

Aspartyl-Asparaginyl- β -Hydroxylase (AAH) Regulation and Function in Liver Disease

By

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LIST OF ABBREVIATIONS

4-MUP	4-methylumbelliferyl phosphate
AAH;ASPH	aspartyl-asparaginyl- β -hydroxylase
Ala/A	alanine
ALD	alcoholic liver disease
AMPK	5' adenosine monophosphate-activated protein kinase
ANOVA	analysis of variance
Asn	asparagine
Asp	aspartic acid
ATF-6 α	activating transcription factor-6 α
BCA	bicinchoninic acid assay
BSA	bovine serum albumin
CHOP	C/EBP homologous protein
CK1	casein kinase 1
CK2	casein kinase 2
DAPI	4',6-diamidino-2-phenylindole
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ERAD	ER associated protein degradation

ERK	extracellular-signal-regulated kinase
ERO-1	endoplasmic oxidoreductin-1
EV	empty vector
F	forward
GFP	green fluorescent protein
Grb-2	growth factor receptor bound protein 2
GRP78	78 kDa glucose-regulated protein
GSK-3 β	glycogen synthase kinase-3 β
H&E	hematoxylin and eosin
HES	hairy-enhancer of split
HIF-1 α	hypoxia inducible factor-1 α
HRP	horseradish peroxidase
IGF	insulin-like growth factor
IGF-1R	insulin-like growth factor-1 receptor
IP3	inositol-1,4,5-triphosphate receptor
IR	insulin receptor
IRE-1 α	inositol-requiring enzyme-1 α
IRS-1	insulin receptor substrate-1
K _d	dissociation constant
LDH	lactate dehydrogenase
LSC	liver slice culture

LSD	least significant difference
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase kinase
MICE	Microtiter immunocytochemical ELISA
NID	notch-1 intracellular domain
ORO	Oil red-O
p70S6K	ribosomal protein S6 kinase
PDI	protein disulfide isomerase
PDK-1	phosphoinositide-dependent kinase-1
PERK	PKR-like ER-localized eIF2 α kinase
PI3K	phosphoinositide 3-kinase
PIP ₂	phosphatidylinositol 4,5-bisphosphate
PIP ₃	phosphatidylinositol (3,4,5)-triphosphate
PH	pleckstrin homology
PKA	protein kinase A
PKC	protein kinase C
PP1	protein phosphatase 1
PPAR	peroxisome proliferator activated receptor
PRAS40	proline-rich Akt substrate of 40 kDa
PTEN	phosphatase and tensin homolog
PVDF	polyvinylidene fluoride

R	reverse
RPLPO	large acidic ribosomal protein
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
Ser/S	serine
SH2	src-homology-2
SOS	son of sevenless
SPT	serine palmitoyltransferase
SR	sarcoplasmic reticulum
SREBP-1	sterol regulatory binding element protein-1
TBB	4,5,6,7-tetrabromo-2-azabenzimidazole
TBS	TRIS buffered saline
TLR	toll-like receptor
TNF- α	tumor necrosis factor- α
Thr/T	threonine
Tyr	tyrosine
UPR	unfolded protein response

CHAPTER 1
INTRODUCTION AND SPECIFIC AIMS

1.1. OVERVIEW

Alcoholic liver disease (ALD) and hepatocellular carcinoma (HCC) have in common the dysregulation of insulin and insulin-like growth factor (IGF) signaling [1–5]. Excessive chronic or binge alcohol consumption causes steatohepatitis, which progresses to ALD by the contribution of various factors including inflammation, oxidative and endoplasmic reticulum stress, mitochondrial dysfunction, and insulin as well as insulin-like growth factor-1 (IGF-1) resistance [6–9]. Ethanol disrupts insulin/IGF-1 signaling networks at various points of the cascade, impairing downstream signaling through ERK mitogen-activated protein kinase (MAPK) and phosphatidyl-inositol-3-kinase (PI3K). Impaired signaling through MAPK disrupts liver growth and repair after toxic injury, while disruption of signaling through PI3K impairs hepatocyte survival, metabolism, and migration, which is needed for liver remodeling. The enzyme aspartyl-asparaginyl- β -hydroxylase (AAH) is a downstream target of insulin/IGF-1 signaling with an essential role in promoting cell adhesion and migration [10,11]. In the liver, ethanol exposure downregulates AAH expression, disrupting hepatocyte migration and remodeling of the liver remodeling [7,12].

On the other hand, insulin/IGF-1 signaling networks are upregulated in HCC, increasing signaling through ERK-MAPK, which promotes mitogenesis and gene expression [13], and through PI3K, which promotes glucose uptake [14], inhibits apoptosis, and stimulates tumor cell migration and invasion [4,5,15]. As a downstream target of insulin/IGF-1 signaling that is also regulated through ERK-MAPK and PI3K, AAH is also overexpressed in HCC, mediating tumor cell migration, invasion, and aggressiveness [16,17]. Several lines of evidence indicate that AAH mediates its function via the activation of Notch signaling networks [3,11,18,19]. The purpose of the present work was to investigate the mechanisms that govern the regulation of AAH

expression and function and its consequences on the activation of Notch signaling in liver diseases.

1.2. INSULIN/IGF-1 SIGNALING

1.2.1. Insulin/IGF-1 signaling activation. Insulin and IGF-1 signaling promotes hepatocyte growth and survival by activation of a signaling cascade of events that start by the interaction of insulin or IGF-1 polypeptide to its corresponding receptor (**Figure 1-1**). The insulin/IGF-1 receptors are composed of two extracellular α and two transmembrane β subunits. Insulin/IGF-1 binds to the α -subunits and triggers a conformational change in the β -subunits, activating the receptor's intrinsic tyrosine (Tyr) kinase activity. Intrinsic kinase activation leads to autophosphorylation at Tyr residues 1158, 1162, and 1163 [20,21]. Phosphorylated Tyr (pTyr) residues are recognized by phosphotyrosine-binding domains present in “docking proteins,” such as the insulin receptor substrate-1 (IRS-1), which are then also phosphorylated at specific Tyr (pTyr) residues. Target proteins bind to pTyr-IRS-1 through their src-homology-2 (SH2) domain, leading to activation of downstream signaling.

Binding of the SH2 domains of growth factor receptor bound protein 2 (Grb-2) and the p85 subunit of PI3K activate two separate arms of insulin/IGF-1 signaling upon binding to pTyr-IRS-1. One arm of insulin/IGF-1 signaling is activated by the interaction of Grb-2 with son of sevenless (SOS), a complex that will then dock to pTyr-IRS-1, which activates SOS. SOS promotes exchange of guanosine diphosphate to guanosine triphosphate in Ras and activates it. Ras activates Raf kinase, which phosphorylates and activates MAPK kinase (MEK), which in turn phosphorylates and activates MAPK [22]. Another arm of insulin/IGF-1 signaling pathway involves activation of PI3K. Binding of SH2 domain of PI3K to pTyr-IRS-1 recruits PI3K to the plasma membrane, where the p110 subunit of PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP₂) to

yield phosphatidylinositol 3,4,5-triphosphate (PIP₃). The pleckstrin homology (PH) domains of phosphoinositide-dependent kinase-1 (PDK-1) and Akt recognize PIP₃, and bring the two proteins into close proximity. PDK-1 phosphorylates and activates Akt, which subsequently phosphorylates and deactivates glycogen synthase kinase 3 β (GSK-3 β), and activates important downstream signaling events.

1.2.2. Ethanol-impaired insulin/IGF-1 signaling. As previously alluded to, excessive chronic or binge alcohol consumption causes steatohepatitis, which progresses to ALD by the contribution of various factors including inflammation, oxidative and endoplasmic reticulum stress, mitochondrial dysfunction, and insulin and IGF-1 resistance [6–9]. Ethanol impairs insulin/IGF-1 signaling at various points of the cascade [12,23]. As displayed in **Figure 1-1**, ethanol impairs binding of insulin to its receptor, although it does not alter their expression. On the other hand, ethanol does affect the expression of IGF polypeptide and in this way impairs downstream receptor signaling [12]. Ethanol also increases phosphatase and tensin homolog (PTEN) by increasing the transcription factor p53, which dephosphorylates PIP₃ and decreases PDK-1-mediated phosphorylation and activation of Akt [1]. Lastly, ethanol increases GSK-3 β activity, independent of insulin signaling, through reactive oxygen species-mediated activation [24,25]. Ethanol increases oxidative stress, and GSK-3 β , as another mechanism of insulin/IGF-1 signaling inhibition. Ethanol impairs insulin signaling, hepatocyte migration needed for remodeling of the liver. A rat model of chronic ethanol exposure demonstrated that ethanol disrupts the liver's ability to remodel, losing its lobular architecture [12]. Ethanol causes this histopathological feature, at least in part, by disrupting insulin/IGF-1 signaling networks in the liver that decrease downstream expression of AAH, which is a molecule with an essential role in promoting cell adhesion and migration.

Migration is a cyclical process that starts with the cell's response to an external signal that leads to the polarization and extension of a protrusion in the direction of movement. The front or leading edge has actin polymerization that protrudes and adheres to the substratum. The rear end of the cell has stable bundles and decreased adhesion. This process is followed by protrusion in which the membrane extends in the direction of migration. The process is divided into three main steps that include the expansion of the plasma membrane, formation of an underlying backbone, and establish contacts with the substratum. The protrusion is produced by formation of actin filaments, a process regulated by calcium [42]. Adhesion occurs mainly through integrin receptors that attach protrusions to the substratum. These adhesions, at least in part, propel the cell forward and it is an integral process of migration. Contraction of the cell moves it forward, accompanied by the release of the attachments at the rear. Cell translocation and retraction of the rear completes the cycle of cell migration.

Ethanol mediates its inhibitory effects on AAH expression through increased glycogen synthase kinase-3 β (GSK-3 β) activity, and inhibition of Akt and MEK. Since ethanol increases GSK-3 β activity through impaired insulin signaling and increased oxidative stress, it is conceivable that the detrimental effects of ethanol on the liver may be mediated through GSK-3 β . GSK-3 β negatively regulates AAH, and in the context of ethanol exposure, in which GSK-3 β is overly active, GSK-3 β promotes the degradation of AAH, as a mechanism of impaired hepatocyte motility, needed for remodeling of the liver [23,26–28].

1.2.3. Upregulation on insulin/IGF-1 signaling in HCC. Insulin/IGF-1 signaling networks are upregulated in HCC, increasing signaling through ERK-MAPK, which promotes mitogenesis and gene expression [13], and through PI3K, which promotes glucose uptake [14], inhibits apoptosis, and stimulates tumor cell migration and invasion (**Figure 1-1**) [4,5,15]. MAPK promotes cell growth through signaling cues it receives

from epidermal growth factor receptor (EGFR) and IGFR, among others [5]. Interestingly, these receptors are upregulated in HCC and lead to over phosphorylation and activation of MAPK, upregulating transcription of genes involved in cell proliferation, angiogenesis, and glycolysis [29,30]. One mechanism of activation involves the downregulation of dual-specificity phosphatase 1, an inhibitor of ERK.

PI3K/Akt is also upregulated in HCC. A study demonstrated that PI3K is overly active in 40% of HCCs [31]. Mechanisms of aberrant PI3K/Akt activation include IGF and IGF receptor overexpression [4], gain-of function mutations in PI3K [32], and impaired expression and/or function of phosphatase and tensin homolog (PTEN), which converts PIP_3 to PIP_2 and prevents recruitment of Akt to the plasma membrane and its activation by PDK-1 [33]. In addition, protein phosphatase, another negative regulator of Akt that dephosphorylates the protein at Thr-450, is downregulated in the human Huh7 hepatoma cell line, contributing to aberrant activation of PI3K/Akt signaling [34].

AAH is one of the few distinct biomarkers of HCC. Overexpressed insulin/IGF-1 signaling increases AAH protein, promoting cell adhesion, migration, and invasion [35]. AAH immunoreactivity is mainly found at margins of infiltrating tumors [17,36,37] to mediate invasiveness of the tumor into surrounding tissue [16,35,37]. AAH is upregulated in HCC, compared to surrounding, cancer-free liver tissue at both the mRNA and protein levels. AAH mRNA was previously reported to be two-fold to ten-fold higher in HCC tumor tissue, compared to the non-HCC surrounding tissue. AAH mRNA upregulation correlated with AAH protein, since protein expression was approximately three-fold higher in HCC compared to surrounding HCC-free liver tissue [3,38].

Since AAH is overexpressed in HCC tissue, but present at low levels in surrounding healthy tissue, it makes AAH an outstanding biomarker for HCC detection, even in the early stages of the disease. Xian, *et al.* found AAH could be detected even in unifocal or small HCC, or in the early stage of the disease. Early HCC detection is

particularly important because at this stage adjuvant care is more effective. More specifically, long-term survival is only possible in patients with small, asymptomatic HCC and treatment options include surgical resection, liver transplantation, ethanol or acetic acid injections, and radiofrequency thermal ablation [39]. Unfortunately, HCC has a very poor prognosis due to the tumor's late stage of discovery and its incessant invasive growth pattern [40], which can be attributed to the actions of AAH protein as it promotes cell migration, tumor infiltration, and metastasis [3,10,16]. HCC is usually discovered when the patient displays symptoms. Unfortunately, at this stage the tumor is severely advanced leaving the patient with a small chance for survival. If the tumor is discovered when the symptoms are already apparent and the tumor is not a candidate for resection, the patient may only have six months left of life. Most studies report a five-year survival rate of less than 5% in symptomatic HCC patients. HCCs are resistant to radio- and chemotherapy.

According to the World Health Organization grading system, HCC tumors can be categorized based on their histological grade as well differentiated, moderately differentiated, and poorly differentiated [39]. Differentiation is a measure of the similarity of a particular tumor cell to the tissue it arose from. Well differentiated HCC is usually observed in small, early-stage tumors with a diameter less than 2 cm, and is the least aggressive form of HCC; moderately differentiated HCC is usually observed in tumors more than 3 cm, arranged in cells of 3 layers or more in thickness; poorly differentiated HCC has a solid pattern of growth and it is the most aggressive. AAH protein expression correlates with tumor differentiation and levels of aggressiveness.

Moreover, AAH levels correlate with intra-hepatic metastasis, suggesting that AAH increases the ability of HCC tumors to spread to other sites within the liver [38]. Intrahepatic metastases occurs by the spread of HCC through the portal vein of the liver and/or to other sites of the liver [41]. This is one of the main predictors of HCC

reoccurrence, rendering it difficult to cure and correlates with a poor prognosis. AAH levels have been shown to be an accurate predictor of survival since it correlates with infiltrative growth pattern. In a study where AAH levels were measured in intrahepatic cholangiocarcinoma tumors, levels of AAH correlated with years of survival after surgical removal of the tumor. There was a 40% patient survival approximately six years after surgery when they had low AAH protein expression (*i.e.* less than 50% of cells) compared to 20% patient survival when they had high levels of AAH protein (>50% cells). Therefore, AAH protein expression correlates with HCC tumor aggressiveness and a poor clinical prognosis [38]. To better understand AAH-mediated regulation of adhesion, migration, and invasion, we need to study more closely AAH gene and protein expression, as well as its regulation and function.

1.3. AAH

1.3.1. Gene. AAH is encoded by a ~200 kb gene in the human A β H-J-J locus, which is located in the long arm (q) of chromosome 8 at band 12 (8q12) [17,42]. The AAH gene diversifies by the use of alternative promoters and alternative splicing mechanisms that produce different gene products with diverse function and tissue distributions [42–45]. The gene is under the control of two promoter sequences, P1 and P2, located at the 5' end (**Figure 1-2**). The P1 promoter is controlled by Specificity protein transcription factor which regulates AAH mRNA expression in several human mature tissues, including liver, pancreas, heart, adrenal gland, brain, and skeletal muscle [46–48]. The P2 promoter, on the other hand, is regulated by myocyte enhancer factor (MEF)-2. P2 activates AAH transcription in excitable tissues, such as skeletal and cardiac muscles, as well as brain, with lower levels of expression in the kidneys [44].

In addition to using two alternative promoter sequences, the AAH gene diversifies its products by alternative splicing of its 24 exon sequences [43]. Two

identical AAH proteins are encoded by two mRNA transcripts that differ at the 3'-untranslated region and results from splicing together exons 1, 3, 5, and 8-17, and 19-24. Generation of two smaller AAH gene products, junctate and junctin, results from splicing of exons 2-5 and 8-18, and 2-5, 6, and 7, respectively [46]. The three AAH protein isoforms have in common exons that encode a fraction of the N-terminal cytosolic region, the transmembrane domain, but differ at the C-terminus in both length and function (**Figure 1-2**).

1.3.2. Protein. AAH is the longest gene product of the A β H-J-J locus and the only one with enzymatic activity when translated into protein. The cDNA that encodes AAH protein was first cloned and characterized in bovine liver, followed by its characterization in a human osteosarcoma cell line [49,50]. The AAH transcript is translated into a 758 amino acid transmembrane, or integral, protein of the endoplasmic reticulum with a calculated molecular weight of ~86 kD. Transmembrane proteins may be classified by their topology as types 1-4, according to the location of its amino (N-) terminus (**Figure 1-3**) [51]. Type 1 and 2 transmembrane proteins span the membrane once with the N-terminus facing the extracellular space (type 1) or the cytosol (type 2). Type 3 transmembrane proteins span the membrane more than once. Lastly, type 4 proteins are composed of n subunits linked by n polypeptides to form a group of domains that form an aqueous channel.

According to this system of classification, AAH is a type 2 transmembrane protein (**Figure 1-4A**) whose structure consists of four domains: an N-terminal (cytoplasmic) domain that faces the cytosol, a transmembrane domain that spans the membrane of the ER, a negatively-charged domain, and a C-terminal catalytic domain. The latter two domains are inside the ER lumen (**Figure 1-4B**). The first, N-terminal domain is mainly composed of positively-charged residues that appear to give this region an intrinsically disordered character. The software IUPred predicts the N-terminal region to have a high

tendency for disorder (>0.5) (**Figure 1-5A**), which correlates to a potentially unstructured region [52]. This IUPred prediction is supported by a decreased sedimentation coefficient and increased stokes radius of full-length AAH, suggesting an extended N-terminal region, compared to the more compact 52-56 kD C-terminal catalytic domain [49,53]. The second region corresponds to the transmembrane domain, which is characterized by a highly hydrophobic stretch of residues that anchor AAH to the ER membrane.

The third region of AAH is largely composed of negatively-charged residues and this region is identical to that of junctate. Although the function of this region has not been elucidated, it may interact with other proteins (>0.5) based on the ANCHOR software, which predicts regions in proteins that cannot form favorable interchain interactions and as a result, may interact with other globular binding partners to reach a more favorable energy state (**Figure 1-5B**). One possibility is that it may bind to other proteins to regulate calcium release from the ER, as it is the cases for the proteins junctate and junctin [42,54,55]. Junctate and junctin are also type 2 transmembrane proteins that are truncated isoforms of AAH (**Figure 1-2** and **Figure 1-4**). Junctate is a 32 kD protein whose C-terminal luminal region is also negatively-charged and has a high capacity to bind calcium (*i.e.* 21 mol calcium/mol protein) with moderate affinity (*i.e.* $K_D = 217 \mu\text{M}$). Interestingly, junctate was previously demonstrated to interact with the ryanodine receptors to modulate the flux of calcium from intracellular stores. Junctin, the smallest of the proteins generated by the AAH gene, regulates the flux of calcium from the ER and sarcoplasmic reticulum (SR) by forming a complex with the ryanodine receptor, triadin, and calsequestrin in excitable tissues, such as cardiac and skeletal muscles [56,57]. The junctate and junctin isoforms lack a fourth domain that is only present in AAH and confers the protein its catalytic activity.

The fourth domain of AAH contains catalytic activity which is essential to promote cell adhesion, migration, and invasion. AAH's catalytic activity, hydroxylation, was first observed in extracts of mouse L-cells and rat liver microsomes [49,53,58]. AAH belongs to the α -ketoglutarate-dependent dioxygenase family of dioxygenases, that includes prolyl-4 and lysyl-hydroxylases, which requires α -ketoglutarate and iron (II) to catalyze their reactions [59,60]. AAH hydroxylation begins with the interaction of iron (II), α -ketoglutarate, and molecular oxygen to form an iron (III) superoxide byproduct (**Figure 1-6**). The reactive oxygen then attacks the α -ketoglutarate at the carbon-2 position, yielding an iron (II) peroxy byproduct. The reactive product interacts with aspartate (Asp) or asparagine (Asn) to yield 3-hydroxyaspartic acid or 3-hydroxyasparagine, succinate, and carbon dioxide. Experimental evidence indicates the His-675 residue of AAH, which is also highly conserved among members of the α -ketoglutarate dependent family of dioxygenases, coordinates iron binding and is therefore essential for hydroxylation [49,61].

AAH undergoes physiological cleavage since addition of microsomal fractions produces a 52-56 kD fragment corresponding to the C-terminal catalytic domain contained inside the lumen of the ER [49,50,53,58]. The catalytic domain of AAH hydroxylates certain Asp/Asn residues present in epidermal growth factor (EGF)-like domains. EGF-like domains are based on the EGF structure that are known to interact with calcium [62]. The domains are 30 to 40 amino acids long, six of which are conserved cysteine (Cys) residues that fold the domain into its proper three-dimensional (3D) structure. The 3D structure of EGF-like domains is essential for AAH hydroxylation since synthetic peptides that only contained the consensus sequence (eight residue minimum) were not hydroxylated by AAH [63]. AAH hydroxylates Asp/Asn residues in the consensus sequence xxCysx**Asp/Asn**xxxxTyr/PhexCysxCysxx of EGF-like domains [64,65]. The consensus sequence for AAH hydroxylation is present in a wide variety of

protein targets including clotting factors [65], proteins that provide structural support in the extracellular matrix [62], and Notch signaling pathway proteins [66,67].

1.3.3. Relation to Notch signaling. Several lines of evidence have pointed to a direct link between AAH and the activation of Notch signaling networks since (1) Notch-1 and, its natural ligand, Jagged-1 contain the consensus sequence for AAH hydroxylation, (2) AAH physically interacts with Notch-1 and Jagged-1 [3], (3) inhibition of AAH decreases Notch-1, Jagged-1, and HES-1 expression, while over-expression yields the opposite effects and the exact mechanism is not currently understood [18,19], and (4) Notch-1 and Jagged-1 were previously demonstrated to promote cell adhesion and migration [68]. Notch signaling is essential for cell differentiation, embryonic development and tissue self-renewal. Notch-1 and Jagged-1 are large transmembrane proteins anchored to the cell membrane with extracellular EGF-like domains that are involved in ligand-binding and protein-protein interactions. Notch-1 contains 36 extracellular EGF-like domain repeats. AAH has been previously demonstrated to interact with Notch-1 and Jagged-1, Notch's natural binding partner, leading to downstream Notch signaling pathway activation (**Figure 1-7**). Binding of Jagged (or Delta-like family of proteins) to the Notch extracellular domain exposes a cleavage site in the extracellular domain cleaved by a protease of a disintegrin and metalloprotease domain family. The remaining transmembrane domain is cleaved by γ -secretase and frees the Notch intracellular domain (NID). At this point, the NID translocates from the plasma membrane to the nucleus, where it complexes with the transcriptional activator CBF1, suppressor of hairless or lag-1 (CSL) and the transcriptional coactivators of the mastermind family of proteins. The complex binds to DNA containing CSL recognition sequences, recruits additional factors and activates transcription of downstream targets such as hairy/enhancer-of-split-1 (HES-1) and hairy/enhancer-of-split related with YRPW motif protein-1 (HEY-1) [69,70].

1.3.4. Regulation of gene and protein expression through insulin and IGF-1 signaling. AAH is strictly regulated at the transcriptional and post-translational levels by hypoxia inducible factor-1 α (HIF-1 α), a transcription factor that activates transcription of hypoxia-inducible genes under conditions of hypoxia or oxidative stress, and by insulin/IGF signaling in the liver. AAH is also regulated by cyclin-dependent kinase-5, but only in the developing central nervous system [11].

The insulin/IGF-1 signaling pathway is essential to promote hepatocyte growth and survival through stimulation of gene expression, energy metabolism, cell survival, regeneration and remodeling, and cell migration. Insulin/IGF-1 signaling regulates AAH expression and cell migration at both mRNA and protein levels through ERK-MAPK and PI3K/Akt signaling cascades, respectively [71–73] (**Figure 1-1**). Previous reports from our laboratory indicated that insulin/IGF-1 stimulation increased MAPK/ERK phosphorylation and activation, and increased AAH mRNA as well as cell motility, while chemical inhibition of the kinase yielded the opposite effects [74]. Therefore, ras/raf/MAPK signaling is an important regulator of AAH expression, and ultimately its function. Furthermore, activation of PI3K/Akt leads to phosphorylation and deactivation GSK-3 β . Interestingly, previous evidence indicated that chemical or si-RNA inhibition of GSK-3 β promoted AAH protein expression as well as its function, while overexpression of the kinase decreased AAH protein expression and inhibited cell adhesion and migration through caspase-mediated degradation [22,75,76]. Taken together, signaling through PI3K/Akt/GSK-3 β modulates AAH protein expression likely through post-translational mechanisms of regulation.

Several lines of evidence indicate AAH is regulated through post-translational mechanisms since, as previously mentioned (1) chemical and si-RNA inhibition of GSK-3 β activity and gene expression increased AAH protein (not mRNA) expression and cell migration; (2) GSK-3 β overexpression decreased AAH protein and impaired motility [77];

(3) ethanol decreases AAH protein, but not its mRNA [28]; (4) it displays an apparent molecular weight between 120-140 kD by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), while its calculated molecular weight is ~86 kD [50], possibly due to post-translational modifications; (5) AAH can be phosphorylated in vitro by GSK-3 β at Ser/Thr residues [28]; and (6) MacVector 8.1 prediction software indicates AAH has several consensus sequences for phosphorylation by various kinases, including GSK-3 β , as well as protein kinase A (PKA), casein kinase 2 (CK2), protein kinase C (PKC) (**Figure 1-8**). The molecular mechanisms that govern the regulation of AAH by phosphorylation are currently unknown.

1.4. SPECIFIC AIMS

My graduate research focused on investigating the role of insulin and IGF signaling regulation of AAH and downstream Notch signaling in the liver. AAH is an ~86 kD transmembrane protein with demonstrated roles in cell adhesion, motility, and invasion. AAH promotes these functions, in part, through hydroxylation of Notch signaling pathway proteins, which upregulates expression of downstream genes involved in motility. Previous studies demonstrated AAH is regulated by insulin and IGF-1 at the post-translational level since (1) GSK-3 β activation inversely correlates with AAH expression and function; (2) it contains multiple predicted Ser/Thr sites of phosphorylation by GSK-3 β , as well as PKA, PKC, and CK2; and (3) it is super-shifted as observed by SDS-PAGE likely due to its phosphorylated state.

To further investigate insulin/IGF-1 regulation of AAH and Notch signaling in the liver, I employed pharmacological tools that are known to modulate insulin/IGF-1 signaling, such as ethanol, myriocin (an inhibitor of ceramide synthesis), and peroxisome proliferator activated receptor (PPAR) agonist treatments. Ethanol impairs insulin/IGF-1 signaling and causes aberrant lipid accumulation, while PPAR agonist treatments and

inhibitors of ceramide synthesis reduce lipid accumulation and improve insulin sensitivity after ethanol exposure. Since PPAR activation and inhibition of ceramide synthesis improve insulin/IGF-1 signaling, we were interested in determining if these treatments restored the downstream expression AAH and Notch signaling proteins. Additionally, we investigated the how AAH may promote Notch signaling activation and the post-translational mechanisms of regulation of AAH. The studies expanded our understanding of insulin/IGF-1 regulation of AAH and Notch signaling pathway activation, as promoters of hepatocyte motility, liver remodeling and regeneration.

The over-arching hypothesis is that insulin/IGF-1 signaling regulates AAH, which has consequences on downstream Notch signaling activation, through post-translational mechanisms of phosphorylation. To demonstrate that insulin/IGF-1 signaling modulates AAH and Notch signaling activation, we investigated (1) the ability of PPAR agonist and ceramide inhibitor treatments to restore the detrimental effects of ethanol on insulin/IGF-1 signaling, AAH, and Notch signaling activation, (2) post-translational regulation of AAH, and (3) the consequences of AAH phosphorylation at specific GSK-3 β sites on AAH expression and Notch signaling activation. The specific aims were organized as follows:

- 1. Determine the effects of treatment with myriocin ceramide inhibitor and insulin sensitizing agents (i.e. PPAR agonists) on the activation of insulin/IGF-1, AAH and Notch signaling activation in ethanol-exposed livers.*
- 2. Characterize IGF-1-stimulated AAH protein expression and kinase inhibition in Huh7 liver cells transfected with N-terminus-Myc-tagged and C-terminus-HA-tagged human wildtype cDNAs regulated by a CMV promoter.*
- 3. Characterize effects of site-directed mutations of AAH at predicted phosphorylation sites [Ser/Thr→Ala to inhibit] on AAH expression, subcellular localization, catalytic activity, Notch signaling, and cell motility and adhesion.*

Figure 1-1

Regulation of AAH Expression and Function

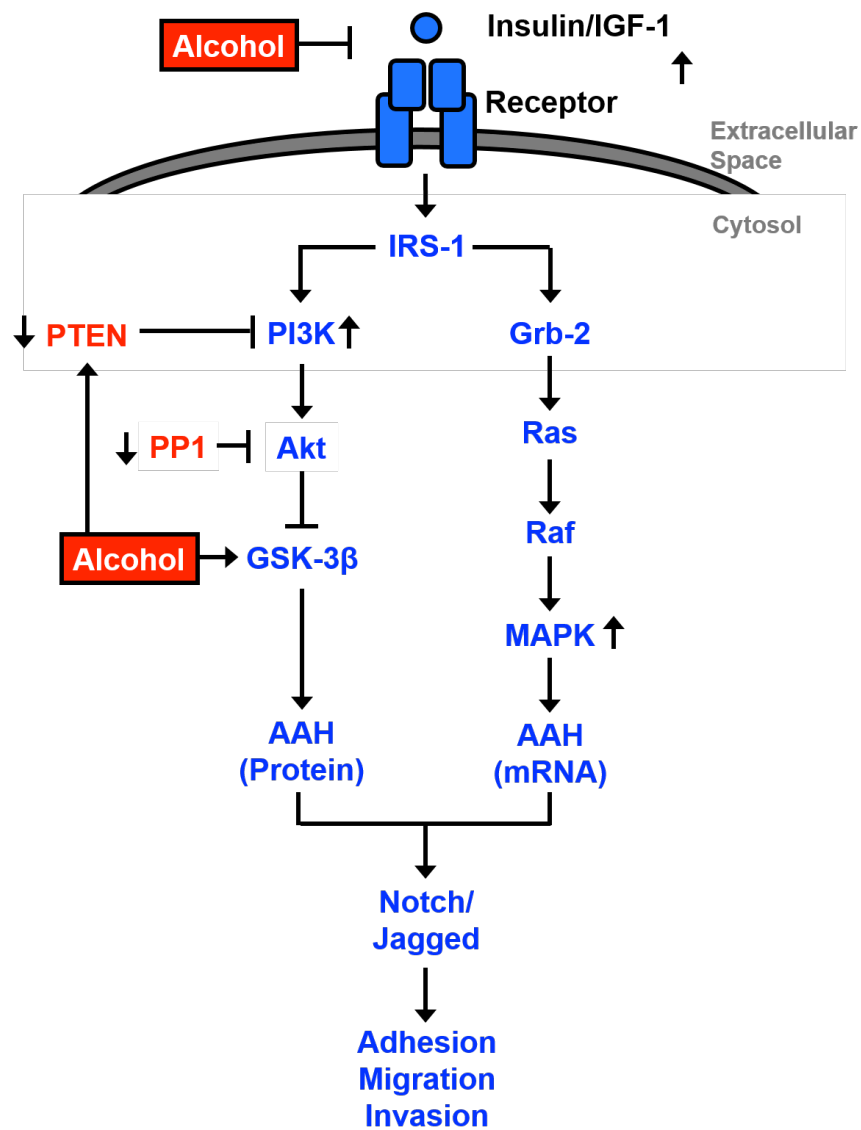


Figure 1-1. Regulation of AAH expression and function. Insulin/insulin-like growth factor-1 (IGF-1) binds to the insulin or IGF-1 receptor and activates the receptor's intrinsic tyrosine (Tyr) kinase activity. Intrinsic kinase activation leads to autophosphorylation at Tyr residues, which are recognized by the insulin receptor substrate-1 (IRS-1), also phosphorylated at specific Tyr (pTyr) residues. One arm of insulin/IGF-1 signaling is activated by the interaction of Grb-2 with son of sevenless (SOS), a complex that docks to pTyr-IRS-1, activating SOS. SOS activates Ras, which in turn activates Raf kinase. Raf phosphorylates and activates mitogen-activated protein kinase (MAPK) kinase, which in turn phosphorylates and activates MAPK [22]. MAPK increases aspartyl-asparaginyl- β -hydroxylase (AAH) gene. On the other hand, the p85 subunit of phosphoinositide-3 kinase (PI3K) binds to pTyr-IRS-1 and promotes phosphorylation and activation of Akt, which phosphorylates and inhibits glycogen synthase kinase-3 β (GSK-3 β). Inhibition of GSK-3 β promotes AAH protein expression. Increased expression of AAH at the mRNA and protein levels promotes cell adhesion and migration. Alcohol disrupts insulin/IGF-1 signaling directly by blocking IRS-1 activation. Alcohol increases the activity of PTEN, which inhibits PI3K activation. Alcohol also promotes the formation of reactive oxygen species that increase activation of GSK-3 β , leading to decreased AAH expression, activation of Notch and Jagged, and hepatocyte adhesion and migration. In the case of hepatocellular carcinoma, IGF-1 receptor is overexpressed, PI3K has gain-of-function mutations, phosphatase and tensin homologue (PTEN) and protein phosphatase 1 (PP1) are downregulated, and MAPK is overly active, leading to aberrant increases in AAH protein expression and increased tumor cell adhesion, migration, and invasion.

