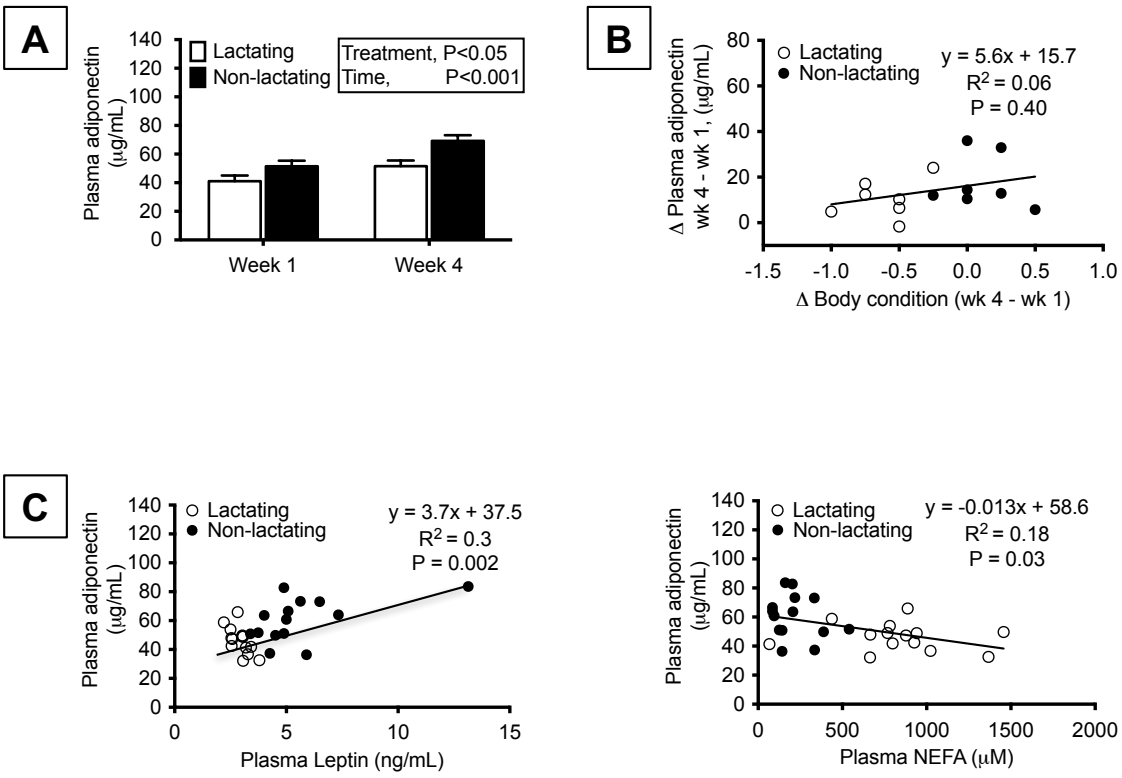


Figure 3.2 Effect of energy balance after parturition on plasma adiponectin. **(A)** After parturition, multiparous dairy cows were milked thrice daily (Lactating) or never milked (Non-lactating). Plasma samples were collected between day +5 and +11 (Week 1) and again between day +26 and +33 (Week 4) and analyzed for adiponectin concentration with a bovine adiponectin ELISA (ELISA). Each bar represents the mean ± SE of 7 cows per treatment. The significant effects of treatment and time are reported. **(B)** Relationship between the change in plasma adiponectin (Δ Plasma adiponectin) and the change in body condition (Δ Body condition) between week 4 and week 1 of the study. **(C)** Relationships between plasma adiponectin and plasma leptin or non-esterified fatty acids (NEFA) over the first 4 weeks following parturition.

Reversal of the insulin and leptin deficits of early lactation

The plasma concentration of leptin and insulin falls over the last few days of pregnancy and reach a nadir after parturition [19,20]. To determine whether a fall in plasma leptin contributes to reduced adiponectin, cows received continuous IV infusions of saline or hLeptin between day 8 and 12 of lactation. After correcting for the bioactivity of hLeptin, the total effective leptin concentration was calculated within range of concentrations found in LP dairy cows [19]. Relative to saline infusion, the hLeptin infusion caused a 3.5-fold increase in the effective plasma leptin concentration (2.3 *vs.* 8 ng/mL, $P < 0.001$) and a 45% increase in plasma T_3 (0.91 *vs.* 1.32 ng/mL, $P < 0.01$), but did not affect energy variables such as feed intake and milk energy output [25]. Plasma adiponectin remained unaffected after over 88 h of hLeptin treatment (Figure 3.1B).

Next, we asked whether insulin had an effect on plasma adiponectin in either LP or EL. Dairy cows underwent hyperinsulinemic-euglycemic clamps for 96 h in LP and for 48 h in EL. Plasma insulin increased within 4 h from 1.8 to 4.0 ng/mL in LP and from 0.7 to 2.5 ng/mL in EL, whereas the plasma glucose concentration remained within 10% of basal concentration at all times. Plasma adiponectin fell between LP and EL (Figure 3.3; Stage, $P < 0.001$), but failed to increase with 48 h of hyperinsulinemia in either LP or EL. Prolonging the period of hyperinsulinemia to 96 h during LP was also without effects on plasma adiponectin (Basal *vs.* 96 h; 54 *vs.* 56 ng/mL).

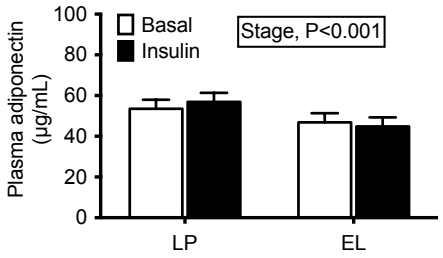


Figure 3.3 Effect of euglycemic-hyperinsulinemia in late pregnancy and early lactation on plasma adiponectin. Multiparous dairy cows during late pregnancy (LP, day -31 ± 1.5, relative to parturition on day 0) and early lactation (EL, day +7 ± 1.6) were studied for 66 h under basal condition (Basal) and for 48 h during euglycemic-hyperinsulinemia (Insulin). Plasma samples collected during the basal period and between 36 and 48 h of euglycemic-hyperinsulinemia were analyzed for adiponectin concentration with a bovine adiponectin ELISA (ELISA). Each bar represents the mean ± SE of 6 cows. The significant effect of physiological stage (Stage) is reported.

Exaggerating the GH increase or mimicking the NEFA increase of early lactation

Plasma GH and NEFA increase in periparturient dairy cows [19,20]. To assess the possible regulatory influence of GH on plasma adiponectin, LP or EL cows were treated with recombinant bST for 5 consecutive days. Plasma IGF-I was lower in EL than LP cows under basal condition (Figure 3.4A, Stage, $P < 0.001$). After 5 days of treatment, plasma IGF-I was increased by GH treatment, but to a lesser extend in EL than LP (Figure 3.4A; Stage × GH, $P < 0.05$). Adipose tissue, on the other hand, remained fully responsive to GH as shown by near identical GH-dependent Stat5 phosphorylation across physiological states (Figure 3.4B). Plasma adiponectin was again reduced in EL relative to LP (Stage, $P < 0.05$), but unaffected by GH in either LP or EL (Figure 3.4C).

Finally, the possibility that plasma NEFA represses plasma adiponectin was evaluated by IV infusion of a lipid emulsion in the absence or presence of glucagon for 16 consecutive hours. Intralipid caused a 6-fold increase in plasma NEFA within 3 h of infusion (Saline *vs.* Intralipid, 102 *vs.* 576 μM; $P < 0.001$) and this increase persisted for the remainder of the infusion; glucagon had no additional effect on plasma NEFA (Intralipid + Glucagon, 613 μM). Plasma adiponectin remained unaffected throughout intralipid infusion irrespective of the presence or absence of glucagon (Figure 3.5).

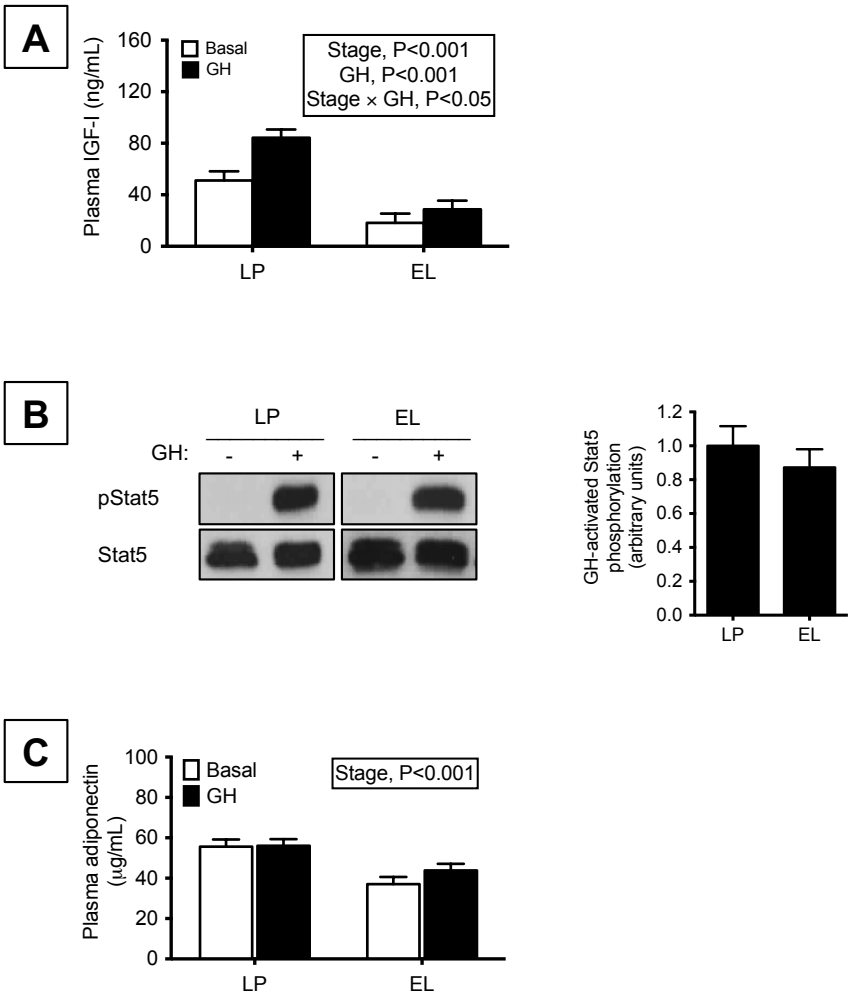


Figure 3.4 Effect of GH in late pregnancy and early lactation on plasma adiponectin. Multiparous dairy cows were studied between days -40 and -36 (relative to parturition on day 0, LP) and between days +7 and +11 (EL) over a 5-day period. On the first day of the study period, basal blood samples were collected hourly over a 3 h period. Adipose tissue was then collected before and 15 min after an intrajugular growth hormone (GH) bolus (20 μg rbST/kg BW). On days 2 to 5, cows received daily injections of GH (40 mg rbST/day). **(A)** Plasma samples collected during the basal period (Basal) and on day 5 of GH treatment (GH) were analyzed for plasma insulin-like growth factor - I (IGF-I). Each bar represents the mean ± SE of 7 cows per treatment and stage. The significant effects of physiological stage (Stage), GH treatment, and their interaction are reported. **(B)** Left: Adipose tissue was obtained on day 1 of the study period immediately before (-) and 15 minutes after an intrajugular bolus of rbST (GH) (+). Total cellular extracts were prepared from tissues and analyzed for tyrosine phosphorylated Stat5 (pStat5) and total Stat5 (Stat5) by western immunoblotting. A representative sample is shown for each stage and treatment. Right: The pStat5 signal was normalized to total Stat5 signal and expressed relative to LP level with each bar representing the mean ± SE of 7 cows. **(C)** Plasma samples collected during the basal period (Basal) and on day 5 of GH treatment (GH) were analyzed for adiponectin concentration with a bovine adiponectin ELISA (ELISA). Each bar represents the mean ± SE of 7 cows per stage. The significant effect of physiological stage (Stage) is reported.

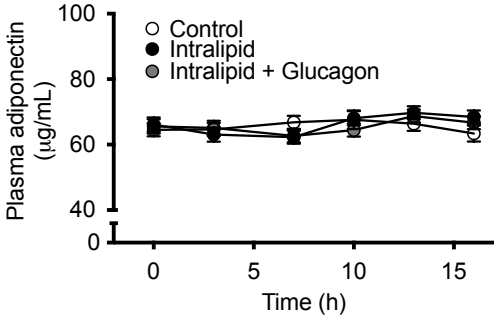


Figure 3.5 Effect of increased NEFA on plasma adiponectin. Non-pregnant, non-lactating dairy cows were treated over 16 h with combinations of intravenous (IV) infusion of saline or intralipid 20% and subcutaneous (SC) injections of saline or glucagon. Treatments were IV infusion and SC injections of saline (Control), IV infusion of intralipid and SC injections of saline (intralipid), or IV infusion of intralipid and SC injection of glucagon (intralipid + glucagon). Plasma samples were collected over the 16 h periods and analyzed for adiponectin concentration with a bovine adiponectin ELISA (ELISA). Each point represents the mean ± SE of 6 cows.

DISCUSSION

IR is a mechanism used by the modern dairy cow to promote glucose partitioning to the mammary gland at the expense of insulin sensitive tissues such as skeletal muscle or adipose tissue [33,34]. Mechanisms regulating IR during EL in transition dairy cows are not well understood. In this context, we and others have shown that plasma adiponectin dropped in parallel with increased IR in periparturient dairy cows [17,18,35]. Adiponectin is an insulin sensitizing hormone and therefore its reduction could contribute to the IR of periparturient dairy cows. Virtually no information is available on mechanisms regulating plasma adiponectin in periparturient dairy cattle, prompting us to examine possible regulatory effects of metabolic and hormonal factors changing in a dynamic manner during this period.

The profile of plasma adiponectin in periparturient dairy cows consists of two reciprocal patterns; a falling phase over the last 2 – 3 weeks of pregnancy followed by a rising phase after parturition [17,35]. The placenta has been suggested to produce adiponectin in rats and humans [36]. Hence, an obvious question is whether the placenta could account for the falling phase through reduced adiponectin production in LP and its expulsion at parturition. More recent studies performed on mid and late gestation human placenta, primary human cytotrophoblasts, and placental choriocarcinoma cell lines (Jeg-3, JAR, and BeWo) have ruled out placental production [36–40]. Moreover, adiponectin expression was less than 0.5% of maternal adipose tissue expression at both day 50 and 135 of gestation in the sheep (Boisclair et al unpublished data). Given the close evolutionary relation between sheep and cattle, it is unlikely that the bovine placenta is a

source of adiponectin and a contributing factor to its falling plasma concentration in late pregnant dairy cows.

In rodents and humans, loss of adiposity and NEB increase plasma adiponectin. The relation between energy balance and adiposity and plasma adiponectin has been investigated in non-lactating, non-pregnant dairy cows and in cows from the last month of gestation through the first 12 weeks of lactation [35,41]. The contribution of these factors to changes in plasma adiponectin around parturition remains unclear. Accordingly, we used dairy cows dried off immediately after parturition to test the possibility that these factors contribute to the recovery of plasma adiponectin after parturition. In particular, the non-lactating dairy cows completely avoided energy insufficiency after parturition and did not experience a loss of adiposity over the next 4 weeks. Against expectations, non-lactating dairy cows not only maintained higher plasma adiponectin than lactating dairy cows, but also recovered plasma adiponectin at similar rates as lactating dairy cows. Measurable amounts of adiponectin are secreted in milk [35], raising the possibility that the higher plasma adiponectin of non-lactating dairy cows relates to absence of adiponectin excretion in milk. The fraction of the steady state plasma adiponectin pool appearing daily in milk is 5% or less [35]. Adiponectin has a relatively short half-life (i.e. 90 min) [42], implying that this fraction would be even less if calculated on the basis of daily adiponectin production. Therefore milk adiponectin secretion [35] cannot account for the 21% difference in plasma adiponectin between lactating and non-lactating dairy cows. Finally, the positive effect of energy balance in EL on plasma adiponectin is in contrast to the lack of any response when dairy cows experience severe energy insufficiency in mid-

lactation [35]. Overall, our data suggest that the reduction in plasma adiponectin after parturition is driven in part by NEB and that adiponectin recovery after parturition occurs independently of loss of adiposity.

The periparturient period is characterized by rapid reductions in plasma leptin and insulin, raising the possibility that either one or both of these hormones are positive regulators of adiponectin production. In apparent support for this model, plasma adiponectin increases after leptin therapy of leptin-deficient humans and mice [21,43]. However, we failed to detect increased plasma adiponectin in EL dairy cows, despite a 3.5-fold increase in plasma leptin. It is also important to mention that the positive effect of leptin therapy seen in leptin-deficient humans and mice is likely a consequence of substantial loss of adiposity rather than a direct leptin effect. In similar fashion to the lack of leptin effects, increasing plasma insulin in either LP or EL was also without any effects on plasma adiponectin. This result is not only inconsistent with a positive role for insulin in the cow, but also discordant with results in humans and rodents showing that loss of function mutations of the insulin receptor increase plasma adiponectin, whereas euglycemic-hyperinsulinemic clamps uniformly reduce plasma adiponectin [23,44–46]. Interestingly, effects of insulin in humans are not seen on HMW adiponectin [23]. Accordingly, it is possible that the lack of insulin effect in dairy cows relate to circulation of adiponectin predominantly in the HMW form [17]. Finally, the periparturient period in dairy cows is associated with a reduction in the plasma concentration of thyroid hormones [25,47]. Hypothyroidism in human patients is associated with reduced plasma adiponectin, whereas hyperthyroidism in both humans and rodents has the opposite

effects [48,49]. The hypothyroid state of EL is also unlikely to account for reduced adiponectin, because it was partially corrected by leptin in the leptin infusion experiment [25]. A similar conclusion applies to the IGF-I deficit of EL, which was also completely reversed after 48 h of euglycemic-hyperinsulinemia in EL [20].