Figure 1-2

Structure of the 5' End of the Human A β H-J-J Locus

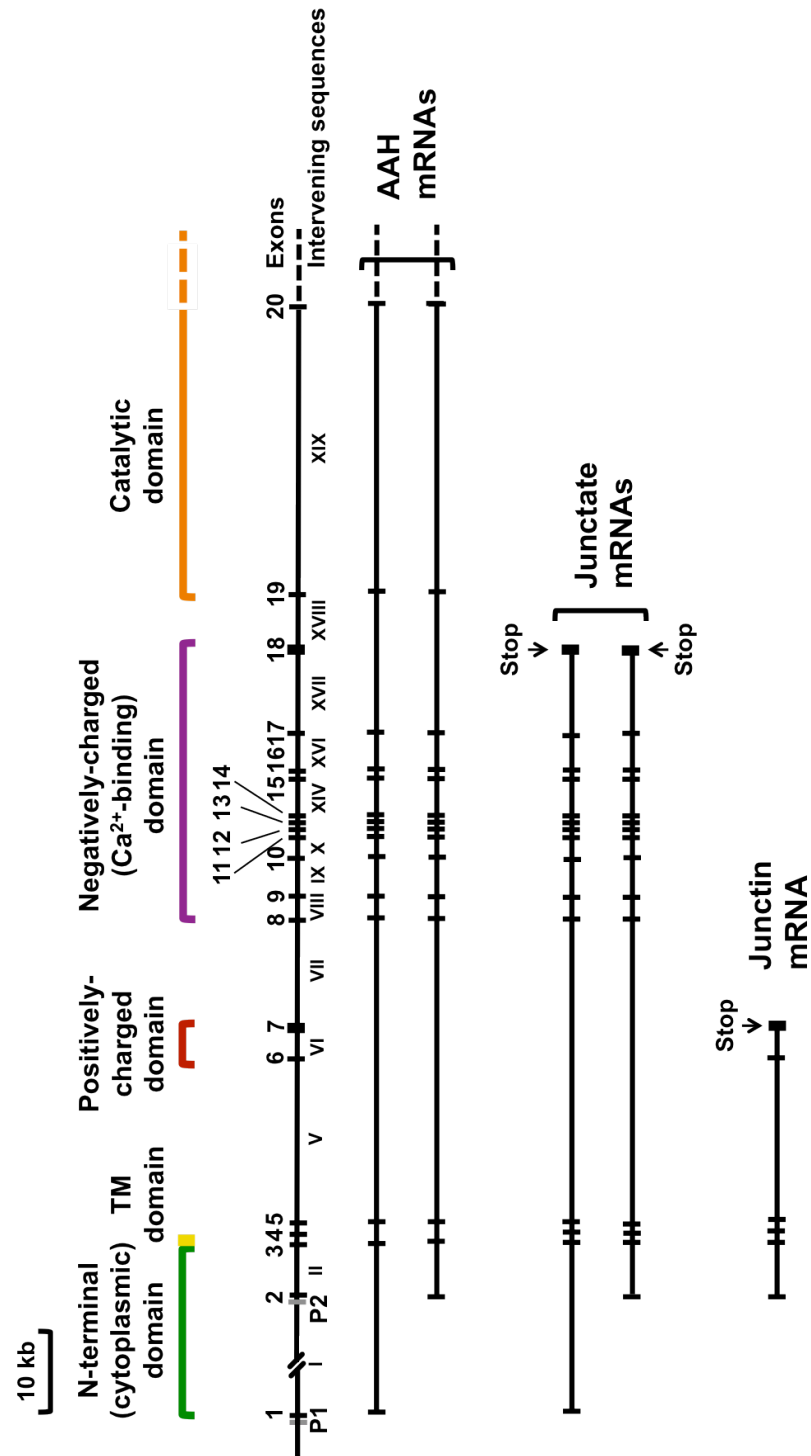


Figure 1-2. Structure of the 5' End of the Human A β H-J-J Locus. Arabic numbers over black boxes indicate exons and roman numerals below indicate intervening intron sequences. The two putative promoters P1 and P2 are indicated in gray. A schematic representation of aspartyl-asparaginyl- β -hydroxylase (AAH), as well as its shorter isoforms juncate and junctin, products of alternative splicing [42,46] are also displayed. N-terminal (cytoplasmic) (green), transmembrane (TM) (yellow), positively charged (red), negatively charged (Ca²⁺-binding) (purple), and catalytic domains (orange) are indicated. The locations of stop codons are also shown. Figure modified from [43].

Figure 1-3

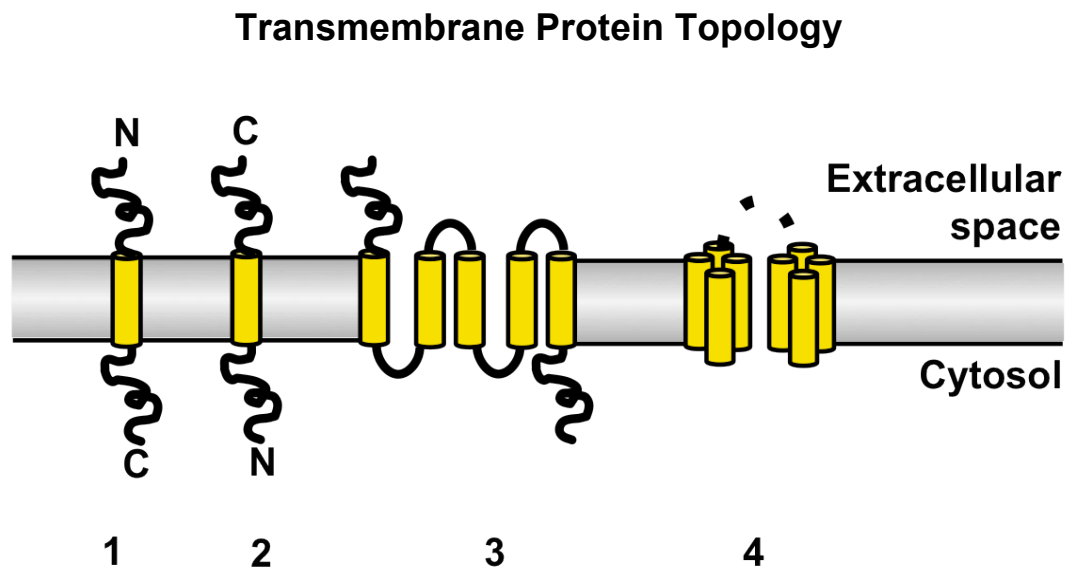


Figure 1-3. Transmembrane protein topology. The extracellular space and cytoplasmic surfaces of the membrane are indicated. Type 1 proteins have a cytosolic C-terminus; type 2 proteins have a cytosolic N-terminus; type 3 proteins span the membrane more than once; type 4 proteins have domains that define an aqueous transmembrane channel, but the number of domains may vary. The dashed arch indicates that the n domains may either be part of a single polypeptide chain, or an aggregate of n independent subunits. Modified from [51].

Figure 1-4

Transmembrane Topology and Structural Domains of AAH, Juncate, and Junctin

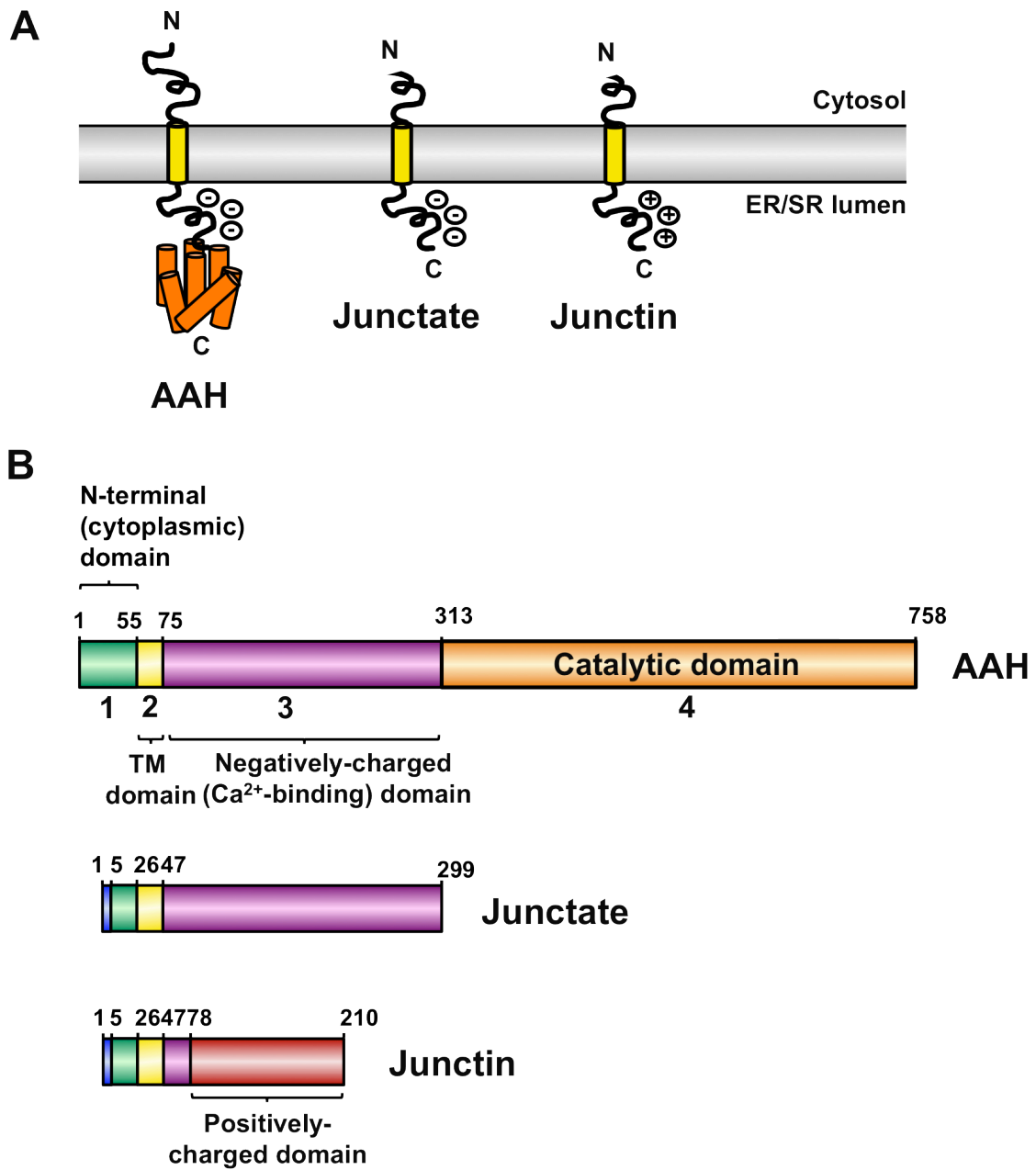


Figure 1-4. Transmembrane topology and structural domains of AAH, juncate, and junction. (A) Schematic representation of cytosolic and luminal surfaces of the endoplasmic reticulum (ER)/ sarcoplasmic reticulum (SR) membranes are indicated. Aspartyl-asparaginyl- β -hydroxylase (AAH), juncate, and junctin type 2 transmembrane topology. (B) Diagram of structural domains of AAH, juncate, and junctin. 1. N-terminal (cytoplasmic) (green and/or blue); 2. Transmembrane (TM) (yellow); 3. Negatively-charged charged (Ca^{2+} -binding) (purple); 4. AAH catalytic (orange) domains are shown. Junctin-specific positively-charged domain (red) is also indicated.

Figure 1-5

Intrinsically Disordered Regions and Probability of Interaction in AAH

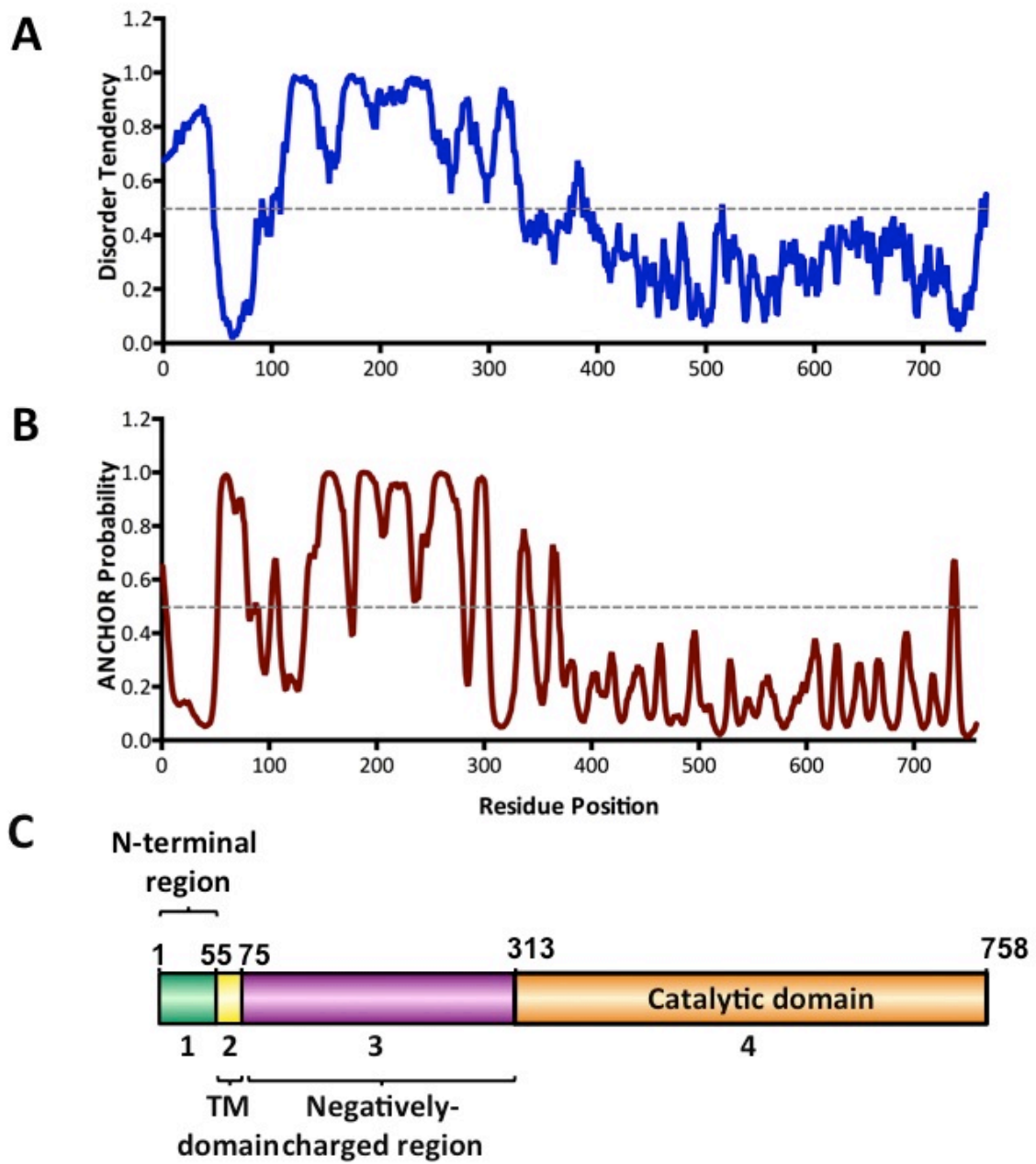


Figure 1-5. Intrinsically disordered regions and probability of interaction in AAH.

AAH amino acid sequence of aspartyl-asparaginyl- β -hydroxylase (AAH) was put through the IUPred and ANCHOR prediction software to determine regions of disorder and probability of these regions to interact with other proteins. (A) Residues flanking the transmembrane (TM) domain have a high disorder propensity (>0.5). (B) Residues in the TM domain and negatively-charged region have a high probability (>0.5) of having protein binding partners.

Figure 1-6

Mechanism of AAH Hydroxylation

X=NH₂ or OH

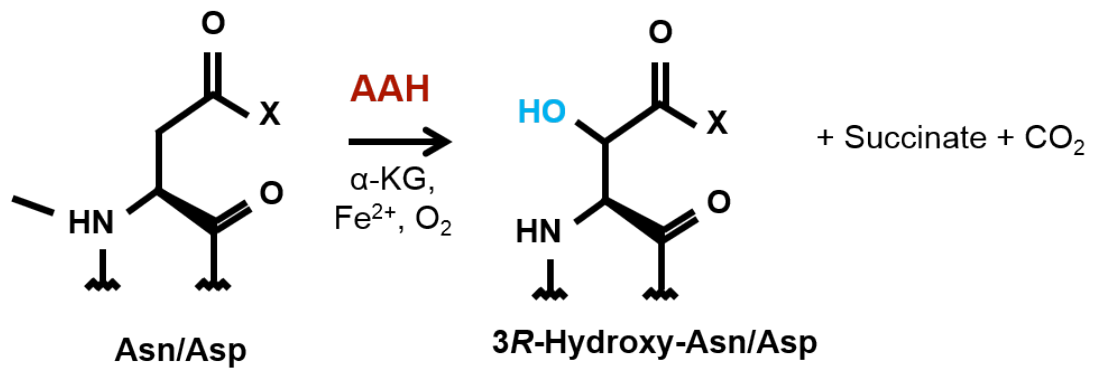


Figure 1-6. Mechanism of AAH hydroxylation. Iron (II) interacts with α -ketoglutarate and molecular oxygen to form an iron (III) superoxide byproduct. The reactive oxygen attacks α -ketoglutarate at the carbon-2 position, yielding an iron (II) peroxy byproduct. The reactive product interacts with aspartate (Asp) or asparagine (Asn) to yield succinate, carboxylic acid, and 3-hydroxyaspartic acid or 3-hydroxyasparagine. Experimental evidence indicates the His-675 residue of AAH coordinates iron binding and is essential for catalytic activity.

Figure 1-7

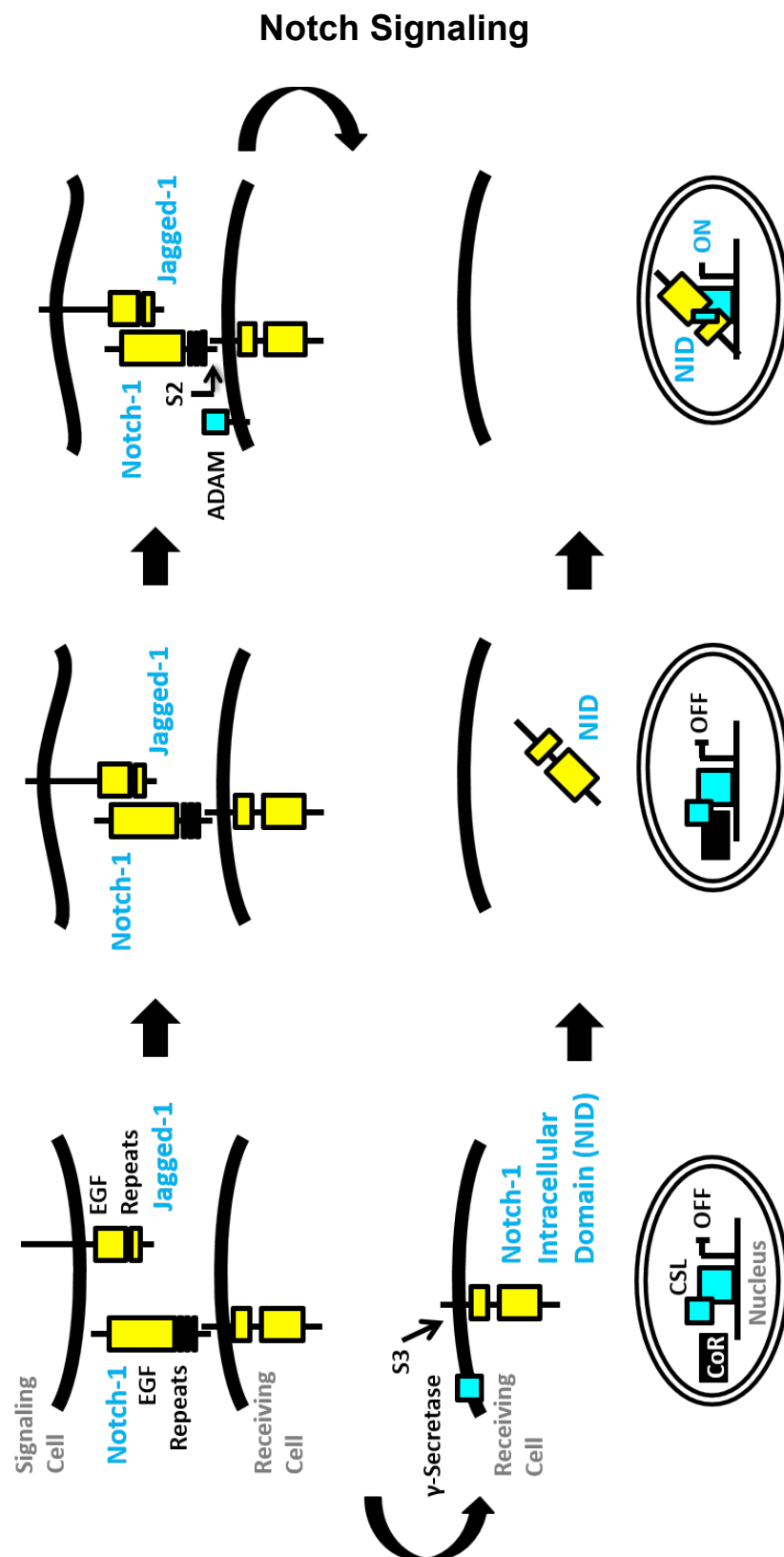


Figure 1-7. Notch signaling. Binding of Jagged (or Delta-like family of proteins) to the Notch extracellular domain at epidermal growth factor (EGF)-like repeats exposes a cleavage site (S2) in the extracellular domain cleaved by a protease of a disintegrin and metalloprotease (ADAM). The remaining transmembrane domain is cleaved by γ -secretase (S3) and frees the Notch intracellular domain (NID). At this point, the NID translocates from the plasma membrane to the nucleus, where it displaces co-repressors (CoR) and complexes with suppressor of hairless or lag-1 (CSL) and the transcriptional coactivators of the mastermind family of proteins. The complex binds to DNA containing CSL recognition sequences, recruits additional factors and activates transcription of downstream target genes (ON) [69,70].

Figure 1-8

Predicted Phosphorylation Sites on AAH

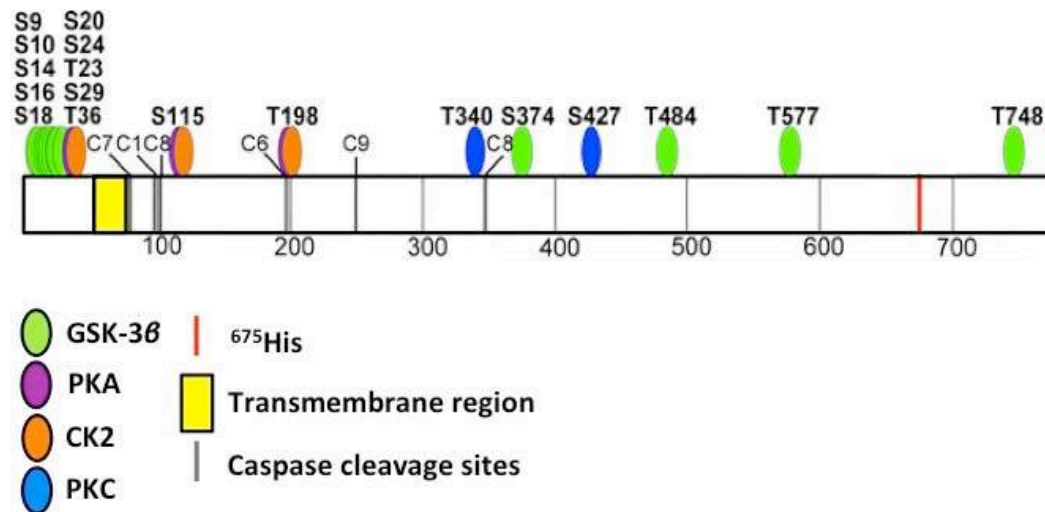


Figure 1-8. Predicted phosphorylation sites on AAH. Aspartyl-asparaginyl- β -hydroxylase (AAH) amino acid sequence was placed through the MacVector 8.1 prediction software and AAH is predicted to have several consensus sequences for phosphorylation by glycogen synthase kinase 3 β (GSK-3 β) (green), protein kinase A (PKA) (magenta), casein kinase 2 (CK2) (orange), and protein kinase C (PKC) (blue). The critical His-675 residue (red line), transmembrane region (yellow), and caspase cleavage sites (gray) are also indicated. Figure obtained from [28].

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CHAPTER 2

MYRIOCIN-CERAMIDE INHIBITOR MODULATES INSULIN/IGF AND ER STRESS PATHWAYS IN EXPERIMENTAL ALCOHOL-RELATED STEATOHEPATITIS

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AUTHOR CONTRIBUTIONS

DL had a significant role in tissue processing and analysis by qRT-PCR, duplex ELISA, and Western blotting and was involved in all phases of the research. MT and VZ generated animal model, collected tissue, assisted in qRT-PCR and multiplex ELISA assays. SMdIM designed the experiments, had a major role in data interpretation, and wrote most of the manuscript. All co-authors contributed to the final manuscript.

2.1. RATIONALE

Alcohol causes alcoholic liver disease (ALD), which is characterized by lipid accumulation in the early stages of the disease. Ethanol impairs insulin and insulin-like growth factor-1 (IGF-1) signaling at various points of the cascade through decreased insulin/IGF-1 receptor binding, activation of negative regulators of Akt, and increased GSK-3 β activity, all of which inhibit downstream signaling transduction. In addition, ethanol can indirectly impair insulin and IGF-1 signaling by increasing ceramides, which negatively regulate insulin receptor substrate-1 (IRS-1) and Akt. Ceramides activate inflammatory signals, such as c-Jun N-terminal kinases that phosphorylate IRS-1 at inhibitory Ser residues and inhibit its activation, activate protein kinase C ζ , which serine-phosphorylates Akt and prevents its activation by 3-phosphoinositide dependent protein kinase-1, and activates protein phosphatase 2, which dephosphorylates and deactivates Akt.

Impaired insulin/IGF-1 signaling disrupts glucose uptake, inducing endoplasmic reticulum stress. ER stress further increases lipid accumulation, and insulin resistance, all of which exacerbate each other. Furthermore, ethanol-induced insulin resistance impairs the expression of aspartyl-asparaginyl- β -hydroxylase (AAH) expression, resulting in decreased hepatocyte migration, impaired liver remodeling, and progression to ALD. The study was initiated to determine if inhibition of ceramide synthesis with

myriocin, decreased ceramide loa and insulin resistance, thereby restoring AAH expression. We used liver slice cultures in particular as a method to evaluate early responses of the ER to myriocin and determine its value as a potential therapeutic option for the treatment of ALD.

2.2. ABSTRACT

Background: ALD is mediated in part by insulin resistance, which dysregulates lipid metabolism, increasing hepatic ceramides, lipotoxicity and endoplasmic reticulum (ER) stress. However, toxic ceramides increase inflammation and worsen insulin resistance. **Aim:** We tested the hypothesis that ceramide inhibitors could reduce ER stress and restore insulin signaling, including expression of insulin responsive genes such as aspartyl- asparaginyl- β -hydroxylase (AAH), which mediates structural remodeling of the liver. **Design:** Using a liver slice culture (LSC) model generated from control and ethanol-exposed rats, we examined effects of myriocin (serine palmitoyl transferase inhibitor) on insulin/IGF signaling, and expression of AAH and ER stress-related proteins in liver. **Results:** Ethanol-induced hepatic steatosis resulted in increased cytotoxicity and triglyceride and ceramide accumulations. Ethanol also inhibited signaling through the insulin and IGF-1 receptors, IRS-1, Akt, and p70S6k, increased activation of GSK-3 β , reduced AAH, Humbug, and β -actin, and altered ER stress pathway protein expression. Myriocin reduced hepatic steatosis and ceramide levels, normalized components of insulin/IGF-1 signaling, and enhanced expression of AAH, but did not reduce cytotoxicity, triglycerides, or ER stress proteins in ethanol exposed LSCs. **Conclusions:** Ceramide accumulation partly mediates liver insulin resistance in ALD. Ceramide inhibitors may provide supplementary treatment for restoring liver function in ALD.

Keywords: alcoholic liver disease; ceramides; steatohepatitis; insulin resistance; Notch; aspartyl-asparaginyl- β -hydroxylase; myriocin

2.3. INTRODUCTION

Alcohol abuse is a leading cause of liver-related morbidity and mortality [1-3]. Excessive chronic or binge alcohol consumption causes steatohepatitis, which progresses to ALD [4] due to sustained insulin and insulin-like growth factor (IGF) resistance [5-7], inflammation, oxidative and endoplasmic reticulum (ER) stress, lipotoxicity with ceramide accumulation, and mitochondrial dysfunction [5-9]. Ethanol-impaired insulin and IGF type 1 (IGF-1) signaling [5,6,10-15] inhibits downstream activation of ERK mitogen-activated protein kinase (MAPK), which supports liver growth and repair after injury, and phosphatidylinositol-3-kinase (PI3K)-Akt [5,16,17], which promotes hepatocellular survival, motility (for remodeling), and metabolism [18]. In addition, ethanol activates glycogen synthase kinase 3 β (GSK-3 β) [11], and phosphatases that negatively regulate receptor tyrosine kinases and PI3K [11,19]. Therefore, inhibition of insulin/IGF signaling accounts for many of the toxic and degenerative effects of ethanol on the liver.

Aspartyl-asparaginyl- β -hydroxylase (AAH) is an important downstream target of insulin signaling because of its role in regulating cell motility, which is needed for liver remodeling, repair, and regeneration [6,20-22]. AAH is an ~86 kD type 2 transmembrane protein that undergoes physiological cleavage, releasing a ~52-54 kD C-terminal fragment that has catalytic (hydroxylase) activity, and a smaller ~32-34 kD N-terminal fragment that is highly homologous to another protein called Humbug [23-25]. AAH's C-terminal catalytic fragment confers cell motility, invasiveness, and adhesion [20,22], whereas both Humbug and the N-terminus of AAH may mediate calcium flux between the endoplasmic reticulum (ER) and cytosol via ryanodine receptors [26], and thereby

modulate cell adhesion [20]. Insulin and IGFs regulate AAH by increasing its mRNA, catalytic activity [20,27-29], and protein [21,27,28]. Insulin/IGF stimulation causes AAH protein to accumulate by inhibiting GSK-3 β phosphorylation, which leads to AAH's proteolytic degradation [28-30]. Ethanol can inhibit AAH's expression and function by impairing insulin/IGF signaling, and increasing GSK-3 β activity [5,29,31,32].

Growing evidence suggests that besides insulin/IGF resistance, ethanol mediates its adverse effects on liver by causing lipid dyshomeostasis [33]. Lipid dyshomeostasis results in increased lipolysis and ceramide accumulation. Attendant lipotoxicity further impairs insulin signaling [34-36], activates pro-inflammatory cytokines, inhibits PI3K-Akt [37-40], and increases ER and oxidative stress [9,41,42]. The contributions of ceramide accumulation to the pathogenesis of ALD are further suggested by the findings that: 1) mice deficient in acidic sphingomyelinase are resistant to ethanol-induced hepatic steatosis [43]; 2) ceramides inhibit 5' adenosine monophosphate-activated protein kinase (AMPK) [44] and promote local hepatocellular injury [45], possibly because AMPK reduces ER stress and apoptosis [46]; and 3) hepatic ceramide levels are elevated and their profiles substantially altered in advanced human ALD [7,47].

Previous studies showed that treatment with peroxisome proliferator activated receptor (PPAR) agonists, which are insulin sensitizers that have anti-inflammatory actions, could prevent structural, biochemical, and molecular pathologic changes in experimental chronic ALD [6,42]. However, not all aspects of ALD were resolved with PPAR agonist treatments. In particular, ceramide load remained elevated [48], setting the stage for ALD to eventually progress due to persistent insulin/IGF resistance and inhibition of target genes and signaling molecules that regulate liver remodeling. Therefore, additional approaches are needed to effectively treat chronic ALD. Herein, we evaluate the therapeutic effects of a ceramide inhibitor with respect to resolution of

hepatic steatosis, insulin resistance, impaired AAH expression, and ER stress.

2.4. MATERIALS AND METHODS

2.4.1. Materials. The Taqman Gene expression master mix, Amplex UltraRed soluble fluorophore, Amplex Red Triglyceride Assay Kit, Akt Pathway Total and Phospho 7-Plex panels, and the 4-methylumbelliferyl phosphate (4-MUP) fluorophore were obtained from Invitrogen (Carlsbad, CA). QIAzol Lysis RNA extraction Reagent was bought from Qiagen, Inc (Valencia, CA). MaxiSorp plates, OptiPlates (96-well) needed for enzyme-linked immunosorbent assays (ELISAs), BD Falcon culture inserts, and Nunc culture supplies were obtained from Thermo Scientific (Rochester, NY). SuperBlock TBS, bicinchoninic assay, and enhanced chemiluminescence reagents were purchased from Pierce (Rockford, IL). Mouse monoclonal anti-ceramide and myriocin were from Sigma-Aldrich Co (St. Louis, MO). Rabbit polyclonal antibody to large acidic ribosomal protein (RPLPO) was from Proteintech, Inc (Chicago, IL). The A85E6, A85G6, and FB50 mouse monoclonal antibodies to AAH were characterized as described [29,31]. All other antibodies and reagents for ELISAs were purchased from Abcam (Cambridge, MA), Upstate (Billerica, MA), Vector Laboratories (Burlingame, CA), Invitrogen (Carlsbad, CA), or Chemicon (Temecula, CA). Other fine chemicals were purchased from CalBiochem (Carlsbad, CA) or Sigma-Aldrich (St. Louis, MO).

2.4.2. Organotypic slice culture. Liver slice cultures (LSCs) were generated with livers from adult Long Evans rats maintained on Lieber-deCarli isocaloric liquid diets containing 0% (control) or 37% ethanol by caloric content. At harvest, livers were placed into ice-cold Hanks Balanced Salt Solution (HBSS). Eight mm diameter cylindrical cores were sliced at a thickness of 150 μ m using a McIlwain tissue chopper (Mickle Laboratory Engineering Co. Ltd, UK). After gentle separation under a dissecting microscope, 2 or 3 slices were placed (non-overlapping) on the bottoms of BD Falcon 8- μ m pore culture

inserts and seeded into 12-well Nunc plates. Each well contained 1 ml of pre-warmed (37°C) Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 10 mM non-essential amino acids, 120 U/mL Penicillin, 120 µg/ml Streptomycin, and either 0 mM or 50 mM ethanol (corresponding with in vivo blood alcohol levels). After 24 hours incubation, cultures were treated with 0.1% DMSO (vehicle) or 10 µM myriocin, a serine palmitoyl transferase (de novo pathway) inhibitor [49]. LSCs were maintained at 37°C in a standard humidified CO₂ incubator and on a rotating platform. LSC medium was changed daily with fresh additions of chemical inhibitors.

We used the LDH Cytotoxicity Assay Kit to assess effects of ceramide inhibitor treatments on culture viability. In brief, duplicate 50 µl aliquots of cell culture medium were transferred to individual wells of a 96-well white OptiPlate. After combining with 50 µl of reaction mixture containing 4 mM resazurin, the samples were incubated at 37°C for 30 minutes. Fluorescence was measured (Ex 530 nm/Em 590 nm) in a SpectraMax M5 microplate reader (Molecular Devices Corp., Sunnyvale, CA) and results were normalized to protein content. LSCs were harvested after 72 h and tissues were snap-frozen in a dry ice/methanol bath and stored at -80°C for protein studies.

2.4.3. LSC studies. LSC homogenates were prepared in lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA (pH 8.0), 50 mM NaF, 0.1% Triton X-100, and protease and phosphatase inhibitors [7]. Protein concentration was measured with BCA reagents. Immunoreactivity was measured by direct binding duplex ELISAs and normalized to large acidic ribosomal protein (RPLPO) measured in the same wells [7]. Protein homogenates (100 ng/50 µl) were adsorbed to the well bottoms of MaxiSorp plates by overnight incubation at 4°C, and then blocked for 3 hours with 1% bovine serum albumin (BSA) in TRIS buffered saline (TBS). After washing, the samples were incubated with primary antibody (0.1-0.4 µg/ml) overnight at 4°C. Immunoreactivity was

detected with horseradish peroxidase-conjugated secondary antibody and Amplex UltraRed soluble fluorophore. Fluorescence was measured (Ex 565 nm/Em 595 nm) in a SpectraMax M5 (Molecular Devices, Sunnyvale, CA). Subsequently, the samples were incubated with biotin-conjugated antibodies to RPLPO, and immunoreactivity was detected with streptavidin-conjugated alkaline phosphatase (1:1000) and 4-Methylumbelliferyl phosphate (4-MUP). Fluorescence (Ex360/Em460) was measured in a SpectraMax M5. Binding specificity was assessed in parallel incubations in which the primary or secondary antibody was omitted. The ratios of specific protein/RPLPO were calculated and used for inter-group statistical comparisons. Ceramide immunoreactivity was quantified by ELISA as previously described [7,42,50]. Triglycerides were measured in frozen tissue using a commercial assay.

2.4.4. Bead-based multiplex ELISA. LSC homogenates (100 µg protein/sample) were used to assess signaling through insulin and IGF-1 receptors, IRS-1, and Akt pathways with the Akt Total and Phospho 7-Plex Panels. The Akt Total 7-Plex panel measured: insulin receptor (IR), IGF-1 receptor (IGF-1R), IRS-1, Akt, proline-rich Akt substrate of 40 kDa (PRAS40), ribosomal protein S6 kinase (p70S6K), and glycogen synthase kinase 3β (GSK-3β). The Akt Phospho 7-Plex panel measured: pYpY1162/1163-IR, pYpY1135/1136-IGF-1R, pS312-IRS-1, pS473-Akt, pT246-PRAS40, pTpS421/424-p70S6K, and pS9-GSK3β. Immunoreactivity was measured in a BioPlex 200 (Hercules, CA).

2.4.5. Statistical analysis. Data depicted in box plots reflect group medians (horizontal bar), 95% confidence interval limits (upper and lower box limits) and range (whiskers). Data (N=4 independent LSCs/group) were analyzed using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA), and inter-group comparisons were made by repeated measures one-way analysis of variance (ANOVA) with Tukey's multiple comparisons post hoc test. P-values are shown within the graph panels.

2.5. RESULTS

2.5.1. Myriocin effects on ethanol-induced cytotoxicity and lipid content. LDH release into the culture supernatant, an index of cytotoxicity, was measured at the 48 h and 72 h culture time points (**Figure 2-1A**). At the 48-hour time point, LDH release was similarly high in control and ethanol exposed, vehicle-treated LSCs. At 72 h, LDH release in control+vehicle LSCs was sharply reduced relative to 48 h, whereas in ethanol+vehicle LSCs, LDH release remained elevated and was significantly higher than control (**Figure 2-1A**). Myriocin reduced cytotoxicity in control but not ethanol-exposed LSCs cultures at the 48 and 72 hour time points (**Figure 2-1A**), rendering the inter-group differences statistically significant ($P < 0.0001$). At the 48 h point, triglyceride levels were similarly elevated in control and ethanol-exposed, vehicle-treated LSCs. In both groups, hepatic triglyceride content spontaneously declined after 72 h in culture, although triglyceride levels were significantly higher in ethanol-exposed LSCs (**Figure 2-1B**). Myriocin significantly reduced triglyceride content in the 48 h control LSCs, but did not further reduce triglycerides at the 72h time point. In contrast, myriocin had no significant effect on triglyceride content in ethanol-exposed LSCs (**Figure 2-1B**). Consequently, ethanol-exposed LSC triglyceride levels were significantly elevated relative to control at both the 48 h content declined spontaneously over time in both groups ($P < 0.001$) and 72 h ($P < 0.05$) time points.

Hepatic ceramide was significantly elevated in ethanol-exposed, vehicle-treated relative to control LSCs, at both the 48 h and 72 h points. However, hepatic ceramides content declined spontaneously over time in both groups (**Figure 2-1C**). Myriocin reduced ceramide levels in control and ethanol-exposed LSCs at the 48 h point, and although the levels were higher in ethanol-exposed LSCs, the inter-group differences were not statistically significant (**Figure 2-1C**). At the 72 h point, hepatic ceramide levels

were similar to those observed in vehicle-treated LSCs, suggesting mainly spontaneous rather than therapeutic responses.

2.5.2. Myriocin restores hepatic insulin and IGF signaling and downstream activation of Akt and Erk MAPK pathways in ALD. Multiplex ELISAs were used to measure total (**Figure 2-2**) and phosphorylated (**Figure 2-3**) levels of insulin receptor, IGF-1 receptor, IRS-1, Akt, GSK-3 β , PRAS40, and p70S6K. In addition, the calculated ratios of phosphorylated to total protein (**Figure 2-4**) were used to assess relative levels of phosphorylation.

Repeated measures ANOVA tests demonstrated significant inter-group differences with respect to GSK-3 β (F=4.73; P=0.021) (**Figure 2-2E**) and p70S6K (F=4.63; P=0.022) (**Figure 2-2G**). There were no significant inter-group differences with respect to the expression levels of insulin receptor (**Figure 2-2A**), IGF-1 receptor (**Figure 2-2B**), IRS-1 (**Figure 2-2C**), Akt (**Figure 2-2D**), or PRAS40 (**Figure 2-2G**). Post-hoc Tukey's multiple comparison test demonstrated significantly increased levels of GSK-3 β (P<0.05) and p70S6K (P<0.05) in vehicle-treated, ethanol-exposed relative to control+vehicle LSCs. Although insulin receptor and IGF-1 receptor levels were higher in the ethanol+vehicle group, the differences from control did not reach statistical significance. Myriocin treatment uniformly abrogated the inter-group differences, rendering expression levels of all 7 signaling proteins similar in control and ethanol-exposed LSCs (Figure 2).

We detected significant effects of ethanol and/or myriocin on expression levels of pYpY1162/11630-insulin receptor (F=5.05; P=0.017) (**Figure 2-3A**), pS312-IRS-1 (F=4.1; P=0.032) (**Figure 2-3C**), and pS473-Akt (F=7.62; P=0.004) (**Figure 2-3D**). Post-hocTukey's multiple comparison test revealed that ethanol+vehicle LSCs had significantly lower levels of pYpY1162/1163-insulin receptor (P<0.05), pS312-IRS-1 (P<0.05), and pS473-Akt (P<0.01) relative to control. Although pYpY1135/1136-IGF-1 receptor was also

reduced in ethanol+vehicle LSCs (**Figure 2-3B**), the difference did not reach statistical significance. Myriocin rendered the mean levels of $pYpY^{1162/1163}$ -insulin receptor, $pYpY^{1135/1136}$ -IGF-1 receptor, pS^{312} -IRS-1, and pS^{473} -Akt similar in control and ethanol-exposed LSCs by reducing the levels in control cultures and/or increasing the levels in ethanol-exposed LSCs. In contrast, ethanol and myriocin had little or no effects on the expression of pS^9 -GSK-3 β (**Figure 2-3E**), pT^{246} -PRAS40 (**Figure 2-3F**), and $pTpS^{421/424}$ -p70S6K (**Figure 2-3G**).

Significant inter-group differences were also observed for the calculated ratios of $pYpY^{1162/1163}/total$ -insulin receptor ($F=4.47$; $P=0.025$) (**Figure 2-4A**), $pS^{473}/total$ -Akt ($F=4.37$; $P=0.027$) (**Figure 2-4D**), $pS^9/total$ -GSK-3 β ($F=4.60$; $P=0.023$) (**Figure 2-4E**), and $pTpS^{421/424}/total$ -p70S6K ($F=5.87$; $P=0.01$) (**Figure 2-4G**), but not for $pYpY^{1135/1136}/total$ -IGF-1 receptor (**Figure 2-4B**), $pS^{312}/total$ -IRS-1 (**Figure 2-4C**), or $pT^{246}/total$ -PRAS40 (**Figure 2-4F**). Myriocin completely abrogated the effects of ethanol, resolving all significant inter-group differences with respect to the relative levels of phospho/total signaling proteins (**Figure 2**).

2.5.3. Myriocin restores ethanol-impaired AAH expression. Duplex ELISAs measured effects of ethanol and myriocin on AAH, Humbug, and β -actin expression in LSCs (**Figure 2-5**). AAH was detected with the A85G6 monoclonal antibody, which binds to an epitope in the C-terminus, in the vicinity of the catalytic domain [31]. The A85E6 and FB50 monoclonal antibodies bind to different epitopes in the N-terminus of AAH [31]. However, because the N-terminus of AAH is virtually identical to Humbug, FB50 and A85E6 detect AAH and Humbug together [31, 51-53]. We observed significant inter-group differences in expression of AAH-A85G6 ($F=65.56$; $P<0.0001$) (**Figure 2-5A**), AAH+Humbug-A85E6 ($F=24.03$; $P<0.0001$) (**Figure 2-5B**), AAH+Humbug-FB50 ($F=63.73$; $P<0.0001$) (**Figure 2-5C**), and β -Actin ($F=31.98$; $P<0.0001$) (**Figure 2-5D**). Post hoc tests demonstrated significantly reduced levels of A85G6 ($P<0.001$), A85E6

($P<0.001$), FB50 ($P<0.001$), and β -actin ($P<0.001$) immunoreactivity in ethanol+vehicle relative to control+vehicle LSCs. Myriocin reduced A85G6 ($P<0.001$), A85E6 ($P<0.001$), FB50 ($P<0.001$), and β -actin ($P<0.001$) in control LSCs, and increased/normalized A85G6 ($P<0.001$) in ethanol-exposed LSCs. In contrast, myriocin had no significant effect on A85E6, FB50 or β -actin expression in ethanol-exposed LSCs.

2.5.4. Myriocin effects on hepatic ER stress in LSCs. We measured immunoreactivity to proteins that mediate ER stress at various levels in the cascade [54]. ANOVA tests demonstrated significant inter-group differences with respect to GRP78 ($F=14.93$; $P<0.0001$) (**Figure 2-6A**), PDI ($F=7.32$; $P=0.0002$) (**Figure 2-6B**), calnexin ($F=5.31$; $P=0.002$) (**Figure 2-6C**), IRE-1 α ($F=7.038$; $P=0.0003$) (**Figure 2-6D**), CHOP ($F=30.11$; $P<0.0001$) (**Figure 2-6E**), ERO1 ($F=0.34$; $P<0.0001$) (**Figure 2-6F**). Ethanol significantly reduced hepatic expression of calnexin ($P<0.01$), IRE-1 α ($P<0.05$), and CHOP ($P<0.001$), and increased ERO1 ($P<0.001$) relative to vehicle-treated controls.

In contrast, GRP78 and PDI were expressed at similar levels in control and ethanol exposed LSCs. Myriocin significantly increased expression of GRP78 ($P<0.001$) and PDI ($P<0.01$) in control LSCs (relative to vehicle), and GRP78 ($P<0.001$), calnexin ($P<0.05$), and IRE-1 α ($P<0.01$) in ethanol-fed rats (relative to vehicle). In contrast, myriocin had no significant effects on PDI, CHOP, or ERO1 expression in ethanol-exposed LSCs, or calnexin, IRE-1 α , CHOP, or ERO1 in control LSCs. Consequently, the major effects of myriocin were to increase and normalize expression of calnexin and IRE-1 α in ethanol-exposed LSCs, similarly increase GRP78 in control and ethanol-exposed LSCs, and selectively increase PDI in control LSCs.

2.6. DISCUSSION

2.6.1. Rationale for using the ceramide-inhibitor approach for ALD. The present study examines the potential therapeutic effects of myriocin on liver function after

chronic in vivo ethanol exposure. Myriocin inhibits serine palmitoyl transferase, which mediates de novo ceramide synthesis. The studies utilized a liver slice culture model to evaluate early responses to myriocin. We focused on insulin/IGF-1 signaling networks and expression of AAH, a downstream target that regulates cell motility and adhesion [6,20-22].

2.6.2. Ceramide inhibitor effects on cytotoxicity and lipid storage in ethanol-exposed LSCs. Therapeutic effectiveness of the myriocin treatments was demonstrated by the lowering of ceramide levels in both control and ethanol exposed LSCs at the 48 h time point. The subsequent spontaneous declines in ceramide levels, irrespective of treatment could reflect general adaptive responses to the LSC environment. Despite early reductions of ceramide levels, the failure of myriocin to reduce cytotoxicity in ethanol-exposed LSCs indicates that the hepatotoxic effects of ethanol and/or acetaldehyde are not ameliorated by this approach. Similarly, hepatic triglyceride storage is not measurably decreased following short-term inhibition of ceramide synthesis.

2.6.3. Short-term effects of myriocin on insulin/IGF-1/IRS-1 signaling in ALD. Ethanol exposure broadly impaired insulin and IGF-1 signaling by inhibiting the absolute and relative levels of phosphorylated receptors and IRS-1. Correspondingly, insulin/IGF-1 signaling through Akt and p70S6K was reduced, whereas PRAS40 was relatively unaffected. In addition, the total levels of GSK-3 β were increased, and relative levels of GSK-3 β phosphorylation were decreased in ethanol-exposed LSCs. These results indicate that ethanol exposure led to GSK-3 β activation since Ser9-phosphorylation of GSK-3 β inhibits the kinase. These broad impairments in insulin/IGF signaling correspond with the decreased levels of AAH and AAH+Humbug expression, which are stimulated by insulin and IGF-1, and decreased by GSK-3 β [30, 31]. Myriocin effectively restored insulin and IGF-1 signaling networks, rendering the total and relative

phosphorylated levels of most proteins similar in control and ethanol-exposed LSCs. Therefore, reducing hepatic ceramide load may be beneficial for restoring hepatic insulin responsiveness needed to maintain liver function in ALD.

2.6.4. Myriocin effects on AAH expression in ethanol-exposed livers. The finding that ethanol inhibits AAH and AAH+Humbug expression corroborates previous in vivo studies of ALD [6, 31]. The significant increases in AAH protein expression (A85G6 immunoreactivity) in ethanol-exposed livers treated with myriocin correlate with the enhancements in insulin/IGF-1 signaling. On the other hand, the modest effects of myriocin on A85E6 and FB50 immunoreactivity suggest that other therapeutic approaches are needed to restore Humbug expression in ALD. The same conclusion could be drawn with respect to β -actin, in which the inhibitory effects of ethanol were not reversed by myriocin.

2.6.5. Myriocin effects on ER stress signaling proteins. Insulin resistance and ceramide accumulation promote ER stress, and ER stress exacerbates insulin resistance, inflammation, oxidative stress, and ceramide accumulation [55-62]. The ER mediates protein synthesis, folding, maturation, and trafficking [63], and is critical for Ca^{2+} homeostasis and triglyceride synthesis. ER stress occurs when homeostatic mechanisms get disrupted and unfolded proteins accumulate, leading to increased formation of reactive oxygen species. Prolonged activation of the unfolded protein response (UPR) leads to inflammation, injury, steatosis, and apoptosis [8,54,63,64].

The ER adapts to stress by activating the UPR [60,65], which quickly increases levels of ER stress sensor proteins including: inositol-requiring enzyme 1 α (IRE-1 α), PKR-like ER-localized eIF2 α kinase (PERK), and the activating transcription factor 6 α (ATF-6 α ; ER membrane-anchored transcription factor). PERK and IRE-1 α transmit stress signals in response to protein misfolding or unfolding, and thereby activate ER stress networks [63]. PERK drives global arrest of protein synthesis and up-regulates the

transcription factor C/EBP homologous protein CHOP, which promotes apoptosis. IRE-1 α increases transcription of chaperones and ER associated protein degradation (ERAD) machinery.

Insulin resistance drives ER stress because vital ER functions such as protein synthesis, modification, and folding, calcium signaling, and lipid biosynthesis utilize glucose as the main source of energy; insulin resistance impairs glucose uptake and metabolism. Moreover, pathogenic lipolysis leading to ceramide accumulation is initiated by critical levels of ER stress and mitochondrial dysfunction [45,66-68]. Ethanol activates all major ER stress sensor cascades [66-69], and the resulting increased levels of ER stress exacerbate insulin resistance and lipolysis. End results include, increased ceramide production [70,71], worsening of inflammation and insulin resistance, and lipotoxicity [8,72,73].

Herein we observed relative inhibition of calnexin, IRE-1 α , and CHOP/GADD153, and increased levels of ERO-1 in ethanol+vehicle relative to control+vehicle LSCs. ERO-1, complexed with the PDI molecular chaperone, mediates oxidation of disulfide bonds in folding proteins. Therefore, the ethanol associated increases in ERO-1 could reflect increased disposal of mal-folded proteins in liver. At the same time, reduced expression of calnexin and IRE-1 α could indicate impaired folding and assembly of newly synthesized proteins in the ER (calnexin) and reduced transcription of chaperones and ER associated protein degradation machinery (ERAD). Consequences would include that in these short-term experiments, the ethanol-exposed hepatocytes respond to increased formation of mal-folded proteins and can utilize homeostatic mechanisms to prevent their accumulation. Myriocin-mediated restoration of hepatic insulin/IGF signaling mechanisms in ethanol-exposed LSCs was associated with slight but significant increases in GRP78, calnexin, and IRE-1 α . This suggests that myriocin-induced reduction in hepatic ceramide levels in ethanol-exposed LSCs was correlated

with increased activation of ER stress responses. While these effects may seem paradoxical, the outcomes suggest they are protective and needed to restore homeostasis vis-à-vis persistent hepatic injury from continuous ethanol exposure. On the other hand, the persistently lower levels of PDI, CHOP, and ERO-1 in ethanol exposed LSCs suggests that, in these short-term experiments, the mechanisms designed to destroy cells with activated UPRs and accumulated mal-folded proteins were not mobilized by the myriocin treatments. This phenomenon could partly account for the persistent cytotoxicity (high-level LDH release) in ethanol-exposed LSCs, irrespective of myriocin treatment.

Figure 2-1

Cytotoxicity and Lipid Assays

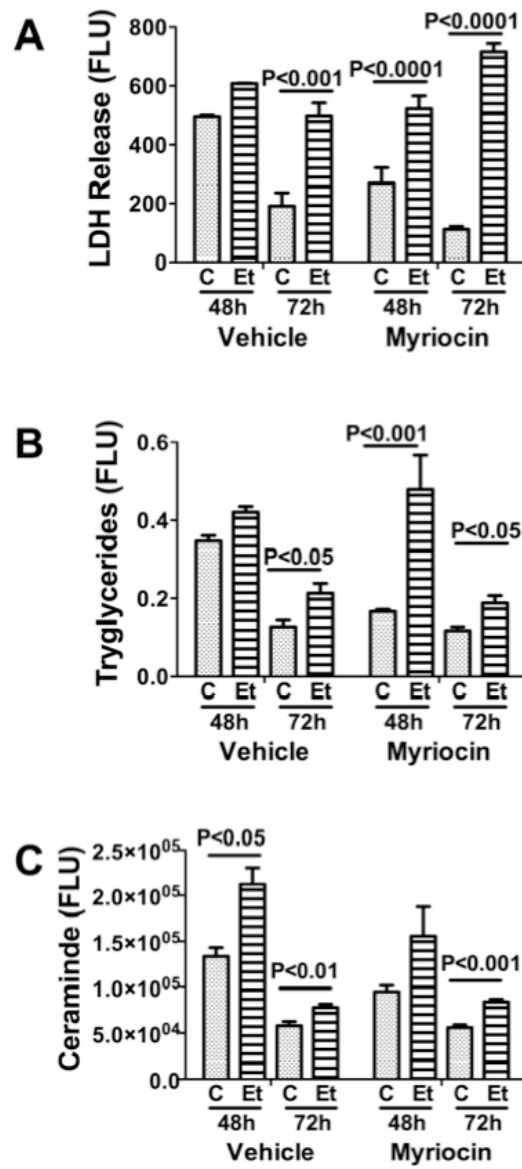


Figure 2-1. Cytotoxicity and lipid assays. Liver slice cultures (LSC) were generated from control (C) and ethanol-exposed (Et) rats (see Methods). LSCs were treated with vehicle (0.1% DMSO) or myriocin for 24 or 48 hours, beginning 24 h after seeding. (A) Culture supernatants were assayed for LDH release as an index of cytotoxicity. (B) LSC tissues were used to measure (B) triglycerides with a fluorescent-based commercial assay, and (C) ceramides immunoreactivity by ELISA. Data from 4 replicate independent cultures were analyzed per group. Results were normalized to protein content in the cultures. Inter-group comparisons were made by repeated measures ANOVA and Tukey's multiple comparisons test. FLU: fluorescent light units.

Figure 2-2

Ethanol and Myriocin Effects on Hepatic Insulin, IGF, and IRS Signaling Mechanisms

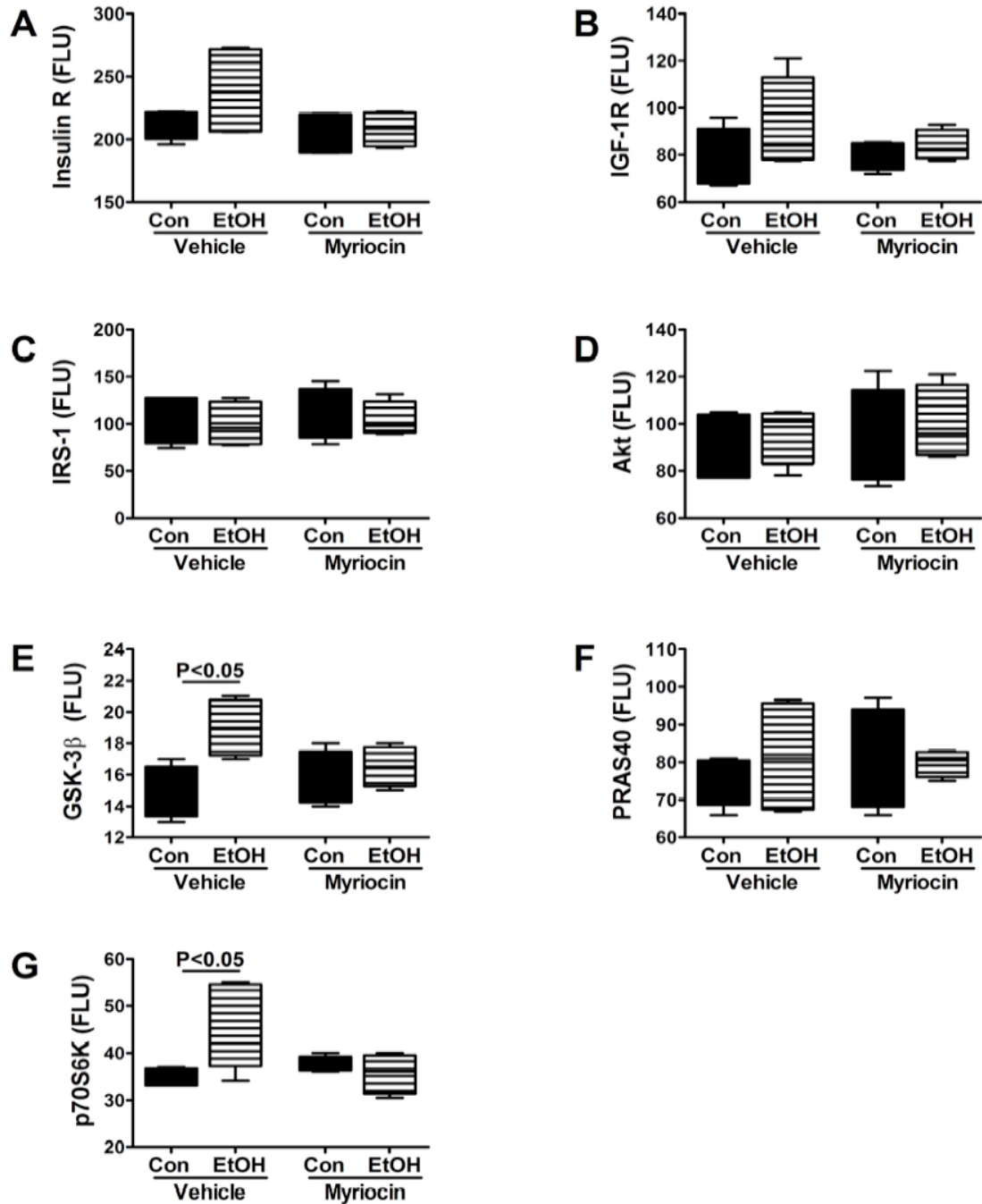


Figure 2-2. Ethanol and myriocin effects on hepatic insulin, IGF and IRS signaling mechanisms. LSCs generated from control (Con) and ethanol-exposed (EtOH) rats (see Methods) were treated with vehicle or myriocin for 48 h. Fresh frozen tissue homogenates were used to measure (A) insulin receptor, (B) IGF-1 receptor, (C) IRS-1, (D) Akt, (E) GSK-3 β , (F) PRAS40, and (G) p70S6K by multiplex ELISA. Results were normalized to protein content. N=4 independent LSCs per group. Box plots depict medians (horizontal bars), 95% confidence intervals (upper and lower limits of boxes), and ranges (stems). Inter-group comparisons were made by repeated measures ANOVA with the post-hoc Tukey's multiple comparisons test.

Figure 2-3

Ethanol and Myriocin Effects on Phosphorylated Insulin, IGF and IRS Signaling Molecules in Liver

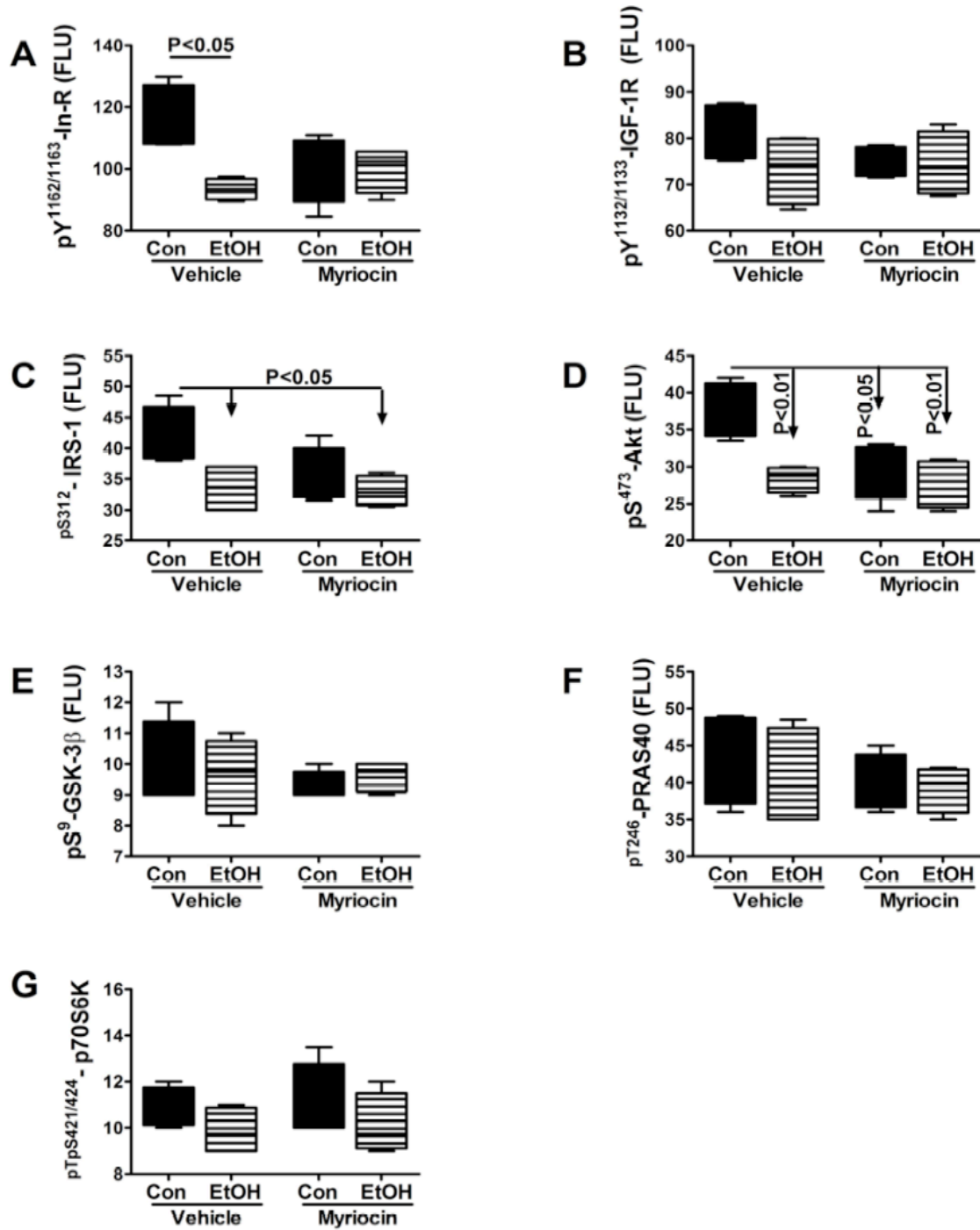


Figure 2-3. Ethanol and myriocin effects on phosphorylated insulin, IGF and IRS signaling molecules in liver. LSCs generated from control (Con) and ethanol-exposed (EtOH) rats were treated with vehicle or myriocin for 48 h. Fresh frozen tissue was used to measure (A) $pYpY^{1162/1163}$ -insulin receptor, (B) $pYpY^{1135/1136}$ -IGF-1 receptor, (C) pS^{312} -IRS-1 (D) pS^{473} -Akt (E) pS^9 -GSK-3 β (F) pT^{246} -PRAS40, and (G) $pT^{246}pS^{112}$ -p70S6K by multiplex ELISA. Results were normalized to protein content. N=4 independent LSCs per group. Box plots depict medians (horizontal bars), 95% confidence intervals (upper and lower limits of boxes), and ranges (stems). Inter-group comparisons were made by repeated measures ANOVA with the post-hoc Tukey's multiple comparisons test.