Plasma GH and NEFA have been implicated as drivers of IR and both are increased in EL dairy cows [20,34]. In the case of both mice and humans, existing data suggest the possibility that adiponectin mediates the effect of GH on IR. Thus, GH receptor knockout mice have elevated plasma adiponectin and insulin sensitivity, despite increased fatness [50,51]. Similarly, increased insulin sensitivity and plasma adiponectin coexist in Laron dwarf patients, but both are reduced in acromegaly patients [52,53]. Therefore, we tested the possibility that GH inhibits adiponectin production by treating LP and EL dairy cows with exogenous GH. As previously shown [19,20], plasma IGF-I was lower in EL than LP and GH increased plasma IGF-I to a larger extent in LP than EL . The attenuated increase in plasma IGF-I reflects reduced hepatic GH responsiveness as a result of loss of GH receptor expression in EL [54]. On the other hand, adipose tissue remains GH-responsive in both LP and EL as demonstrated by equally robust GH-dependent Stat5 activation across physiological states. Despite this, GH treatment for 5 consecutive days had no impact on plasma adiponectin irrespective of physiological state.

Plasma NEFA also contributes to IR [55,56], particularly when their levels are excessively elevated in EL as a consequence of over nutrition and/or obesity in LP [57,58]. Plasma NEFA promotes IR by promoting synthesis of lipid intermediates (i.e. ceramide species, diacylglycerol, etc.) and by activating the production of pro-inflammatory

cytokines such as tumor necrosis factor α (TNFα) [59–61]. Interestingly, intralipid, ceramide, and TNFα have been shown to repress adiponectin production both *in vivo* and *in vitro* [62–64]. Despite these data implicating NEFA as a repressor of adiponectin production, a 6-fold increase in plasma NEFA had no effect on plasma adiponectin.

In summary, we show that changes in energy balance during the onset of lactation mediate plasma adiponectin. Rapid and sudden changes in plasma levels of leptin insulin, GH, and NEFA occur in parallel to this effect, and were shown to regulate plasma adiponectin in rodents or humans [21–23]. However, our results suggest that these factors have no effect on plasma adiponectin in periparturient dairy cows. Future work in dairy cows could aim to explore what regulates the variation in plasma adiponectin in the periparturient period.

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CHAPTER 4

IDENTIFYING FACTORS REGULATING ADIPONECTIN SECRETION IN PRIMARY BOVINE ADIPOCYTES[2]

[2]Krumm C.S., Giesy S.L., Orndorff C.L., and Boisclair Y.R. (2016) Identifying factors regulating adiponectin secretion in primary bovine adipocytes. *In preparation*.

INTRODUCTION

Early lactating (EL) dairy cows suffer from major deficits in virtually all major class of organic nutrients, including the lactose precursor glucose [1]. Dairy cows cope with this nutritional insufficiency by calling on adaptations such as insulin resistance (IR). IR creates a metabolic environment promoting lactose synthesis, because mammary glucose uptake occurs in an insulin-independent manner [2]. Mechanisms leading to IR in periparturient dairy cows are not completely understood. Increased growth hormone (GH) secretion in EL likely plays a role given its ability to suppress insulin-stimulated glucose uptake in adipose tissue and skeletal muscle [2–4]. The increased circulating non-esterified fatty acid (NEFA) of EL may also be involved through induction of signals interfering with insulin signaling such as ceramide and the cytokine tumor necrosis factor α (TNFα) [5–9]. One hormone that has not been extensively studied in the context of IR of EL is adiponectin. We and others have shown that the plasma concentration of adiponectin drops in parallel with the rise of IR in transition dairy cows [10–12]. This observation may be relevant to the IR of EL because work in other species have implicated adiponectin as an insulin-sensitizing hormone [13–15].

Adiponectin is a 30-kDa protein secreted exclusively by white adipose tissue (WAT) [16–18]. Unlike most other adipokines, adiponectin circulates as low molecular weight (LMW), medium molecular weight (MMW), and high molecular weight (HMW) complexes containing 3, 6, or 18 or more adiponectin monomers [19,20]. Adiponectin oligomer assembly occurs within the endoplasmic reticulum (ER) and involves a subset of ER chaperone proteins [i.e. glucose-regulated protein, 78 kDa (Grp78), endoplasmic

reticulum protein 44 (Erp44), endoplasmic reticulum oxidoreductase α (ERO1-Lα), protein disulfide isomerase associated 6 (Pdia6), disulfide bond A oxidoreductase-like protein (DsbA-L), and gamma adaptin ear containing, ARF binding protein 1 (Gga1)] [21,22]. Interestingly, the periparturient reduction in plasma adiponectin in dairy cows occurred in the absence of any changes in adiponectin mRNA, but was associated with reduced expression of Grp78, Erp44, and Pdia6 in WAT [10]. These chaperones are regulated in part through the master regulator of ER homeostasis x-box binding protein 1 (Xbp1) [23,24].

These data led us to hypothesize that Xbp1 expression is reduced in WAT in EL, leading to reduced ER chaperone expression and adiponectin secretion. Accordingly, we first determined whether Xbp1 is regulated in adipose tissue during the transition from late pregnancy (LP) to EL. Second, we asked whether Xbp1 overexpression in adipocytes increases ER chaperone expression and adiponectin secretion. Finally, we examined the possibility that effects of other factors on adiponectin secretion are explained in part by regulation of ER chaperones.

MATERIALS & METHODS

Source of adipose tissue

All experiments were performed on multiparous Holstein cows and were approved by the Cornell Institutional Animal Care and Use Committee. A previously described cow study was used to measure changes in Xbp1 gene expression in WAT during the transition period [25]. WAT was obtained from the tail head subcutaneous depot of ten cows in LP (day -29 ± 2, relative to parturition on day 0) and again in EL (day +8). WAT samples were frozen at -80°C until used for total RNA isolation.

WAT used for isolation of bovine stromovascular (SVC) cells were obtained from dairy cows at the start of the dry off period approximately 7 weeks before expected parturition. All cows were of parity ≥ 1 and were in good body condition (body condition score ≥ 3.0). Immediately after biopsy, WAT was immersed in growth media [DMEM/F12 (1:1 vol; Thermo Fisher; Waltham, MA) supplemented with 10% fetalgro (Rocky Mountain Biologicals, Inc.; Missoula, MT), penicillin-streptomycin (50 U/mL; Thermo Fisher; Waltham, MA), GlutaMAX (2 mM; Thermo Fisher; Waltham, MA), and amphotericin B (1 μg/mL; Thermo Fisher; Waltham, MA)] and transported to the laboratory.

Isolation, differentiation, and study of bovine stromovascular cells (SVC)

Bovine SVCs were isolated and differentiated into adipocytes as previously described [26]. In brief, WAT was rinsed in a solution of 20% betadine and growth media, followed by a single wash with fresh growth media. Pieces of WAT (~ 1 mm^3) were

placed onto 100 mm dishes and kept in place with a coverslip attached to the growth surface of the cell dish with vacuum grease. After 10 days, bovine SVCs growing out of the explants were recovered by trypsinization and seeded into 100 mm dishes. They were reseeded when 90% confluent into 60 mm dishes pre-coated with gelatin (Sigma Aldrich; St. Louis, MI). SVC were incubated over the first 48 h with adipocyte differentiation media [DMEM/F12 (1:1 vol) + insulin (10 μg/mL; Sigma Aldrich; St. Louis, MI), rosiglitazone (1 μM; Sigma Aldrich; St. Louis, MI), intralipid (2%; Sigma Aldrich; St. Louis, MI), penicillin-streptomycin (50 U/mL; Thermo Fisher; Waltham, MA), GlutaMAX (2 mM; Thermo Fisher; Waltham, MA), and amphotericin B (1 μg/mL; Thermo Fisher; Waltham, MA)] supplemented with dexamethasone (0.25 μM; Sigma Aldrich; St. Louis, MI) and 3-isobutyl-1-methylxanthine (IBMX; 0.5 mM; Sigma Aldrich; St. Louis, MI), and adipocyte differentiation media alone thereafter. WAT explants and SVC were kept at 37°C and 5% CO_2 at all times, with media changed every 2 days.

For experiments involving adenoviruses, SVC were infected at 1700 h on day 6 of differentiation followed by media change 15 h later. Media and total RNA were collected as indicated in figure legends. In other studies, differentiated SVC were incubated between day 6 and 8 with either PBS (Control), 100 ng/mL bovine GH (Protiva; St. Louis, MI), or 10 ng/mL of human TNFα (R&D systems; Minneapolis, MN). Media and total RNA were collected on day 8 of differentiation. Oil-red O staining was used to confirm lipid accumulation in differentiated SVC. In brief, cells were washed with PBS and fixed with 10% formalin for 1 hour at room temperature. Following two washes with

60% isopropanol, bovine adipocytes were stained with Oil-red O (Sigma Aldrich; St. Louis, MO) for 10 min at room temperature and analyzed by Axiovert 40 Microscope bright light microscopy (Carl Zeiss; Oberkochen, Germany) at 10X magnification.

Construction of an adenovirus expressing bovine Xbp1$_S$

Bovine spliced variant (Xbp1$_S$) cDNA was generated by reverse transcription of total mRNA from bovine WAT followed by high fidelity amplification using previously described procedures [27]. Primers used to amplify bovine Xbp1$_S$ were 5'-CTGGTACCCGCCACCATGGTGGT GGTTGCACCC-3' and 5'-AAGCGGCCGCTTAGACACTAATCAGCTGG-3'. The resulting cDNA was subcloned into the adenoviral shuttle vector pAd-CMV and shown by sequencing to correspond exactly to bovine Xbp1$_S$ (Ref Seq NM_001034727.3). The bovine Xbp1$_S$ adenovirus was generated using the AdEasy system as previously described [28]. In brief, the plasmid was linearized using the restriction enzyme PmeI and transformed into AdEasier bacteria containing the adenoviral backbone plasmid pAdEasy-1. As a result, the bovine Xbp1$_S$ cDNA was recombined into the pAdEasy-1, giving pAdEasy-Xbp1$_S$. Adenovirus particles were generated by transfecting human embryonic kidney 293a (HEK293a) cells with Pac-I-linearized pAdEasy-Xbp1$_S$ using Lipofectamine 2000 (Thermo Fisher; Waltham, MA). The virus was then amplified via 5 rounds of HEK293a infection. Adenoviruses expressing mCherry (Cherry) under the control of the CMV promoter (Cherry; Vector Biolabs; Malvern, PA) were amplified in parallel using the exact same procedure.

Western blot analysis

Media were pooled from four individual 60 mm dishes and concentrated to 100 μL using centrifugal units with a molecular weight cutoff of 10 kDa (Amicon ultra-4; Merck; Darmstadt, Germany). Total cellular extracts were prepared from bovine adipocytes by lysis with radioimmunoprecipitation assay buffer [10 mM tris-HCl (pH 7.4), 150 mM NaCl, 1% nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 mM NaF, 0.25% sodium deoxycholate, and 10% glycerol, supplemented with protease and phosphatase inhibitors (Halt TM phosphatase inhibitor mixture EDTA-free; Thermo Fisher; Waltham, MA)]. Protein concentrations of cellular extracts were determined with the BCA assay (Thermo Fisher; Waltham, MA). Concentrated media (18 μL) or fixed amounts of cellular extract (20 – 40 μg) were separated on 13 % polyacrylamide gels and transferred onto nitrocellulose membranes (Protran, Schleicher, and Schuell Bioscience; Dassel, Germany). Membranes were blocked in 5% non-fat dried skim milk with tween-20 (0.05 M Tris-HCl (pH 7.4), 0.2 M NaCl and 0.1% Tween-20). Membranes were then immunodecorated with a mouse primary antibody against human adiponectin (A gift from Dr. Tohru Funahashi, Osaka University; Suita, Japan) and a rabbit primary antibody against human β-actin (Cell Signaling; Danvers, MA). Primary antibodies were diluted at 1:1,000 (β-actin) or 1:20,000 (adiponectin) in blocking solution. Signals were developed with 1:20,000 dilution of either IR Dye 800 goat anti-rabbit or goat anti-mouse secondary antibody (LI-COR Biotechnology, Lincoln, NE) and visualized with the LI-COR Odyssey infrared imaging system.

RNA extraction and analysis of gene expression

WAT biopsies and bovine adipocytes were lysed with Qiazol (Qiagen; Hilden, Germany) followed by total RNA purification using RNeasy Mini columns and on-column RNase-free DNase treatment (Qiagen; Hilden, Germany). Reverse transcription reactions were performed with 1 μg of mRNA in a total 20 μL volume with the high-capacity cDNA reverse transcription kit and RNase inhibitor (Applied Biosystems; Foster City, CA). Gene expression was analyzed with quantitative real-time PCR assays using Power SYBR Green Mix (Applied Biosystems; Foster City, CA). Real-time PCR assays were performed in duplicate with a total 25 μL reaction volume containing 500 nM concentration of each primer and reverse transcribed mRNA (25 ng except 2.5 ng for the internal standard gene 18S). The sequences of all primers used are given in Appendix I. mRNA data were analyzed using a relative standard curve based on serial dilution of pooled cDNA from bovine adipocytes. Unknown sample expression levels were calculated from the standard curve and adjusted to the geometric mean expression of β-Actin and 18S.

Statistical analysis

Data were analyzed by a mixed model using the fit model procedure of JMP Pro 11.0 statistical software (SAS Institute, Cary, NC). For $Xbp1_S$ mRNA abundance during the transition period, the model accounted for physiological stage (LP *vs.* EL) as the fixed effect and animal as the random effect. For the time course of differentiation, the model accounted for time (0, 4, 6, 8, and 10 days) as the fixed effect and cell dish as the random effect. If significant, the effect of time was partitioned into linear, quadratic, and cubic

contrasts. For the effect of $Xbp1_S$ overexpression, the model accounted for adenovirus (Cherry *vs.* $Xbp1_S$) as the fixed effect and cell dish as the random effect. For the intralipid experiment, the model accounted for treatment (- *vs.* + intralipid) as the fixed effect and cell dish as the random effect. For the TNFα and GH experiments, the model accounted for treatment (either Control *vs.* TNFα or GH) as the fixed effect and cell dish as the random effect. The level of statistical significance was set at $P < 0.05$.

RESULTS

Reduced Xbp1 expression in adipose tissue of transition dairy cows

In previous work, we showed that plasma adiponectin dropped by 40% between LP and EL [10]. This reduction occurred in absence of changes in adiponectin mRNA in adipose tissue, but was associated with attenuated expression of the Xbp1-dependent ER chaperones Grp78, Erp44, and Pdia6 [10]. Accordingly, we asked whether Xbp1 expression is regulated in WAT of transition dairy cows. WAT expression of both total Xbp1 ($Xbp1_T$) and its active spliced variant Xbp1 ($Xbp1_S$) was reduced by 42 and 50% respectively between LP and EL (Figure 4.1; $P < 0.001$). These data raise the possibility that lower expression of Xbp1 expression in EL contributes to reduced plasma adiponectin.

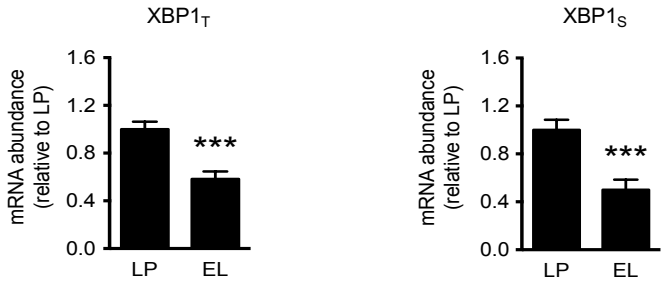

Figure 4.1 Effect of the transition period on Xbp1 expression in adipose tissue. Adipose tissue was collected from multiparous cows on day -29 ± 2 (relative to parturition on day 0, LP) and again on day +8 of lactation (EL). **(A)** Total mRNA was isolated and analyzed by quantitative real-time PCR for expression of total x-box binding protein 1 ($Xbp1_T$) and spliced variant Xbp1 ($Xbp1_S$). The expression of each gene is relative to LP. Each bar represents the mean ± SE of mRNA abundance for the indicated gene (n = 10). ***, $P < 0.001$.

Capacity of primary bovine adipocytes to secrete adiponectin

As a first step to determine whether $Xbp1_S$ can regulate adiponectin production, we asked whether SVC differentiated into adiponectin-secreting adipocytes. Bovine SVC were grown out of WAT explants and upon confluence, incubated in differentiation media over 10 days. Lipid accumulation was not detected by Oil-red O on day 0, but became obvious by day 6 of differentiation and peaked in abundance and intensity by day 8 (Figure 4.2A). Acquisition of the adipogenic phenotype was confirmed by maximal expression of the adipogenic marker fatty acid binding protein-4 (Fabp4) within 4 days of incubation in differentiation media (Figure 4.2B; $P < 0.01$). Leptin and adiponectin mRNA were undetectable on differentiation day 0, but increased in a linear fashion over the next 10 days (Figure 4.2B; $P < 0.05$ or less). Finally, we asked whether adiponectin was produced by assessing its presence in cells and media collected during differentiation (Figure 4.2C). Adiponectin was undetectable at all times in cellular extracts and in media collected between days 0 and 4 of differentiation. Adiponectin was first seen in media collected between day 4 and 6 of differentiation and kept increasing in abundance over the next 2 collection periods (Figure 4.2C). Together, these findings demonstrate that bovine SVC differentiated into adipocytes produce and secrete adiponectin.