Figure 2-4

Ethanol and Myriocin Effects on Relative Levels of Phosphorylated Insulin, IGF and IRS Signaling Molecules In Liver

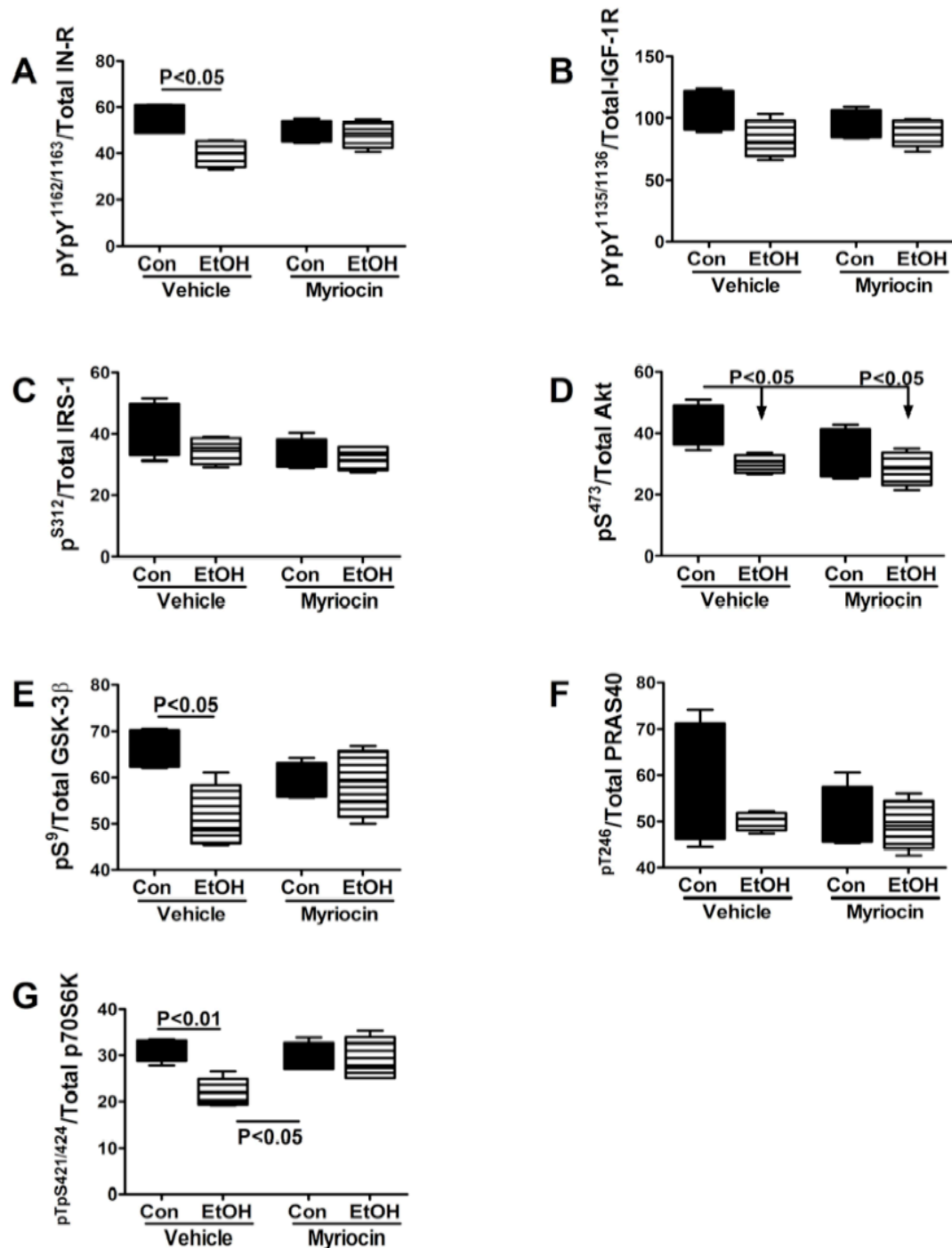


Figure 2-4. Ethanol and myriocin effects on relative levels of phosphorylated insulin, IGF and IRS signaling molecules in liver. LSCs generated from control (Con) and ethanol-exposed (EtOH) rats were treated with vehicle or myriocin for 48 h. Graphs depict calculated ratios of (A) $pYpY^{1162/1163}/\text{total-insulin receptor}$ (B) $pYpY^{1135/1136}/\text{total-IGF-1 receptor}$, (C) $pS^{312}/\text{total-IRS-1}$ (D) $pS^{473}/\text{total Akt}$, (E) $pS^9/\text{total GSK3b}$, (F) $pT^{246}/\text{total PRAS40}$, and (G) $pT^{246}pS^{112} / \text{total p70S6K}$ measured by multiplex ELISAs. Results were normalized to protein content. N=4 LSCs per group. Box plots depict medians (horizontal bars), 95% confidence intervals (upper and lower limits of boxes), and ranges (stems). Inter-group comparisons were made by repeated measures ANOVA with the post-hoc Tukey's multiple comparisons test.

Figure 2-5

Effects of Ethanol and Myriocin on AAH and Humbug
Expression in LSCs

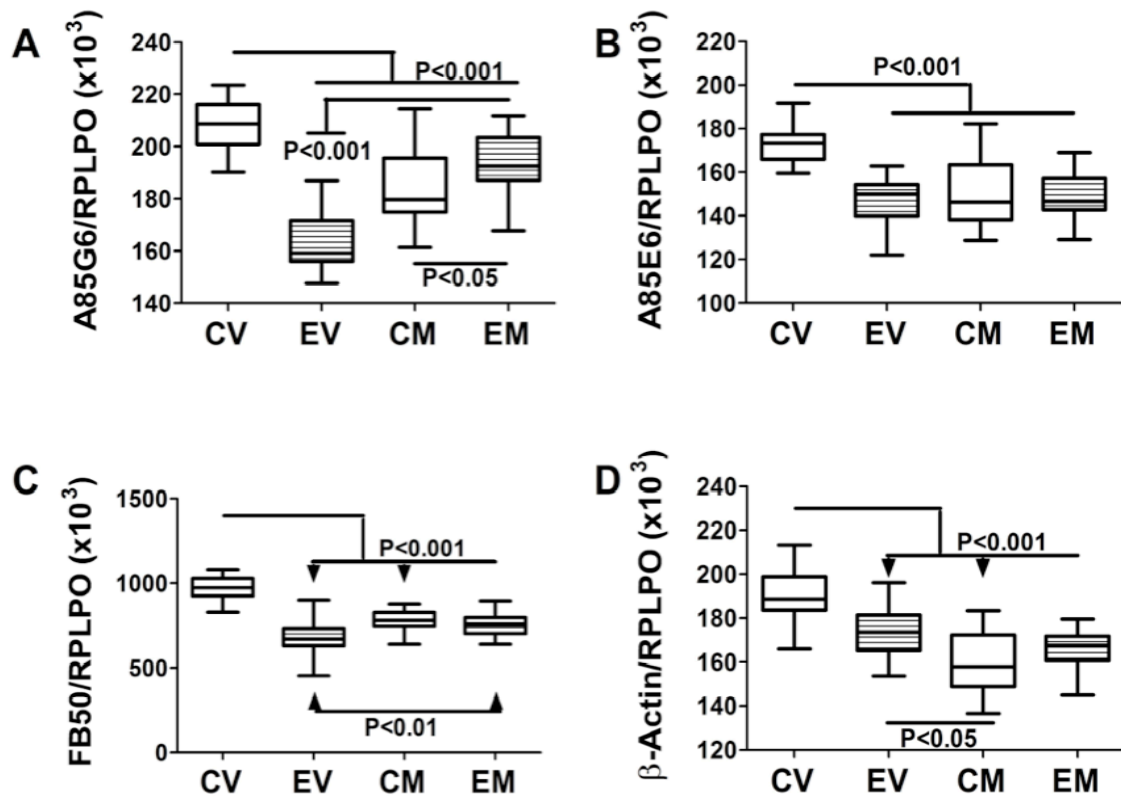


Figure 2-5. Effects of ethanol and myriocin on AAH and Humbug expression in LSCs. LSCs generated from control (C) and ethanol-exposed (E) rats were treated with vehicle or myriocin for 48 hours. Duplex ELISAs measured (A) AAH-A85G6, (B) AAH+Humbug-A85E6, (C) AAH+Humbug-FB50, and (D) β -actin. Results were normalized to RPLPO measured in the same wells. N=4 LSCs per group. Box plots depict medians (horizontal bars), 95% confidence intervals (upper and lower limits of boxes), and ranges (stems). Inter-group comparisons were made by repeated measures ANOVA with the post-hoc Tukey's multiple comparisons test. CV: control+vehicle; EV: ethanol+vehicle; CM: control+myriocin; EM: ethanol+myriocin.

Figure 2-6

Effects of Ethanol and Myricetin on ER Stress Proteins in LSCs

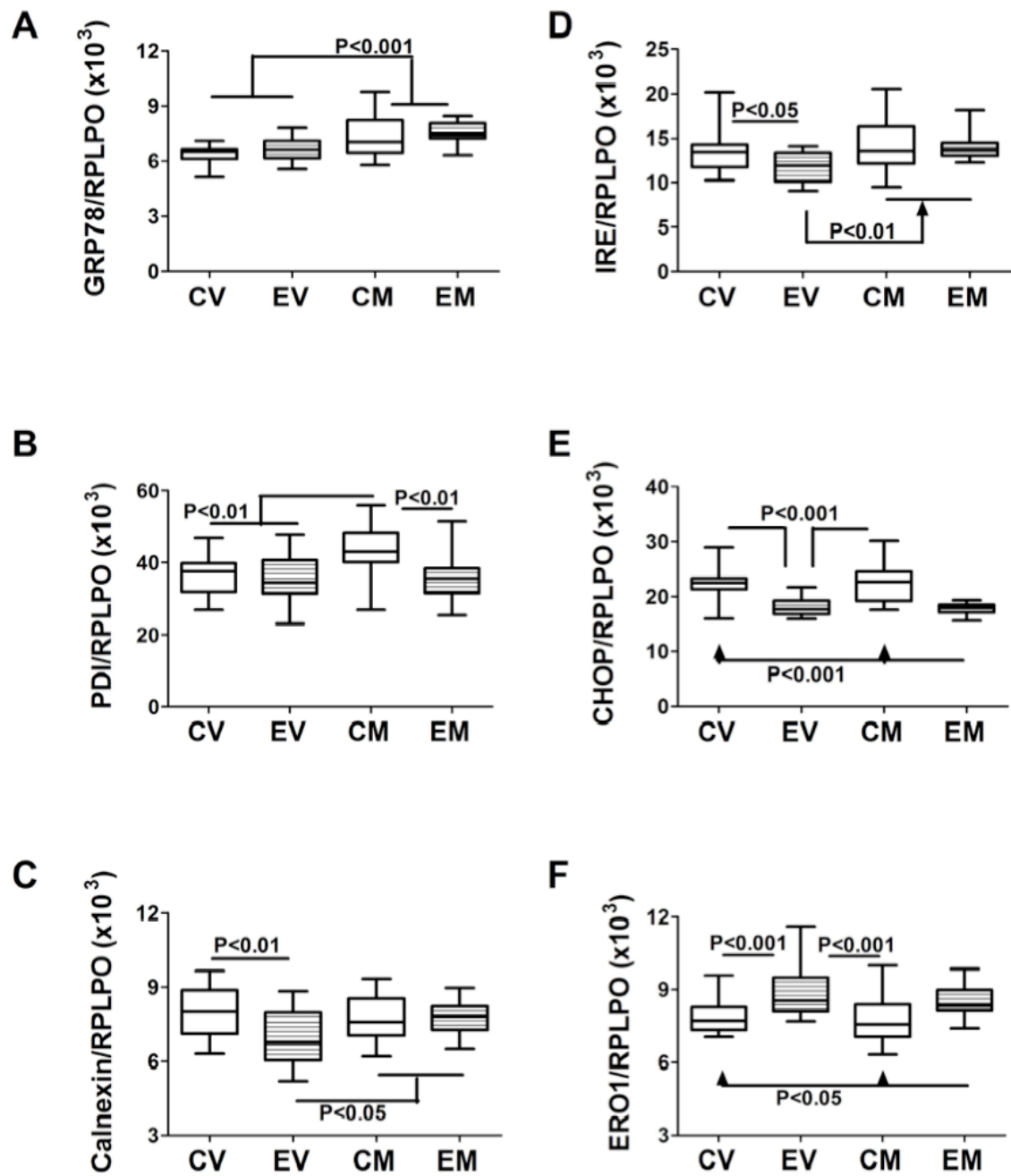


Figure 2-6. Effects of ethanol and myriocin on ER stress proteins in LSCs. LSCs generated from control (C) and ethanol-exposed (E) rats were treated with vehicle (0.1% DMSO) or myriocin for 48 hours. Duplex ELISAs measured (A) Bip/GRP78, (B) protein disulphide isomerase (PDI), (C) calnexin, (D) inositol requiring enzyme-1 (IRE-1a), (E) CHOP, and (F) endoplasmic oxidoreductin-1 (ERO-1). Results were normalized to RPLPO. Data from 4 replicate cultures were analyzed per group. Box plots depict medians (horizontal bars), 95% confidence intervals (upper and lower limits of boxes), and ranges (stems). Inter-group comparisons were made using repeated measures ANOVA with post-hoc Tukey's multiple comparison test. CV: control+vehicle; EV: ethanol+vehicle; CM: control+myriocin; EM: ethanol+myriocin.

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CHAPTER 3

CERAMIDE INHIBITOR-MYRIOCIN RESTORES INSULIN/IGF SIGNALING FOR LIVER REMODELING IN EXPERIMENTAL ALCOHOL-RELATED STEATOHEPATITIS

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alterations to keep the dissertation format consistent.

AUTHOR CONTRIBUTIONS

DL had a significant role in tissue processing and analysis by qRT-PCR, duplex ELISA, and Western blotting and was involved in all phases of the research. VZ, MT, and LL generated animal model, collected tissue, assisted in qRT-PCR and multiplex ELISA assays. SMdIM designed the experiments, had a major role in data interpretation, and wrote most of the manuscript. Co-authors all contributed to the final manuscript.

3.1. RATIONALE

Alcohol abuse is the primary cause of alcohol-related liver disease (ALD), characterized by steatosis in the early stages of the disease. Ethanol has been shown to impair insulin signaling by disrupting activation at various points of the cascade. In addition, ethanol causes aberrant accumulation of toxic lipids known as ceramides, which activate pro-inflammatory signals that negatively regulate the activation of insulin receptor substrate-1 (IRS-1), and activate protein kinase C ζ (PKC ζ) and protein phosphatase 2 (PP2), which inhibit Akt activation. Ethanol-induced insulin resistance decreases aspartyl-asparaginyl- β -hydroxylase (AAH) protein expression and function, as it relates to the activation of Notch signaling networks, via hydroxylation of Notch-1 and Jagged-1 to promote hepatocyte adhesion and migration. Impaired hepatocyte migration disrupts liver remodeling after alcohol injury, thereby contributing to progression to ALD. Since ethanol promotes aberrant ceramide accumulation, it was of interest to determine if treatment with myriocin, an inhibitor of de novo ceramide synthesis, reduced ceramide load, ameliorated insulin resistance, and restored the expression of AAH and Notch signaling activation, to restore hepatic architecture in ALD.

3.2. ABSTRACT

Background: Alcohol-related liver disease (ALD) is mediated in part by insulin

resistance. Attendant dysregulation of lipid metabolism increases accumulation of hepatic ceramides that worsen insulin resistance and compromise the structural and functional integrity of the liver. Insulin and IGF stimulate aspartyl-asparaginyl- β -hydroxylase (AAH), which promotes cell motility needed for structural maintenance and remodeling of the liver. AAH mediates its effects by activating Notch, and in ALD, insulin/IGF signaling, AAH, and Notch are inhibited. **Design:** To test the hypothesis that in ALD, hepatic ceramide load contributes to impairments in insulin, AAH, and Notch signaling, control and chronic ethanol-fed adult Long Evans rats were treated with myriocin, an inhibitor of serine palmitoyl transferase. Livers were used to assess steatohepatitis, insulin/IGF pathway activation, and expression of AAH-Notch signaling molecules. **Results:** Chronic ethanol-fed rats had steatohepatitis with increased ceramide levels, impairments in signaling through the insulin receptor, insulin receptor substrate, and Akt, and decreased expression of AAH, Notch, Jagged, HES-1, HIF-1 α , and proliferating cell nuclear antigen. Myriocin abrogated many of these adverse effects of ethanol, particularly hepatic ceramide accumulation, steatohepatitis, and impairments of insulin signaling through Akt, AAH, and Notch. **Conclusions:** In ALD, the histopathology and impairments in insulin/IGF responsiveness can be substantially resolved by ceramide inhibitor treatments, even in the context of continued chronic ethanol exposure.

Key terms: Alcoholic liver disease; ceramides; steatohepatitis; insulin resistance; Notch; aspartyl-asparaginyl- β -hydroxylase-myriocin.

3.3. INTRODUCTION

Alcohol abuse is a leading cause of liver-related morbidity and mortality [1]. Excessive alcohol consumption causes steatohepatitis, which progresses to chronic

alcohol-related liver disease (ALD) [2] due to combined effects of insulin and insulin-like growth factor (IGF) resistance [3-5], inflammation, oxidative and endoplasmic reticulum (ER) stress, lipotoxicity with ceramide accumulation, and mitochondrial dysfunction [3-7]. Ethanol-impaired insulin and IGF type 1 (IGF-1) signaling [3,4,8-10] inhibits downstream activation of mitogen-activated protein kinase (MAPK), which supports liver growth and repair, and phosphatidylinositol-3-kinase (PI3K) and Akt [3], which promote hepatocellular survival, motility, and metabolism [11]. Ethanol increases activation of glycogen synthase kinase 3 β (GSK-3 β) [8] and phosphatases that negatively regulate receptor tyrosine kinases and PI3K [8]. Therefore, disruption of insulin/IGF signaling accounts for many long-term toxic and degenerative effects of ethanol on the liver [3, 9, 12, 13].

Aspartyl-asparaginyl- β -hydroxylase (AAH) is an important downstream target of insulin signaling because of its role in regulating cell motility, which is needed for liver remodeling, repair, and regeneration [4,14-16]. AAH is an ~86 kD type 2 transmembrane protein that undergoes physiological cleavage, releasing a ~52-54 kD C-terminal fragment that has catalytic (hydroxylase) activity, and a smaller ~32-34 kD N-terminal fragment that is highly homologous to another protein called Humbug [17-19]. AAH's C-terminal catalytic fragment confers cell motility, invasiveness, and adhesion [14,16] whereas the N-terminus of AAH, which is highly homologous to Humbug, mediates calcium flux between the endoplasmic reticulum (ER) and cytosol via ryanodine receptors [20], and thereby modulate cell adhesion [14].

The consensus sequence for AAH hydroxylation resides in epidermal growth factor (EGF)-like domains that are present in proteins such as Notch and Jagged [21,22]. AAH hydroxylation of Notch leads to its proteolytic cleavage and release of the intracellular domain, which translocates to the nucleus where it binds to transcriptional regulators, displacing co-repressors and recruiting co-activators [23,24]. The net effect is

to increase expression of target genes such as, Hairy-Enhancer of Split (HES) and HES-related proteins [25]. AAH's roles in cell motility and invasion have been linked to activation of Notch networks [14,26,27]. Insulin and IGFs regulate AAH by increasing its mRNA levels, catalytic activity [14,26,28,29], and protein expression [15,26,28]. Insulin/IGF stimulation causes AAH protein to accumulate in cells, due to inhibition of GSK-3 β phosphorylation, which renders AAH susceptible to proteolytic degradation [27]. Ethanol inhibits AAH expression and function by impairing insulin/IGF signaling and increasing GSK-3 β activity [3,29-31].

Consequences of insulin resistance, including dysregulated lipid metabolism [12,32-34], inflammation [32], oxidative and ER stress [6,34] metabolic and mitochondrial dysfunction [35], decreased DNA synthesis [4], and increased cell death [12] contribute to hepatocellular injury and degeneration, and cause ALD to progress [13]. Dysregulation of lipid metabolism is of particular interest because the attendant lipolysis with ceramide accumulation establishes a lipotoxic state. Consequences include, further compromise of insulin signaling networks [36], activation of pro-inflammatory cytokines, inhibition of PI3K-Akt [37-39], and increases ER and oxidative stress [7,33,40].

Ceramides are likely to have an important role in the pathogenesis and progression of ALD based on reports demonstrating that: 1) mice deficient in acidic sphingomyelinase are resistant to ethanol-induced hepatic steatosis [41]; 2) ceramides inhibit AMPK [42] and promote local hepatocellular injury [43], possibly because AMPK reduces ER stress and apoptosis [44]; and 3) in humans with advanced ALD, hepatic ceramide levels are increased [5,13]. The present study tests the hypothesis that ALD can be treated effectively by inhibiting hepatic ceramide accumulation to dis-inhibit signaling through insulin/IGF and inter-related pathways. Using an established experimental model of chronic ALD, we assessed the therapeutic effects of myriocin, a potent serine palmitoyl transferase (de novo pathway) inhibitor [45], on steatohepatitis,

insulin signaling, ER stress markers, and activation of AAH-Notch signaling networks in liver.

3.4. MATERIALS AND METHODS

3.4.1. Materials. The Taqman Gene expression master mix, Amplex UltraRed soluble fluorophore, Akt Pathway Total and Phospho 7-Plex panels, and the 4-Methylumbelliferyl phosphate (4-MUP) fluorophore were purchased from Invitrogen (Carlsbad, CA). ERK1/2 and ^{pT202/Y204}-ERK single-plex bead-based assays were purchased from EMD Millipore (Billerica, MA). QIAzol Lysis Reagent for RNA extraction was obtained from Qiagen, Inc (Valencia, CA). MaxiSorp plates and OptiPlates (96-well) used for enzyme-linked immunosorbent assays (ELISAs) were purchased from Thermo Fisher Scientific (Rochester, NY). SuperBlock TBS, bicinchoninic assay reagents, and enhanced chemiluminescence (ECL) solutions were purchased from Pierce Chemical Company (Rockford, IL). Mouse monoclonal anti-ceramide and myriocin were purchased from Sigma-Aldrich Co (St. Louis, MO). Rabbit polyclonal antibody to large acidic ribosomal protein (RPLPO) was purchased from Proteintech Group, Inc (Chicago, IL). The A85E6 and A85G6 mouse monoclonal antibodies to AAH were generated, purified, and characterized as previously described [29,30]. All other antibodies and immunodetection reagents for ELISAs were purchased from Abcam (Cambridge, MA), Upstate (Billerica, MA), Invitrogen (Carlsbad, CA), or Chemicon (Temecula, CA). Fine chemicals were purchased from CalBiochem (Carlsbad, CA) or Sigma-Aldrich (St. Louis, MO).

3.4.2. In vivo experimental model. Adult male Long Evans rats (Harlan Sprague Dawley, Inc., Indianapolis, Indiana) were pair-fed with isocaloric liquid diets (BioServ, Frenchtown, NJ) containing 0% or 37% (caloric content; 9.2% v/v) ethanol for 8 weeks [4]. Two weeks prior to initiating the study, rats were adapted to the liquid diets by incrementing ethanol from 0% to 11.8%, 23.6% and then 37% of the caloric content.

Controls were adapted to ethanol-free liquid diets over the same period. After 3 weeks on the full liquid diets, rats in each group were treated on Mondays, Wednesdays, and Fridays by intraperitoneal (i.p.) injection of myriocin (0.3 mg/kg) or saline. The myriocin and vehicle treatments were continued through the last 5 weeks of the study. Rats were monitored daily to ensure adequate nutritional intake and maintenance of body weight. Rats were housed under humane conditions and kept on a 12-hour light/dark cycle with free access to food. All experiments were performed in accordance with protocols approved by Institutional Animal Care and Use Committee at the Lifespan-Rhode Island Hospital and they conform to guidelines established by the National Institutes of Health.

3.4.3. Liver tissue processing. At the end of the experiment, rats were fasted overnight, and then sacrificed by isoflurane inhalation. Livers were divided for freezing and fixation. Fresh tissue samples were snap-frozen in a dry ice/methanol bath and stored at -80°C for protein, ceramide, and RNA studies. Alternatively, tissue samples were immersion-fixed in 4% buffered paraformaldehyde and embedded in paraffin. Histologic sections (5-microns thick) were stained with Hematoxylin and Eosin (H&E). Cryostat sections (10 µm thick) of paraformaldehyde fixed, non-embedded tissues were stained with Oil Red-O (ORO) to assess hepatic steatosis. Steatosis severity across the entire tissue sample (1 cm²) was graded under code and at 100x magnification as follows: 0= not detectable staining; 1= staining confined to a 3-4 cell thick radius of hepatocytes in Zone 3; 2= hepatocyte ORO staining extends up to Zone 2; 3=ORO staining extends into and somewhat beyond Zone 2; 4= panlobular ORO staining. Inter-group comparisons were made by ANOVA tests with post hoc Tukey tests.

3.4.4. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assays. RNA was extracted from frozen liver using the RNeasy Mini Kit. Total RNA was reverse transcribed with random oligonucleotide primers and the AMV 1st Strand cDNA Synthesis Kit. Gene expression was measured using a hydrolysis probe-based duplex

qRT-PCR assay with β -actin as a reference gene as previously described ⁴⁶. Gene-specific primers and matched probes were determined with the ProbeFinder Software (Roche, Indianapolis, IN). PCR amplifications were performed in a LightCycler 480 machine (Roche, Indianapolis, IN) and results were analyzed using the LightCycler® Software 4.0.

3.4.5. Enzyme-linked immunosorbent assay (ELISA). Immunoreactivity was measured by direct binding duplex ELISAs, and normalized to large acidic ribosomal protein (RPLPO) measured in the same wells ⁵. Liver tissue homogenates were prepared in lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA (pH 8.0), 50 mM NaF, 0.1% Triton X-100, and protease and phosphatase inhibitors ⁵. Protein concentration was measured with BCA reagents. Direct binding ELISAs were performed in 96-well MaxiSorp plates. Protein homogenates (100 ng/50 μ l) were adsorbed to the well bottoms by overnight incubation at 4°C, and then blocked for 3 hours with 1% bovine serum albumin (BSA) in TRIS buffered saline (TBS). After washing, the samples were incubated with primary antibody (0.1-0.4 μ g/ml) for 1 hour at 37°C. Immunoreactivity was detected with horseradish peroxidase (HRP)-conjugated secondary antibody and Amplex UltraRed soluble fluorophore. Fluorescence intensity was measured (Ex 565 nm/Em 595 nm) in a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA). Subsequently, the samples were incubated with biotin-conjugated antibodies to RPLPO, and immunoreactivity was detected with streptavidin-conjugated alkaline phosphatase (1:1000) and the 4-Methylumbelliferyl phosphate (4-MUP) fluorophore. Fluorescence (Ex 360 nm/Em 450 nm) intensity was measured in a SpectraMax M5 microplate reader. Binding specificity was determined from parallel negative control incubations in which the primary or secondary antibody was omitted. The ratios of specific protein/RPLPO were calculated and used for inter-

group statistical comparisons. Ceramide immunoreactivity was quantified by ELISA as previously described [5,47].

3.4.6. Bead-based multiplex ELISA. Liver protein homogenates (100 µg samples) prepared as described earlier, were used to assess signaling through insulin and IGF-1 receptors, IRS-1, and Akt pathways with the Akt Total and Phospho 7-Plex Panels. The Akt Total 7-Plex panel measured: insulin receptor (IR), IGF-1 receptor (IGF-1R), IRS-1, Akt, proline-rich Akt substrate of 40 kDa (PRAS40), ribosomal protein S6 kinase (p70S6K), and glycogen synthase kinase 3β (GSK-3β). The Akt Phospho 7-Plex panel measured: pYpY1162/1163-IR, pYpY1135/1136-IGF-1R, pS312-IRS-1, pS473-Akt, pT246-PRAS40, pTpS421/424-p70S6K, and pS9-GSK3β. Total ERK1/2 and pT202/Y204-ERK1/2 were measured in single-plex assays. Immunoreactivity was measured in a BioPlex 200 (BioRad, Hercules, CA).

3.4.7. Statistical analysis. Data depicted in box plots reflect group medians (horizontal bar), 95% confidence interval limits (upper and lower box limits) and range (whiskers). Tabulated data reflect means ± SEMs for each group. Data (N=6/group) were analyzed using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA), and inter-group comparisons were made using repeated measures one-way analysis of variance (ANOVA) with Tukey's multiple comparisons post hoc test. Computer software generated P-values are shown within the graph panels or table.

3.5. RESULTS

3.5.1. Myriocin ameliorates ALD. Control livers had the expected chord-like architecture and were free of inflammation, steatosis, fibrosis, and cholestasis (**Figure 3-1A**). Myriocin treatment produced subtle changes in control livers, such that hepatocytes were rendered more polygonal with discrete borders relative to control+vehicle livers

(**Figure 3-1B**). Chronic ethanol-fed rats had steatohepatitis characterized by diffuse hepatocellular cytoplasmic micro-vesicular lipid vacuoles, dilated sinusoids, multiple foci of lymphomononuclear cell inflammation, and scattered hepatocytes undergoing apoptosis or necrosis (**Figure 3-1C**). Myriocin reduced sinusoidal dilation, inflammation, and hepatic steatosis. However, foci of apoptosis, low-level inflammation, and evidence of increased cell turnover persisted (**Figure 3-1D**).

Oil red O staining revealed zonal and relatively low levels of hepatocellular lipid accumulation in control livers, irrespective of myriocin treatment (**Figures 3-2A-2B**). Chronic ethanol feeding caused striking pan-lobular hepatic steatosis, with both coarse and fine cytoplasmic lipid droplets (**Figure 3-2C**). Myriocin conspicuously reduced hepatic steatosis, as was evident by Oil red O staining (**Figure 3-2D**). Results of semi-quantitative ORO grading (Supplementary (S) Methods) revealed significantly higher levels of hepatic steatosis in the ethanol+vehicle group relative to all others ($P<0.0001$), and similarly low levels of hepatocellular ORO staining in the control+vehicle, control+myriocin, and ethanol+myriocin groups (**STable 3-1**). Correspondingly, higher levels of hepatic ceramide were measured in ethanol+vehicle relative to control+vehicle livers ($P<0.001$), whereas myriocin normalized hepatic ceramide levels in the ethanol fed rats, despite continuous ethanol exposure. In contrast, myriocin treatment had no significant effect on hepatic triglyceride content (**STable 3-1**).

3.5.2. Myriocin restores hepatic insulin and IGF signaling and downstream activation of Akt pathways in ALD. Multiplex ELISAs were used to measure total (**Figure 3-3**) and phosphorylated (**Figure 3-4**) insulin receptor, IGF-1 receptor, IRS-1, Akt, GSK-3 β , PRAS40, and p70S6K, and single-plex assays were used to measure ERK1/2 and $p^{T202/Y204}$ -ERK1/2. The calculated ratios of phosphorylated to total protein (**SFigure 3-1**) were used to assess relative levels of phosphorylation.

ANOVA tests demonstrated significant inter-group differences with respect to

insulin receptor ($F=3.1$; $P<0.05$), IRS-1 ($F=33.46$; $P<0.0001$), Akt ($F=27.74$; $P<0.0001$), proline-rich Akt substrate 40 kDa (PRAS40) ($F=4.04$; $P=0.01$), p70S6K ($F=20.06$; $P<0.0001$), and ERK ($F=15.37$; $p<0.0001$). In contrast, no significant inter-group differences were observed with respect to IGF-1 receptor (**Figure 3-5-3B**) or GSK-3 β (**Figure 3-3F**). Post-hoc Tukey-Kramer tests demonstrated that chronic ethanol feeding reduced hepatic expression of the insulin receptor ($P<0.05$), IRS-1 ($P<0.001$), Akt ($P<0.05$), and p70S6K ($P<0.001$) (**Figure 3-3**). Although PRAS40 expression was also lower in ethanol-exposed livers, the difference from control did not reach statistical significance. Myriocin abolished the inter-group differences with respect to hepatic expression of insulin receptor and PRAS40, significantly increased expression of Akt ($P<0.001$) and p70S6K ($P<0.05$) relative to control, and similarly increased ERK in control and ethanol-exposed livers relative to vehicle (**Figure 3-3**). In contrast, myriocin failed to increase and normalize IRS-1 expression in ethanol-exposed livers.

ANOVA tests demonstrated significant inter-group differences with respect to pYpY1162/1163-Insulin receptor ($F=4.58$; $P=0.013$) (**Figure 3-4A**), pS473-Akt ($F=8.2$; $P=0.0009$) (**Figure 3-4E**), pS9-GSK-3 β ($F=6.89$; $P=0.0023$) (**Figure 3-4F**), and pT421/S424-p70S6K ($F=7.94$; $P=0.001$) (**Figure 3-4H**). In contrast, ethanol or myriocin treatment had no significant effects on the expression levels of pYpY1135/1136-IGF-1R (**Figure 3-4B**), pS312-IRS-1 (**Figure 3-4C**), pT202/Y204-ERK1/2 (**Figure 3-4D**), or pT246-PRAS40 (**Figure 3-4G**). Post hoc Tukey's multiple comparison test revealed significantly reduced levels of pS473-Akt ($P<0.01$) and increased levels of pS9-GSK-3 β ($P=0.05$) and pT421/S424-p70S6k ($P<0.01$) in ethanol+vehicle exposed relative to control+vehicle livers. The reduced levels of pT246-PRAS40 in ethanol-exposed livers did not reach statistical significance. Myriocin increased pS473-Akt and decreased pYpY1162/1163-Insulin receptor ($P<0.05$) and pT421/S424-p70S6k ($P<0.01$) in ethanol-treated livers (relative to vehicle). Phosphorylated levels of insulin receptor, IGF-1 receptor, IRS-1, ERK1/2, Akt, PRAS40, and p70S6K were similar

in myriocin-treated control and ethanol-exposed livers. In contrast, p^{S9} -GSK-3 β expression was significantly higher in ethanol-exposed, myriocin-treated versus corresponding control livers (**Figure 3-4F**).

ANOVA tests demonstrated significant inter-group differences with respect to the calculated $p^{YpY1162/1163}$ /total-Insulin receptor ($F=4.96$; $P=0.009$) (**SFigure 3-1A**), p^{S312} /total-IRS-1 ($F=23.82$; $P<0.0001$) (**SFigure 3-1C**), p^{S473} /total-Akt ($F=12.02$; $P=0.001$) (**SFigure 3-5-1E**), p^{S9} /total-GSK-3 β ($F=3.11$; $P=0.049$) (**SFigure 3-1F**), and $p^{T421/S424}$ /total-p70S6K ($F=43.44$; $P<0.0001$) (**SFigure 3-1H**). In contrast, ethanol or myriocin treatment had no significant effects on the expression levels of $p^{YpY1135/1136}$ /total-IGF-1R (**SFigure 3-1B**), $p^{T202/Y204}$ /total-ERK1/2 (**SFigure 3-1D**), or p^{T246} /total-PRAS40 (**SFigure 3-1G**). Post hoc Tukey's multiple comparison test revealed that myriocin significantly reduced the calculated relative levels of $p^{YpY1162/1163}$ /total-insulin receptor ($P<0.01$), p^{S473} /total-Akt ($P<0.001$), and $p^{T421/S424}$ /total-p70S6K ($P<0.001$) relative to ethanol+vehicle and/or control+vehicle. The mean relative levels of p^{S312} /total-IRS-1 were significantly higher in ethanol+vehicle and ethanol+myriocin treated relative to both control groups ($P<0.001$). Otherwise, the relative levels of protein phosphorylation were similar in myriocin-treated control and ethanol-exposed livers (**SFigure 3-1**).

3.5.3. Effects of myriocin on AAH-Notch signaling networks in chronic ALD.

Duplex probe-based qRT-PCR assays revealed significant effects of ethanol and/or myriocin treatment on AAH ($F=4.11$; $P=0.017$) (**Figure 3-5A**), Jagged ($F=8.25$; $P=0.0006$), HIF-1 α ($F=5.25$; $P=0.063$) (**Figure 3-5C**), and HES-1 ($F=2.99$; $P=0.05$) (**Figure 3-5E**). Post hoc Tukey multiple comparison tests demonstrated that chronic ethanol feeding inhibited hepatic expression of AAH ($P<0.05$; **Figure 3-5A**) and HES-1 ($P<0.05$; **Figure 3-5E**), but increased expression of Jagged-1 ($P<0.05$) and HIF-1 α ($P<0.05$) relative to control (+vehicle). Myriocin treatment resulted in similar (not significantly different) levels of AAH, HIF-1 α , and HES-1 in control and ethanol-exposed

livers, but did not increase AAH mRNA in the ethanol+vehicle group. In contrast, Jagged-1 (**Figure 3-5B**) and Notch 1 (**Figure 3-5D**) expressions were not significantly modulated by myriocin.

Duplex ELISAs, in which immunoreactivity was normalized to RPLPO, were used to measure AAH (A85G6 antibody), Humbug (A85E6 antibody), Notch-1, Jagged-1, and proliferating cell nuclear antigen (PCNA) (**Figure 3-6**). ANOVA tests demonstrated significant effects of ethanol or myriocin on AAH ($F=37.24$; $P<0.0001$) (**Figure 3-6A**), Humbug ($F=38.14$; $P<0.0001$) (**Figure 3-6B**), Notch 1 ($F=17.64$; $P<0.0001$) (**Figure 3-6C**), Jagged 1 ($F=41.20$; $P<0.0001$) (**Figure 3-6D**), and PCNA ($F=36.02$; $P<0.0001$) (**Figure 3-6E**). Tukey's multiple comparison tests demonstrated that chronic ethanol feeding significantly reduced hepatic expression of AAH ($P<0.001$), Humbug ($P<0.001$), Notch 1 ($P<0.05$), Jagged 1 ($P<0.001$), and PCNA ($P<0.001$) relative to vehicle-treated control livers. Myriocin significantly increased the expression of all five proteins in the ethanol group, and all but PCNA in the control group. The net effect was to render the expression of all five proteins similar in control and ethanol-exposed, myriocin-treated livers. The findings with respect to AAH were corroborated by Western blot analysis (**SFigure 3-2**).

3.6. DISCUSSION

3.6.1. Study rationale and objectives. Progressive liver degeneration is mediated by compromised hepatocellular growth, survival, glucose utilization, energy metabolism and protein synthesis [9,13] which are needed for regeneration, repair, and remodeling after injury. Although peroxisome proliferator activated receptor (PPAR) agonists, which are insulin sensitizers that have anti-inflammatory actions, can prevent or reverse histopathologic, ultrastructural, biochemical, and molecular abnormalities in chronic ALD [4,47,48], all aspects of ALD are not resolved. In particular, ceramide load and ER stress

remain elevated [40] setting the stage for ALD to progress due to persistent insulin/IGF resistance and inhibition of target genes and signaling molecules that regulate liver remodeling. Therefore, additional therapeutic approaches are needed. Herein, we evaluated responses to a ceramide inhibitor in an established model of ALD.

3.6.2. Ceramide inhibitor effects on ALD pathology. Myriocin treatment, which significantly reduced hepatic ceramide levels, substantially reduced the severity of steatohepatitis, despite continued ethanol exposure. Myriocin resolved the hepatic steatosis, architectural disarray, sinusoidal dilatation, and to a large extent, inflammation. Persistence of inflammatory and necrotic/apoptotic foci (although small and scattered) corresponds with ongoing injury caused by daily ethanol exposures. Overall, the findings suggest that ceramide accumulation in liver is a critical mediator of ALD. This conclusion is consistent with the known cytotoxic effects of ceramides, including activation of pro-inflammatory cytokines, and inhibition of insulin signaling through Akt, which regulates hepatocellular metabolism, survival and growth [37,38]. Therefore, by reducing hepatic ceramide, liver structure and function in ALD can be restored. Furthermore, since many drugs require liver metabolism for detoxification, ceramide inhibitor therapy could raise the threshold of drug toxicity in ALD.

3.6.3. Effects of myriocin on ethanol-impaired signaling through insulin receptor, IRS-1, Akt, and ERK. Previous studies demonstrated that chronic ALD is associated with hepatic insulin and IGF resistance [9,13,40]. The spectrum of abnormalities includes alterations in trophic factor, receptor, and/or insulin receptor substrate (IRS) protein and/or gene expression, and reduced activation of ERK MAPK and PI3K-Akt. Ethanol-impaired signaling through ERK is associated with reduced regenerative capacity of the liver [8], whereas reduced Akt leads to impaired cell survival, remodeling, motility, and metabolism [4,8,12]. Herein we again demonstrate that chronic ethanol feeding broadly impairs hepatic insulin signaling mechanisms, from receptor to IRS-1, and beyond,

including molecules downstream of Akt, i.e. PRAS40 and P70S6K [10,47]. Myriocin reduced or abolished most ethanol-associated impairments in insulin signaling, suggesting that in chronic ALD, impairments in insulin signaling are mediated in part by hepatic ceramide accumulation. These findings highlight the importance of targeting dysregulated lipid metabolism and lipotoxicity in the clinical management of ALD.

3.6.4. Myriocin reverses ethanol-mediated inhibition of AAH expression. Previous studies demonstrated that AAH, and its highly homologous truncated N- terminus homologue, Humbug [18,19] are stimulated by insulin and IGF [14,26,28,29], and inhibited by ethanol [3,29-31]. Although ethanol inhibits AAH's mRNA, it more prominently decreases AAH's protein expression due to activation of GSK-3 β . GSK-3 β phosphorylates AAH and renders it susceptible to proteolytic degradation [27,30]. PPAR agonist enhancement of insulin signaling in experimental ALD restores AAH protein expression and hepatic architecture [4,15,47]. Herein, we show similar positive effects of myriocin on AAH protein in ALD. However, since GSK-3 β activation was not significantly modulated by myriocin, the mechanism of enhanced AAH protein expression most likely differed from the effects of PPAR agonists. One potential mechanism is that the myriocin effect on AAH may have been mediated by reduced hepatocellular stress along with pro-apoptotic, inflammatory, and proteolytic functions due to increased signaling through Akt.

3.6.5. Myriocin reverses ethanol-mediated inhibition of Notch signaling mechanisms. Notch signaling is activated by ligand e.g. Delta or Serrate (Jagged) binding to Notch receptors on the cell surface [23]. Subsequent proteolytic cleavage of Notch releases its intracellular domain, which translocates to the nucleus where it binds to transcriptional regulators, e.g. CSL, displacing co-repressors and recruiting co-activators [23,24]. The net effect is to increase expression of target genes including, Hairy- Enhancer of Split (HES) and HES-related proteins [50]. Notch signaling is regulated by AAH [14,26,27], and both Notch and Jagged have the consensus sequence

for AAH hydroxylation [21,22]. AAH's catalytic activity promotes Notch's translocation to the nucleus, cell adhesion, and cell motility [17,29], yet AAH expression and function are stimulated by insulin/IGF [15,28] and inhibited by ethanol [30]. In addition, previous studies showed that AAH and Notch cross-talk with hypoxia-inducible factor-1 α (HIF-1 α), and that HIF-1 α expression is regulated by insulin/IGF and stimulated by oxidative stress and ethanol [26].

Herein, we show that myriocin had no significant effects on the mRNA levels of AAH, Notch-1, Jagged-1, or HIF-1 α , and instead increased expression of AAH, Humbug, Notch-1 and Jagged-1 proteins in both control and ethanol-exposed livers. Corresponding with the anticipated increases in Notch signaling, HES-1 mRNA levels were increased by myriocin treatment of ethanol-exposed livers. HES-1 is a downstream target of Notch signaling, and the finding of normalized/increased HES-1 expression in ethanol+myriocin treated livers reflects increased Notch activation by myriocin. The increased expression of HIF-1 α in ethanol-exposed livers is consistent with previous observations [26]. In essence, ethanol impaired AAH-Notch signaling mechanisms and growth potential of the liver (PCNA expression) were restored by myriocin treatment.

3.7. CONCLUSION

The findings suggest that aberrant ceramide accumulation plays a major role in the pathogenesis of ALD. Moreover, although insulin sensitizer drugs, e.g. PPAR agonists [4,47,48] and anti-inflammatory agents [10] can prevent or reverse many aspects of ALD, their failure to completely resolve the disease process could be due to persistently high levels of hepatic ceramides [47]. The results suggest that mono-therapy with ceramide inhibitors can restore hepatic architecture and function in chronic ALD because they normalize hepatic ceramide levels, and thereby reduce lipotoxicity,

inflammation, and impairments in insulin/IGF-1 signaling and gene expression. Moreover, ceramide inhibitors support signaling through pathways that activate AAH-Notch networks utilized for structural remodeling of the liver after injury.

Figure 3-1

**Histopathologic Features of Experimental Alcohol-Induced
Steatohepatitis-Effects of Myriocin Treatment**

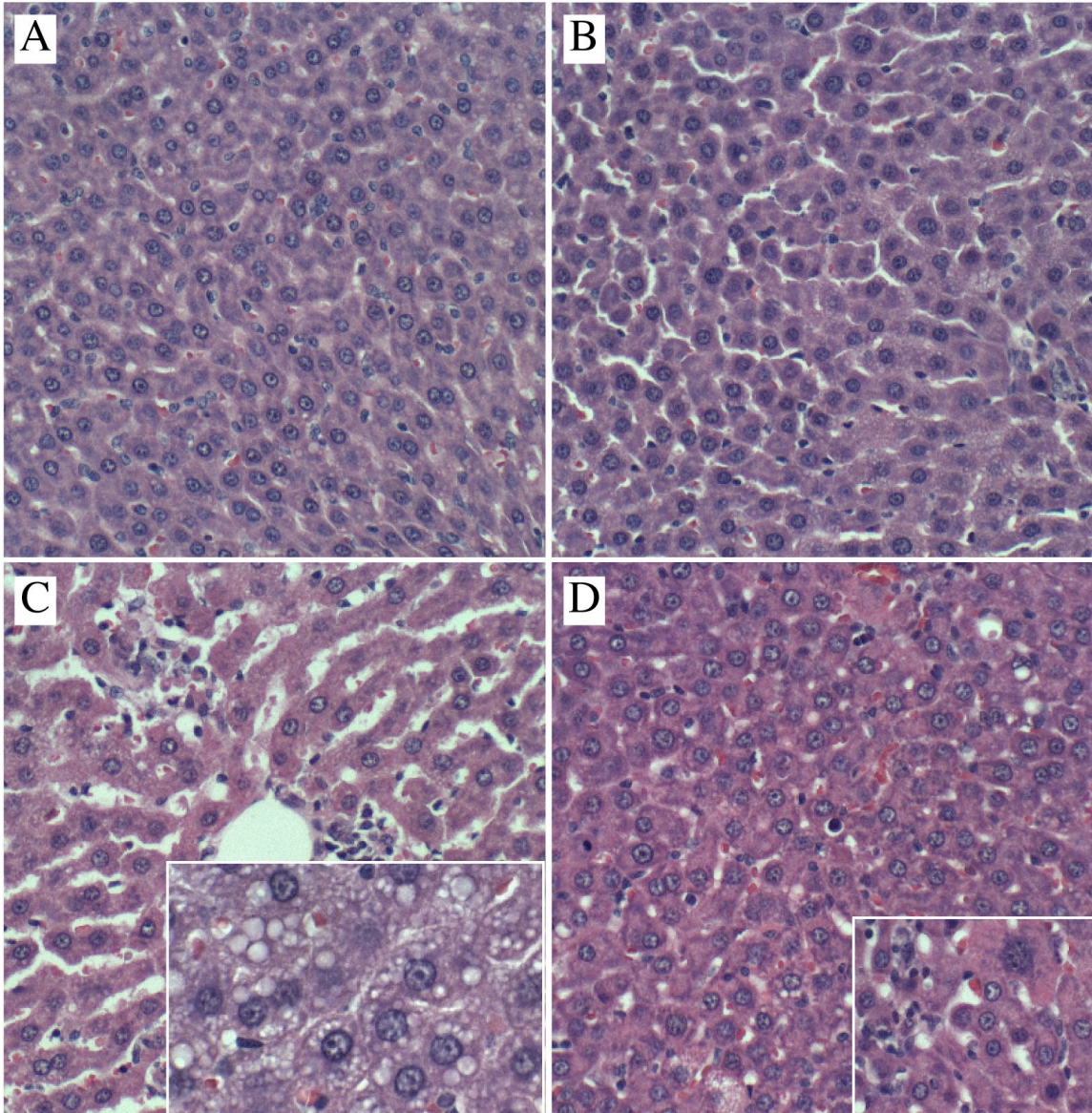


Figure 3-1. Histopathologic features of experimental alcohol-induced steatohepatitis-effects of myriocin treatment. Adult male Long Evans rats were fed with isocaloric liquid diets containing 0% (control) or 37% ethanol (caloric content) for 8 weeks, and treated with vehicle or myriocin 3 times per week over the last 5 weeks of the experiment. Paraformaldehyde-fixed paraffin-embedded 5 micron thick sections of liver were stained with H&E. (A) Control livers had regular chord-like architectures and uniform hepatocyte structure. (B) Myriocin treatment had minimal discernible effect on hepatic architecture in control rats. (C) Ethanol-exposed livers exhibited foci of lymphomononuclear cell inflammation (upper left and center), apoptotic bodies, and prominent micro-vesicular steatosis (clear cytoplasmic vacuoles-inset). (D) Myriocin treatment nearly normalized hepatic architecture in ethanol-fed rats, although apoptosis and mild inflammation persisted (inset).

Figure 3-2

**Increased Hepatic Steatosis in Experimental Chronic ALD-
Effects of Myriocin Treatment**

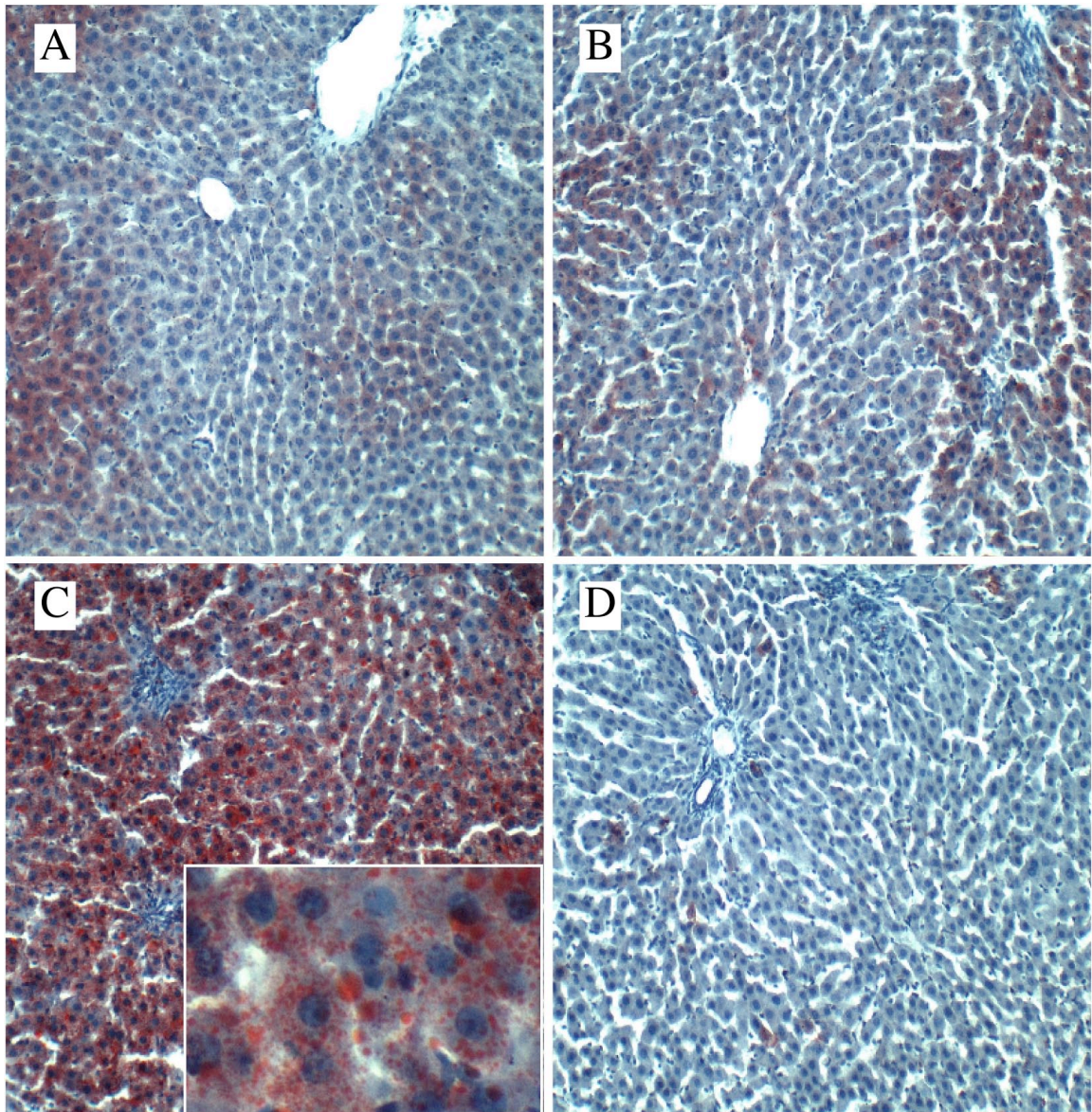


Figure 3-2. Increased hepatic steatosis in experimental chronic ALD-effects of myriocin treatment. Cryostat sections of paraformaldehyde-fixed livers from (A) control + vehicle, (B) Control + myriocin, (C) ethanol + vehicle, and (D) ethanol + myriocin were stained with Oil Red O to detect cytoplasmic lipid accumulation (red punctate or vesicular labeling-inset in C).

Figure 3-3

Chronic Ethanol Feeding and Myriocin Treatment Effects on Hepatic Insulin, IGF and IRS Signaling Mechanisms

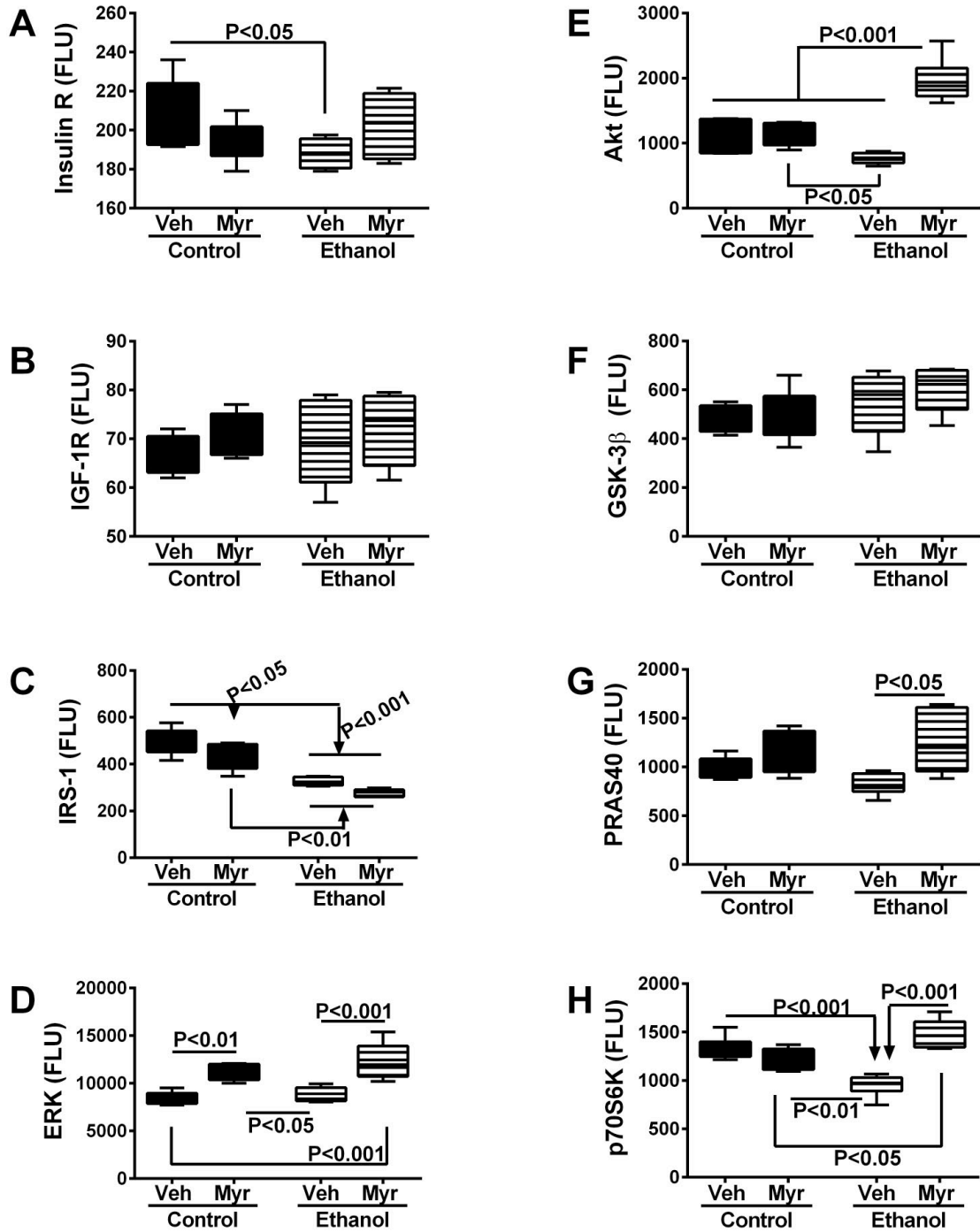


Figure 3-3. Chronic ethanol feeding and myriocin treatment effects on hepatic insulin, IGF and IRS signaling mechanisms. Liver tissue from control and chronic ethanol-fed rats that were treated with vehicle (Veh) or myriocin (Myr) was used to measure (A) insulin receptor (R), (B) IGF-1R, (C) IRS-1, (D) ERK1/2, (E) Akt, (F) GSK-3 β , (G) PRAS40, and (H) p70S6K by multiplex or single-plex ELISA. Results were normalized to protein content in reactions. N=8 or 10 samples per group. Box plots depict medians (horizontal bars), 95% confidence intervals (upper and lower limits of boxes), and ranges (stems). Inter-group comparisons were made by repeated measures one-way ANOVA with post-hoc Tukey tests.

Figure 3-4

Chronic Ethanol Feeding and Myriocin Treatment Effects on Phosphorylated Insulin, IGF and IRS Signaling Molecules in Liver

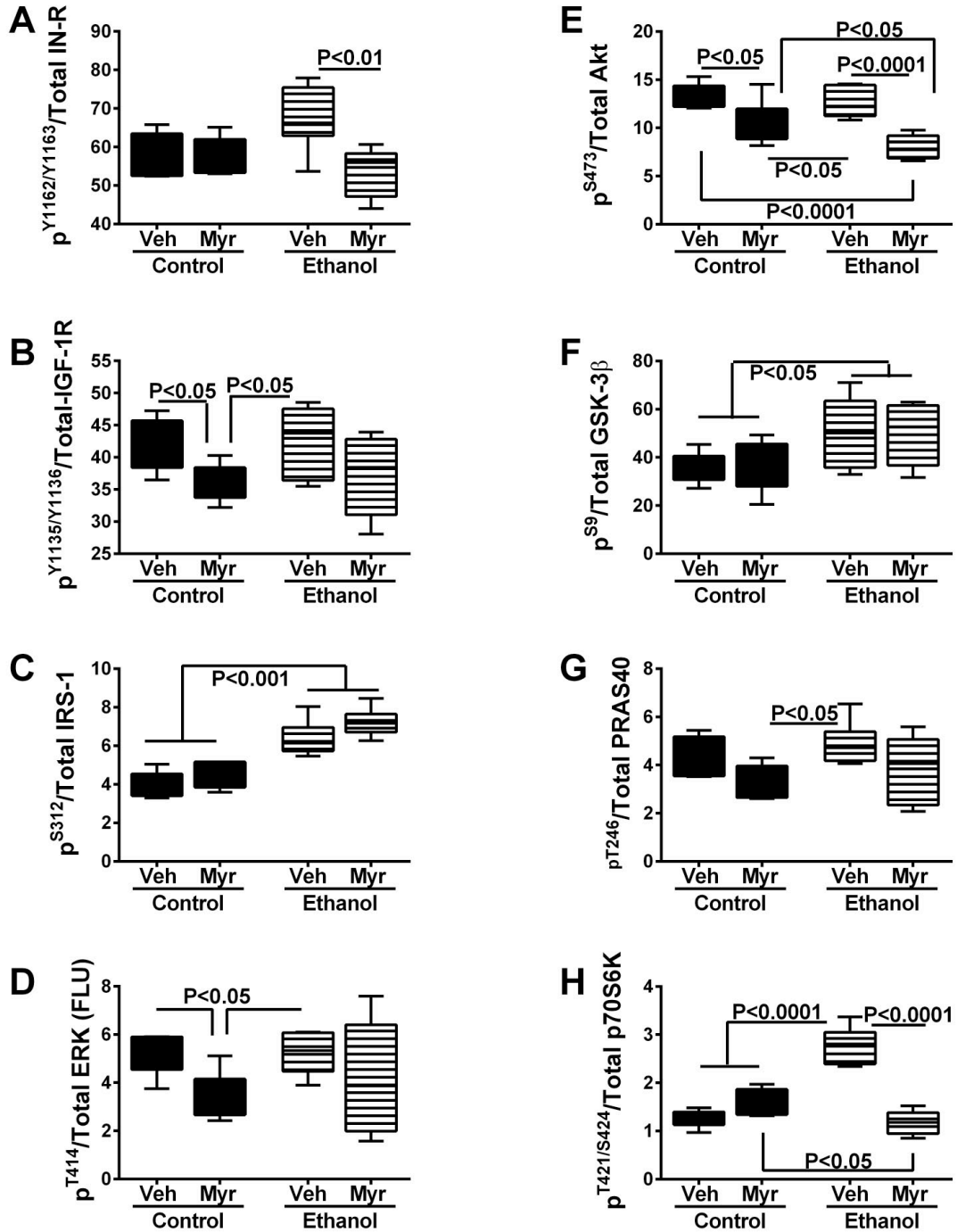


Figure 3-4. Chronic ethanol feeding and myriocin treatment effects on phosphorylated insulin, IGF and IRS signaling molecules in liver. Liver tissue from control and chronic ethanol-fed rats that were treated with vehicle (Veh) or myriocin (Myr) was used to measure (A) $pYpY^{1162/1163}$ -insulin receptor (R), (B) $pYpY^{1135/1136}$ -IGF-1R, (C) pS^{312} -IRS-1, (D) $pT^{202/Y204}$ -ERK1/2, (E) pS^{473} -Akt (F) pS^9 -GSK-3 β , (G) pT^{246} -PRAS40, and (H) $pT^{246}pS^{112}$ -p70S6K by multiplex or single-plex ELISA. Results were normalized to protein content in reactions. Box plots depict medians (horizontal bars), 95% confidence intervals (upper and lower limits of boxes), and ranges (stems). Inter-group comparisons were made by repeated measures one-way ANOVA with post-hoc Tukey tests of significance.

Figure 3-5

Effects of Chronic Ethanol Exposure and Myriocin Treatment on AAH and Notch Signaling-Related mRNAs in Liver

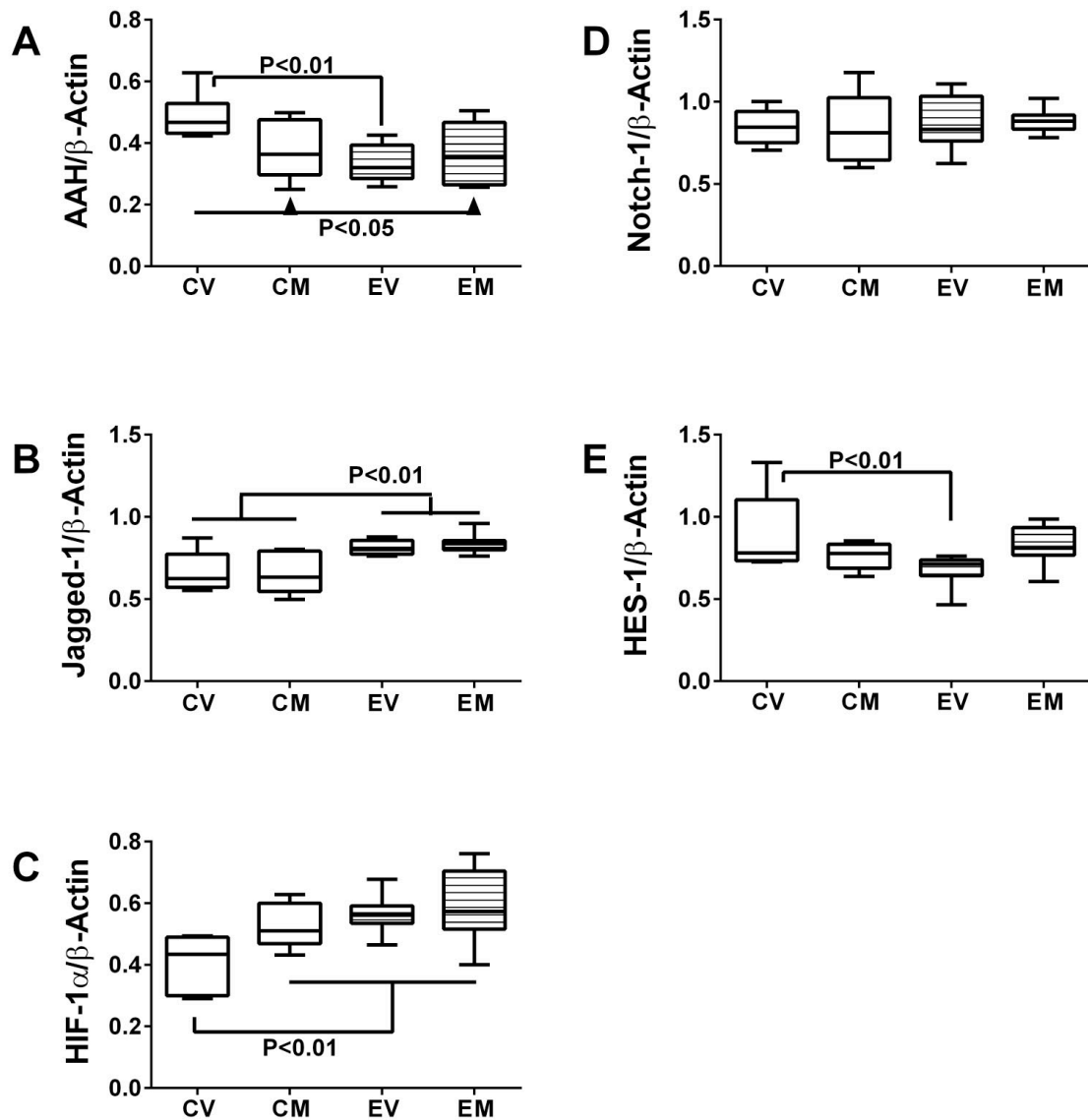


Figure 3-5. Effects of chronic ethanol exposure and myriocin treatment on AAH and Notch signaling-related mRNAs in liver. Livers from control and chronic ethanol-fed rats that were treated with vehicle or myriocin were used to measure (A) AAH, (B) Jagged 1, (C) HIF-1 α , (D) Notch-1, and (E) HES-1 mRNA levels in probe hydrolysis-based qRT-PCR assays. Results were normalized to β -actin, which was measured simultaneously. Inter-group comparisons were made using the calculated mRNA/ β -actin ratios. CV=control+vehicle; CM= control+myriocin; EV=ethanol+vehicle; EM=ethanol+myriocin. N=8 or 10 samples per group. Box plots depict medians (horizontal bars), 95% confidence intervals (upper and lower limits of boxes), and ranges (stems). Inter-group comparisons were made by repeated measures one-way ANOVA with post-hoc Tukey tests of significance.