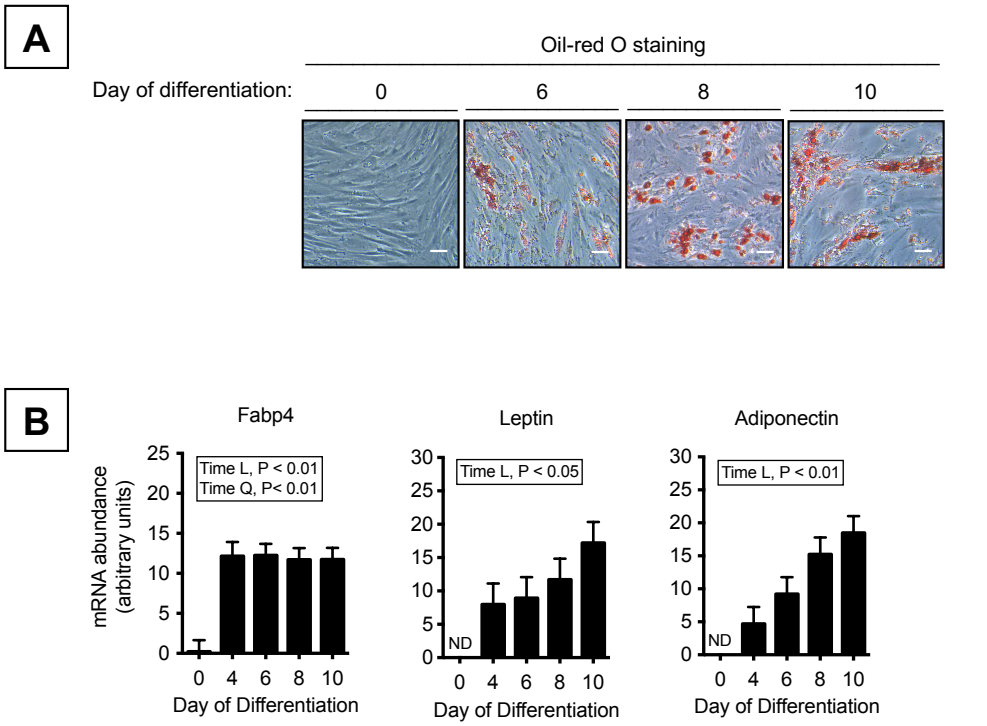

Figure 4.2 Capacity of primary bovine adipocytes to secrete adiponectin. Stromovascular cells isolated from white adipose tissue were grown to confluence and then incubated in differentiation media for 0 to 10 days. (**A)** Cells were stained over the course of differentiation with Oil-red O and photographed at 10X magnification. Scale bar = 50 μM. **(B)** Total mRNA was isolated over the course of differentiation and analyzed by quantitative real-time PCR for expression of fatty acid binding protein 4 (Fabp4), leptin, and adiponectin. Each bar represents the mean ± SE of mRNA abundance for the indicated gene (n = 3 per time point). Not detectable (N.D). The significance levels of the linear (L) and quadratic (Q) effects of time are given. **(C)** Cellular extracts prepared at the indicated day of differentiation and media conditioned by cells over indicated periods of differentiation were analyzed for adiponectin abundance by western immunoblotting. Actin was used as a loading control for total cellular extracts. Similar results were observed in a second experiment for variables shown in A to C.

Effect of Xbp1 overexpression on ER chaperones and adiponectin secretion

First, we asked whether adenoviruses could efficiently infect cells undergoing adipogenic differentiation. Bovine cells were infected with adenoviruses expressing Cherry on day 6 of differentiation, followed by determination of fluorescence 96 h later. As shown in Figure 4.3A, fluorescence was detected in virtually all cells, and was particularly strong in lipid-accumulating cells.

Next, cells were infected on day 6 of differentiation with adenoviruses expressing either Cherry or $Xbp1_S$ and analyzed 72 h later. Infection with $Xbp1_S$ adenovirus caused a 1.5 to 5-fold increase in the expression of the ER chaperones Grp78, Erp44, and Pdia6. (Figure 4.3B; $P < 0.05$ or less). $Xbp1_S$ overexpression did not alter adiponectin expression (Figure 4.3C).

Finally, effect of $Xbp1_S$ overexpression was assessed by measuring adiponectin secreted in media. As shown in Figure 4.3D, $Xbp1_S$ overexpression did not impact the amount of adiponectin secreted in media between 48 and 72 h following infection. Similarly, prolonging the period of infection and doubling the adenovirus dose did not affect adiponectin secretion in media between 72 and 96 h following infection. Overall, these data show that $Xbp1_S$ is a positive regulator of Grp78, Erp44, and Pdia6 expression, but do not support a role for $Xbp1_S$ in regulating adiponectin production in bovine adipocytes.

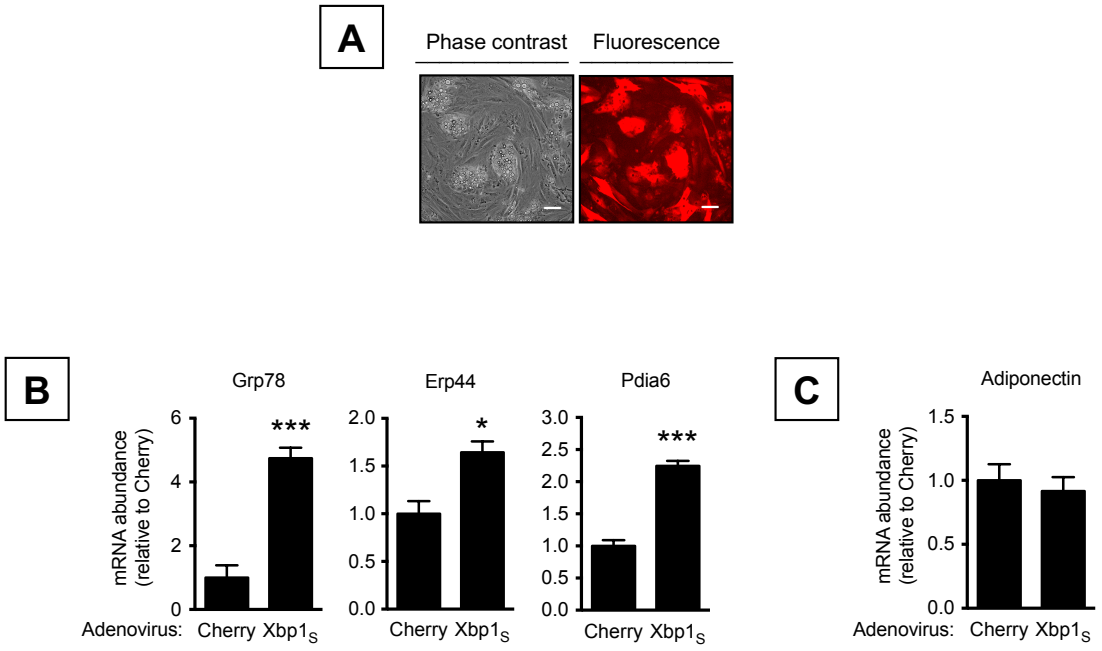

Figure 4.3 Effect of $Xbp1_S$ on ER chaperone expression and adiponectin secretion in bovine adipocytes. Stromovascular cells isolated from white adipose tissue were grown to confluence and then incubated in differentiation media. Cells were infected on day 6 of differentiation with adenoviruses encoding mCherry (Cherry) or spliced variant Xbp1 ($Xbp1_S$). **(A)** Cells were photographed at 10X magnification under phase contrast or fluorescence microscopy after 96 h of infection. **(B-C)** Total RNA was isolated after 72 h of infection and analyzed by quantitative real-time PCR for expression of ER chaperones [glucose-regulated protein,78 kDa (Grp78), endoplasmic reticulum protein 44 (Erp44), and protein disulfide isomerase associated 6 (Pdia6)] **(B)** or adiponectin **(C)**. Expression of each gene is relative to Cherry-infected cells. Each bar represents the mean ± SE of mRNA abundance for the indicated gene (n = 4). *, $P < 0.05$, and ***, $P < 0.001$. **(D)** Cells were infected for 72 or 96 h with a 1X or 2X dose of adenoviruses and media was collected over the last 24 h of infection. Media were analyzed for adiponectin abundance by western immunoblotting (n = 2 per adenovirus and time point). Similar results were observed in a second experiment for variables shown in A to D.

Effect of intralipid on ER chaperone expression and adiponectin secretion

Conditions used for converting SVC to adipocytes are very similar across species, except for the presence of exogenous lipid when using bovine SVC [26,29–31]. Accordingly, we asked whether intralipid supplementation was necessary for adiponectin secretion by differentiated SVC. SVCs were cultured for 8 days in differentiation media supplemented with or without intralipid. The presence of intralipid increased the density and intensity of lipid-containing cells, but had no effect on mRNA abundance of the adipocyte differentiation marker Fabp4 (Figure 4.4A). Surprisingly, absence of intralipid abrogated adiponectin secretion in media, even though adiponectin mRNA was unaffected by the presence or absence of intralipid (Figure 4.4B). Accordingly, we asked whether absence of adiponectin secretion was associated with reduced expression of ER chaperones and their transcriptional regulators Xbp1 and ATF6. As shown in Figure 4.4C, absence of intralipid had no effect on the mRNA abundance of ER-chaperones (Grp78, Erp44, ERO1-Lα, Pdia6, DsbA-L, and Gga1), $Xbp1_S$ or ATF6α . These data show that intralipid is required for adiponectin production by a mechanism that does not involve $Xbp1_S$, ATF6α, and ER chaperones.

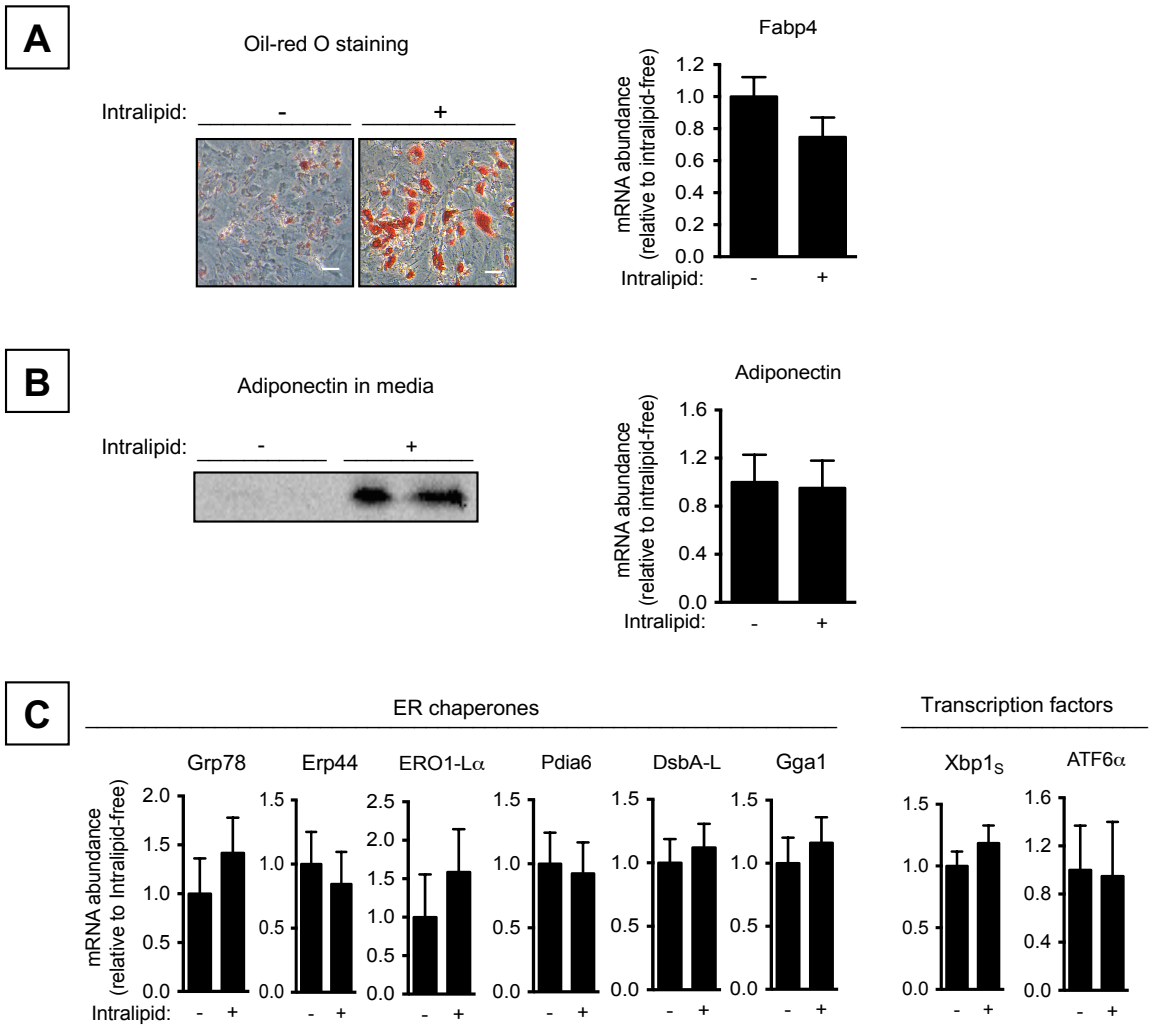

Figure 4.4 Effect of intralipid on ER chaperone expression and adiponectin secretion in bovine adipocytes. Stromovascular cells isolated from white adipose tissue were grown to confluence and then incubated in differentiation media in the presence (+) or absence (-) of intralipid. Cells and media were collected on day 8 of differentiation. **(A)** Left: Cells were stained with Oil-red O and photographed at 10X magnification. Scale bar = 50 μM. Right: Total mRNA was analyzed by quantitative real-time PCR for expression of fatty acid binding protein 4 (Fabp4). Expression is given relative to intralipid-free treatment. Each bar represents the mean ± SE of Fabp4 mRNA abundance (n = 3 per treatment). **(B)** Left: Media conditioned between day 6 and 8 of differentiation were analyzed for adiponectin abundance by western immunoblotting (n = 3 per treatment). Right: Total mRNA was analyzed by quantitative real-time PCR for expression of adiponectin. Expression is given relative to intralipid-free treatment. Each bar represents the mean ± SE of adiponectin mRNA abundance (n = 3 per treatment). **(C)** Total mRNA was analyzed by quantitative real-time PCR for mRNA abundance of ER chaperones [glucose-regulated protein, 78 kDa (Grp78), endoplasmic reticulum protein 44 (Erp44), endoplasmic reticulum oxidoreductase 1-like α (ERO1-Lα), protein disulfide isomerase associated 6 (Pdia6), glutathione S-transferase kappa 1 (DsbA-L), and golgi associated, gamma adaptin ear containing, ARF binding protein 1 (Gga1)] and transcription factors [spliced variant x-box binding protein 1 ($Xbp1_S$) and activating transcription factor-6 α (ATF6α)]. The expression of each gene is given relative to intralipid-free treatment. Each bar represents the mean ± SE of mRNA abundance for the indicated gene (n = 3 per treatment). Similar results were observed in a second experiment for variables shown in A to C.

Effect of TNFα and GH on ER chaperone expression and adiponectin secretion

TNFα and GH suppress adiponectin secretion in 3T3-L1 adipocytes and human adipose tissue explants respectively [32,33]. To determine whether similar effects are seen in differentiated bovine SVC, cells were treated with TNFα or GH between day 6 and 8 of differentiation. TNFα treatment did not alter lipid accumulation in cells differentiated for 8 days, but caused a 27% reduction in the mRNA abundance of the adipocyte differentiation marker Fabp4 (Figure 4.5A; $P < 0.05$). Treatment with TNFα decreased adiponectin mRNA abundance by 86% (Figure 4.5B; $P < 0.05$) and adiponectin in media by 29% (Figure 4.5C; $P < 0.01$). TNFα also reduced expression of Grp78 by 44%, but otherwise had no effect on the expression of other ER chaperones or the expression of $Xbp1_S$ or ATF6α (Figure 4.5D). On the other hand, GH treatment had no effect on indices of differentiation (Oil-red O staining and Fabp4 expression) or adiponectin production (mRNA level and adiponectin secretion in media) [Figures 4.6B – D]. These data suggest that effects of TNFα on adiponectin secretion are mediated predominantly through reduced adiponectin mRNA rather than repression of ER chaperone expression.

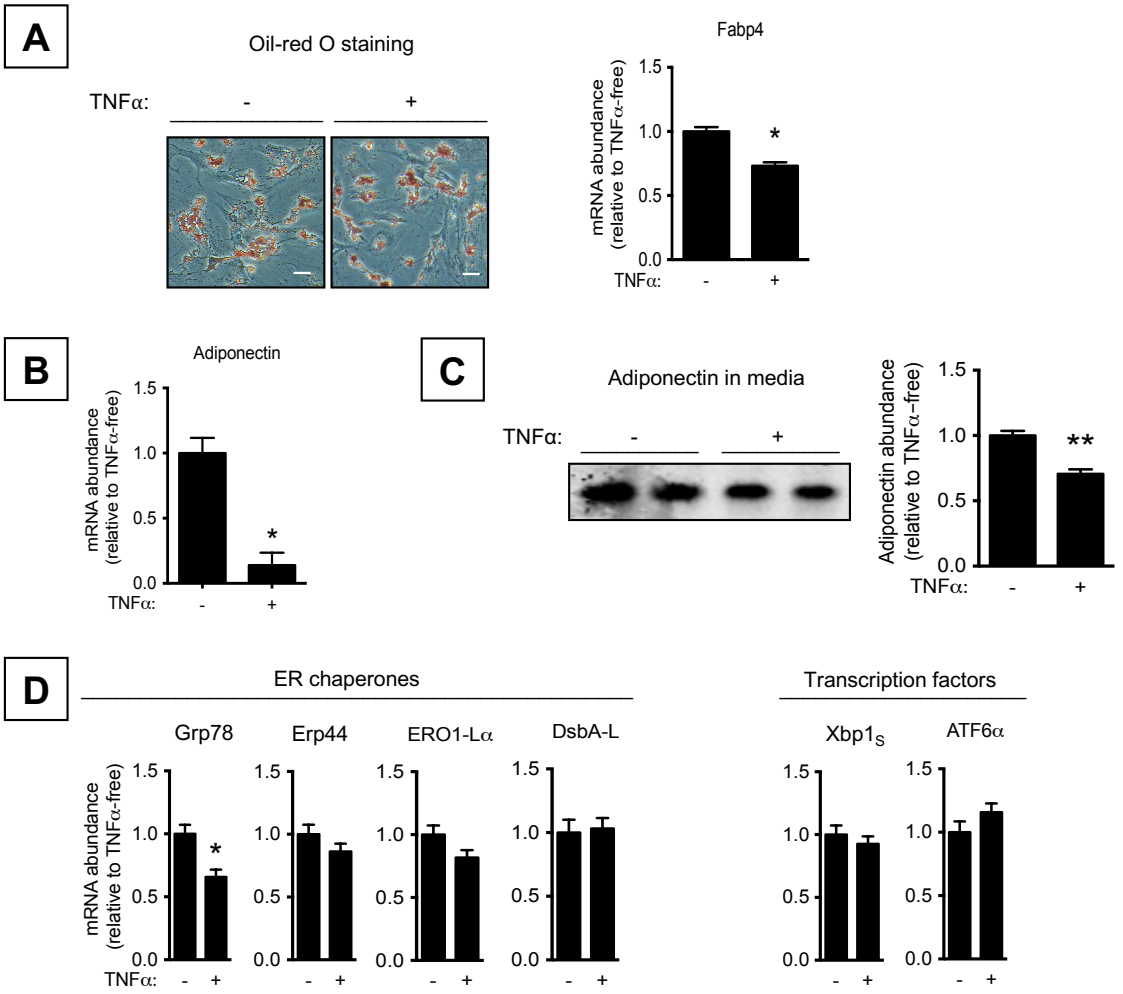

Figure 4.5 Effect of TNFα on ER chaperone expression and adiponectin secretion in bovine adipocytes
Stromovascular cells isolated from white adipose tissue were grown to confluence and then incubated in differentiation media for 6 days. Cells were then incubated between day 6 and 8 with differentiation media supplemented with (+) or without (-) human tumor necrosis factor α (TNFα). Cells and media were collected on day 8 of differentiation. **(A)** Left: Cells were stained with Oil-red O and photographed at 10X magnification. Scale bar = 50 μM. Right: Total mRNA was analyzed by quantitative real-time PCR for expression of fatty acid binding protein 4 (Fabp4). Expression is given relative to TNFα-free treatment. Each bar represents the mean ± SE of Fabp4 expression (n = 4 per treatment). *, $P < 0.05$. **(B)** Total mRNA was analyzed by quantitative real-time PCR for expression of adiponectin. Expression is given relative to TNFα-free treatment. Each bar represents the mean ± SE of adiponectin mRNA abundance (n = 4 per treatment). *, $P < 0.05$. **(C)** Left: Media conditioned between day 6 and 8 of differentiation were analyzed for adiponectin abundance by western immunoblotting (n = 2 per treatment). Right: The adiponectin signal was quantified and expressed relative to TNFα-free treatment. Each bar represents the mean ± SE of adiponectin protein abundance (n = 2 per treatment). **, $P < 0.01$. **(D)** Total mRNA was analyzed by quantitative real-time PCR for mRNA abundance of ER chaperones [glucose-regulated protein,78 kDa (Grp78), endoplasmic reticulum protein 44 (Erp44), endoplasmic reticulum oxidoreductase 1-like α (ERO1-Lα), and glutathione S-transferase kappa 1 (DsbA-L)] and transcription factors [spliced variant x-box binding protein 1 ($Xbp1_S$) and activating transcription factor-6 α (ATF6α)]. The expression of each gene is given relative to TNFα-free treatment. Each bar represents the mean ± SE of mRNA abundance for the indicated gene (n = 4 per treatment). *, $P < 0.05$. Similar results were observed in a second experiment for variables shown in A to D.