Figure 3-6

Effects of Chronic Ethanol Exposure and Myricetin Treatment on
AAH and Notch Signaling Molecules in Liver

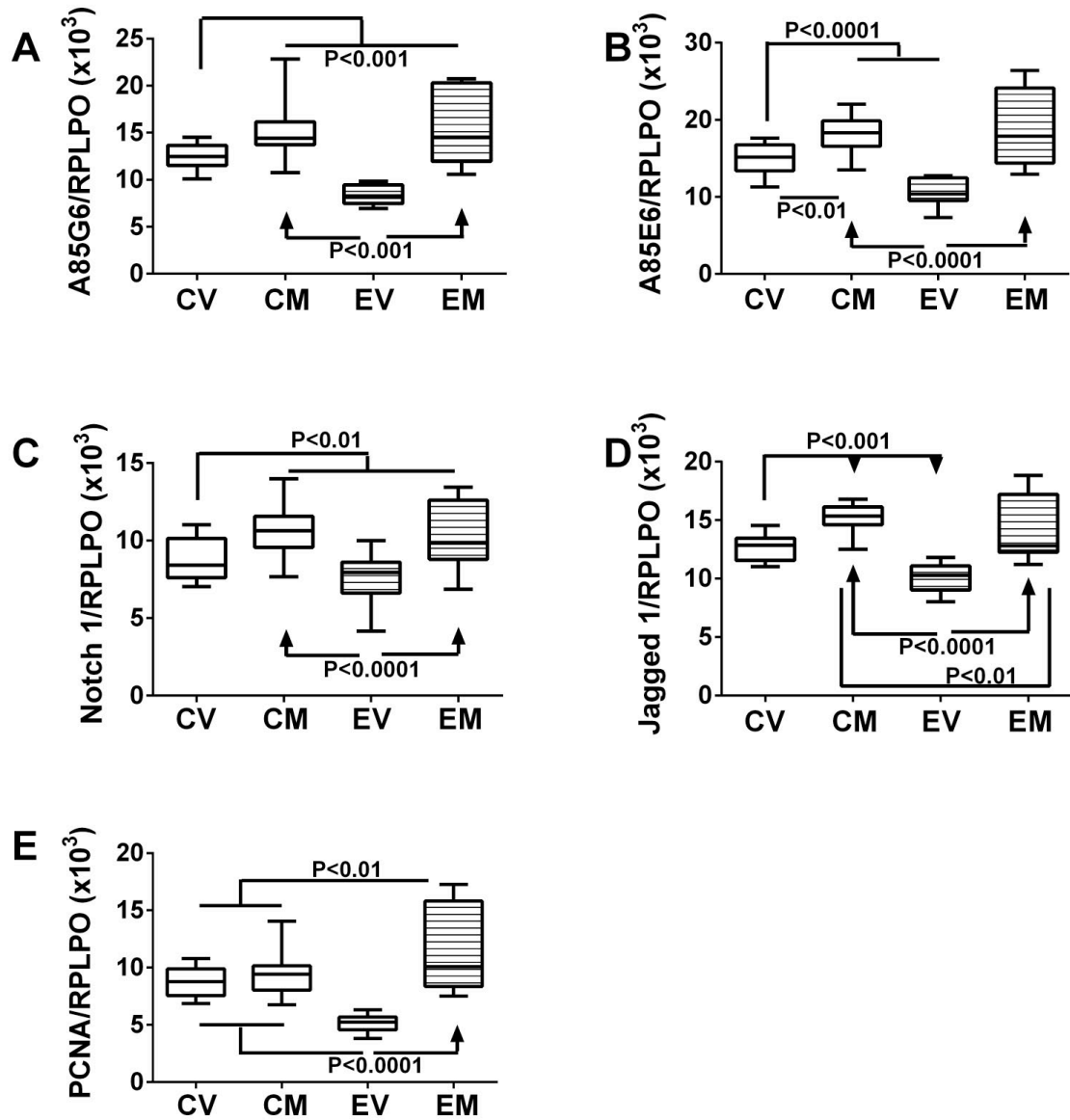


Figure 3-6. Effects of chronic ethanol exposure and myriocin treatment on AAH and Notch signaling molecules in liver. Livers from control and chronic ethanol- fed rats that were treated with vehicle or myriocin were used to measure (A) AAH-A85G6, (B) AAH/Humbug-A85E6, (C) Notch-1, (D) Jagged 1, and (E) PCNA immunoreactivity by duplex ELISAs. Results were normalized to RPLPO, which was measured in the same wells. Inter-group comparisons were made using calculated protein/RPLPO ratios. CV=control+vehicle; CM= control+myriocin; EV=ethanol+vehicle; EM=ethanol+myriocin. N=8 or 10 samples per group. Box plots depict medians (horizontal bars), 95% confidence intervals (upper and lower limits of boxes), and ranges (stems). Inter-group comparisons were made by repeated measures one-way ANOVA with post-hoc Tukey tests of significance.

Supplementary Table 3-1

Effects of Ethanol and Myriocin on Hepatic Lipids

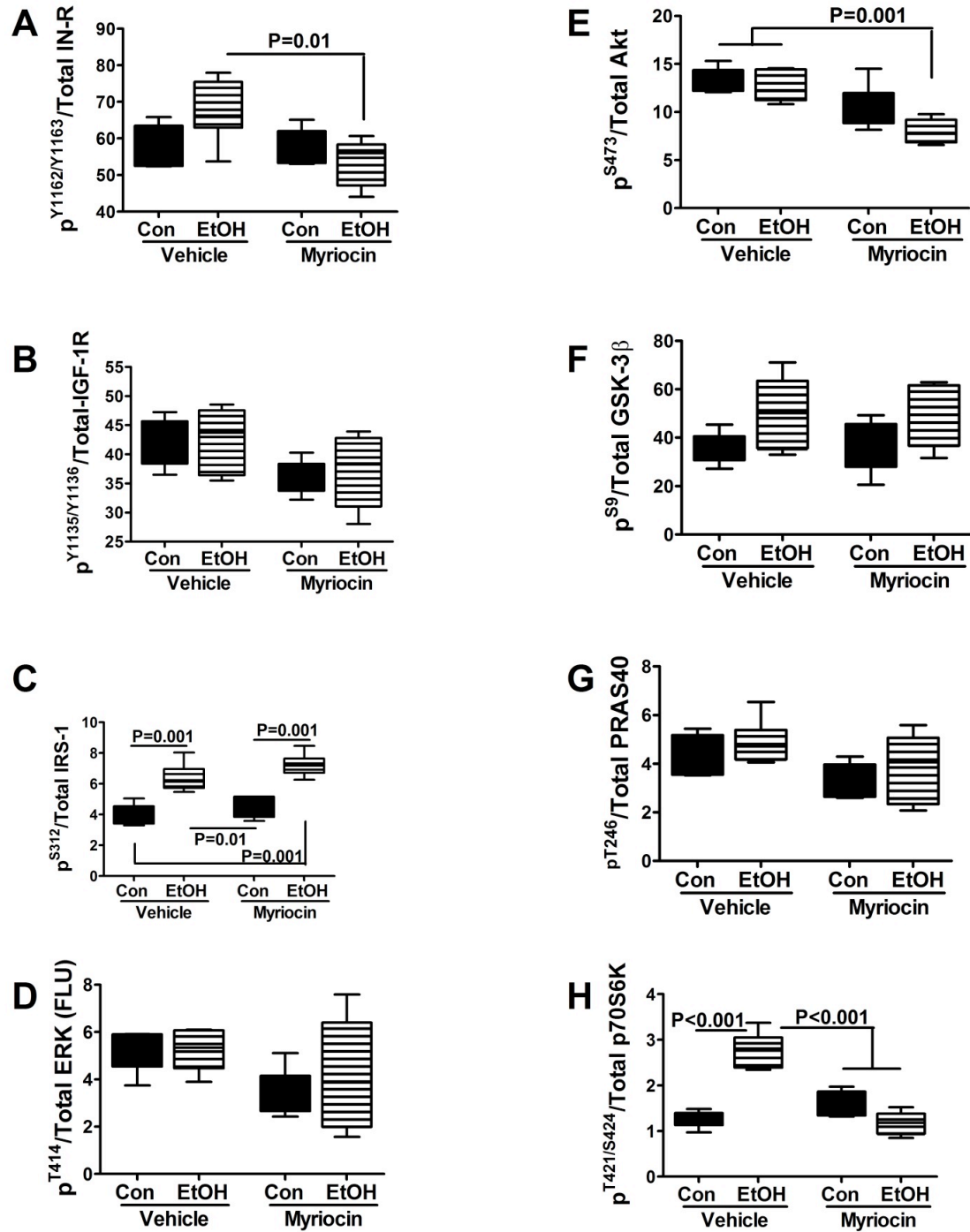
Lipid	C+V	C+M	Et+V	Et+M	F-Ratio	P-Value
ORO Grade	2.0 ± 0.0	1.25 ± 0.16	3.75 ± 0.16	1.13 ± 0.29	53.01	<0.0001
Triglycerides	8.4 ± 0.6	7.8 ± 0.5	7.3 ± 0.9	5.5 ± 0.7	2.76	0.066
Nile Red	1027 ± 53.4	1227 ± 120.4	1045 ± 53.1	997.7 ± 77.1	1.65	0.19
Ceramide	195651 ± 10301	198474 ± 14901	296502 ± 18904**	176868 ± 11409	14.65	<0.0001

Supplementary Table 3-1. Effects of ethanol and myriocin on hepatic lipids*.

Hepatic Oil red O (ORO) staining was graded on in histological sections using a semi-quantitative scale from 0 to 4 (see Supplementary Methods). Triglycerides and Nile red fluorescence were measured in fresh frozen tissue using commercial assays. Ceramide immunoreactivity was measured by ELISA. Values corresponding to triglycerides, Nile red, and ceramides reflect relative fluorescence units (arbitrary) normalized to protein content in the samples. M= myriocin treatment (see Methods). **Post hoc Tukey's multiple comparison test demonstrated significantly higher ceramide levels in Et+V livers compared with all other groups ($P<0.001$). *C+V = control + vehicle; Et + V = chronic ethanol fed + vehicle treatment.

Supplementary Figure 3-1

Chronic Ethanol Feeding And Myriocin Treatment Effects on Relative Levels of Phosphorylated Insulin, IGF and IRS Signaling Molecules in Liver

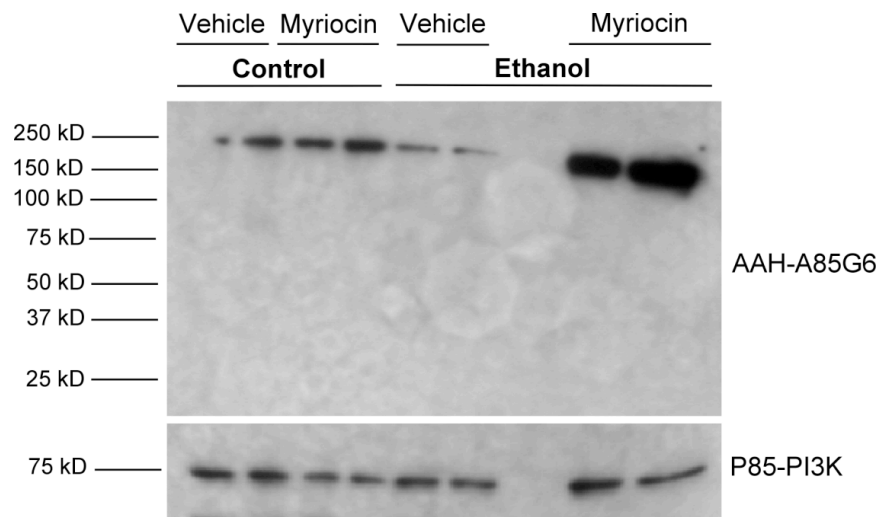


Supplementary Figure 3-1. Chronic ethanol feeding and myriocin treatment effects on relative levels of phosphorylated insulin, IGF and IRS signaling molecules in liver. Liver tissue from control and chronic ethanol-fed rats that were treated with vehicle (Veh) or myriocin (Myr) was used to assess relative activation of insulin/IGF-1/IRS upstream and downstream signaling networks. Graphs depict the calculated ratios of (A) $\text{pYpY}^{1162/1163}/\text{total insulin receptor (R)}$, (B) $\text{pYpY}^{1135/1136}/\text{total IGF-1R}$, (C) $\text{pS}^{312}/\text{total IRS-1}$, (D) $\text{pT}^{202/Y204}/\text{total ERK1/2}$, (E) $\text{pS}^{473}/\text{total Akt}$, (F) $\text{pS}^9/\text{total GSK3}\beta$, (G) $\text{pT}^{246}/\text{total PRAS40}$, and (H) $\text{pT}^{246}\text{pS}^{112} / \text{total p70S6K}$ measured by multiplex or single-plex ELISAs. Box plots depict medians (horizontal bars), 95% confidence intervals (upper and lower limits of boxes), and ranges (stems). Inter-group comparisons were made by repeated measures one-way ANOVA with post-hoc Tukey tests of significance.

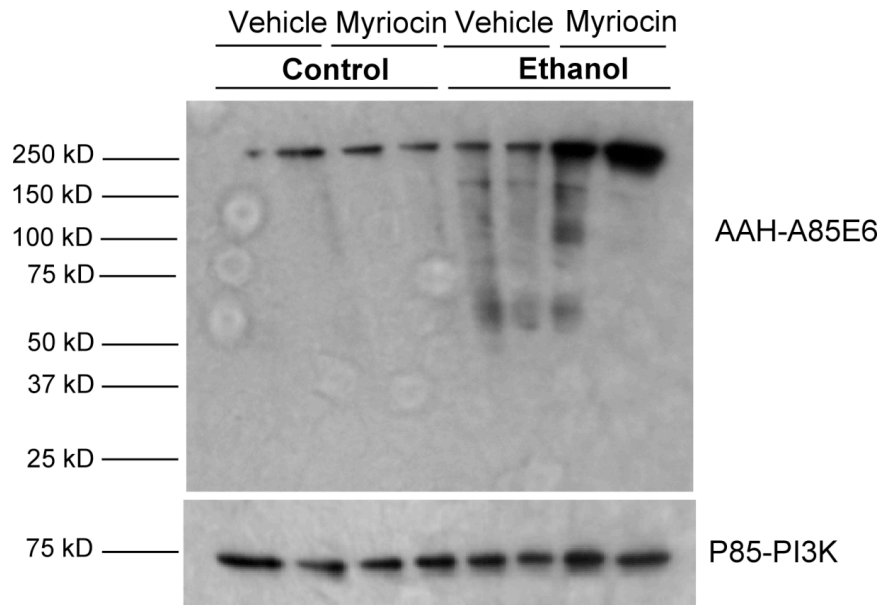
Supplementary Figure 3-2

Myriocin Increases Hepatic AAH Expression in Chronic Ethanol-Fed Rats

A



B



Supplementary Figure 3-2. Myriocin increases hepatic AAH expression in chronic ethanol-fed rats. Adult male Long Evans rats were fed with isocaloric liquid diets containing 0% (control) or 37% ethanol (caloric content) for 8 weeks, and treated with vehicle or myriocin 3 times per week over the last 5 weeks of the experiment. Western blot analysis was performed with 30 µg liver protein homogenate per lane (measured using the BCA assay). Blots were probed with monoclonal antibodies to AAH (A85G6) or AAH/Humbug (A85E6). The latter recognizes the full-length AAH, Humbug, and N-terminal cleavage fragments of AAH. Immunoreactivity was revealed with HRP-conjugated secondary antibody, ECL reagents, and film autoradiography. Blots were re-probed with rabbit polyclonal antibodies to the p85 subunit of PI3 Kinase as a loading control. In A and B, the upper bands represent AAH, which migrate higher than the predicted ~86 kD due to post-translational modification by phosphorylation. The lower bands in B represent cleavage products of AAH or Humbug. Note sharply increased levels of AAH in myriocin-treated ethanol-exposed livers. Effects in control livers are subtle, corresponding to the already lower levels of ceramide in liver.

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CHAPTER 4

**PPAR AGONIST EFFECTS ON NOTCH SIGNALING MEDIATORS IN
EXPERIMENTAL CHRONIC ALCOHOL-INDUCED STEATOHEPATITIS**

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AUTHOR CONTRIBUTIONS

DL measured target protein expression by duplex ELISA, interpreted results, and wrote the manuscript; MT set up animal model; TR assisted with duplex ELISA measurements; JRW provided insightful feedback; and SMdIM originated ideas, designed the experiments with co-authors, directed the research, and was closely involved in the data interpretation.

4.1. RATIONALE

Alcoholic liver disease (ALD) is mediated, at least in part, by sustained insulin and insulin-like growth factor (IGF) resistance. Alcohol impairs insulin signaling by various mechanisms, including disruption of lipid membrane composition, which is essential for insulin receptor binding, decreased IGF-1 polypeptide expression and insulin receptor substrate-1 activation [1–3], upregulation of phosphatases that inhibit Akt activation [4,5], and aberrant glycogen synthase kinase-3 β activation [6]. Previous reports indicated that treatment with a PPAR- δ agonist successfully restored insulin/IGF signaling and increased expression of aspartyl-asparaginyl- β -hydroxylase (AAH), a protein with a functional role in cell motility [7]. Increased AAH expression by PPAR- δ agonist treatment could explain the restoration of hepatic architecture observed by Ramirez, *et al.* [8]. The purpose of the study was to determine if the therapeutic effects of PPAR agonists, particularly of PPAR- δ , on restoring liver structure and function induced by chronic ethanol exposure was due to increased expression of AAH and Notch signaling proteins, promoting hepatocyte migration needed for proper liver remodeling and regeneration.

4.2. ABSTRACT

Background: Chronic alcohol consumption causes insulin resistance and impairs the expression of the insulin-responsive molecule aspartyl-asparaginyl- β -hydroxylase (AAH). AAH promotes cell motility, needed for liver remodeling, through Notch signaling and these functions are impaired by alcohol exposure, contributing to alcoholic liver disease progression. Agonists of peroxisome proliferator activated receptor (PPAR)- δ , and to a lesser extent of PPAR- γ , were previously demonstrated to restore insulin/IGF-1 signaling, AAH expression, hepatic morphology and function. The purpose of the present work was to investigate whether PPAR agonist treatments could also restore Notch signaling expression, downstream of AAH, in a model of chronic ethanol exposure.

Methods: Long Evans rats were chronically-fed control or ethanol-containing diets and administered agonists of PPAR- α , PPAR- δ , or PPAR- γ by intraperitoneal injections. Liver tissue homogenates were used to examine expression of insulin-responsive AAH, Notch signaling proteins, hypoxia inducible factor-1 α (HIF-1 α), which is a positive regulator of AAH, and proliferating cell nuclear antigen (PCNA), to measure the potential for hepatic growth.

Results: PPAR- δ agonist treatment restored the expression of Notch-1 despite continued chronic ethanol exposure.

Conclusion: Preliminary findings suggest that PPAR- δ agonists may restore Notch-1 protein to activate hepatocyte migration, which is essential to restore ethanol-impaired hepatic structure and function in ALD.

Keywords: alcoholic liver disease; aspartyl-asparaginyl- β -hydroxylase; Notch; peroxisome proliferator activated receptor.

4.3. INTRODUCTION

Alcohol abuse is a major public health concern as it is the leading cause of liver-related morbidity and mortality [9,10]. Excessive and continued alcohol consumption

sets the stage for progression from steatosis to alcoholic liver disease (ALD) due to sustained mitochondrial dysfunction, oxidative and endoplasmic reticulum stress [11,12], inflammation [13,14], and insulin and insulin-like growth factor (IGF) resistance [1–3]. ALD encompasses a spectrum of disorders that range from steatosis to fibrosis, cirrhosis, and, in some cases, liver failure or development of hepatocellular carcinoma [15]. Ethanol disrupts insulin/IGF receptor binding, phosphorylation of insulin receptor substrate-1, and downstream signaling through mitogen-activated protein kinase, which promotes liver growth and repair, and phosphoinositide-3 kinase (PI3K), which is needed for metabolism, cell survival, and motility. Furthermore, ethanol upregulates the expression of negative regulators of PI3K, further impairing activation of downstream signaling events [16].

The molecule aspartyl-asparaginyl- β -hydroxylase (AAH) is a downstream target of insulin/IGF signaling with an essential role in promoting cell adhesion and motility, necessary for hepatic remodeling and regeneration. AAH is an ~86 kD type 2 transmembrane protein located in the endoplasmic reticulum (ER). AAH is physiologically cleaved inside the cell to yield N-terminal and C-terminal fragments. The N-terminal fragment is virtually identical to Humbug, a truncated isoform that supports cell adhesion through regulation of Ca^{2+} flux from intracellular stores [17,18]. The C-terminal fragment contains the catalytic domain that promotes cell adhesion and motility by hydroxylating epidermal growth factor-like domains of Notch signaling proteins [19,20].

The Notch signaling proteins Notch-1 and Jagged-1 have demonstrated roles in cell migration, differentiation, and adhesion [21]. AAH activates Notch signaling networks by hydroxylating Notch-1 and, its ligand, Jagged-1 at their epidermal growth factor-like domains. Binding of Jagged (or Delta-like family of proteins) to the Notch extracellular domain triggers two sequential cleavage events. The first cleavage event occurs close to

the transmembrane region on the extracellular face of the membrane, which is carried out by a disintegrin and metalloprotease protease. The second cleavage event occurs within the transmembrane domain of Notch, triggered by a complex formed between γ -secretase and presenilin proteases, which causes the Notch intracellular domain (NID) to translocate from the plasma membrane to the nucleus. Once in the nucleus, the NID removes co-repressors and recruits transcriptional co-activators near specific DNA sequences to activate the transcription of target genes, such as hairy and enhancer of split-1 (HES-1) and HES-related proteins [22,23]. Through this mechanism, insulin/IGF, AAH, and Notch signaling promote cell migration needed for liver remodeling and regeneration.

Peroxisome proliferator activated receptors (PPARs) are transcription factors and nuclear receptors that heterodimerize with retinoid x receptors and bind to peroxisome proliferator response elements throughout the genome. Complex binding activates the transcription of genes involved in various biological functions, including lipid metabolism [24,25]. PPARs are expressed as three isoforms: PPAR- α , PPAR- δ , and PPAR- γ . PPAR- α plays an essential role in the β -oxidation of fatty acids and it is expressed in the liver and, to a lesser extent, in the kidney, heart, skeletal muscle, and brown adipose tissue [26]. PPAR- α is naturally activated by polyunsaturated fatty acids and fibrates. The PPAR- δ isoform regulates lipid catabolism and oxidative phosphorylation and is mainly expressed in muscle, kidney, heart, and liver [27]. Natural ligands include both saturated and unsaturated fatty acids. The PPAR- γ isoform promotes adipocyte differentiation and is generally involved in lipogenesis [28]. PPAR- γ is primarily present in adipose tissue and it prefers activation by polyunsaturated fatty acids [29]. Thiazolidinediones are a class of synthetic PPAR- γ agonists with insulin-sensitizing properties approved by the Food and Drug Administration for treatment of human type 2 diabetes [30].

We previously showed PPAR- δ and PPAR- γ agonists also ameliorated ethanol-induced insulin resistance and restored hepatic structure [7,8,31]. PPAR- δ , and to a lesser extent PPAR- γ , agonist treatments strikingly restored the adverse effects of alcohol on insulin signaling, AAH expression, and hepatic architecture. Since AAH promotes cell migration through activation of Notch signaling [19,32–34], we evaluated the effects of PPAR agonist treatments on AAH and downstream Notch signaling protein expression, as a mechanism that mediates the previously observed restoration of hepatic architecture. We also examined the effects of PPAR agonist treatments on hypoxia inducible factor-1 α (HIF-1 α), a positive regulator of AAH expression, and proliferating cell nuclear antigen (PCNA), involved in liver growth. The preliminary findings suggest that treatment with a PPAR- δ agonist restores Notch-1 protein expression to promote cell motility and restore hepatic architecture in the livers of chronic ethanol-fed rats.

4.4. MATERIALS AND METHODS

4.4.1. Materials. Long Evans (LE) rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN, USA), liquid diets from BioServ (Frenchtown, NJ, USA), and PPAR- α , PPAR- δ , and PPAR- γ agonists from CalBiochem (Carlsbad, CA, USA). Bicinchoninic assay (BCA) reagent kit was purchased from Pierce (Rockford, IL, USA) and reagents for duplex enzyme-linked immunosorbent (ELISA) assay amplex ultrared reagent and 4-methylumbelliferyl phosphate (MUP) were purchased from Invitrogen (Carlsbad, CA, USA). 96-well FluoroNunc maxisorp and optiplates were purchased from Thermo Scientific (Rochester, NY, USA), rabbit polyclonal antibody to large acidic ribosomal protein (RPLPO) from Proteintech (Chicago, IL, USA), alkaline phosphatase conjugated to streptavidin from Vector Laboratories (Burlingame, CA, USA), and SpectraMax M5

Microplate Reader from Molecular Dynamics (Sunnyvale, CA, USA). GraphPad Prism 5 software was purchased from GraphPad Software (San Diego, CA, USA).

4.4.2. Chronic ethanol exposure model. Adult (~220-250 g) male LE rats were pair-fed with isocaloric liquid diets containing 0% (control) or 37% ethanol (9.2% v/v) for six weeks [35]. Starting on the second week of feeding, the rats in the control and ethanol groups were administered intraperitoneal injections of vehicle (saline), a PPAR- α (GW7647; 2.5 μ g/Kg), a PPAR- δ (L-160,043; 2 μ g/Kg), or a PPAR- γ (Fmoc-Leu; 1.8 mg/Kg) agonist twice weekly for 5 weeks. The PPAR doses, route of administration, and frequency were based on previously established experimental protocols [7,36]. At the end of the protocol liver tissue from (N=8 per treatment group) was harvested, snap-frozen, and stored at -80°C. The rats were monitored daily to ensure proper nutritional intake and maintenance of a healthy body weight. The rats lived under humane conditions, were kept in a 12-hour light/dark cycle, and had free access to the liquid diets. All experiments were conducted in accordance with guidelines established by the National Institutes of Health and approved by the institutional Animal Care and Use Committee at the Lifespan-Rhode Island Hospital.

4.4.3. Duplex enzyme-linked immunosorbent assay (ELISA). Frozen liver tissue was homogenized in weak lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid pH 8.0, 50 mM NaF, 0.1% triton x-100) in the presence of protease and phosphatase inhibitors [1,35]. Homogenates were centrifuged and protein concentration in the supernatant was measured by the BCA assay. Target immunoreactivity was measured by ELISA and normalized to RPLPO directly measured in the same wells, as previously described [37,38]. Protein homogenates (100 ng/50 μ l) were adsorbed onto the bottom surfaces of maxisorp 96-well plates by incubation on a rotating platform overnight. Non-specific protein binding was prevented by incubation with 1% bovine serum albumin in TRIS buffered saline. The samples were incubated

with monoclonal and polyclonal primary antibodies (0.5-1 µg/ml) overnight at 4°C and immunoreactivity was detected with secondary antibodies conjugated to horseradish peroxidase and Amplex UltraRed soluble fluorophore (*Ex* 530 nm/*Em* 590 nm). Subsequently, the samples were incubated with biotinylated RPLPO and immunoreactivity was detected with streptavidin-conjugated alkaline phosphatase (1:1000) and the 4-MUP fluorophore (*Ex* 360 nm/*Em* 460 nm). Fluorescence was detected with the SpectraMax M5 microplate reader. Non-specific binding was assessed by parallel incubations in which the primary or secondary antibodies were omitted. The ratios of target protein/RPLPO fluorescence were calculated to allow inter-well comparisons and at least 6 biological replicates, in quadruplicates, were analyzed for each experiment.

4.4.4. Statistical analysis. Data depicted in the graphs represent the means ± standard error of the mean (S.E.M.) for each group. Data (N=6 animals/group in quadruplicates) were analyzed using GraphPad Prism 5 software. Inter-group comparisons were made using repeated measures of two-way analyses of variance (ANOVA) with Fisher's Least Significant Difference (LSD) post hoc test. Computer software generated P-values (P<0.05 or better) are indicated above the bars.

4.5. RESULT

4.5.1. Effects of PPAR agonist treatments on AAH-Notch signaling networks during chronic ethanol exposure. Duplex ELISA measured the levels of Humbug (A85E6 monoclonal antibody), AAH (A85G6 monoclonal antibody), Notch-1, Jagged-1, HIF-1α, and PCNA (**Figure 4-1**). Two- way ANOVAs demonstrated significant inter-group differences in ethanol-exposed (0.0001<P<0.0015) and PPAR agonist treated groups (P<0.05). Consistent with previous reports [7,38,39], Fisher's LSD post hoc test demonstrated chronic ethanol feeding (+vehicle) significantly decreased Humbug

($P < 0.01$) (**Figure 4-1A**), Notch-1 ($P < 0.001$) (**Figure 4-1C**), Jagged-1 ($P < 0.01$) (**Figure 4-1D**), and HIF-1 α ($P < 0.0001$) relative to control (+vehicle). However, no effects were visible on AAH (**Figure 4-1B**) and PCNA (**Figure 4-1F**) under these conditions. Treatment with PPAR- δ agonist abolished inter-group differences in the levels of Notch-1 and PPAR- γ agonist abolished inter-group differences in the levels of Humbug expression. Since ethanol increases lipid peroxidation, we measured the levels of 4-hydroxyenonal (HNE) (**Figure 4-2**). In this set of experiments, no significant differences were found between control (+vehicle) and ethanol (+vehicle) treatments for 4-HNE levels. PPAR- δ agonist treatment significantly decreased the levels of 4-HNE ($P < 0.05$) relative to livers exposed to ethanol and PPAR- α agonist treatments.

4.6. DISCUSSION

4.6.1. Study rationale and objectives. Chronic alcohol consumption promotes ALD progression, mediated in part by sustained insulin resistance [40,41]. Ethanol disrupts insulin/IGF signaling at various points of the signaling cascade. Ethanol disrupts lipid membrane composition, which is essential for insulin receptor binding, decreases IGF polypeptide expression, decreases IRS-1 activation [1–3], and promotes the expression of negative regulators of insulin/IGF signaling [4,5]. Previous reports indicated that treatment with the insulin sensitizing agents PPAR- δ and, to a lesser extent PPAR- γ , agonists remarkably restored ethanol-impaired insulin/IGF signaling, stimulating the expression of AAH and other insulin-responsive genes [7]. Increased AAH expression by PPAR- δ agonist treatment could explain the restoration of hepatic architecture observed by Ramirez, *et al.* since AAH promotes cell motility, necessary for liver remodeling and regeneration [8]. AAH promotes cell migration through activation of Notch signaling networks [19,32–34]. The purpose of the present study was to determine if the therapeutic effects of PPAR agonists, particularly of PPAR- δ , on restoring insulin/IGF-1

signaling, AAH expression, and liver morphology was mediated by increased expression of Notch signaling proteins.

4.6.2. PPAR- δ agonist promotes AAH and Notch signaling protein expression.

Several lines of evidence indicate AAH promotes cell motility through the activation of Notch signaling since (1) Notch-1 and, its natural ligand, Jagged-1 contain the consensus sequence for AAH hydroxylation, (2) AAH physically interacts with Notch-1 and Jagged-1 [32], (3) inhibition of AAH decreases Notch-1, Jagged-1, and HES-1 expression, while over-expression yields the opposite effects [19,34], and (4) Notch-1 and Jagged-1 were previously demonstrated to promote cell adhesion and migration [42]. Since PPAR- δ and, to a lesser extent PPAR- γ , agonist treatments improve insulin sensitivity and AAH expression, we extended our investigations to determine if they enhanced expression of the downstream targets of AAH, Notch-1 and Jagged-1 as a mechanism to promote hepatocyte migration necessary for liver remodeling. Our findings suggest that PPAR- δ agonists restore Notch-1 protein expression. It is worth noting that AAH protein expression was not significantly reduced in the ethanol (+vehicle) group relative to control in this set of experiments, contrary to the results previously published by Pang, *et al.* in 2009 [7]. The difference could be to the age of the protein samples since the samples were prepared approximately seven years ago. Some of the protein might have degraded from the time these samples were prepared and used for Western blot analysis. Nevertheless, the results from this experiment suggest that in ethanol-treated livers, PPAR- δ agonist could possibly restore Notch-1 protein expression. Whether this is truly the case, I am not able to conclude from the data reported herein.

The results suggest the therapeutic effects of PPAR- δ agonist treatment on hepatic structure and function during chronic alcohol exposure are mediated by

improved insulin/IGF signaling, AAH, and Notch signaling protein expression to restore hepatic architecture during liver remodeling and regeneration [7].

4.7. CONCLUSION

The study provides preliminary evidence into the mechanism by which PPAR- δ agonists restore ethanol-impaired liver structure and function in ALD. PPAR- δ may aid in the process of hepatic remodeling and regeneration by enhancing the activation of signaling pathways that promote AAH-Notch signaling networks, which promote hepatocyte migration after toxic injury.

4.8. FUTURE DIRECTIONS

Newer samples could be used to perform the analysis and the experiments reported herein could be repeated.

Figure 4-1

Effects of Chronic Ethanol Exposure and PPAR Agonist Treatments on AAH and Notch Signaling Molecules in the Liver

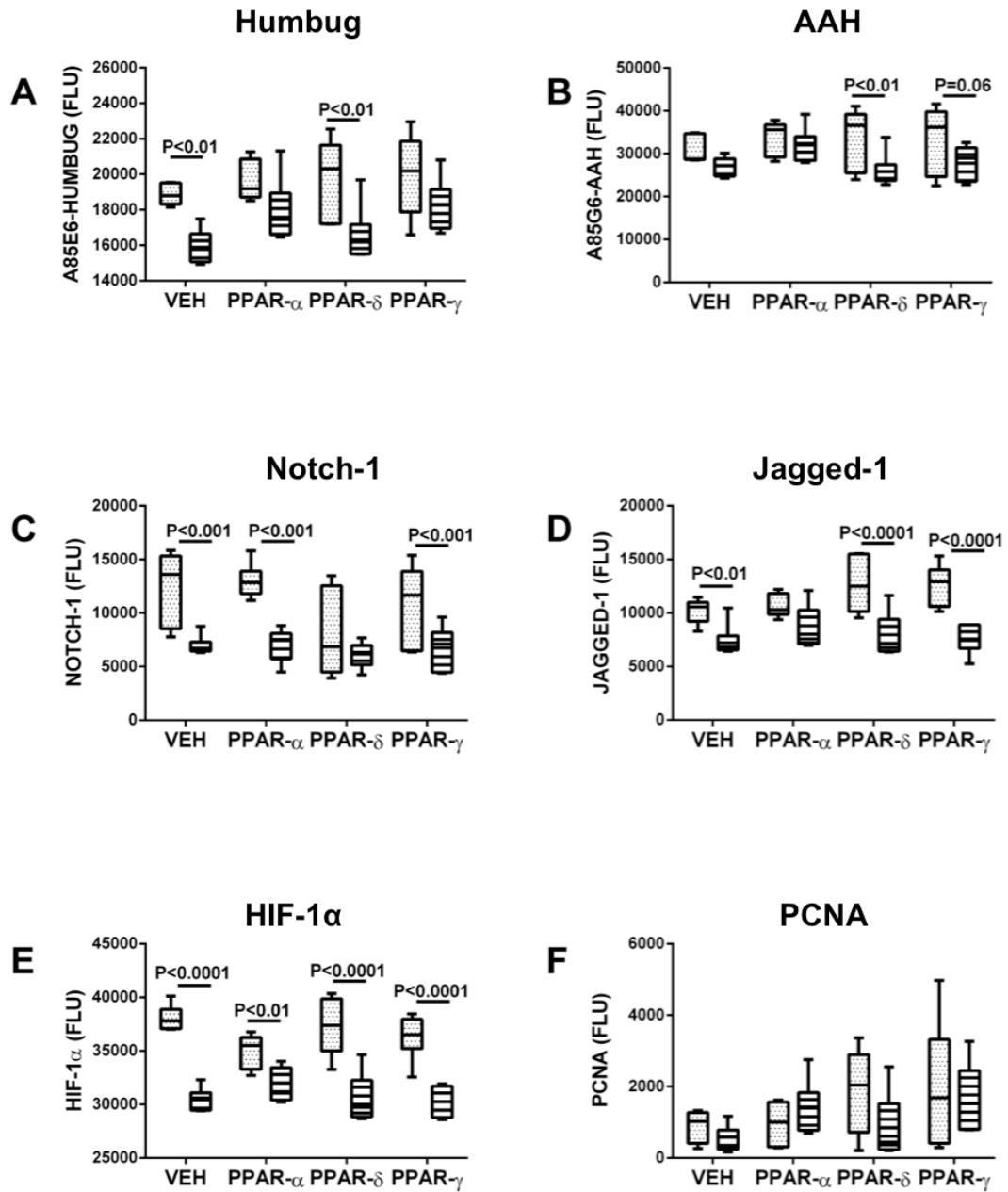


Figure 4-1. Effects of chronic ethanol exposure and PPAR agonist treatments on AAH and Notch signaling molecules in the liver. Rats were chronically-fed control or ethanol-containing (37%) liquid diets and administered vehicle (saline) or agonists of peroxisome proliferator activated receptor (PPAR)- α , PPAR- δ , or PPAR- γ via intraperitoneal injections for six weeks. Livers homogenates were prepared in weak lysis buffer from (A) Humbug-A85E6, (B) AAH-A85G6, (C) Notch-1, (D) Jagged-1, (E) hypoxia inducible factor-1 α (HIF-1 α), and (F) proliferating cell nuclear antigen (PCNA) immunoreactivities by duplex ELISA. Results were normalized to large ribosomal protein (RPLPO) measured in the same wells. Data depicted in the graphs represent the means $\pm$ standard error of the mean (S.E.M.) for each group. Data (N=6 animals/group in quadruplicates) were analyzed using GraphPad Prism 5 software. Inter-group comparisons were made using repeated measures of two-way analyses of variance (ANOVA) (see **Table 4-1**) with Fisher's Least Significant Difference post hoc test. Computer software generated P-values ($P < 0.05$ or better) are indicated above the bars.

Figure 4-2

Effects of Chronic Ethanol Exposure and PPAR Agonist Treatments on 4-HNE

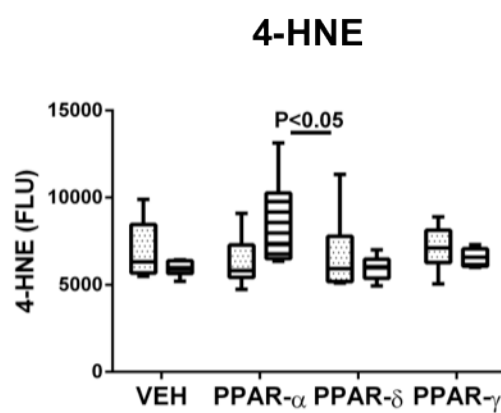


Figure 4-2. Effects of chronic ethanol exposure and PPAR agonist treatments on 4-HNE. Rats were chronically-fed control or ethanol-containing (37%) liquid diets and administered vehicle (saline) or agonists of peroxisome proliferator activated receptor (PPAR)- α , PPAR- δ , or PPAR- γ via intraperitoneal injections for six weeks. Livers homogenates were prepared in weak lysis buffer to detect 4-hydroxynonenal (HNE), which measures lipid peroxidation, immunoreactivity by duplex ELISA. Results were normalized to large ribosomal protein (RPLPO) measured in the same wells. Data depicted in the graphs represent the means $\pm$ standard error of the mean (S.E.M.) for each group. Data (N=6 animals/group in quadruplicates) were analyzed using GraphPad Prism 5 software. Inter-group comparisons were made using repeated measures of two-way analyses of variance (ANOVA) (see **Table 4-1**) with Fisher's Least Significant Difference post hoc test. Computer software generated P-values ($P < 0.05$ or better) is indicated above the bar.

Table 4-1

**Rat Livers from Control and Ethanol-Fed Rats Chronically
Treated with PPAR Agonists**

Gene/Protein	Ethanol		PPAR Agonist		Ethanol x Agonist	
	F-Ratio	P-Value	F-Ratio	P-Value	F-Ratio	P-Value
AAH-A85G6	19.62	0.0015	7.85	N.S.	4.53	N.S.
Humbug-A85E6	34.57	<0.0001	10.30	0.063	3.11	N.S.
NOTCH-1	38.86	<0.0001	11.02	0.032	4.94	N.S.
JAGGED-1	51.11	<0.0001	4.62	N.S.	5.82	N.S.
HIF-1	71.77	<0.0001	1.60	N.S.	5.11	0.035
PCNA	2.43	N.S.	16.03	0.048	6.98	N.S.
4-HNE	0.08	N.S.	5.54	N.S.	14.40	0.08

Table 4-1. Rat livers from control and ethanol-fed rats chronically treated with PPAR agonists. F-Ratio=variance, the strength of which reports differences between the groups. P-values are significant $p < 0.05$. However, they represent a trend if $0.05 < p < 0.10$. In two-way ANOVA tests, different treatments are tested, along with interactions between the two factors.

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CHAPTER 5

**POST-TRANSLATIONAL REGULATION OF AAH: RELEVANCE TO
HEPATOCELLULAR CARCINOMA MOTILITY AND INVASIVENESS***

***Manuscript in preparation**

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AUTHOR CONTRIBUTIONS

DL conducted the experiments pertaining to transfection, protein detection by ELISA, Western blotting, and fluorescence microscopy, and drafted the manuscript. J-SG designed the AAH constructs in collaboration with SMdIM. MT generated and validated expression and function of AAH constructs; NR performed immunofluorescence and confocal microscopy of HCC cells treated with different kinase inhibitors;; JRW provided insightful feedback and support; and SMdIM originated ideas, designed the experiments and was very closely involved with data analysis and interpretation, and drafting of the manuscript.

5.1. RATIONALE

Insulin and insulin-like growth factor (IGF) signaling is upregulated in HCC, aberrantly increasing AAH expression, cell adhesion, motility, and invasion [1]. Previous evidence demonstrated insulin/IGF stimulates AAH protein expression and function through glycogen synthase kinase-3 β (GSK-3 β) inhibition, suggesting AAH is post-translationally regulated by phosphorylation [2]. Furthermore, AAH contains several predicted consensus sequences of phosphorylation by GSK-3 β , as well as protein kinase A (PKA), protein kinase C (PKC, and casein kinase 2 (CK2). AAH activates migration, at least in part, by hydroxylation of Notch-1 and, its ligand, Jagged-1, leading to the activation of Notch signaling networks [3–5]. In the present work we investigated how IGF-1 promotes AAH expression and its consequences on Notch signaling activation, through post-translational mechanisms in HCC.

5.2. ABSTRACT

Background: Hepatocellular carcinoma (HCC) is associated with upregulation of insulin/insulin-like growth factor (IGF) signaling. Insulin/IGF signaling stimulates the

expression of aspartyl-asparaginyl- β -hydroxylase (AAH), promoting tumor cell adhesion, migration, and invasion via Notch signaling. AAH is likely post-translationally regulated since chemical inhibition of glycogen synthase kinase-3 β (GSK-3 β) promotes AAH expression and motility, while overexpression yields the opposite effects. Furthermore, AAH contains multiple sites predicted to be phosphorylated by GSK-3 β , protein kinase A (PKA), protein kinase C (PKC), or casein kinase 2 (CK2). Herein, we investigated the effects of IGF-1 stimulation on the expression of AAH as well as subcellular trafficking of AAH and Notch-1; we also studied the post-translational regulation of AAH. **Methods:** N-terminally tagged and C-terminally tagged AAH constructs were transiently expressed in human Huh7 hepatoma cells and stimulated with IGF-1 to track trafficking of AAH and Notch-1 intracellular domain (NID). The post-translational regulation of AAH protein was assessed by chemical inhibition of GSK-3 β , PKA, PKC, and CK2. **Results:** IGF-1 stimulated recombinant AAH protein expression and trafficking to the plasma membrane, while also promoting the nuclear translocation of the NID. GSK-3 β , PKC, and CK2 inhibition enhanced IGF-1-stimulated AAH protein expression. **Conclusion:** IGF-1 stimulation promotes the subcellular trafficking of AAH and Notch-1, possibly leading to Notch signaling activation. The effects on AAH are mediated through post-translational mechanisms of phosphorylation.

Key Terms: Hepatocellular carcinoma; insulin; insulin-like growth factor; aspartyl-asparaginyl- β -hydroxylase; Notch

5.3. INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide [6,7] and it has recently become the fastest growing cause of cancer-related mortality among males in the United States [8]. Molecular mechanisms of HCC

carcinogenesis include dysregulation of cell cycle checkpoints, of apoptosis [9–11], and of growth factor signaling [12–14]. Insulin/insulin-like growth factor (IGF) signaling is significantly upregulated in HCC through mechanisms that include gain-of-function mutations in phosphoinositide 3-kinase (PI3K) [15], impaired expression/function of phosphatase and tensin homolog [16], or over-expression of IGF and the IGF receptor [14].

Aspartyl-asparaginyl- β -hydroxylase (AAH) is an important target of insulin/IGF signaling

that promotes cell migration and invasion [17–19], necessary for HCC tumor infiltration and intrahepatic metastatic spread [4]. AAH is an ~86 kD [17] type 2 transmembrane protein located in the endoplasmic reticulum (ER) [20,21]. AAH is physiologically cleaved into a ~30-34 kD N-terminal fragment that is identical to Humbug (Junctate), a truncated isoform that binds calcium and promotes adhesion [22,23], and a ~52-56 kD C-terminal fragment that contains catalytic activity [24]. The C-terminal catalytic domain of AAH promotes cell motility by hydroxylating specific aspartate (Asp) and asparagine (Asn) residues contained within a specific sequence in epidermal growth factor (EGF)-like domains of the Notch signaling pathway proteins Notch-1 and Jagged-1 [4,25,26]. AAH was previously linked to Notch signaling networks in HCC [4].

Notch signaling starts by binding of Jagged (or Delta-like family of proteins) to the Notch extracellular domain triggering two rapid cleavage events. The Notch extracellular domain is first cleaved from the transmembrane domain in a metalloprotease-dependent manner, which rapidly promotes cleavage of the Notch intracellular domain (NID) in a protease-dependent manner [27]. At this point, the NID translocates to the nucleus, where it complexes with the transcriptional activator CBF1, suppressor of hairless or lag-1 (CSL), and the transcriptional co-activators of the mastermind-like family of proteins. The complex binds to CSL consensus sequences on DNA, displaces co-repressors and

recruits additional co-activators to promote the transcription of Notch signaling targets, such as hairy and enhancer of split-1 (HES-1) and hairy/enhancer-of-split related with YRPW motif protein-1 (HEY-1) [27,28].

Insulin/IGF regulates AAH at the post-translational level by signaling through PI3K, which promotes phosphorylation and activation of Akt, followed by phosphorylation and inhibition of glycogen synthase kinase-3 β (GSK-3 β) [29–31]. Evidence suggests AAH protein expression and function are likely regulated by post-translational mechanisms of phosphorylation since (1) chemical and siRNA-mediated GSK-3 β inhibition promotes AAH protein expression and activates migration, while GSK-3 β overexpression yields the opposite effects; (2) AAH contains several sites predicted consensus sequences for phosphorylation that belong to GSK-3 β , protein kinase A (PKA), protein kinase C (PKC), and casein kinase 2 (CK2); and (3) AAH migrates at a higher molecular weight than the predicted ~86 kD possibly due to its phosphorylated state. First, we investigated the effects of insulin/IGF stimulation on AAH protein expression and the implications in Notch signaling activation. Additionally, we examined the role of kinase activity in the regulation of AAH by inhibiting the activity of kinases predicted to phosphorylate AAH. Our results suggest that IGF-1 promotes AAH expression and NID translocation to the nucleus; these effects may be regulated at the post-translational level by direct or indirect mechanisms of phosphorylation.

5.4. MATERIALS AND METHODS

5.4.1. Materials. Recombinant IGF-1 was purchased from Sigma-Aldrich (St. Louis, MO, USA). pcDNA 3 vector, *Escherichia coli* DH5 α cells, Dulbecco's Modified Eagle Medium, Lipofectamine 2000 Transfection Reagent, Hoechst 33342, and Amplex UltraRed were purchased from Invitrogen (Carlsbad, CA, USA). Non-essential amino acid mixture was purchased from Gibco-BRL (Grand Island, NY, USA). pcDNA 3 vector with a 6x Myc-tag

was a gift from Dr. Y. Eugene Chin from Brown University (Providence, RI, USA) [32]. QIAquick Gel Extraction Kit and QIAprep Spin Miniprep Kit were purchased from Qiagen (Valencia, CA, USA). MaxiSorb plates, OptiPlates (96-well), BD Falcon culture inserts, and Nunc culture supplies were obtained from Thermo Scientific (Rochester, NY, USA). Polyvinylidene fluoride membranes were purchased from Perkin-Elmer (Waltham, MA, USA). Myc antibody purchased from Cell Signaling Technologies (Danvers, MA, USA) and HA antibody purchased from Santa Cruz Biotechnologies (Dallas, TX, USA). The A85G6 and FB50 mouse monoclonal antibodies to AAH were characterized as previously described [2,33]. SuperBlock, bicinchoninic assay, enhanced chemiluminescence reagents, and Dylight 547 Conjugated to Streptavidin were purchased from Pierce (Rockford, IL, USA). Fisherbrand Superfrost Plus Stain Slides were purchased from Fisher Scientific (Pittsburgh, PA, USA) and SpectraMax M5 Microplate Reader from Molecular Dynamics (Sunnyvale, CA, USA). Histofix was purchased from Amresco (Solon, Ohio, USA) and Shandon Cytospin Centrifuge 3 from Thermo Shandon (Pittsburgh, PA, USA). Other fine chemicals were purchased from CalBiochem (Carlsbad, CA, USA) or Sigma-Aldrich (St. Louis, MO, USA).

5.4.2. Recombinant AAH plasmid constructs. The coding region of human AAH was amplified from a 293T cell cDNA library by the polymerase chain reaction (PCR) using the forward primer 5'-CGGAATTCATGGCCCAGCGTAAGAATGCCA-3', reverse primer 5'-CCGCTCGAGCTAAATTGCTGGAAGGCTGC-3' and *Pfu* DNA polymerase [17]. The AAH PCR product was digested with EcoRI and XhoI restriction enzymes and gel purified with the QIAquick Gel Extraction Kit. A pcDNA 3 vector with a 5'-end 6x Myc-tag insert was received as a gift and original pcDNA 3 vector was also engineered to contain a 3'-end 2x HA tag using the forward primer 5'-GCAGGATCCTACCCATACGATGTTCTGACTAT-3' and reverse primer 5'-TAACGGTACCAAGCTTGCATAGTC-3'. The AAH PCR product was cloned into the

Myc-modified (pCMV-N-Myc-AAH) or the HA-modified pcDNA 3 vector (pCMV-C-HA-AAH). The recombinant plasmids were transformed into *Escherichia coli* DH5 α competent cells and positive clones cultured in *Luria Broth* media. The plasmids were purified with the QIAprep Spin Miniprep Kit and correct insert sequence and orientation was verified by DNA sequencing. Lastly, protein expression was verified in 293T and Huh7 cells by Western blot.

5.4.3. Cell culture. Huh7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with heat-inactivated 5% fetal bovine serum (FBS), 10 mM non-essential amino acid mixture, and 2 mM L-glutamine in 5% CO₂ at 37°C. Huh7 cells were transiently transfected with pCMV-N-Myc-AAH, pCMV-C-HA-AAH, or empty vector (EV) control at semi-confluency using Lipofectamine 2000 and co-transfected with a green fluorescent protein plasmid to monitor transfection efficiency. To determine the effects of growth-factor stimulation on recombinant AAH protein, 24-hr cultures expressing N-Myc-AAH or C-HA-AAH were placed in media with 1% FBS for 4 hrs and treated with vehicle or stimulated with IGF-1 (50 ng/mL) for up to 60 min. To assess the role of kinase inhibition on IGF-1-stimulated AAH, Huh7 were pre-treated with inhibitors of GSK-3 β (20 mM LiCl), casein kinase 2 (CK2; 5 μ M TBCA/TBB), protein kinase A (PKA; 20 μ M H-89), protein kinase C (PKC; 1 μ M Gö6983), or MAPK/ERK (20 μ M PD98059) control for 4 hrs, followed by stimulation with IGF-1 (50 ng/ml). To assess the role of long-term of insulin stimulation and GSK-3 β inhibition on N-Myc-AAH, cells were placed in 1% FBS media for 4 hrs, followed by insulin stimulation and 20 mM LiCl treatment for 16 hrs.

5.4.4. Protein studies. Huh7 protein homogenates were prepared in radio-immunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.5, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 2 mM EGTA) in the presence of protease (1mM PMSF, 0.1 mM TPCK, 1 mg/ml aprotinin, 1 mg/ml pepstatin A, 0.5 mg/ml leupeptin, 1 mM NaF, 1mM Na₄P₂O₇) and phosphatase (2mM Na₃VO₄) inhibitors [1].

Protein homogenates were centrifuged at 14000xg for 10 min at 4°C and protein concentration in supernatant was measured by the bicinchoninic acid (BCA) assay. For Western blot analysis, 30 µg of protein were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes that were subsequently incubated with SuperBlock in TRIS buffered saline (TBS) to prevent non-specific binding. After washing, the membranes were incubated with primary antibodies (0.5-1 µg/ml) overnight at 4°C. Immunoreactivities were detected with horseradish peroxidase (HRP)-conjugated secondary antibody, SuperSignal enhanced chemiluminescence reagents, and film autoradiography.

5.4.5. Microtiter immunocytochemical ELISA (MICE) assay. The MICE assay is an efficient method to rapidly and directly quantify protein under several treatment conditions in 96-well plate format [34]. For the MICE assay, Huh7 cells were fixed with Histofix, permeabilized with 0.05% saponin in TBS, and endogenous peroxidase activity blocked with 0.03% hydrogen peroxide in phosphate buffered saline (PBS). Non-specific protein binding was prevented by incubation with SuperBlock-TBS. After washing, the cells were incubated with primary antibodies (0.5-1 µg/ml) overnight at 4°C and target protein immunoreactivity was detected with HRP-conjugated secondary antibodies and Amplex UltraRed fluorophore (*Ex* 530 nm/*Em* 590 nm). Ratios of target protein/ cell density with Hoechst 33342 dye (*Ex* 360 nm/*Em* 460 nm) and fluorescence measured with SpectraMax M5 microplate reader. Ratios of target protein/cell density allowed for inter-well comparisons. 4-6 replicate cultures were analyzed for each experiment.

5.4.6. Statistical analysis. Data depicted in the graphs represent the means ± standard error of the mean (S.E.M.) for each group. Data (N=4 independent cultures/group) were analyzed using GraphPad Prism 5 software. Inter-group comparisons were made using repeated measures of one-way or two-way analyses of variance (ANOVA) with Fisher's

Least Significant Difference (LSD) post hoc test. Computer software generated P-values are indicated over the bars or time-points (* $P<0.05$; ** $P<0.01$; *** $P<0.001$; and **** $P<0.0001$).

5.5. RESULTS

5.5.1. Effects of IGF-1 stimulation on AAH protein expression. Western blot analysis was used to measure the effects of IGF-1 stimulation on AAH protein expression (**Figure 5-1**). Short-term stimulation with IGF-1 significantly and progressively increased endogenous AAH (FB50+A85G6) expression over time ($0.01<P<0.001$). The ~56 kD species corresponds to the C-terminal catalytic domain of AAH, which is the main cleavage product usually detected in Huh7 cells. The results suggest endogenous AAH accumulates in response to IGF-1 stimulation.

To increase the signal of full-length AAH, N-terminally tagged AAH (N-Myc-AAH) or C-terminally tagged AAH (C-HA-AAH) cDNAs, under the control of CMV promoters, were transiently expressed in Huh7 cells (**Figure 5-2**). Differences from 0 time-point of stimulation were found to be significant by ANOVA ($P=0.007$). The Post hoc Fisher's LSD test demonstrated significantly higher levels of AAH (FB50+A85G6) protein than Myc ($0.01<P<0.00001$) (**Figure 5-2A,B**). The lower Myc than FB50/A85G6 levels could reflect differences in signal intensity resulting from antibody binding. Myc immunoreactivity did not increase over time, compared to the increase from the 0 time point for the control culture (**Figure 5-1**). No significant increases in Myc levels over time could be due to the presence of a CMV promoter, and possibly other factors such as cleavage and maintenance of steady-state turnover levels of recombinant protein. Although AAH has a calculated molecular weight of ~86 kD, it migrated at an apparent molecular weight of ~120-140 kD by SDS-PAGE, possibly due to post-translational modifications. In contrast, HA levels were higher than A85G6 as illustrated (**Figure 5-2**

C,D) and both graphs depict progressive increases in AAH expression over time. The differences from the 0 time point are significant for both by ANOVA tests ($P < 0.001$). Similar levels of p85 subunit of PI3K (loading control) were observed in all samples.

5.5.2. Effects of IGF-1 stimulation on the subcellular trafficking of AAH and Notch-

1. In addition to examining the effects of IGF-1 stimulation on total AAH protein expression, we investigated the consequences on the subcellular trafficking of N-terminally tagged AAH (N-Myc-AAH) and C-terminally tagged AAH (C-HA-AAH). The N-terminus (Myc) exhibited a different intracellular trafficking pattern than the C-terminus (HA) in response to IGF-1 stimulation. Note the initial peripheral localization of Myc at time 0 (**Figure 5-3A**), accumulations of Myc-AAH in the ER/perinuclear zone at 10 (**Figure 5-3B**) and 30 min (**Figure 5-3C**), followed by its return to the cell periphery and processes at 60 minutes (**Figure 5-3D**). C-HA-AAH exhibited perinuclear and nuclear labeling at time 0 (**Figure 5-3F**), followed by a speckled microsomal or mitochondrial pattern (10 min) (**Figure 5-3G**), increased perinuclear, nuclear, and cell process (podocyte) labeling (30 min) (**Figure 5-3H**), then finally tight perinuclear and some nuclear localization at 60 minutes (**Figure 5-3I**). Note the C-HA-AAH contains the catalytic subunit which hydroxylates Notch. Notch-1 translocation to the nucleus follows the trafficking of HA-AAH to the perinuclear zone and nucleus (**Figure 5-3K-N**). Negative control studies were parallel incubations in which the primary antibodies were omitted (**Figure 5-3E, J, O**).

5.5.3. Effects of short-term IGF-1 and kinase inhibitor treatments on AAH protein.

Insulin and IGF-1 regulate AAH protein through expression through post-translational mechanisms [2]. More specifically, AAH may be regulated by phosphorylation since the protein contains several predicted consensus sequences of phosphorylation, most of which belong to GSK-3 β , but also PKA, PKC, and CK2. To gain a better understanding the post-translational regulation of AAH, we inhibited the activity of GSK-3 β (LiCl)

[35,36], CK2 (TBCA/TBB), PKA (H89), PKC (Gö6983), PD98059 (MEK inhibitor) or CK1 (D4476), the two latter serving as negative controls.

MICE assays, in which target protein immunoreactivity was normalized to cell density (H33342), were used to measure AAH (A85G6 antibody) and β -actin (**Figure 5-4**). ANOVA tests demonstrated significant inter-group differences occurred in cells treated with kinase inhibitors and stimulated with IGF-1 on AAH ($F=18.72$; $P=0.0042$) (**Figure 5-4A**) and β -actin ($F=18.44$; $P<0.0001$) (**Figure 5-4B**) relative to 0 min of stimulation. Post hoc Fisher's LSD test showed AAH was significantly increased over basal levels by IGF-1 stimulation in the presence of LiCl ($P<0.05$), H-89 ($P<0.0001$), and Gö6983 ($P<0.01$). It is noteworthy that predicted GSK-3 β , PKA, and PKC phosphorylation sites are present on AAH [2]. Both basal and IGF-1 stimulated levels of β -Actin were significantly increased in cultures treated with Gö6983 ($P<0.01$) or PD98059 ($P<0.0001$), while it was significantly decreased in cultures treated with H-89 ($P<0.05$).

5.5.4. Effects of IGF-1 stimulation and kinase inhibitor treatments on AAH protein.

The MICE assay was used to examine the effects of kinase inhibitor treatments and IGF-1 stimulation for up to 60 min on AAH protein (A85G6 antibody) (**Figure 5-5**). ANOVA tests demonstrated significant inter-group differences in AAH protein expression in cells stimulated with IGF-1 and treated with LiCl ($F=8.92$; $P=0.0008$) (**Figure 5-5A**), TBCA ($F=9.18$; $P=0.032$), (**Figure 5-5B**), Gö6983 ($F=6.42$; $P<0.0001$) (**Figure 5-5C**), H89 ($F=25.81$; $P<0.016$) (**Figure 5-5D**), and PD98059 ($F=24.42$; $P<0.0001$) (**Figure 5-5E**). Fisher's LSD post hoc test revealed that both LiCl ($0.05<P<0.0001$) and Gö6983 ($P<0.0001$) pre-treatments result in significantly higher basal and IGF-1 stimulated AAH immunoreactivity relative to vehicle. H-89 shows a significant stimulatory effect ($P<0.01$) at the 15-minute time point only; otherwise the curve is flat. TBCA, the CK2 inhibitor, shows striking time-dependent IGF-1 stimulated AAH expression ($0.05<P<0.0001$),

whereas PD98059 had an early (15 min) positive effect on AAH ($P < 0.0001$), but not later. To summarize, treatment with LiCl, Gö6983, and TBCA sustain AAH protein expression relative to vehicle.

5.5.5. Effects of kinase inhibitor treatments on IGF-1-stimulated AAH subcellular trafficking. Immunofluorescence staining and confocal microscopy imaging showed that both vehicle and CK1-pretreated cultures exhibit the same trends with diffuse cytoplasmic and focal perinuclear labeling after 5 minutes (**Figure 5-6A** and **Figure 5-6P**, respectively), and progressive increases in perinuclear labeling over time (**Figure 5-6B-D** and **Figure 5-6Q-S**, respectively). LiCl (**Figure 5-6E-H**), H89 (**Figure 5-6I-L**), and TBB (**Figure 5-6M-P**) prominently increased the levels of FB50 immunoreactivity throughout the cytoplasm, with eventual conspicuous labeling of the perinuclear zone.

5.5.6. Effects of GSK-3 β activity on AAH expression. Since most of the sites predicted to be phosphorylated on AAH are consensus sequences for GSK-3 β and it is a downstream target of insulin/IGF-1 signaling, we examined the long-term consequences of insulin and LiCl treatments on N-Myc-AAH and C-HA-AAH by Western blot. The representative Western blot showed that insulin increased N-Myc-AAH (**Figure 5-7A**) and C-HA-AAH (**Figure 5-7B**) protein and the positive effects were further enhanced by treatment with LiCl. The positive effects of LiCl on N-Myc-AAH were corroborated by immunofluorescence staining and confocal microscopy, as demonstrated by increased Myc expression in podocytes (**Figure 5-7D**) relative to vehicle-treated cells (**Figure 5-7C**).

5.6. DISCUSSION

5.6.1. Study rationale and objectives. The aggressive behavior of HCC is due to its unrelenting growth and highly infiltrative growth pattern. This is, at least in part, mediated by aberrant upregulation of insulin/IGF signaling cascades, which significantly increase

AAH expression. Consequences of increased AAH expression include increased tumor cell migration, subsequent infiltration into surrounding tissue, and increased intrahepatic metastatic potential [1]. AAH promotes cell migration, partly, by activation of Notch signaling networks [3–5]. Evidence suggests that AAH is regulated by post-translational mechanisms of phosphorylation since AAH contains several predicted consensus sequences of phosphorylation by GSK-3 β , PKA, PKC, and CK2 and it was previously shown to be regulated by GSK-3 β in PNET2 cells [2]. These studies were initiated to investigate the role of kinase activity on modulating AAH protein expression.

5.6.2. Insulin/IGF signaling stimulates AAH protein expression and activates Notch signaling. Insulin/IGF signaling networks are significantly upregulated in HCC [14,37,38]. Up-regulation of insulin/IGF-1 signaling increases AAH protein expression, which is highly expressed at the margin of infiltrating HCC tumors, mediating infiltration into surrounding tissue, and promoting intrahepatic metastatic spread [39]. Herein we demonstrate that stimulation with insulin/IGF-1 promotes both endogenous and recombinantly expressed AAH protein. Since insulin/IGF-1 stimulated recombinant AAH protein expression, the results suggest AAH is regulated by insulin/IGF signaling through post-translational mechanisms.

5.6.3. Insulin/IGF signaling stimulates subcellular trafficking of AAH and Notch-1. AAH is linked to the activation of Notch signaling networks since (1) Notch-1 and, its natural ligand, Jagged-1 contain the consensus sequence of AAH hydroxylation; (2) AAH physically interacts with Notch-1 and Jagged-1; and (3) AAH, Notch-1, and Jagged-1 are upregulated in HCC [4]. Since insulin/IGF-1 promotes AAH expression, it was of interest to investigate the consequences of IGF-1 stimulation on AAH and Notch signaling activation. First, we demonstrate that IGF-1 promotes a different intracellular trafficking pattern for the N-terminus and the C-terminus of AAH. IGF-1 causes the C-terminal catalytic domain of AAH to translocate from the perinuclear zone or ER to the

plasma membrane, where it may, as previously reported, interact with and hydroxylate Notch-1 and Jagged-1 at their EGF-like domains [4,40]. We propose that hydroxylation by AAH facilitates the interaction between Notch-1 and Jagged-1 and triggers two rapid cleavage events that release the Notch-1 intracellular domain (NID) from the plasma membrane [27]. At this point, the NID translocates to the nucleus, possibly chaperoned by the AAH catalytic domain, where it displaces co-repressors and recruit co-activators to initiate the transcription of Notch signaling target genes involved in cell adhesion, cell migration, and invasion [3,5]. Taken together, IGF-1 promotes AAH protein expression and trafficking to the plasma membrane to facilitate the activation of Notch signaling as a mechanism that promotes HCC tumor cell migration and invasion.

5.6.4. Kinases regulate AAH in the short-term of IGF-1 stimulation. Insulin/IGF-1 signaling was previously demonstrated to regulate AAH as the transcriptional level through ERK/MAPK and at the post-translational level through PI3k/Akt signaling [4,31]. PI3K/Akt was previously demonstrated to promote its stimulatory effects on AAH protein through inhibition of GSK-3 β [2]. Since GSK-3 β modulates AAH expression, AAH is likely regulated by post-translational mechanisms of phosphorylation. This is supported by the fact that AAH contains several predicted consensus sequences of phosphorylation by GSK-3 β , as well as PKA, PKC, and CK2 [2]. The findings suggest that AAH is post-translationally regulated since inhibition of GSK-3 β , PKA, and PKC promotes AAH expression during short-term stimulation with IGF-1. Additionally, basal and IGF-1 stimulated levels of β -Actin when PKA and PKC are inhibited, but the explanation is not immediately evident.

5.6.5. Kinases regulate AAH during IGF-1 stimulation. Additional investigations on the role of kinase activity during a longer time-course of IGF-1 stimulation demonstrate that inhibition of GSK-3 β , CK2, and PKC promote AAH protein expression; the GSK-3 β and PKC inhibitors function in the absence of IGF-1, whereas the CK2 inhibitor effects

seem dependent upon or responsive to IGF-1 stimulation. Since CK2 can regulate the activity of GSK-3 β , the effects on AAH could be mediated via both kinases. For instance, AAH may need to be phosphorylated by CK2 before it can be phosphorylated by GSK-3 β , following a well-known priming mechanism of phosphorylation [41]. The relevance of these data to the predicted CK2, GSK-3 and PKC phosphorylation sites on AAH was explored in mutagenesis studies (**Chapter 6**). The minimal response to the inhibition of PKA suggests that the predicted PKA site on AAH may not be functionally important. The results from the inhibition of MEK suggest the responses with respect to IGF-1 stimulated AAH are indirect, given the lack of predicted MEK sites on AAH. Correspondingly, one alternative interpretation from these data is that all of the kinase inhibitor effects are indirect rather than directly regulating AAH.

5.6.6. Effects of kinase inhibition on the intracellular trafficking on IGF-1-stimulated AAH. In addition to examining the role of kinase inhibition during the time-course of IGF-1 stimulation, we investigated the effects on the subcellular trafficking of AAH. Herein, we demonstrate that kinase inhibition increases total AAH, as well as Humbug expression, probably corresponding to increased stabilization (reduced degradation) of the proteins. Whether these effects are direct or indirect require further study in which the predicted phosphorylation sites corresponding to GSK-3 β , PKA, CK2, and also PKC are deleted. If the effects are direct, then deletion of the relevant phosphorylation sites should increase basal levels of AAH in transfected cells (**Chapter 6**).

5.6.7. Insulin/IGF inhibits GSK-3 β and promotes AAH. Upon binding of insulin/IGF to its corresponding tyrosine kinase transmembrane receptor, a cascade of phosphorylation events activate Akt, which phosphorylates GSK-3 β at Ser-9 and inhibits its activity. GSK-3 β inhibition was previously shown to increase AAH protein expression and induce neuronal migration [2]. We extended our investigations to examine the long-

term effects of insulin stimulation and GSK-3 β inhibition on AAH protein expression. Long-term insulin stimulation and GSK-3 β inhibition promoted recombinant AAH protein expression. Since GSK-3 β increased AAH protein in the absence of growth-promoting levels of trophic factors, the results suggest that inhibition of GSK-3 β is sufficient to increase AAH levels, probably due to protein stabilization or reduced degradation [2]. The immunofluorescence/confocal microscopy studies also demonstrate higher levels of AAH when GSK-3 β is inhibited in Huh7 cells. The distribution of labeling overlaps with vehicle-treated cells, but includes podocytes. This could be an effect of higher levels of protein being more readily detected in those compartments rather than differential trafficking per se.