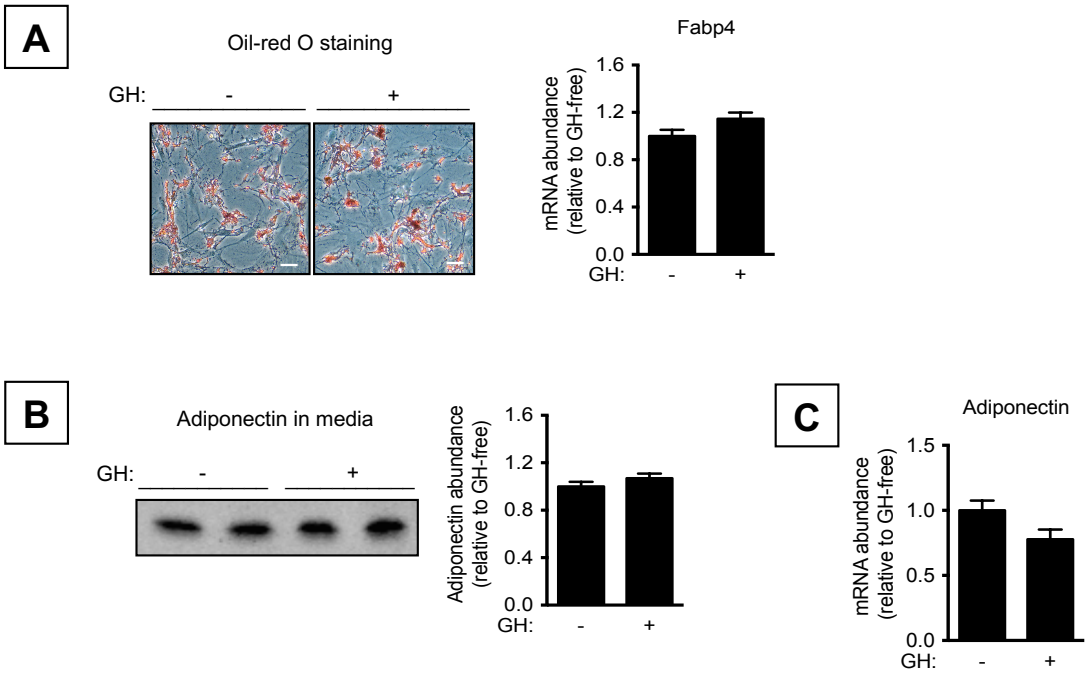

Figure 4.6 Effect of GH on adiponectin secretion in bovine adipocytes. Stromovascular cells isolated from white adipose tissue were grown to confluence and then incubated in differentiation media for 6 days. Cells were then incubated between day 6 and 8 with differentiation media supplemented with (+) or without (-) bovine GH. Cells and media were collected on day 8 of differentiation. **(A)** Left: Cells were stained with Oil-red O and photographed at 10X magnification. Scale bar = 50 μM. Right: Total mRNA was analyzed by quantitative real-time PCR for expression of fatty acid binding protein 4 (Fabp4). Expression is given relative to GH-free treatment. Each bar represents the mean ± SE of Fabp4 mRNA abundance (n = 4 per treatment). **(B)** Left: Media conditioned between day 6 and 8 of differentiation were analyzed for adiponectin abundance by western immunoblotting (n = 2 per treatment). Right: The adiponectin signal was quantified and expressed relative to GH-free treatment. Each bar represents the mean ± SE of adiponectin protein abundance (n = 2 per treatment). **(C)** Total mRNA was analyzed by quantitative real-time PCR for expression of adiponectin. Expression is given relative to GH-free treatment. Each bar represents the mean ± SE of adiponectin mRNA abundance (n = 4 per treatment). Similar results were observed in a second experiment for variables shown in A to C.

DISCUSSION

The plasma adiponectin concentration in transition dairy cows drops by 40% during the last 2 – 3 weeks before parturition, and is followed by a gradual rise during lactation [10–12]. Periparturient changes in plasma adiponectin, however, occur in absence of changes in adiponectin mRNA expression [10,34]. This is best illustrated by a recent study where changes in plasma adiponectin and indices of adiponectin production in fat depots were studied between parturition and day 105 of lactation. This work showed that adiponectin recovered fully in both plasma and individual depots by day 42 of lactation in complete absence of any changes in mRNA [35,36]. A lack of relation between changes in adiponectin mRNA and plasma adiponectin is not unique to transition dairy cows. For example, human participants undergoing extensive weight loss experienced increased plasma adiponectin in absence of changes in adiponectin mRNA [37]. Longitudinal studies following rhesus monkeys through the development of obesity and type 2 diabetes showed a drop in plasma adiponectin, independent of changes in adiponectin mRNA [38]. Therefore, mechanisms other than changes in WAT adiponectin mRNA are involved in reducing the plasma concentration of adiponectin in many conditions including the transition dairy cow. In this context, there is evidence of a role by ER chaperone in controlling the assembly of adiponectin into oligomeric complexes [22,39–42]. For example, ERO1-Lα overexpression increased adiponectin secretion in 3T3-L1 adipocytes [39]. Overexpressing DsbA-L in 3T3-L1 adipocytes and adipose tissue in mice enhanced adiponectin secretion [41,43]. Consistent with a reduced capacity of WAT to assemble adiponectin complexes after parturition, we previously showed that ER chaperone

proteins including Grp78, Erp44, and Pdia6 expression were lower in EL than LP in transition dairy cows [10]. These and other chaperones (e.g. DsbA-L) are positively regulated by the master regulator of ER homeostasis Xbp1. Moreover, $Xbp1_S$ overexpression in mouse WAT led to increased ER chaperone expression and increased adiponectin production and secretion [44]. Consistent with a role for Xbp1, we observed reduced $Xbp1_S$ expression during the transition from LP to EL. This prompted us to assess the possibility that $Xbp1_S$ regulates adiponectin production through effects on key ER chaperones.

We evaluated this possibility using SVC differentiated into adipocytes. Others had previously shown that this system faithfully recapitulated lipogenic regulation [26], but whether it was capable of producing and secreting adiponectin was unknown. Adiponectin mRNA was undetectable on day 0 of differentiation, but increased in a linear fashion over the next 10 days. Detection of adiponectin in media showed a similar increase over 10 days of differentiation. We next tested whether overexpressing $Xbp1_S$ could increase adiponectin production and secretion through increased ER chaperone expression. Accordingly, adipocytes were infected with adenovirus encoding bovine $Xbp1_S$. Overexpressing $Xbp1_S$ increased the expression of ER chaperones Grp78, Erp44, and Pdia6 1.5 to 5-fold, but had no effect on adiponectin mRNA and more importantly on adiponectin secretion in media. It is possible that ER chaperone expression in bovine adipocytes is already sufficient for oligomer assembly and secretion of adiponectin, and that further increasing their expression has no effect. It will be important in future work to assess whether knockdown of $Xbp1_S$ or its dependent set of ER chaperones (Grp78,

Erp44, or Pdia6) affect adiponectin secretion. Finally, also involved is the transcription factor ATF6α in regulating ER homeostasis [24], and some ER chaperones such as Grp78 are more dependent on ATF6α rather than $Xbp1_S$ [45]. Moreover, transcriptional activation of several ER chaperone proteins require both $Xbp1_S$ and ATF6α [46]. These data suggest that experiments knocking out $Xbp1_S$, ATF6α, or both should be pursued in this system.

Differentiation of SVC to adipocytes requires a conserved cocktail of reagents across all species [30,31]. Ruminants differ somewhat in that this cocktail is usually supplemented by exogenous lipid such as intralipid [26,29]. This prompted us to ask whether exogenous lipid impacted adiponectin production and secretion, and whether variation in ER chaperones was involved in this effect. Differentiated adipocytes secreted adiponectin in media only in the presence of intralipid, even though adiponectin mRNA was unaffected. We next asked whether intralipid-mediated adiponectin secretion was associated with changes in ER chaperone expression. Intralipid had no effect on expression of ER chaperones (Grp78, Erp44, ERO1-Lα, DsbA-L, and Gga1). Intralipid had a similar lack of effect on expression of transcription factors mediating ER chaperone expression ($Xbp1_S$ and ATF6α). In direct agreement with our studies, treatment with fatty acids including palmitic acid, oleic acid, linoleic acid, eicosapentaenoic acid, or docosahexaenoic acid increased adiponectin secretion in absence of changes in the expression of ER chaperones in 3T3-L1 adipocytes [47].

The periparturient period is characterized by rapid increases in drivers of IR including plasma GH and adipose TNFα, suggesting a model whereby either one or both of these hormones are negative regulators of adiponectin production and secretion [8,48].

We observed that TNFα suppressed adiponectin mRNA by 86% and adiponectin secretion in media by 29% in bovine adipocytes. The ability of TNFα to inhibit adiponectin mRNA abundance and secretion has also been demonstrated in 3T3-L1 adipocytes and mice [49–51]. Moreover, TNFα exerted this effect in part by antagonizing expression of the ER chaperone proteins Erp44 and DsbA-L [51]. Accordingly, we asked whether inhibition of adiponectin secretion in media is exclusively due to inhibition of adiponectin transcription or in part due to changes in expression of ER chaperones. However, TNFα failed to inhibit expression of Erp44 and DsbA-L in bovine adipocytes. A similar lack of effect was found on expression of transcription factors mediating ER chaperone expression ($Xbp1_S$ and ATF6α). Therefore, it is likely that suppression of adiponectin mRNA transcription, rather than post-transcriptional modifications account for most of the TNFα-induced suppression of adiponectin secretion in bovine adipocytes. In contrast to TNFα-mediated suppression of adiponectin secretion, GH treatment failed to induce any effects on adiponectin mRNA or secretion in media in bovine adipocytes. This result is inconsistent with an inverse relation between GH and adiponectin in rodents and humans [52–55], but in agreement with the failure of chronic GH treatment to alter plasma adiponectin in transition dairy cows (Chapter 3).

In summary, we determined that $Xbp1_S$ expression in adipose tissue drops from LP to EL in the transition dairy cow. Using a primary bovine adipocyte system, we showed that $Xbp1_S$ overexpression regulates expression of the ER chaperone proteins Grp78, Erp44, and Pdia6, but not adiponectin expression or secretion. Finally, we showed

that while TNFα suppressed adiponectin secretion, these effects were not due to regulation by ER chaperones.

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CHAPTER 5

DEFECTIVE LIPID METABOLISM IN LIVER-SPECIFIC SEL1L KNOCKOUT MICE FED AN ATHEROGENIC DIET[3]

[3]Krumm C.S., Francisco A.B.., Chang W.H.., Deng K.Y., Boisclair Y.R., and Long Q. (2016) Defective lipid metabolism in liver-specific sel1l knockout mice fed an atherogenic diet.

INTRODUCTION

Protein misfolding and aggregation have been associated with an array of diseases including type 1 diabetes, chronic viral hepatitis, and nonalcoholic fatty liver [1,2]. Endoplasmic reticulum (ER) stress resulting from protein misfolding or ER malfunction can activate one of several quality control systems. For example, activation of the unfolded protein response (UPR) can transcriptionally activate a subset of ER chaperones to increase the folding capacity of the ER [3]. In addition, activation of the endoplasmic-reticulum associated degradation (ERAD) system results in the retrotranslocation of unfolded or misfolded proteins across the ER membrane, followed by their degradation by the cytosolic proteasomal degradation system [3]. Several reports have shown cell-specific differences in UPR and ERAD responses to ER stress. Animal models are therefore required to further elucidate the tissue-specific functions of UPR and ERAD. Moreover, while many models of defective UPR have been developed, few models of ERAD deficiencies exist.

In yeast, hydroxymethylglutaryl reductase degradation protein 3 (Hrd3p) is critical in maintaining stability and communication of different components of the ERAD system [4,5]. Sel-1 suppressor of lin-12-like protein (Sel1l), the mammalian homolog of Hrd3p, is an integral ER resident glycoprotein and is also required for ERAD-mediated dislocation of misfolded proteins [4]. Sel1l expression is highly induced by the activating transcription factor-6 α (ATF6α) branch of the UPR in response to ER stress [6]. The essential role of Sel1l in mammals has recently been demonstrated using Sel1l knockout mice. For example, mice lacking Sel1l exhibited early lethality as well as increased

activation of UPR in the pancreas [7]. Together, these studies highlight the conserved significance of Sel1l in sustaining ER homeostasis across species.

The liver is involved in involved in plasma protein synthesis, cholesterol biosynthesis, and lipoprotein assembly and as a result, is highly enriched in ER organelles [1]. ER stress and UPR activation are elevated in a myriad of liver diseases traced to defective lipid metabolism (i.e. steatosis, choleostasis, and alcohol-induced liver injury) [8,9]. However, mechanisms connecting ER stress and lipid homeostasis are poorly understood. In this context, a recent study implicated Sel1l in the regulation of lipid metabolism in adipose tissue [10]. Accordingly, the present study was designed to ask whether Sel1l regulates lipid metabolism in liver.

MATERIALS & METHODS

Animals

All procedures were approved by the Cornell Institutional Animal Care and Use Committee. Sel1l$^{f/f}$ mice with loxP sites on either side of exon 6 in the Sel1l gene were generated as recently described and maintained on a C57BL/6 background [7]. Albumin-Cre mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and maintained on a C57BL/6 background. Experimental animals were generated by intercrossing Sel1l$^{f/f}$ female mice with Sel1l$^{f/f}$ male mice carrying the albumin-Cre transgene. Offspring retained for studies were either Sel1l$^{f/f}$ (designated WT) or Sel1l$^{f/f}$ carrying the albumin-Cre transgene (designated ΔSel1l^{Liver}). Litters were weaned and genotyped at 3 weeks of age. All terminal procedures involved euthanasia by CO_2 asphyxiation.

Mice were routinely housed on a 12-h light/12-h dark cycle and fed a normal chow diet (19.1% protein, 44.3% carbohydrate and 5.8% fat; LM-485). In some experiments, mice were fed the normal chow diet until 8 weeks of age and then switched to a high fat diet (HFD; 23.5% protein, 27.3% carbohydrate, and 34.3% fat; TD.06414) or an atherogenic diet (AD; 19.7% protein, 40.7% carbohydrate, 15.8% fat, 1.25% cholesterol, and 0.5% sodium cholate; TD.88051)). The fatty acid profile of the diets can be found in Appendix II. All diets were purchased from Envigo (Indianapolis, IN).

Statin treatment

Effect of the statin, simvastatin, was assessed in WT and ΔSel1l^{Liver} mice offered AD. Eight-week old male WT and ΔSel1l^{Liver} mice received either a daily oral gavage of

phosphate-buffered saline (PBS) or progressively increasing doses of simvastatin over an 8-week period (0.5 mg/kg during weeks 1 - 2, 1 mg/kg during weeks 2 - 4, 2 mg/kg during weeks 4 - 6, and 4 mg/kg during weeks 6 - 8). Simvastatin was purchased from Merck Sharp and Dohme (Rahway, NJ) and dissolved in PBS. Body mass and survival rate were monitored weekly.

Biochemical analyses

Routine determination of blood glucose involved sampling the tail artery and analysis with an Ascensia Contour glucometer (Bayer Health Care; Tarrytown, NY). The insulin tolerance test (ITT) was performed in 10-week old mice fed a normal chow diet. Briefly, after being fasted for 8 hours, a basal blood sample was obtained from the tail artery. This was followed by an ip injection of human insulin (0.75 IU/kg body weight, Humulin R, Eli Lilly Co.; Indianapolis, IN) and blood sampling 15, 30, 60, and 120 min later.

Plasma and liver were obtained from 16-week old WT and $\Delta Sel1l^{Liver}$ mice (n = 5 per genotype and diet). Liver tissue was homogenized twice in 100 mg/ml PBS with a Polytron tissue homogenizer for 30 seconds. The protein concentration of the resulting lysate was quantified with the bicinchoninic acid assay (BCA) protein assay kit (Thermo Fisher; Waltham, MA). The lysates were mixed with 2:1 chloroform: methanol mixture. PBS was then added and the lysates were spun at 1200 g for 5 minutes at 4°C. The resulting lower layer consisting of lipids was reconstituted in a 1% triton/ethanol solution and assayed for triglyceride, total cholesterol, and total bile acids using commercial kits

(L-Type Triglyceride M Color-A & L-Type TG M Color-B, Wako Cholesterol E, and Total Bile Acids Assay Kit, Wako; Richmond, VA). Plasma was prepared from blood samples and analyzed with the same kits for triglyceride, total cholesterol, and total bile acids. In addition, alanine transaminase (ALT) was assayed from plasma using a commercial kit (Cayman Chemical Company; Ann Arbor, MI).

Histology

WT and $\Delta Sel1l^{Liver}$ mice were euthanized at 16 weeks of age (n = 3 per genotype and diet). Liver tissues were fixed in 4% paraformaldehyde overnight at 4°C followed by dehydration in 95% ethanol overnight at 4°C. Liver sections were then sent to the Cornell Core Facility for processing and staining with hematoxylin-eosin (H&E). All histologically stained sections were analyzed through an Axiovert 40 Microscope bright light microscopy (Carl Zeiss; Oberkochen, Germany) at 10X and 40X magnification.

mRNA extraction and analysis of gene expression

WT and $\Delta Sel1l^{Liver}$ mice were euthanized at 16 weeks of age (n = 8 per genotype). Liver tissue was lysed with Qiazol (Qiagen; Hilden, Germany) followed by total mRNA purification using RNeasy Mini columns and on-column RNase-free DNase treatment (Qiagen; Hilden, Germany). Reverse transcription reactions were performed with 2 μg of mRNA in a total 20 μL volume with the SuperScript III Reverse Transcriptase (Thermo Fisher; Waltham, MA). Gene expression was analyzed with quantitative real-time PCR assays using Power SYBR Green Mix (Applied Biosystems; Foster city, CA). Real-time

PCR assays were performed in duplicate with a total 25 μL reaction volume containing 500 nM concentration of each primer and reverse transcribed mRNA (25 ng except 2.5 ng for the internal standard gene 18S). The sequences of all primers used are given in appendix III. mRNA data were analyzed using the $2^{-\Delta/\Delta Ct}$ method with 18S expression as the internal standard [11].

Western immunoblotting

Frozen liver tissues from 16-week old WT and $\Delta Sel1l^{Liver}$ mice (n = 8 per genotype) were homogenized in radioimmunopreciptation assay buffer [10 mM tris-HCl (pH 7.4), 150 mM NaCl, 1% nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 mM NaF, 0.25% sodium deoxycholate, and 10% glycerol, supplemented with Halt™ protease and phosphatase inhibitors (Thermo Fisher; Waltham, MA)]. Alternatively, liver samples were processed for preparation of subcellular fractions as previously described [12]. Briefly, fresh liver tissue was homogenized on ice with a dounce pestle in 20% (w/v) organelle purification buffer [250 mM sucrose, 1 mM EDTA pH 7.4, 2 mM $MgCl_2$, 2 mM KCl, 1 M dithiothreitol (DTT), and Halt™ protease and phosphatase inhibitors].

The homogenate was subjected to 3 rounds of centrifugation (twice at 600 g for 15 min; twice at 10,000 g for 30 min; once at 100,000 g for 3 h). The pellet collected after the final centrifugation was denoted as the microsomal fraction (100,000 g). The pellet was solubilized in microsomal buffer [20 mM Imidazole, 5 mM DTT, and 50 mM NaCl supplemented with Halt™ protease and phosphatase inhibitors].

The protein concentration of liver lysates and microsomal fractions was determined using the BCA protein assay. Samples (20 – 40 μg protein) were separated on 5 – 12 % gels and transferred onto polyvinylidene flouride membranes (0.2 μM; Bio-Rad; Hercules, CA). Membranes were blocked in either 5% non-fat dried skim milk (Bio-Rad; Hercules, CA) or 2% bovine serum albumin (BSA, Sigma Aldrich; St. Louis, MI) in tris-based saline with tween-20 [0.05 M Tris-HCl (pH 7.4), 0.2 M NaCl and 0.1% Tween-20]. Membranes were then immunodecorated with primary antibodies against human Sel1l (Abcam; Cambridge, United Kingdom), human 3-hydroxy-3-methylglutaryl-Co A reductase (Hmgcr; Santa Cruz Biotech; Santa Cruz, CA), human cytochrome P450, family 7, subfamily a, polpeptide 1 (Cyp7a1; Santa Cruz Bioteh; Santa Cruz, CA), human low density lipoprotein receptor (Ldlr; Gene Tex; Irvine, CA), human solute carrier family 10 (sodium/bile acid cotransporter family), member 1 (NTCP; Santa Cruz Biotech; Santa Cruz, CA), human heat shock protein 90 (Hsp90; Cell Signaling; Danvers, MA), human endoplasmic reticulum (ER) to nucleus signaling 1 (IRE1α; Cell Signaling; Danvers, MA), human glucose-regulated protein, 78 kDa (Grp78; Cell Signaling; Danvers, MA), human synovial apoptosis inhibitor 1, synoviolin (Hrd1; Novus Biologicals; Littleton, CO), and human protein disulfide isomerase (PDI; Cell Signaling; Danvers, MA). All primary antibodies were diluted in blocking solution at 1:1,000 except Cyp7a1, which was diluted at 1:400. Signals were developed with 1:10,000 dilution of goat anti-rabbit (Thermo Fisher; Waltham, MA) secondary antibody and visualized by chemiluminescence (Super Signal West Pico Chemiluminescent Substrate, Thermo Fisher; Waltham, MA).

Statistical analysis

Data were analyzed by a mixed model using the fit model procedure of JMP Pro 11.0 statistical software (SAS Institute; Cary, NC). Data collected over time (growth curve, plasma glucose, ITT, and body weight during statin treatment) were analyzed by the fit model procedure, which included genotype, time, and their interaction as fixed effects and animal as the random effect. Survival curves were compared by the log-rank (Mantel-Cox) test. All other variables were analyzed by the fit model procedure, which included genotype as the fixed effect and animal as the random effect. The level of statistical significance was set at $p < 0.05$.

RESULTS

Liver-specific deletion of Sel1l in ΔSel1l^{Liver} mice

Sel1l protein expression was expressed ubiquitously, with the highest level of Sel1l protein found in liver, brain, and spleen (Figure 5.1A). ΔSel1L^{Liver} mice were generated by crossing Sel1l$^{f/f}$ mice with Sel1l $^{f/f}$;albumin-Cre mice. To assess liver specific deletion, PCR was performed on DNA isolated from various tissues with primers flanking exon 6. These primers bracket an 800 bp fragment in WT Sel1l and a 200 bp fragment after Cre-mediated recombination (Appendix IV). An expected 800 bp DNA fragment was obtained in WT tissues. The smaller fragment of 200 bp was detected only in the liver of ΔSel1l^{Liver} mice (Figure 5.1B).

Finally, Sel1l expression was assessed in the liver of WT and ΔSel1^{Liver} mice. Sel1l expression was reduced by 75% at the mRNA level and by 58% at the protein level (Figure 5.1C & D; $P < 0.001$). Together, these observations show selective Sel1l deletion in the liver of ΔSel1l^{Liver} mice.

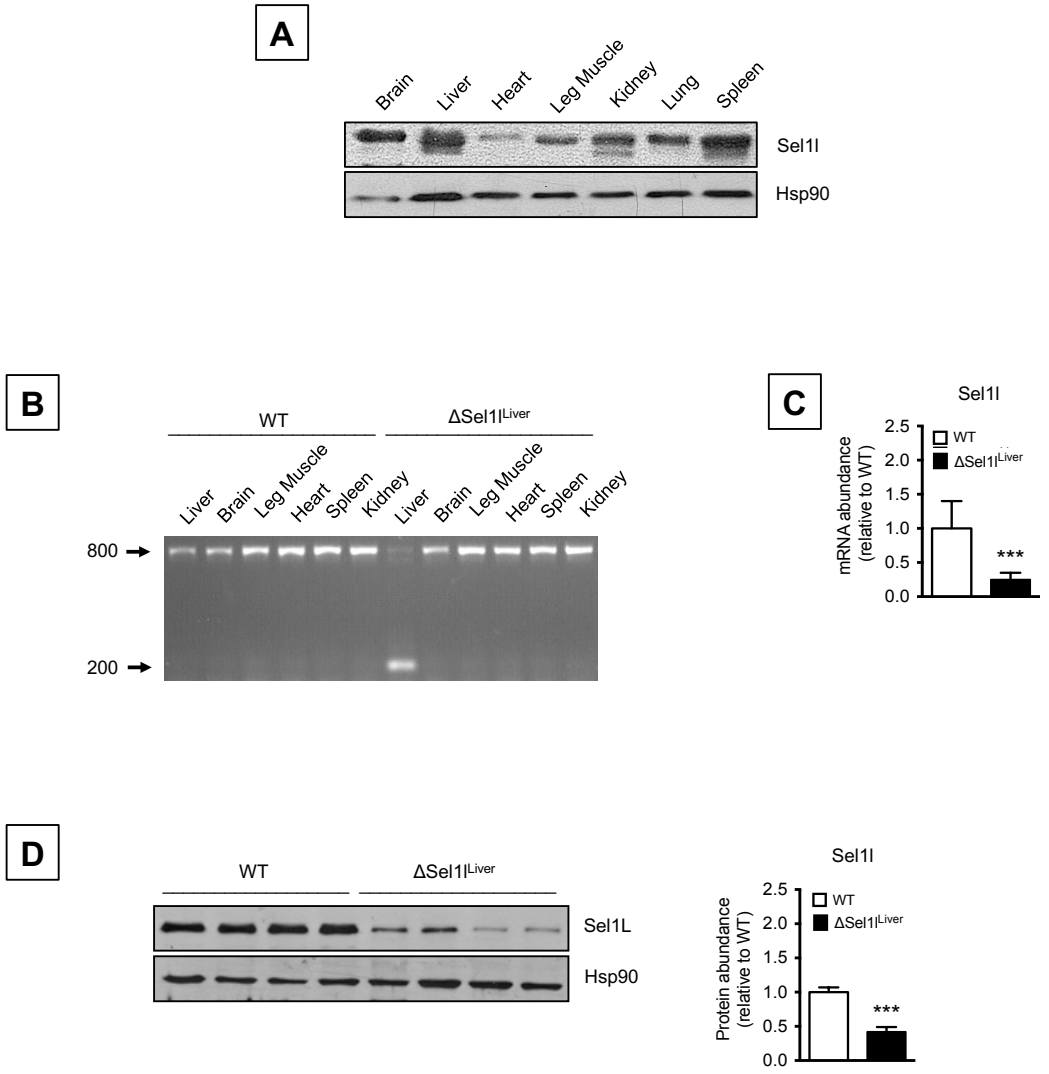

Figure 5.1 Liver-specific deletion of Sel1l in ΔSel1l^{Liver} mice. **(A)** Indicated tissues were collected from a 16-week old wild type (WT) mouse. Total protein extracts were prepared and analyzed by western immunoblotting for Sel-1 suppressor of lin-12-like protein (Sel1l) abundance and the ubiquitous protein heat shock protein-90 (HSP90). (**B**) Genomic DNA was isolated from indicated tissues collected from WT and ΔSel1l^{Liver} mice and analyzed by a PCR strategy yielding an 800 bp fragment for the WT Sel1l allele and a 200 bp fragment for the mutated allele. (**C**) Total mRNA was isolated from liver tissue collected from 16-week old WT and ΔSel1l^{Liver} mice fed normal chow. Sel1l mRNA abundance was measured by quantitative real time-PCR. Gene expression was normalized to 18S expression. Sel1l mRNA abundance is relative to WT level with each bar representing the mean ± SE of 8 mice. ***, $P < 0.001$. **(D)** Liver tissue was isolated from 16-week old WT and ΔSel1l^{Liver} mice fed normal chow. Protein extracts were prepared from liver tissue and analyzed by western immunoblotting for Sel1l abundance. The Sel1l abundance was normalized to the heat shock protein 90 (Hsp90) signal and expressed relative to WT level with each bar representing the mean ± SE of 8 mice. ***, $P < 0.001$.

Effects of Sel1l ablation in liver on growth and glucose metabolism under normal feeding conditions

Male mice were fed a normal chow diet and monitored for body weight gain from 3 to 17 weeks of age. $\Delta Sel1l^{Liver}$ mice experienced a small reduction in body weight (Figure 5.2A; Genotype, $P < 0.01$). This effect appeared to be more prominent between 3 and 8 weeks of age than at 17 weeks of age.

Under fed conditions, $\Delta Sel1l^{Liver}$ mice had lower plasma glucose than WT mice (Genotype, $P < 0.01$). This effect in plasma glucose was more pronounced at 4 and 5 weeks of age than between 14 and 17 weeks of age (Figure 5.2B; Genotype x Time, $P < 0.05$). An ITT test was performed at 10 weeks of age. The glucose response area did not differ between genotypes (Figure 5.2C).

Since Sel1l has been recently shown to play a critical role in lipid metabolism in adipose tissue, we next addressed whether Sel1l deletion affected lipid and cholesterol metabolism [10]. No drastic changes in triglyceride, total cholesterol, or total bile acids were observed in $\Delta Sel1l^{Liver}$ mice when assayed in plasma or liver tissue (Table 5.1). Similarly, liver morphology did not differ between WT and $\Delta Sel1l^{Liver}$ mice when assessed by histology (Figure 5.2D). Similar effects were seen in female mice. Accordingly, all subsequent studies were performed on male mice.

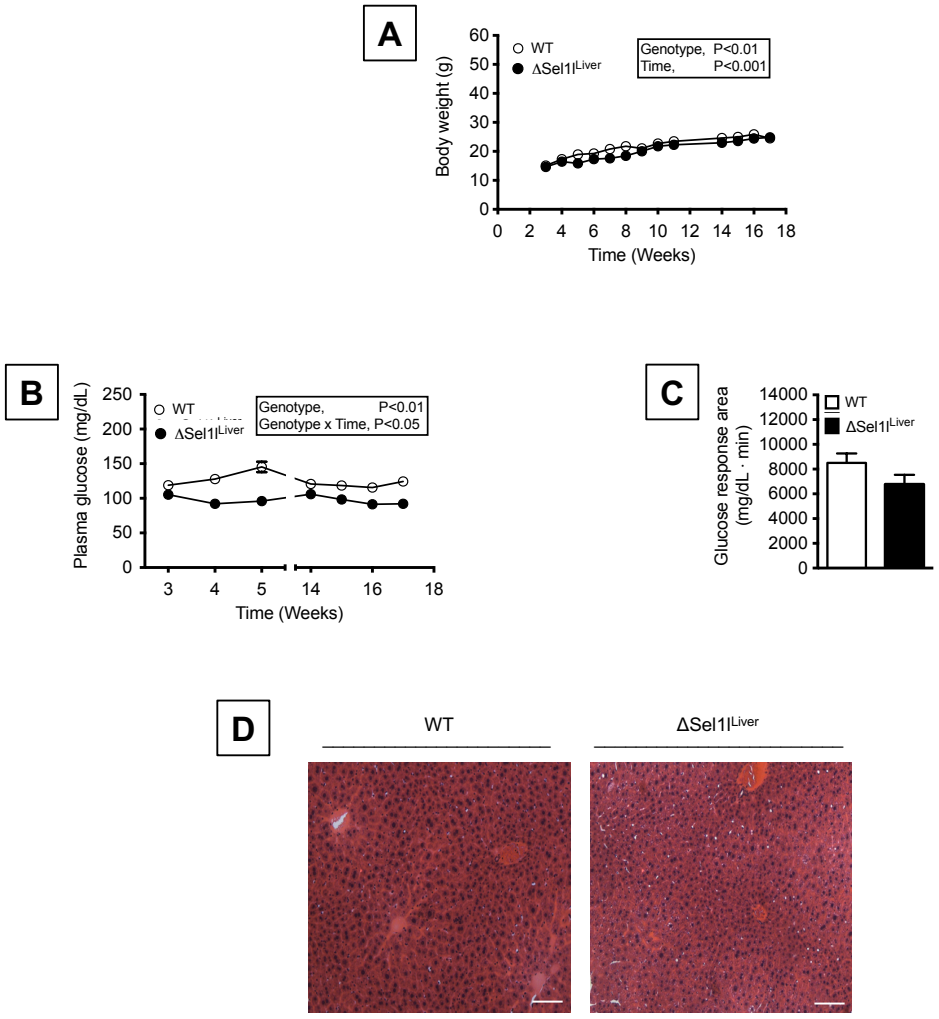

Figure 5.2 Effects of Sel1l ablation in liver on growth and glucose metabolism under normal feeding conditions. Male mice harboring wild type Sel1l (WT) or null Sel1l in liver (ΔSel1l^{Liver}) were fed a normal chow diet and studied between 3 and 17 weeks of age. (**A)** Growth curves representing the average weight of individual mice from each genotype (n = 11 per genotype). The significant effects of genotype and time are reported. (**B)** Random glucose levels are shown. Each curve represents the average plasma glucose levels of individual mice from each genotype (n = 10 -12 per genotype). The significant effects of genotype and genotype x time are reported. (**C)** An insulin tolerance test (ITT) was performed at 10 weeks of age. Each bar represents the mean ± SE of the glucose response area (n = 5 per genotype). (**D)** Hematoxylin-Eosin (H&E)-stained liver sections were prepared from 16-week old mice (n = 3 per genotype) and photographed at 10X magnification. A single representative photograph is shown for each genotype. Scale bar, 100 μm.

Table 5.1 Effect of liver-specific ablation of Sel1l in mice fed a normal chow diet on metabolic variables

	Genotype[1]			
	WT	ΔSel1l^{Liver}	SE	P Value[2]
Plasma				
Triglyceride, mg/dL	58.9	48.5	3.8	NS
Cholesterol, mg/dL	84.4	117.8	12.4	NS
Total Bile Acids, μM	1.1	1.1	0.4	NS
Liver				
Triglyceride, mg/g liver	4.7	4.0	0.43	NS
Cholesterol, mg/g liver	3.5	2.9	0.19	NS
Total Bile Acids, μM/g liver	190.0	249.3	37.4	NS

[1]Male mice harboring wild type (WT) or null Sel1l in liver (ΔSel1l^{Liver}) were fed a normal chow diet and studied at 16 weeks of age (n = 5 per genotype).

[2]Type I error probability for genotype. NS, non-significant at P ≥ 0.05.

Effects of Sel1l deletion in liver on ER Stress under normal feeding conditions

Perturbations in ER homeostasis have been observed in several Sel1l mouse models [7,10,13,14]. We next addressed whether ER homeostasis was altered in liver of $\Delta Sel1l^{Liver}$ mice fed normal chow diet by measuring mRNA levels of several ER stress markers. Sel1l ablation increased the expression of the DnaJ heat shock protein family (Hsp40) member C3 (p58IPK), Hrd1, homocysteine-inducible endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1 (Herp), stress-associated endoplasmic reticulum protein 1 (RAMP4), spliced variant x-box binding protein-1 ($Xbp1_S$), DNA-damage inducible transcript 3 (Chop), Grp78, and DnaJ heat shock protein family (Hsp40) member B11 (ERdj3) ($P < 0.05$ or less). Expression of gamma-aminobutyric acid (GABA) A receptor subunit alpha-1 (Gabra1), a non-UPR-related gene, was not altered between genotypes (Figure 5.3A). Protein expression of Grp78 and IRE1α were increased in liver of $\Delta Sel1l^{Liver}$ mice ($P < 0.05$), whereas no effect was observed on Hrd1 and PDI (Figure 5.3B). Accordingly, ER stress is activated in the liver of $\Delta Sel1l^{Liver}$ mice.

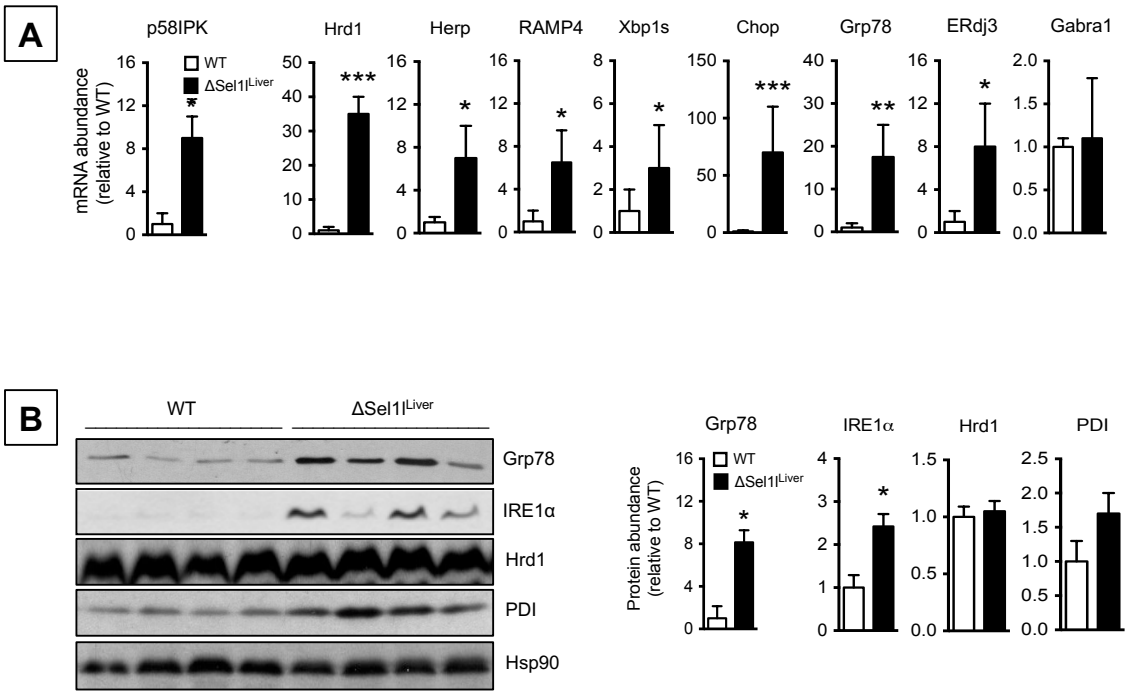

Figure 5.3 Effects of Sel1l deletion in liver on ER Stress under normal feeding conditions. Male mice harboring wild type Sel1l (WT) or null Sel1l in liver (ΔSel1l^{Liver}) were fed a normal chow diet and studied at 16 weeks of age. **(A)** Total mRNA was analyzed by quantitative real-time PCR for expression of genes indicative of DnaJ heat shock protein family (Hsp40) member C3 (p58IPK), synovial apoptosis inhibitor 1, synoviolin (Hrd1), homocysteine-inducible endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1 (Herp), stress-associated endoplasmic reticulum protein 1 (RAMP4), spliced variant x-box binding protein 1 ($XBP1_s$), DNA-damage inducible transcript 3 (Chop), glucose-regulated protein, 78 kDa (Grp78), and DnaJ heat shock protein family (Hsp40) member B11 (ERdj3). Gamma-aminobutyric acid (GABA) A receptor subunit alpha-1 (Gabra1) mRNA was measured as a non-ER stress gene. Gene expression was normalized to 18S expression. The expression of each gene is relative to WT level with each bar representing the mean ± SE of 8 mice. *, P < 0.05, **, P < 0.01, ***, P < 0.001. **(B)** Protein extracts were prepared from liver and analyzed by western immunoblotting for Grp78, endoplasmic reticulum (ER) to nucleus signaling 1 (IRE1α), Hrd1, and protein disulfide isomerase (PDI) abundance. Protein expression was normalized to the heat shock protein 90 (Hsp90) signal. The abundance of each protein is relative to WT level with each bar representing the mean ± SE of 8 mice. *, P < 0.05.

Effect of high fat diet on growth and metabolic variables of ΔSel1l^{Liver} mice

Mice were switched from a normal chow diet to a HFD at 8 weeks of age and studied for the next 9 weeks. As previously observed on a normal chow diet, ΔSel1l^{Liver} mice suffered a slight growth deficit (Figure 5.4A; Genotype, $P < 0.01$). There was no genotype x time interaction indicating that the HFD did not exacerbate the growth deficit of ΔSel1l^{Liver} mice. ΔSel1l^{Liver} mice suffered lower plasma glucose (Genotype, $P < 0.001$) until week 13 of life, which then rose to levels comparable to WT mice thereafter (Figure 5.4B; Genotype x Time, $P < 0.001$).

We also asked whether the HFD impacted lipid and cholesterol metabolism. No genotype effects were seen for triglyceride or total bile acids when assayed in plasma or liver tissue (Table 5.2). In contrast, ΔSel1l^{Liver} mice exhibited a significant increase in total cholesterol in plasma ($P < 0.05$), but not liver. By liver histology, ΔSel1l^{Liver} mice appeared to have fewer hepatic lipid droplets than WT mice (Figure 5.4C).

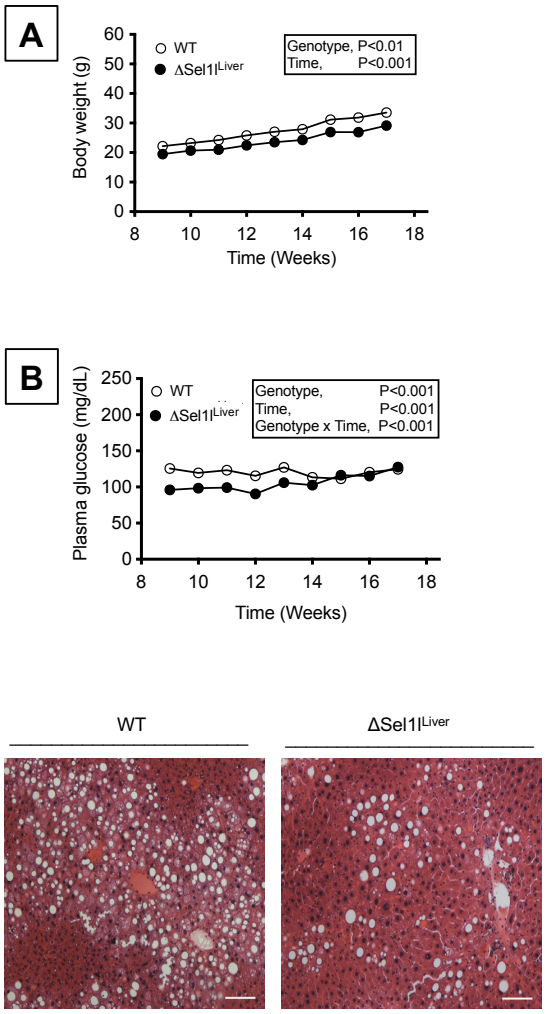

Figure 5.4 Effect of high fat diet on growth and metabolic variables of ΔSel1l^{Liver} mice. Male mice harboring wild type Sel1l (WT) or null Sel1l in liver (ΔSel1l^{Liver}) were fed high fat diet (HFD) between 8 and 17 weeks of age. **(A)** Growth curves are shown. Each curve represents the average weight of individual mice from each genotype (n = 15 per genotype). The significant effects of genotype and time are reported. **(B)** Random glucose levels are shown. Each curve represents the average plasma glucose levels of individual mice from each genotype (n = 8 -11 per genotype). The significant effects of genotype, time, and genotype x time are reported. **(C)** Livers were collected at 16 weeks of age and analyzed by hematoxylin-eosin (H&E)-staining (n = 3 per genotype). All liver sections were photographed at 10X magnification. A single representative photograph is shown for each genotype. Bar scale, 100 μm.

Table 5.2 Effect of high fat diet on growth and metabolic variables of ΔSel1l^{Liver} mice

	Genotype[1]			
	WT	ΔSel1l^{Liver}	SE	P Value[2]
Plasma				
Triglyceride, mg/dL	224.2	241.2	33.3	NS
Cholesterol, mg/dL	93.7	128.3	7.5	0.02
Total Bile Acids, μM	2.2	2.3	0.26	NS
Liver				
Triglyceride, mg/g liver	15.6	17.4	2.9	NS
Cholesterol, mg/g liver	10.1	10.3	0.8	NS
Total Bile Acids, μM/g liver	91.9	94.7	7.6	NS

[1]Male mice harboring wild type (WT) or null Sel1l in liver (ΔSel1l^{Liver}) were fed a high fat diet between 8 and 17 weeks of age (n = 3 per genotype). Mice were studied at 16 weeks of age.

[2]Type I error probability for genotype. NS, non-significant at $P \geq 0.05$. *, significant when $P < 0.05$.

Effect of atherogenic diet on growth and survival of ΔSel1l^{Liver} mice

To address further the possibility that Sel1l plays a role in hepatic lipid metabolism, mice were switched from a normal chow diet to the AD at 8 weeks of age. Body weights were comparable at 8 weeks of age, but a growth deficit became obvious in ΔSel1l^{Liver} mice by week 12 (Figure 5.5A). Between 8 and 16 weeks of age, WT mice gained an average of 3.7 g, whereas ΔSel1l^{Liver} mice lost 5.6 g (Genotype x Time, $P < 0.001$). By week 16, WT mice weighed 24.3 g, versus 12.8 g for ΔSel1l^{Liver} mice (Genotype, $P < 0.001$). Moreover, mortality was observed in a subset of ΔSel1l^{Liver} mice, whereas no such effect was seen in WT mice (Figure 5.5B).

At the end of treatment, the liver was larger and paler in ΔSel1l^{Liver} mice than WT mice. In line with this observation, the liver to body weight ratio increased by 47% in ΔSel1l^{Liver} mice (Figure 5.5C; $P < 0.01$). By H&E histology, hepatocytes in ΔSel1l^{Liver} mice appeared enlarged and "balloon-shaped" (Figure 5.5D). Finally, plasma ALT levels increased by 91% in ΔSel1l^{Liver} mice when compared to WT mice, suggesting liver damage (Figure 5.5E; $P < 0.001$). Thus, our data shows that ΔSel1l^{Liver} mice are intolerant to AD, resulting in liver damage and occasional death.

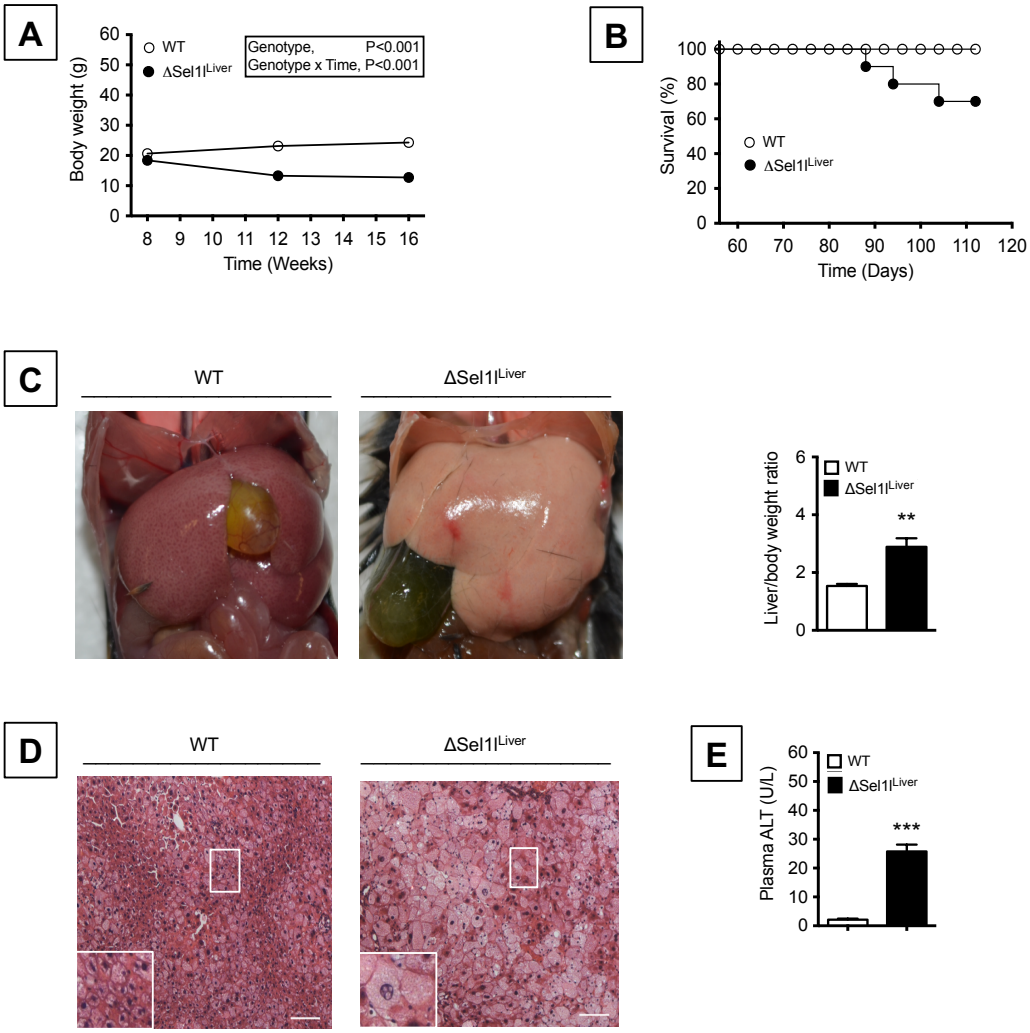

Figure 5.5 Effect of the atherogenic diet on growth and survival of ΔSel1l^{Liver} mice. Male mice harboring wild type Sel1l (WT) or null Sel1l in liver (ΔSel1L^{Liver}) were fed an atherogenic diet between 8 and 16 weeks of age. **(A)** Growth curves of each genotype (n = 10 -13 per genotype). The significant effects of genotype and genotype x time are reported. **(B)** Survival curves of each genotype (n = 10 - 13 per genotype). **(C)** Appearance and fractional weight of liver in 16-week old mice. Each bar represents the mean ± SE of 7 – 10 mice. **, P < 0.01. **(D)**. Hematoxylin-eosin (H&E)-stained liver sections from 16-week old mice (n = 3 per genotype) were photographed at 10X magnification and 40X magnification (bottom left hand corner). A single representative photograph is shown for each genotype. Scale bar, 100 μm. **(E)** Plasma alanine transaminase (ALT) content was measured in 16-week old mice. Each bar represents the mean ± SE of 5 mice. ***, P < 0.001.

Effect of atherogenic diet on hepatic cholesterol metabolism in ΔSel1l^{Liver} mice

We next asked whether alterations in cholesterol metabolism accounted for abnormal liver parameters in ΔSel1l^{Liver} mice fed an AD diet. Total cholesterol levels increased by 63% in plasma and by 54% in liver in ΔSel1l^{Liver} mice (Figure 5.6A; $P < 0.001$). We then focused on mechanisms that could account for abnormally elevated cholesterol in plasma and liver of ΔSel1l^{Liver} mice. We measured the liver mRNA and protein levels of Hmgcr, the rate-limiting enzyme for endogenous cholesterol synthesis. Hmgcr mRNA and protein levels were increased in ΔSel1l^{Liver} mice (Figure 5.6B & data not shown; $P < 0.001$). These data suggest that the liver is unable to reduce cholesterol synthesis in absence of Sel1l.

To test this possibility, we asked whether simvastatin could rescue ΔSel1l^{Liver} mice. Simvastatin reduces endogenous cholesterol synthesis by exhibiting a higher binding affinity to Hmgcr than HMG-CoA [15]. WT and ΔSel1l^{Liver} mice were fed AD between 8 and 16 weeks of age and treated concurrently with either PBS or simvastatin solution. In WT mice, there was no difference in body weight between control and simvastatin groups (Figure 5.6C). As before, ΔSel1l^{Liver} mice lost body weight over time (Genotype x time, $P < 0.001$) and simvastatin was unable to prevent this effect. Similarly, simvastatin was unable to normalize the liver to body weight ratio of ΔSel1l^{Liver} mice to that of WT mice (Figure 5.6D) ΔSel1l^{Liver} mice.

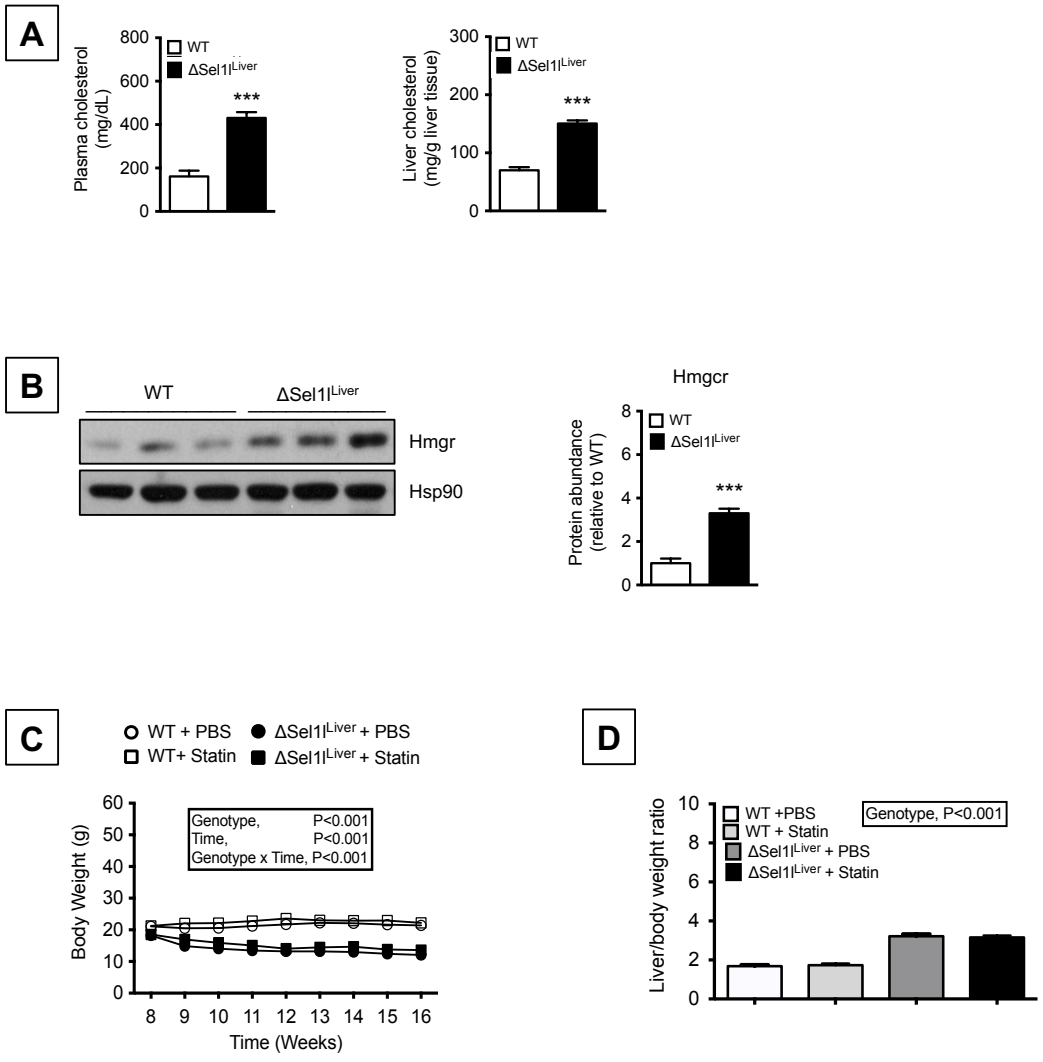

Figure 5.6 Effect of the atherogenic diet on hepatic cholesterol metabolism in ΔSel1l^{Liver} mice. Male mice harboring wild type Sel1l (WT) or null Sel1L in liver (ΔSel1l^{Liver}) were fed atherogenic diet between 8 and 16 weeks of age. **(A)** Total cholesterol was measured in plasma and liver (n = 5 per genotype). Each bar represents the mean ± SE. ***, P < 0.001. **(B)** Liver tissue protein extracts prepared from 16-week old mice were analyzed by western immunoblotting for 3-hydroxy-3-methylglutaryl-Co A reductase (Hmgcr) abundance. Protein expression was normalized to the heat shock protein 90 (Hsp90) signal. The abundance of each protein is relative to WT level with each bar representing the mean ± SE of 8 mice. ***, P < 0.001. **(C)** Mice received an oral gavage of either phosphate-buffered saline (PBS) or an increasing dosage of simvastatin (0.5 mg/kg during week 1 and 2, 1 mg/kg during week 2 to 4, 2 mg/kg during week 4 to 6, and 4 mg/kg during week 6 to 8) (n = 6 -10 per genotype and treatment). Body weight during treatment is shown. The significant effects of genotype, time, and genotype x time are reported. **(D)** The ratio of liver to body weight was calculated at the end of treatment (n = 6 per genotype and treatment). The significant effect of genotype is reported.

Effect of atherogenic diet on hepatic lipid metabolism and low-density lipoprotein receptor in ΔSel1l^{Liver} mice

Next, we asked whether deletion of Sel1l in liver tissue led to alterations in lipid metabolism. The plasma of ΔSel1l^{Liver} mice was "butter-like" yellow and viscous, unlike the clear, fluid appearance of WT plasma (Figure 5.7A), suggesting elevated lipid levels. Moreover, triglyceride was increased 84% in plasma and 67% in liver in ΔSel1l^{Liver} mice (Figure 5.7B; $P < 0.001$). These data suggest that ΔSel1l^{Liver} mice exhibit defects in lipid metabolism.

Ldlr is a plasma membrane glycoprotein primarily expressed in liver that is critically required for uptake and metabolism of the circulating lipoproteins intermediate-density lipoprotein (Idl) and low-density lipoprotein (Ldl) [16]. Genetic deficiency of Ldlr in humans and in mice has been implicated in the pathogenesis of hypercholesterolemia [17]. To assess this, we investigated whether hepatic Ldlr contributes to altered lipid and cholesterol homeostasis in ΔSel1L^{Liver} mice. Hepatic mRNA and protein levels of Ldlr were reduced in ΔSel1l^{Liver} mice ($P < 0.001$ & Data not shown). In addition, ΔSel1l^{Liver} mice displayed reduced hepatic Ldlr protein levels in microsomal fractions (Figure 5.7C; $P < 0.001$). Taken together, these data suggest that ΔSel1l^{Liver} mice have abnormally low levels of hepatic Ldlr.

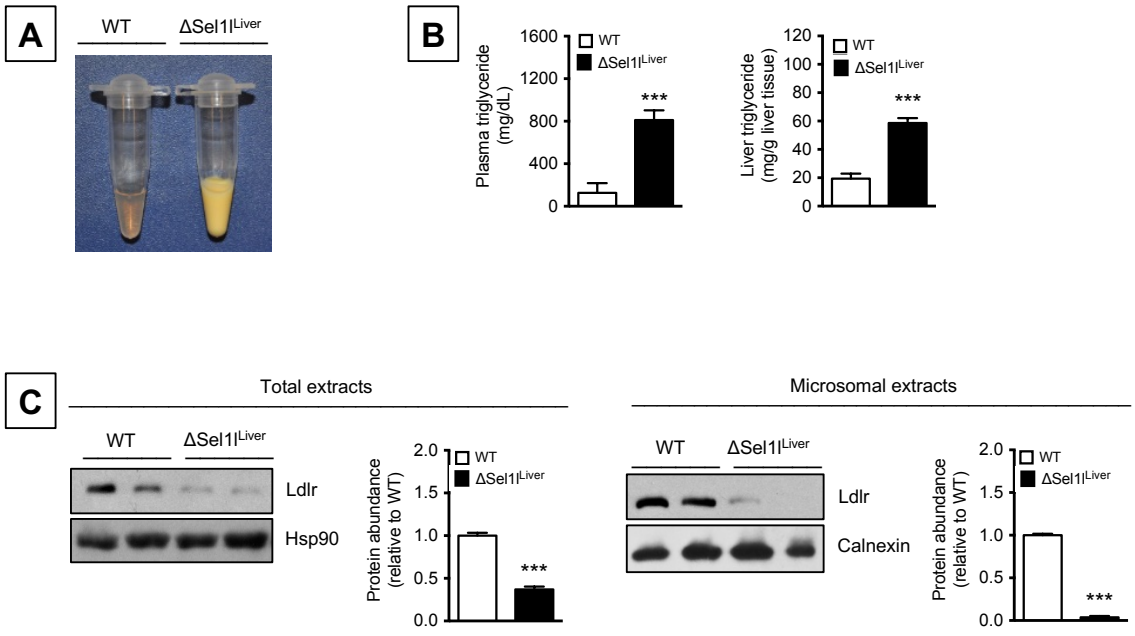

Figure 5.7 Effect of the atherogenic diet on hepatic lipid metabolism and low-density lipoprotein receptor in ΔSel1l^{Liver} mice. Male mice harboring wild type Sel1l (WT) or null Sel1l in liver (ΔSel1l^{Liver}) were fed the atherogenic diet between 8 and 16 weeks of age. **(A)** Plasma was prepared at the time of sacrifice (n = 5 per genotype). A single representative photograph of plasma is shown for each genotype. **(B)** Triglyceride was measured in plasma and liver (n = 5 per genotype). Each bar represents the mean ± SE. ***, P < 0.001. **(C)** Protein extracts were prepared from liver and analyzed by western immunoblotting for low-density lipoprotein receptor (Ldlr) abundance. Protein expression was normalized to the heat shock protein 90 (Hsp90) signal. The abundance of each protein is relative to WT level with each bar representing the mean ± SE of 8 mice. ***, P < 0.001. A microsomal subcellular fraction was purified from differential centrifugation by velocity from total liver extracts and analyzed by western immunoblotting for Ldlr abundance. Ldlr signal was normalized to the calnexin signal. Two representative samples are shown for every subcellular fraction. The abundance of Ldlr is relative to WT level with each bar representing the mean ± SE of 6 mice. ***, P < 0.001.

ΔSel1l^{Liver} mice exhibit defects in bile acid metabolism

Sel1l was recently identified in a genome wide association study as a candidate gene in controlling the plasma concentration of total bile acid [18]. This led us to ask whether ΔSel1l^{Liver} mice fed the AD diet would have altered bile acid metabolism. ΔSel1l^{Liver} mice exhibited a 91% increase in total bile acids when assayed in plasma ($P < 0.001$), whereas no such effect was observed in liver (Figure 3.8A).

We then focused on mechanisms that could account for abnormally elevated total bile acids in plasma of ΔSel1l^{Liver} mice. In liver tissue, we measured the protein abundance of the rate-limiting enzyme of bile acid synthesis (Cyp7a1) and of NTCP a transporter protein involved in the reuptake of plasma bile acids in liver respectively. Cyp7a1 was decreased ($P < 0.01$), whereas NTCP was not altered in ΔSel1l^{Liver} mice (Figure 5.8B). These data suggest defects in bile acid metabolism in absence of Sel1l in liver.

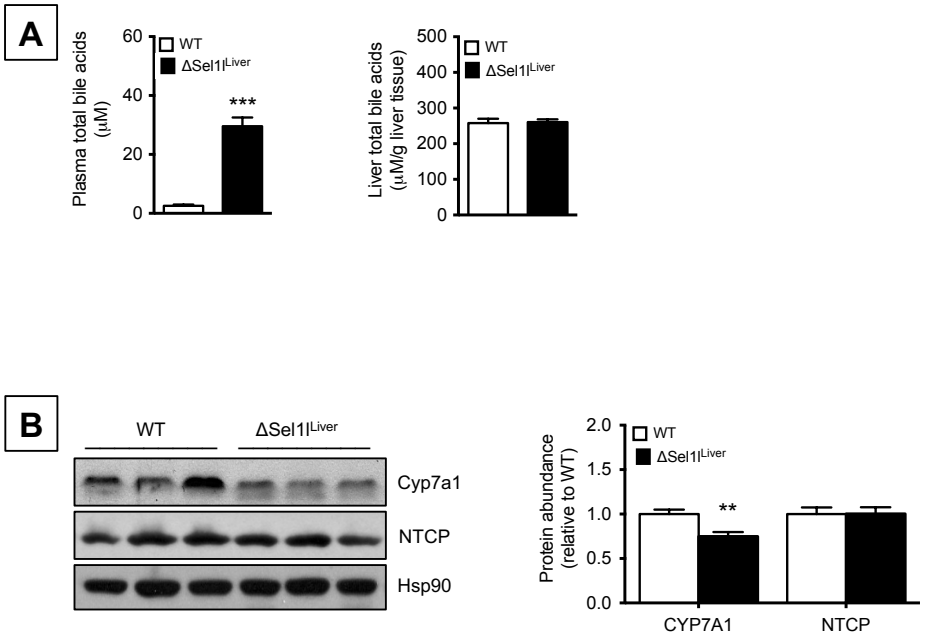

Figure 5.8 ΔSel1l^{Liver} mice exhibit defects in bile acid metabolism. Male mice harboring wild type Sel1l (WT) or null Sel1L in liver (ΔSel1l^{Liver}) were fed the atherogenic diet between 8 and 16 weeks of age. Total bile acid was measured in plasma and liver (n = 5 per genotype). Each bar represents the mean ± SE. ***, P < 0.001. **(C)** Liver tissue protein extracts were analyzed by western immunoblotting for family 7, subfamily a, polpeptide 1 (Cyp7a1) and solute carrier family 10 (sodium/bile acid cotransporter family), member 1 (NTCP) abundance. Protein expression was normalized to the heat shock protein 90 (Hsp90) signal. The abundance of each protein is relative to WT level with each bar representing the mean ± SE of 8 mice. ***, P < 0.001.

DISCUSSION

Cells actively involved in the synthesis and secretion of proteins utilize the ERAD system to target mis-folded or improperly assembled proteins for proteasomal degradation [19]. Sel1l has been identified as an indispensable component of the ERAD system involved in maintaining ER homeostasis [7]. Sel1l is known to form a 1:1 stoichiometric complex with Hrd1 within the ERAD system [20]. Sel1l, however, is six-fold more abundant than Hrd1, leading to the speculation that it is involved in roles independent of the ERAD system [21]. Until recently, however, the only evidence suggestive of an ERAD system-independent role of Sel1l was provided by mice with adipose-specific ablation. In these mice, Sel1l was found to be essential for lipoprotein lipase maturation and storage of lipid in adipose tissue [10]. These observations led us to ask whether deletion of Sel1l in hepatocytes would impact lipid homeostasis.

We first validated that Sel1l mRNA and protein abundance in liver were significantly decreased in $\Delta Sel1l^{Liver}$ mice. As anticipated from other tissue-specific Sel1l knockout mouse models [7,10,13], hepatic Sel1l ablation was associated with substantial increases in markers of ER stress. When placed on a normal chow diet, $\Delta Sel1l^{Liver}$ mice exhibited a slight growth deficit and decreased plasma glucose levels during the first few weeks of life, but did not have any alterations in lipid metabolism [10]. Feeding a HFD resulted in similar effects on growth and early life glycemia, but also led to increased plasma cholesterol levels, suggesting a role for liver Sel1l in lipid metabolism.

To probe this role further, mice were offered an AD diet between 8 and 16 weeks of life. AD is frequently used to amplify defects in lipid metabolism and is particularly

effective in altering the plasma lipoprotein profile in mice. For example, Ldlr knockout mice fed AD experienced reduced high-density lipoprotein (HDL) and increased very low-density lipoprotein (Vldl), Idl, and Ldl in plasma [22].

As expected, the AD revealed major defects in the lipid metabolism of $\Delta Sel1L^{Liver}$ mice. Specifically, AD feeding to $\Delta Sel1l^{Liver}$ mice led to a pale and enlarged liver with 'balloon-shaped' hepatocytes and increased the plasma levels of the marker of liver damage, ALT. $\Delta Sel1l^{Liver}$ mice suffered from increased levels of triglyceride and cholesterol in liver and plasma.

We first attempted to understand the basis for increased liver and plasma cholesterol in $\Delta Sel1l^{Liver}$ mice. Hmgcr has been previously implicated as a critical enzyme of cholesterol homeostasis in liver [23,24]. This enzyme is normally down regulated when dietary cholesterol is in excess. Indeed, feeding AD to WT mice suppressed the mRNA expression of genes involved in cholesterol biosynthesis including Hmgcr [25]. Surprisingly, our data showed increased Hmgcr protein expression in $\Delta Sel1l^{Liver}$ mice fed AD, suggesting loss of the cholesterol negative feedback. To test this mechanism in a functional manner, we asked whether statins could rescue the growth phenotype and mortality of $\Delta Sel1l^{Liver}$ mice. Statins are a class of pharmacological drugs that repress cholesterol synthesis by competing HMG-CoA binding to Hmgcr [26]. Treatment of $\Delta Sel1l^{Liver}$ mice fed AD with statins, however, did not correct the phenotype of $\Delta Sel1l^{Liver}$ mice. An important question that was not addressed is whether statin normalized plasma and liver TG and cholesterol levels in $\Delta Sel1l^{Liver}$ mice.

A second possible contributor to abnormal lipid metabolism of ΔSel1l^{Liver} mice could be inappropriate hepatic uptake of lipoproteins. The Ldlr is involved in the uptake of lipoprotein-enriched cholesterol molecules [chylomicron remnants, Idl, and Ldl] [24]. Mice with targeted disruption of Ldlr experienced significant increases in plasma cholesterol in absence of changes in plasma triglyceride when placed on AD [27]. This phenotype was due to decreased clearance of plasma chylomicron remnants, Vldl, Idl, and Ldl from the circulation [27,28]. As shown by our data, ΔSel1l^{Liver} mice fed AD exhibited decreased hepatic Ldlr mRNA and protein abundance, suggesting that this defect contributes to their increased plasma cholesterol. Deficiency in Ldlr, however, may not be solely responsible as liver also removes chylomicron remnants, Idl, and Ldl via another receptor known as low density lipoprotein-related protein (LRP) [29]. LRP can increase uptake of lipoproteins when Ldlr is downregulated or absent [30]. Unlike Ldlr, LRP abundance is not subject to cholesterol negative feedback [31]. Mice with targeted deletion of LRP in liver experienced increased plasma total cholesterol, reflecting an increase of plasma lipoproteins [32]. It will be important in future work to determine the impact of ΔSel1l^{Liver} mice on all possible lipoprotein receptors as well as impact on the profile of circulating lipoproteins.

Another critical factor to consider in explaining the abnormal lipid phenotype of ΔSel1l^{Liver} mice involves defective machinery involved in lipoprotein hydrolysis. For example, hepatic lipase is a lipolytic enzyme in liver that is synthesized, processed, and secreted from the ER [33]. It is primarily found in the circulation, but also localizes on the surface of hepatocyte sinusoid capillaries. Hepatic lipase exerts a hydrolytic effect on

triglyceride present in Vldl, promoting its conversion to Idl or Ldl in circulation [34]. Rat livers perfused with hepatic lipase antibodies have reduced hepatic uptake of triglycerides [35]. Humans with loss of function mutations of hepatic lipase were predisposed to hyperlipidemia [36]. In contrast, transgenic mice overexpressing hepatic lipase showed reduced plasma Ldl on a normal chow diet and reduced plasma Vldl, Idl, and Ldl on a HFD [37]. As Sel1l was previously shown to be essential for secretion of mature lipoprotein lipase from adipose tissue, it may play a similar role for hepatic lipase secretion from liver.

Conversion of excess cholesterol to bile acids is a mechanism whereby cholesterol homeostasis is maintained [38]. Accordingly, diets with excessive cholesterol and cholic acid can induce alterations in bile acid homeostasis [39]. Interestingly, dogs with a missense mutation in Sel1l (S658P) exhibit increased levels of plasma total bile acid [18]. Very recently, a genome wide association study (GWAS) was performed on C57BL/6J-chr12$^{A/J}$/NaJ consomic mice fed the AD in order to identify loci influencing liver-related metabolic phenotypes. Through this study, Sel1l was identified as a mediator of bile acid homeostasis [18]. C57BL/6J-chr12$^{A/J}$/NaJ consomic mice on the AD showed decreased Sel1l protein expression, increased plasma total bile acids, and increased plasma cholesterol when compared to C57Bl/6 control mice [18]. We found similar results of increased total bile acid in plasma of ΔSel1l^{Liver} mice fed AD.

Hepatocyte basolateral bile acid transporters are responsible for hepatic reuptake of bile acids from the enterohepatic circulation. Wu et al observed a significant decrease in hepatocyte basolateral bile acid transporters including NTCP and solute carrier family

22 (organic anion transporter), member 7 (slc22a7) by feeding AD to C57BL/6J-chr12$^{A/J}$/NaJ consomic mice [18]. In contrast, we observed no differences in NTCP abundance in liver of ΔSel1l^{Liver} mice fed AD. Further experiments are required to determine if additional bile acid transporters including slc22a7 are altered in ΔSel1l^{Liver} mice.

Increasing evidence continues to support that chronic hypercholesterolemia is a contributor to the onset of metabolic diseases including fatty liver, hepatic steatosis, and atherosclerosis [17,40,41]. Our findings therefore have potential therapeutic implications. To the best of our knowledge, this is the first direct evidence of a role for hepatic Sel1l in regulating lipoprotein metabolism. Further studies are required to gain a more complete understanding of the molecular mechanisms linking Sel1l, lipid, and cholesterol homeostasis.

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CHAPTER 6

SUMMARY AND CONCLUSIONS

Part I: Adiponectin

The mammary gland of early lactating (EL) dairy cows uses over 80% of the whole animal glucose flux. A key mechanism promoting mammary glucose utilization during this period is insulin resistance (IR) [1–3]. However, we know surprisingly little about factors responsible for the development of IR in transition dairy cows. We and others have demonstrated a sudden drop in the plasma concentration of the insulin-sensitizing hormone adiponectin between late pregnancy (LP) and EL in transition dairy cows [4–6]. However, factors regulating the periparturient drop in plasma adiponectin remain unknown.

In our first experiment, we assessed whether metabolic and hormonal factors [i.e. energy balance, adiposity, insulin, growth hormone (GH), and non-esterified fatty acids (NEFA)] previously shown to regulate adiponectin in rodents and humans, contribute to the variation in plasma adiponectin in transition dairy cows. Dairy cows dried off at parturition and remaining in positive energy balance maintained higher plasma adiponectin than lactating cows in negative energy balance (NEB). This study also showed that the periparturient recovery of plasma adiponectin occurred independently of changes in adiposity. Increasing circulating levels of leptin, insulin, GH, and NEFA did not have any effects on plasma adiponectin.

We previously demonstrated that the periparturient drop in plasma adiponectin in dairy cows occurred in absence of changes in adiponectin mRNA. In rodents, adiponectin oligomer assembly is coordinated by a subset of endoplasmic reticulum (ER) chaperone proteins [i.e. glucose-regulated protein, 78 kDa (Grp78), endoplasmic reticulum protein

44 (Erp44), endoplasmic reticulum oxidoreductase α (ERO1-Lα), protein disulfide isomerase associated 6 (Pdia6), disulfide bond A oxidoreductase-like protein (DsbA-L), and gamma adaptin ear containing, ARF binding protein 1 (Gga1)] [7,8]. ER chaperone expression is in part coordinated by the master regulator of ER homeostasis x-box binding protein 1 (Xbp1) [9,10]. Xbp1 increased plasma adiponectin by raising ER chaperone expression in white adipose tissue (WAT) of mice [11]. In dairy cows, several ER chaperone proteins (i.e. Grp78, Erp44, and Pdia6) are reduced in WAT during EL. These data led us to hypothesize in our second experiment that Xbp1 is decreased in white adipose tissue (WAT) in EL and that this reduction accounts for decreased ER chaperone expression and adiponectin production. We showed that Xbp1 was decreased in WAT of EL dairy cows. Using a primary bovine adipocyte system, we assessed whether Xbp1 impacted ER chaperone expression and adiponectin production and secretion. Raising Xbp1 expression increased several ER chaperones including Grp78, Erp44, and Pdia6, but had no effect on adiponectin mRNA or secretion in media. Next, we assessed whether other factors regulating adiponectin in other species [exogenous lipid, tumor necrosis factor α (TNFα), and GH] were involved in bovine adipocytes, and if the case, whether the effect related to ER chaperone regulation. TNFα and absence of exogenous lipid reduced adiponectin secretion, but these effects were not associated with changing ER chaperone expression. Similar to our work in dairy cows (Chapter 3), GH had no effect on adiponectin mRNA or secretion in media in bovine adipocytes.

In rodents and humans, adiponectin production and secretion varies between adipose tissue depots (i.e. visceral *vs*. subcutaneous). For example, adiponectin secretion

from human adipose tissue is higher in the visceral depot, than the subcutaneous fat depot after treatment with thiazolidinedione [12]. Another study suggested that subcutaneous white adipose tissue (scWAT) accounts for only 8% of plasma adiponectin in human subjects [13]. In the mouse, the contribution of scWAT was inferred to be 30% based on studies of a transgenic mouse model lacking scWAT [14]. However, studies in dairy cows are limited to scWAT, due to difficulty of obtaining biopsies from other adipose tissue depots. Adiponectin protein abundance measured between adipose tissue depots (visceral and subcutaneous) increased in a similar manner across lactation in dairy cows in absence of changes in adiponectin mRNA [15,16]. However, it is important to note that this experiment did not measure adiponectin mRNA and protein abundance from various adipose tissue depots in transition dairy cows during LP. Future mechanistic studies involving the periparturient drop and recovery of plasma adiponectin in transition dairy cattle need to consider other major fat depots.

In rodents and humans, prolonged calorie restriction and/or weight loss increase plasma adiponectin in absence of changes in both adiponectin expression and secretion from adipose tissue [17,18]. Transplanting adipose tissue into fat-less A-ZIP/F-1 mice increased plasma leptin, but had little effect on adiponectin [19]. Additional sources of adiponectin other than adipose tissue must therefore regulate its plasma concentration. Bone marrow adipose tissue (MAT) has been suggested to produce and secrete adiponectin in mice, rabbits, and humans [20]. Detectable adiponectin mRNA and protein were reported *in vivo* (i.e. mouse and human bone marrow) and *ex vivo* (i.e. rabbit and human MAT explants) [20–23]. Primary bone marrow adipocytes isolated from rabbit and

human bone marrow secrete adiponectin [20]. Two independent groups developed an *ex vivo* system to culture adipocytes derived from bone marrow of fetal calves [24,25]. Unfortunately, both studies did not assess adiponectin production or secretion. Future studies should be performed to determine whether MAT contributes to the quadratic variation of plasma adiponectin in the periparturient dairy cow.

Part II: Sel1l

The liver is an extraordinarily efficient organ in terms of synthesis and secretion of both proteins and lipids. Accordingly, sustaining endoplasmic reticulum (ER) homeostasis is critical for secretory cells. Imbalances in ER function occur in secretory cells undergoing increased synthesis [26]. Cells therefore rely on key quality control systems including the unfolded protein response (UPR) and endoplasmic reticulum-associated degradation (ERAD) to maintain ER homeostasis under conditions of induced stress [27]. A myriad of liver diseases including fatty liver and cholestasis are associated with increased activation of UPR and ERAD [28,29]. Recent evidence implicated the ERAD transmembrane protein sel-1 suppressor of lin 12-like protein (Sel1l) in maintaining lipid homeostasis in adipose tissue [30].

In this work, we assessed whether Sel1l plays a similar role in sustaining lipid homeostasis in liver. To this end, we generated an experimental mouse model whereby Sel1l was specifically ablated in liver ($\Delta Sel1l^{Liver}$). On a normal chow diet, $\Delta Sel1l^{Liver}$ mice showed a slight growth deficit during the first few weeks of life. $\Delta Sel1l^{Liver}$ mice did not exhibit defective indices of lipid metabolism, despite exhibiting increased expression

of ER stress markers. ΔSel1l^{Liver} mice placed on a HFD showed a slight growth deficit. When placed on an atherogenic diet (AD), ΔSel1l^{Liver} mice exhibited hepatomegaly with 'ballooned' hepatocytes, increased triglyceride, and cholesterol in liver and plasma, and total bile acid in plasma. Although this study provides unambiguous evidence of Sel1l playing a role in lipid metabolism in liver, the exact mechanisms are still unknown.

Future studies on ΔSel1l^{Liver} mice could focus on the profile of circulating lipoproteins. This profile would help to identify factors accounting for hypertriglyceridemia and/or hypercholesterolemia in plasma and narrow down possible hepatic pathways affected by absence of Sel1l. Mouse models exhibiting defects in lipid metabolism are characterized by changes in the lipoprotein profile compared to healthy controls. For example, Ldlr knockout (ΔLdlr) mice fed an AD showed increased intermediate-density lipoprotein (Idl), low-density lipoprotein (Ldl), and very low-density lipoprotein (Vldl), and decreased high-density lipoprotein (Hdl) [31]. Humans, non-human primates and other species suffering from hypercholesterolemia showed increased Vldl, Idl, and/or Ldl [32]. Measurement of circulating lipoproteins should therefore be performed as a first step to understand the defects in lipid metabolism of ΔSel1l^{Liver} mice fed AD.

Rodent models of hepatic steatosis showed increased expression of transcription factors and enzymes involved in *de novo* lipogenesis [33]. Increased ER stress was previously linked to increased hepatic lipogenesis in mice [34]. Sha et al reported that elevated plasma triglyceride and hepatic lipids were associated with increased expression of lipogenic genes in liver of ΔSel1l^{Adipose} mice on a high fat diet [30]. It will be important

in future studies to address the possibility that increased hepatic lipogenesis contributes to the hypertriglyceridemia of $\Delta Sel1l^{Liver}$ mice fed AD.

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Appendix I. ***Bovine primers used in real-time PCR analysis***

Gene[1]		Sequence[2]	Product (bp)	Accession No.
Fabp4				
	F	TGCACTTCTTTCTCACCTTGA	150	NM_174314.2
	R	CAGCCACTTTCCTGGTAGC		
Leptin				
	F	TCACCAGGATCAATGACATCTCA	96	NM_173928.2
	R	ACCAGTGACCCTCTGTTTGGA		
Adiponectin				
	F	GTGGCTCTGATTCCACACCT	83	NM_174742.2
	R	GCCATGACTGGGTAAGGCTA		
Erp44				
	F	TGATTGTGCCTTCCTAGCTGCGT	100	NM_001035032.2
	R	ATCTGGAGCAGAATGCCCTGGTG		
ERO1-Lα				
	F	CTACAAGTATTCTGAAGAAGCC	88	NM_001103348.1
	R	CTCACTCAGAGATTCATCCA		
Pdia6				
	F	ACCAGGTTCTCGCCAGCCGAT	96	NM_001206345.1
	R	TGCGTCCCCCGTCGTAATCCA		
Grp78				
	F	CATCACGCCGTCTTATGTGG	76	NM_001075148.1
	R	GGTGAGCTGGTTCTTGGCTG		
DsbA-L				
	F	CTCCGACCGGCTGGAGTTGC	60	NM_001079616.2
	R	GGCACAGGGCCCATCCACTT		
Gga1				
	F	GGCCAGCATCAACGGCTTCTGTG	99	NM_001035408.1
	R	TCCTGCGGGGACTGGATCTTGT		
$Xbp1_T$				
	F	TTGTCACCCCTCCAGAACATC	97	NM_001034727.3
	R	TCCAAGTTGAACAGAATGCCC		

$Xbp1_S$				
	F	TGCTGAGTCCGCAGCAGG	94	NM_001271737.1
	R	CATCAGAGTCCATGGGGAGA		
18S				
	F	GATCCATTGGAGGGCAAGTCT	74	NR_036642.1
	R	GCAGCAACTTTAATATACGCTATTGG		

[1]Primers were designed to measure the abundance of fatty acid binding protein 4 (Fabp4), leptin, adiponectin, endoplasmic reticulum resident protein 44 (Erp44), endoplasmic reticulum oxidoreductase 1-like α (ERO1-Lα), protein disulfide isomerase family associated 6 (Pdia6), glucose-regulated protein, 78 kDa (Grp78), glutathione S-transferase kappa 1 (DsbA-L), golgi associated, gamma adaptin ear containing, ARF binding protein 1 (Gga1), x-box binding protein 1 total ($Xbp1_T$), spliced variant Xbp1 ($XBP1_S$), and 18S ribosomal RNA (18S).
[2]Primer sequences are shown in a 5' to 3' orientation.

Appendix II. Fatty acid profile of diets

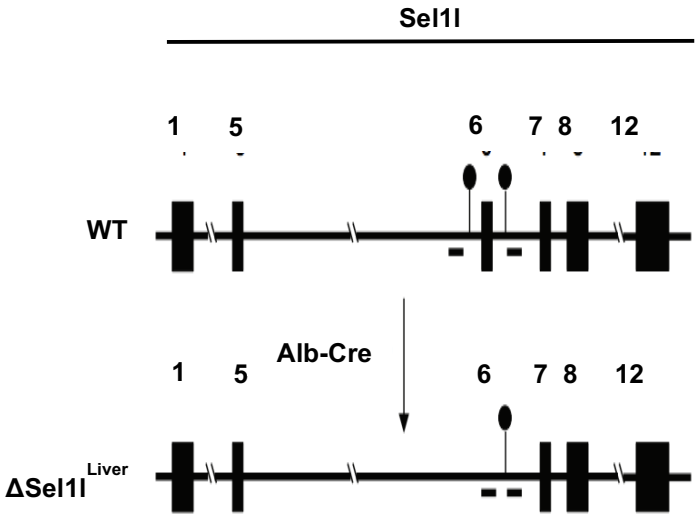
	Normal chow (LM-485)	High fat diet (TD.06414)	Atherogenic diet (TD.88051)
Fatty acids (g/kg)			
14:0 Myristic	-	4.7	-
16:0 Palmitic	0.6	80.2	19.4
18:0 Stearic	0.2	39.3	25.9
18:1 Oleic	1.3	146.8	26.5
18:2 Linoleic	2.6	47.0	17.6
18:3 Linolenic	0.3	5.5	1.28
16:1 Palmitoleic	-	9.6	0.23
20:0 Arachidic	-	94.7	0.83
Saturated fatty acid	0.8	124.8	74.0
Monounsaturated fatty acid	1.3	160.5	55.7
Polyunsaturated fatty acid	2.9	54.0	20.4
n-3	-	-	1.28
n-6	-	-	17.6

Appendix III. ***Murine primers used in real-time PCR analysis***

Gene[1]		Sequence[2]	Product (bp)	Accession No.
Sel1l				
	F	GATGAGTGCACCTCAGACGG	135	NM_001039089.1
	R	TGCTTCCTGCATCTGTCGTC		
p58IPK				
	F	TTGACGGTGCCGATTACACT	97	NM_008929
	R	CATTCTGCTCGCAGTTCACG		
Hrd1				
	F	AGCTACTTCAGTGAACCCCACT	169	NM_001164709
	R	CTCCTCTACAATGCCCACTGAC		
Herp				
	F	TCATGGGTGCCACTGTAGTC	104	NM_022331.1
	R	AGCATCTCGAGGACCACCAT		
RAMP4				
	F	AGAAAAGGCGTCGGTAGGAC	112	NM_030685.3
	R	TCAGTCACTTCACATGCCCA		
$Xbp1_S$				
	F	AACAGAGTAGCAGCGCAGAC	195	NM_001271730.1
	R	AGGATCCAGCGTGTCCATTC		
Chop				
	F	ATGTTGAAGATGAGCGGGTGG	109	NM_007837
	R	GCTTTCAGGTGTGGTGGTGTA		
Grp78				
	F	GATCAGCGGGTCATGGAACA	111	NM_001163434.1
	R	AGCCTTTTCTACCTCACGCC		
ERdj3				
	F	AGGAGCGAAGAGAACTGGACT	116	NM_026400.5
	R	AAATGACTCCAATCCCCAGCC		
Gabra1				
	F	ATTCTGAGCACACTGTCGGG	110	NM_010250.5
	R	TCATAACCGTCCAGCAGTCG		

Hmgcr				
	F	GTAAGCGCAGTTCCTTCCGC	105	NM_008255.2
	R	TTGTAGCCTCACAGTCCTTGG		
Ldlr				
	F	CACTCGCCCAAATTCACCTG	165	NM_010700.3
	R	ATCCTCACTGTGCTTCGGTG		
18S				
	F	GTGGGCCTGCGGCTTAAT	171	NR_003278
	R	GCCAGAGTCTCGTTCGTTATC		

[1]Primers were designed to measure the abundance of sel-1 suppressor of lin-12-like protein (Sel1l), DnaJ heat shock protein family (Hsp40) member C3 (p58IPK), synovial apoptosis inhibitor 1, synoviolin (Hrd1), homocysteine-inducible endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1 (Herp), stress-associated endoplasmic reticulum protein 1 (RAMP4), spliced variant x-box binding protein 1 (Xbp1s), DNA-damage inducible transcript 3(Chop), Glucose-regulated protein, 78 kDa (Grp78), DnaJ heat shock protein family (Hsp40) member B11 (ERdj3), gamma-aminobutyric acid (GABA) A receptor subunit alpha-1 (Gabra1), 3-Hydroxy-3-methylglutaryl-Co A reductase (Hmgr), low-density lipoprotein receptor (Ldlr), and 18S ribosomal RNA (18S).
[2]Primer sequences are shown in a 5' to 3' orientation.



Appendix IV. Schematic representation of ΔSel1l^{Liver} mice. The black filled boxes represent exons. The black circles with straight lines represent lox p sites placed specifically between exon 6. Genotyping primers are shown as thin black lines and bracket an 800 bp region for the WT gene and a 200 bp region for the mutated Sel1l gene.