5.7. CONCLUSION

The findings suggest that insulin/IGF signaling promotes the trafficking of AAH to the plasma where it may facilitate the activation of Notch signaling networks. The effects may occur through the post-translational regulation of AAH mainly by GSK-3 β , CK2, and PKC. These proteins have the potential to serve as promising targets for controlling aggressive growth of HCC.

5.8. FUTURE DIRECTIONS

We will investigate the co-localization of the AAH C-terminal catalytic domain, using C-HA-AAH and Notch-1 intracellular domain (NID) by double-staining. We will also investigate the co-localization of Myc and NID. It will also be important to identify the molecular mechanisms by which the kinases negatively regulate AAH and elucidate whether this occurs through direct or indirect mechanisms of phosphorylation. In other words, investigate whether the kinases directly phosphorylate and target AAH for degradation, or phosphorylate and activate other negative regulators of AAH (*i.e.*

caspases and/or proteases) that degrade the protein. A pitfall of the experiments in which AAH is under the control of a CMV promoter is that the promoter overexpresses AAH and it may not be significantly affected by IGF-1 stimulation because the protein is constantly overexpressed. Therefore, in this case the cDNAs expressing AAH may be acting as dominant negative forms of AAH that do not allow us to examine what happens endogenously to AAH expression upon stimulation with insulin/IGF-1.

Figure 5-1

Short-term IGF-1 Stimulated AAH Protein Expression in Huh7 Cells

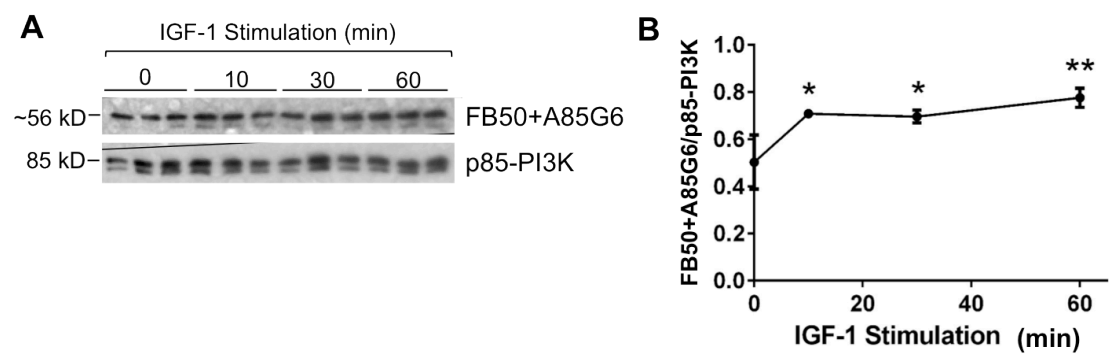


Figure 5-1. Short-term IGF-1 Stimulated AAH protein expression in Huh7 cells.

Sub-confluent Huh7 cell cultures were incubated in medium containing 1% fetal bovine serum for 4 hrs, and then stimulated with 10 ng/ml IGF-1 for 0-60 min. Cell homogenates prepared in radio-immunoprecipitation lysis buffer were used in Western blot analysis to assess shifts in AAH protein expression in response to IGF-1 stimulation. AAH was detected with the (A) FB50+A85G6 monoclonal antibodies, horseradish peroxidase-conjugated secondary antibodies, enhanced chemiluminescence reagents, and film autoradiography. The blots were stripped and re-probed with rabbit polyclonal antibodies to the p85 subunit of phosphoinositide-3-kinase (PI3K) as a loading control. Immunoreactivity was detected as described above. Western blot signals were quantified using the Kodak Digital Science Imaging station (arbitrary units). (B) The calculated mean $\pm$ S.E.M. ratios of FB50+A85G6/p85 signal intensities, corresponding to relative levels of AAH immunoreactivity, are graphed. Significant P-values are shown above each time-point of stimulation (*P<0.05; **P<0.01).

Figure 5-2

Effects of Short-term IGF-1 Stimulation on Endogenous Versus Transfected AAH cDNAs

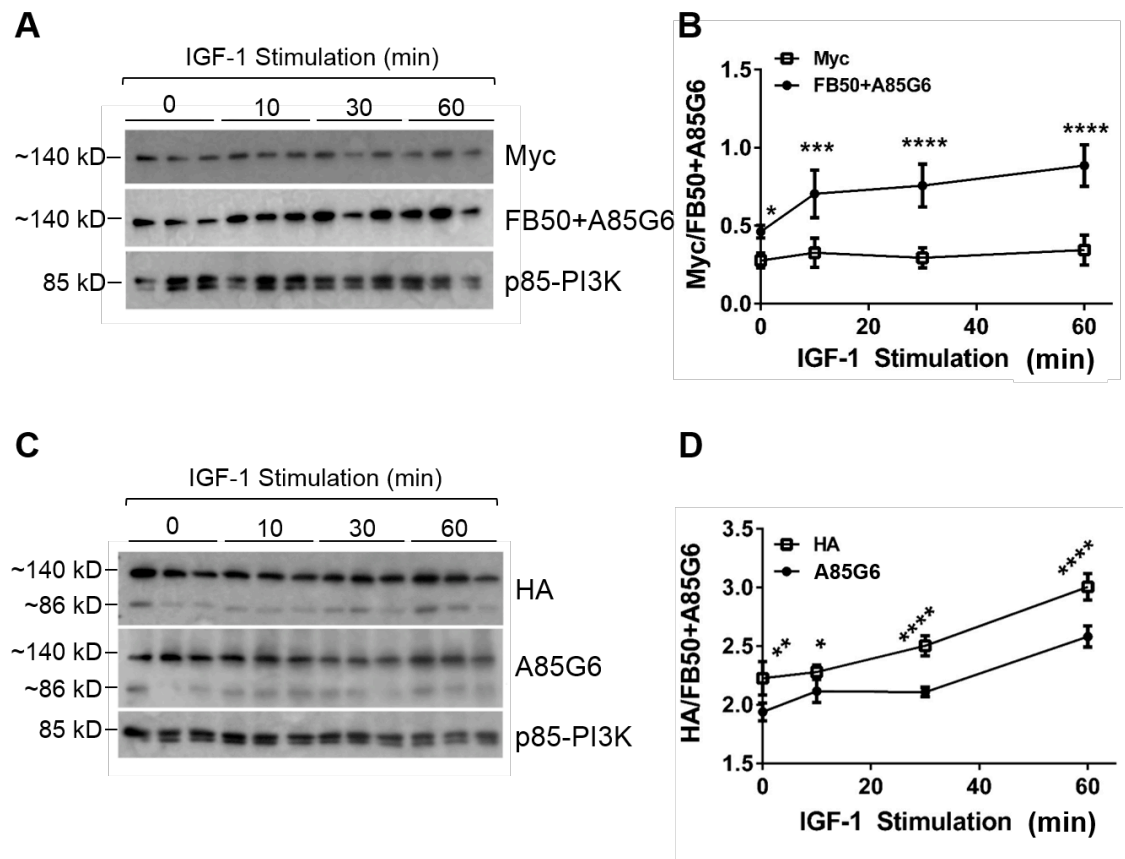


Figure 5-2. Effects of short-term IGF-1 stimulation on endogenous versus transfected AAH cDNAs. Huh7 cells were transiently transfected with recombinant plasmid DNA carrying the full-length human AAH cDNA that was fused in-frame with either an N-terminal Myc tag (N-Myc-AAH) or C-terminal HA tag (C-HA-AAH). Gene expression was under control of a cytomegalovirus promoter. Sub-confluent cultures, 24 hrs post-transfection, were incubated in medium containing 1% fetal bovine serum for 4 hrs, and then stimulated with 10 ng/ml IGF-1 for 0-60 min. Culture homogenates prepared in radio-immunoprecipitation buffer were used in Western blot analysis to assess shifts in the levels of endogenous (A) AAH versus Myc- or (B) HA-tagged protein in response to IGF-1 stimulation. The blots were sequentially probed with FB50+A85G5 (endogenous AAH), Myc or HA (recombinant AAH), and the p85 subunit of PI3K as a loading control. Immunoreactivity was detected with HRP-conjugated secondary antibodies, enhanced chemiluminescence reagents, and film autoradiography. The blots were stripped before being re-probed. (C, D) Western blot signals were quantified using the Kodak Digital Science Imaging station (arbitrary units). The calculated mean $\pm$ S.E.M. ratios of FB50+A85G6/p85, Myc/p85, and HA/p85 signal intensities are graphed in relation to duration of IGF-1 stimulation. Inter-group comparisons were made using two-way repeated measures of ANOVA and the post hoc Fisher's Least Significance Difference test. Significant P-values are shown above each time-point of stimulation (*P<0.05; **P<0.01; ***P<0.001; and ****P<0.0001).

Figure 5-3

Differentially Stimulates intracellular trafficking of the N- and C-terminal portions of AAH

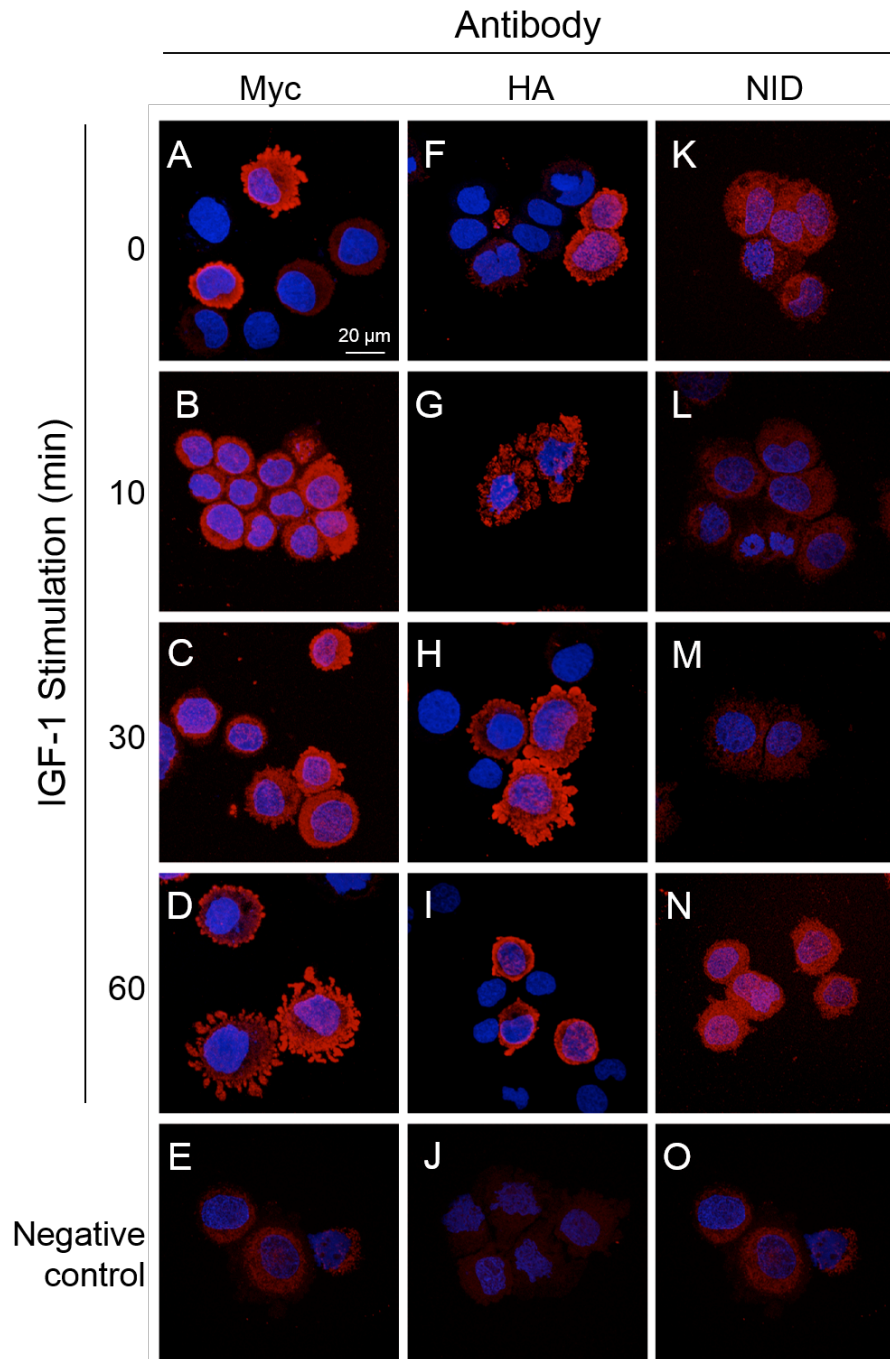


Figure 5-3. IGF-1 Differentially Stimulates intracellular trafficking of the N- and C-terminal portions of AAH. Huh7 cells were transfected with recombinant plasmid expressing (A-E, K-O) N-Myc-AAH or (F-J) C-HA-AAH. Sub-confluent cultures were pre-incubated for 4 hrs in medium containing 0.5% FBS, then stimulated with 10 ng/ml IGF-1 for 0-60 minutes. Cells were harvested and used to generate cytopsin slides that were immunostained with antibodies to Myc (A-D), HA (F-I), or Notch-1 intracellular domain (NID) (K-N). Immunoreactivity was detected with biotinylated secondary antibody and Streptavidin-conjugated Dylight 547 fluorophore (shown in red). (E, J, O) Negative control studies were parallel incubations in which the primary antibodies were omitted. Cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI-blue) and imaged by confocal microscopy (600x original magnification, 2x digital zoom). Representative results are shown.

Figure 5-4

Differential Effects of Kinase Inhibitors on Basal and Short-Term IGF-1 Stimulated AAH Immunoreactivity

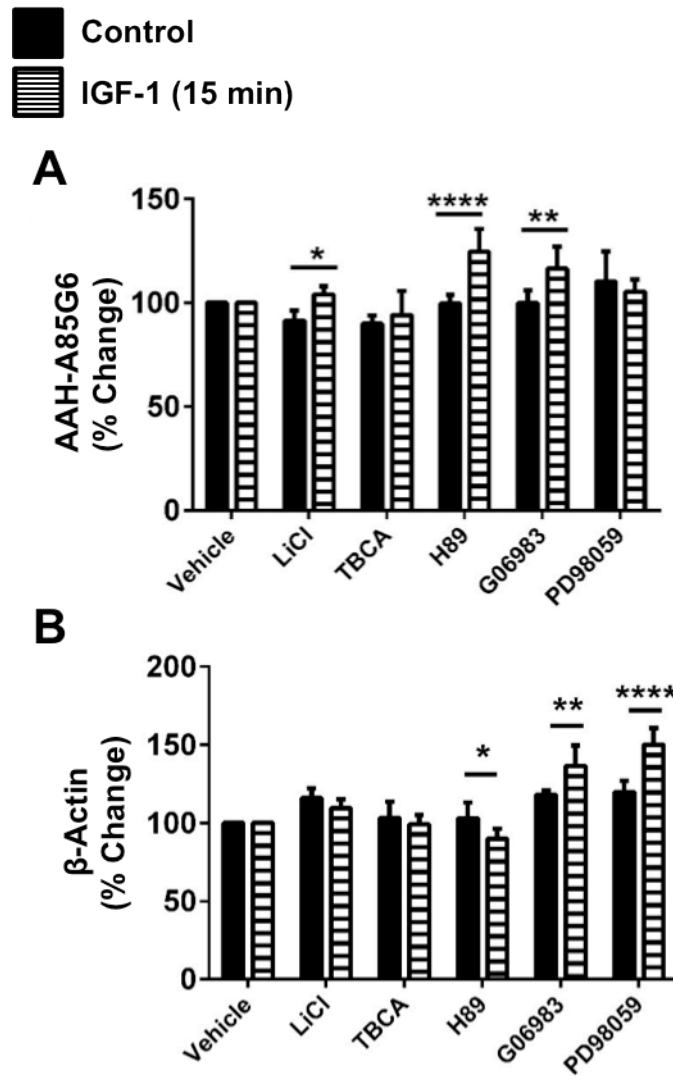


Figure 5-4. Differential effects of kinase inhibitors on basal and short-term IGF-1 stimulated AAH immunoreactivity. Huh7 cells were seeded in 96-well micro-cultures (2×10^4 /well) and treated with vehicle (0.1% DMSO), LiCl (glycogen synthase kinase-3 β inhibitor), TBCA (casein kinase 2 inhibitor), Gö6983 (pan-protein kinase C inhibitor), H-89 (protein kinase A inhibitor), or PD98059 (MEK-1 inhibitor) for 4 hrs in media containing 0.5% FBS. Cells were then stimulated with nothing or 10 ng/ml of IGF-1 for 15 min. The culture media was quickly aspirated and cells were fixed over night with Histofix. Immunoreactivity to (A) AAH-A85G6 and (B) β -Actin was detected using the microtiter-immunocytochemical ELISA-MICE Assay, i.e. cellular ELISA, using horseradish peroxidase conjugated secondary and the Amplex UltraRed fluorophore. The cells were counterstained with Hoechst H33342 (which is linearly correlated with cell density), and results were used to correct differences in levels of immunoreactivity with respect to culture cell density. Fluorescence (Amplex: Ex/Em; H33342: Ex/Em) was measured in an M5 SpectraMax plate reader (Molecular Dynamics). Data in each panel were normalized with respect to vehicle-treated control to enable comparisons among the different antibodies. Graphs depict the mean $\pm$ S.E.M of 4 replicate cultures per experimental group. Inter-group comparisons were made using repeated measures of two-way ANOVA (see **Table 5-1**) and the post hoc Fisher's Least Significance Difference test. Significant P-values are shown above the bars (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; and **** $P < 0.0001$).

Table 5-1**Differential effects of kinase inhibitors on basal and short-term IGF-1 stimulated AAH immunoreactivity**

Gene/Protein	IGF1 Stimulation		Kinase Inhibitor		IGF-1x Inhibitor	
	F-Ratio	P-Value	F-Ratio	P-Value	F-Ratio	P-Value
A85G6	13.99	0.0003	35.18	<0.0001	18.72	0.0042
A85E6	12.41	<0.0001	59.28	<0.0001	14.88	<0.0001
FB50	3.57	N.S.	57.67	<0.0001	3.18	N.S.
β -Actin	1.40	N.S.	66.43	<0.0001	18.44	<0.0001

Table 5-1. Differential effects of kinase inhibitors on basal and short-term IGF-1 stimulated AAH immunoreactivity. F-Ratio=variance, the strength of which reports differences between the groups. P-values are significant $p < 0.05$. However, they represent a trend if $0.05 < p < 0.10$. In two-way ANOVA tests, different treatments by time or gene transfected are tested, along with interactions between the two factors.

Figure 5-5

Differential Effects of Kinase Inhibitors on IGF-1-Stimulated AAH Immunoreactivity

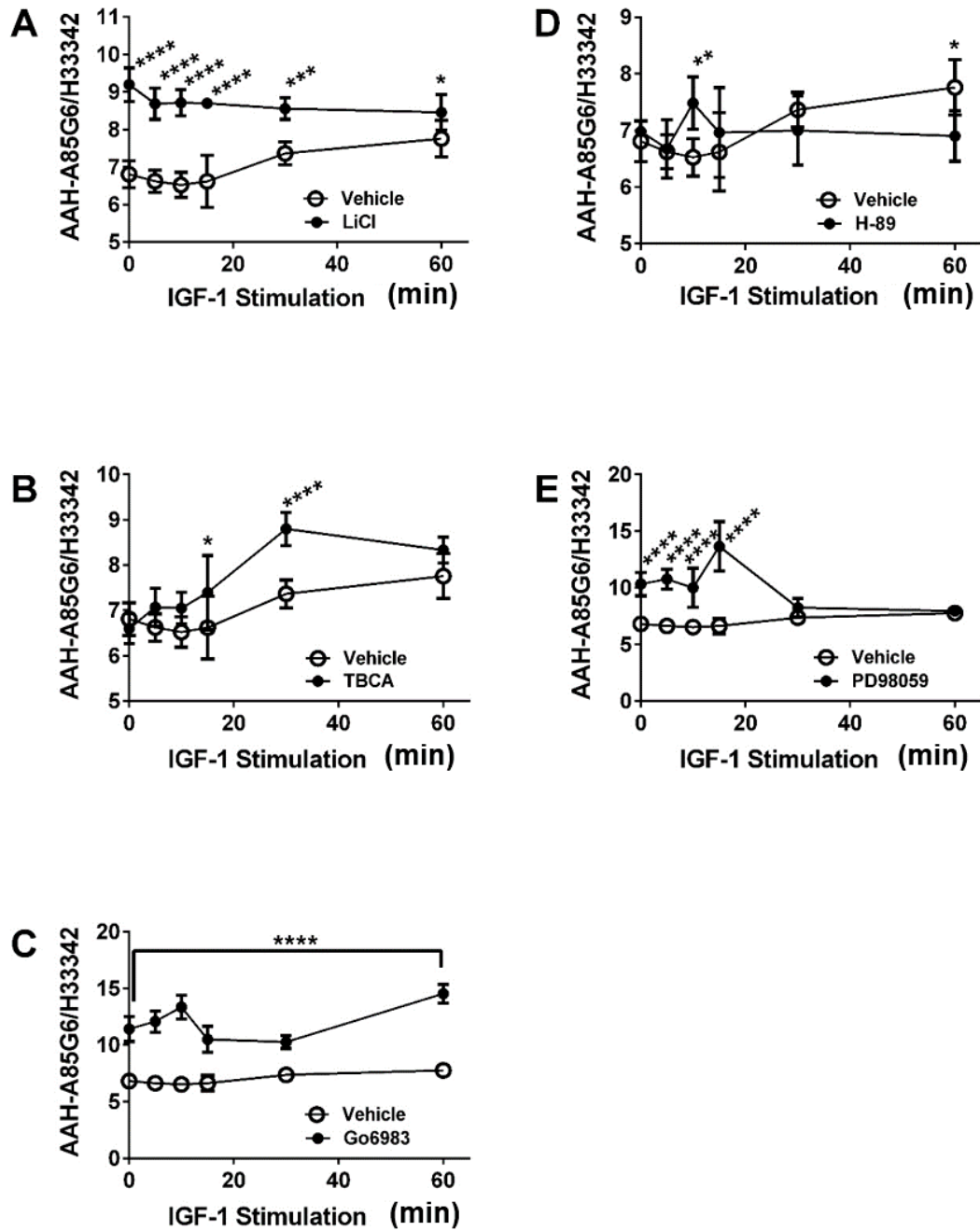


Figure 5-5. Differential effects of kinase inhibitors on IGF-1 stimulated AAH immunoreactivity. Huh7 cells were seeded in 96-well micro-cultures (2×10^4 /well) and half of each plate was treated with vehicle (0.1% DMSO) or (A) LiCl (glycogen synthase kinase-3 β inhibitor), (B) TBCA (casein kinase 2 inhibitor), (C) Gö6983 (pan-protein kinase C inhibitor), (D) H-89 (protein kinase A inhibitor), or (E) PD98059 (MEK-1 inhibitor) for 4 hrs in media containing 0.5% FBS. Cells were then stimulated with 10 ng/ml of IGF-1 for 0-60 min. The culture media was quickly aspirated and cells were fixed over night with Histofix. Immunoreactivity to AAH-A85G6 was detected using the Microtiterimmunocytochemical ELISA-MICE Assay, i.e. cellular ELISA with horseradish peroxidase conjugated secondary and the AmplexUltraRed fluorophore. The cells were counterstained with Hoechst H33342 (which is linearly correlated with cell density), and results were used to correct differences in levels of immunoreactivity with respect to culture cell density. Fluorescence (Amplex: Ex/Em; H33342: Ex/Em) was measured in an M5 SpectraMax plate reader (Molecular Dynamics). Graphs depict the mean $\pm$ S.E.M of 4 replicate cultures per experimental group. Inter-group comparisons were made using repeated measures of two-way ANOVA (see **Table 5-2**) and the post hoc Fisher's Least Significance Difference test. Significant P-values are shown above the bars (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; and **** $P < 0.0001$).

Table 5-2**Differential effects of kinase inhibitors on IGF-1 stimulated AAH immunoreactivity**

Inhibitor	IGF1 Stimulation		Kinase Inhibitor		IGF-1x Inhibitor	
	F-Ratio	P-Value	F-Ratio	P-Value	F-Ratio	P-Value
H-89	16.95	0.083	0.21	N.S.	25.81	0.016
PD98059	11.84	0.0003	49.81	<0.0001	24.42	<0.0001
LiCl	75.57	<0.0001	3.69	0.071	8.92	0.0008
Go6983	9.09	<0.0001	79.37	<0.0001	6.42	<0.0001
TBCA	53.54	<0.0001	13.55	<0.0001	9.18	0.032

Table 5-2. Differential effects of kinase inhibitors on IGF-1 stimulated AAH immunoreactivity. F-Ratio=variance, the strength of which reports differences between the groups. P-values are significant $p < 0.05$. However, they represent a trend if $0.05 < p < 0.10$. In two-way ANOVA tests, different treatments by time or gene transfected are tested, along with interactions between the two factors.

Figure 5-6

IGF-1 Stimulation Promotes AAH Intra-cellular Trafficking-
Effects of Kinase Inhibitor Pre-Treatments

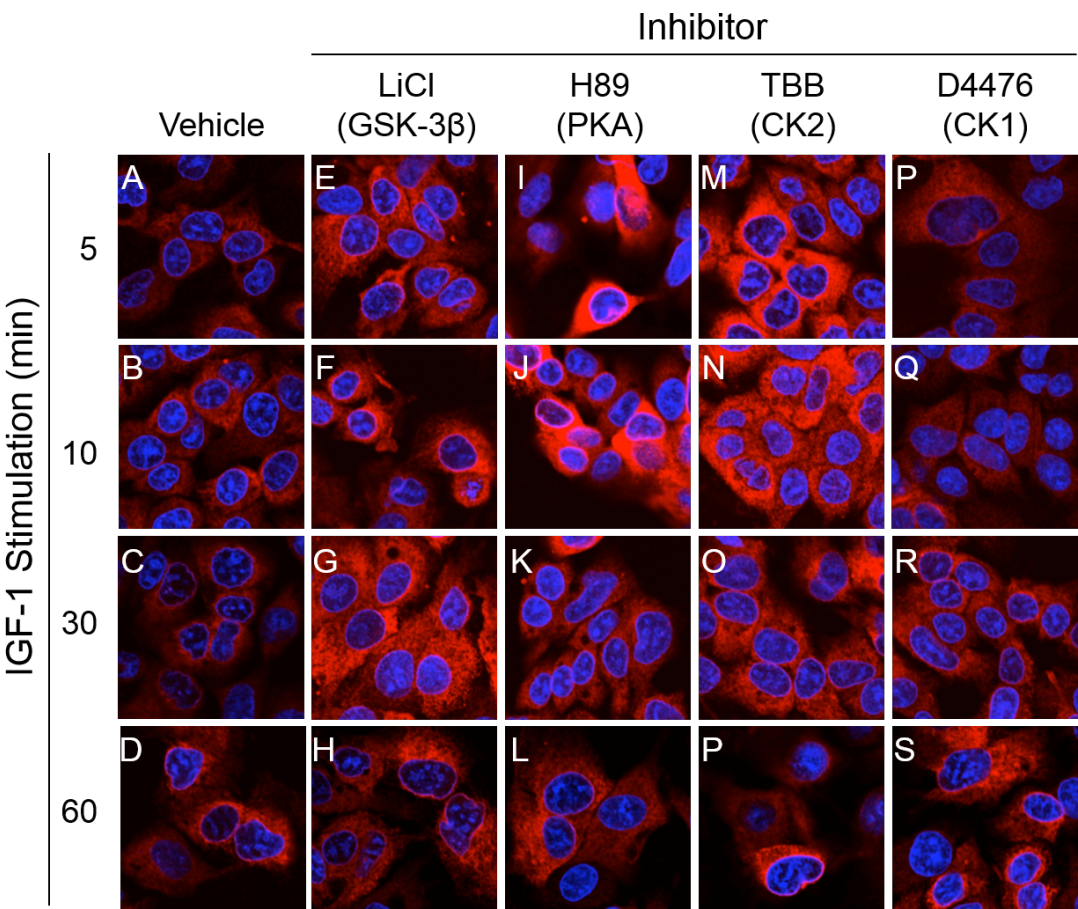


Figure 5-6. IGF-1 stimulation promotes AAH intra-cellular trafficking-effects of kinase inhibitor pre-treatments. Huh7 cells were seeded in 12-well Tissue-Tek Chamber slides (1x10⁵/chamber) and pre-treated for 4 hours with vehicle (0.1% DMSO), LiCl, H-89, TBB (casein kinase 2 inhibitor), or D4476 (CK1 inhibitor; non-relevant) for 4 hrs in media containing 0.5% FBS. Cells were then stimulated with 10 ng/ml of IGF-1 for 5-60 min. The culture media was quickly aspirated and cells were immersion-fixed overnight with Histofix. AAH+Humbug expression was detected by immunofluorescence with the FB50 monoclonal antibody, biotinylated secondary antibody, and Cy-Dye fluorophore (shown in red). Cells were counterstained with DAPI and imaged by confocal laser microscopy. Merged images are depicted.

Figure 5-7

GSK-3 β inhibition and long-term insulin stimulation increase Myc-AAH and HA-AAH on AAH protein expression

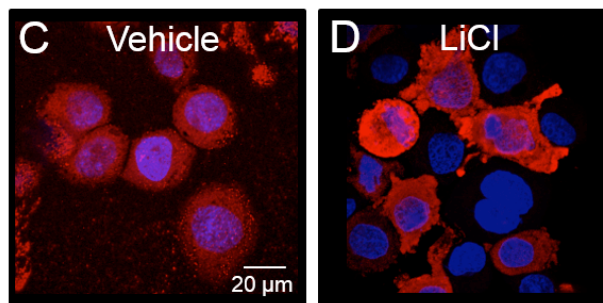
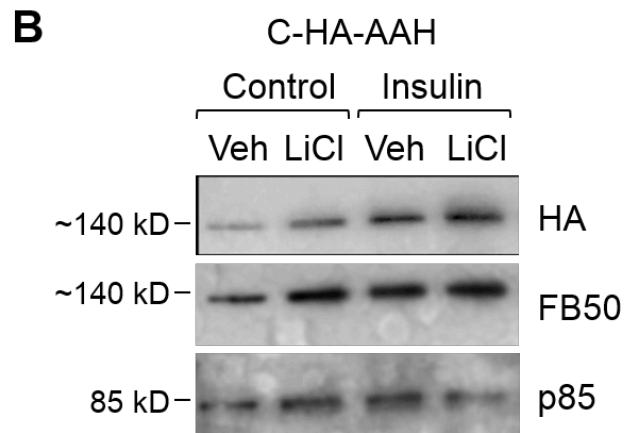
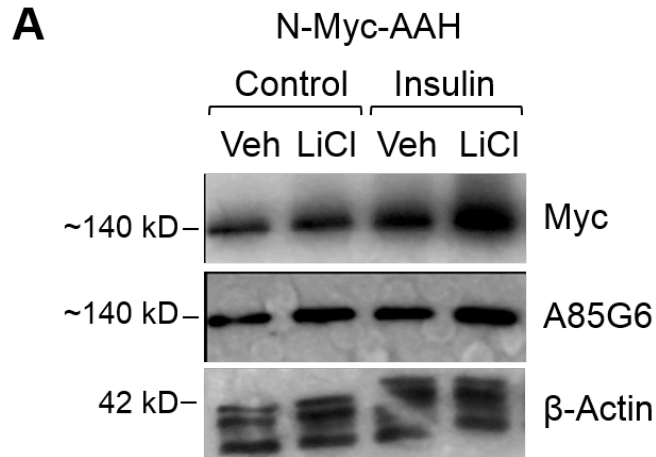


Figure 5-7. GSK-3 β inhibition and long-term insulin stimulation increase N-Myc-AAH and C-HA-AAH on AAH protein expression. Huh7 cells were transfected with the N-Myc-AAH or C-HA-AAH cDNA, pre-treated with vehicle (0.1% DMSO) or 20 mM LiCl in medium containing 0.5% FBS for 4 hrs, then stimulated with 10 nM insulin for 16 hrs. Cultures were harvested for (A, B) Western blot analysis and (C, D) immunofluorescence staining of N-Myc-AAH transfected cytocentrifuge preparations (see Methods). Blots were probed with antibodies to Myc or HA (according to cDNA transfected), then stripped and re-probed with the A85G6 or FB50 (C, D). Myc-AAH immunoreactivity was detected by immunofluorescence (shown in red) with DAPI counter-staining (shown in blue). Cells were imaged by confocal laser microscopy and representative results are shown.

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CHAPTER 6

REGULATION OF AAH EXPRESSION AND FUNCTION

IN HEPATOCELLULAR CARCINOMA CELLS

***Manuscript in preparation**

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and Suzanne M. de la Monte

AUTHOR CONTRIBUTIONS

DL conducted the in vitro experiments using the AAH cDNAs and monoclonal antibodies and drafted the manuscript; JG orchestrated the sub-cloning and mutagenesis of AAH; MT subcloned human AAH cDNA and generated the N-Myc-tagged wildtype and mutant constructs and validated the sequences, expression, and function; JRW provided insightful feedback on the results; and SMdIM originated ideas, designed the experiments, designed the mutagenesis approach, supervised the work, helped analyze the data, and prepare the manuscript.

6.1. RATIONALE

AAH could be regarded as a distinct biomarker of hepatocellular carcinoma. Previous evidence demonstrated that insulin and insulin-like growth factor (IGF) signaling promote AAH expression as well as cell adhesion and motility through inhibition of glycogen synthase kinase-3 β (GSK-3 β). Carter, *et al.* demonstrated GSK-3 β modulates AAH since constitutively active GSK-3 β decreased AAH protein expression and impaired motility of central nervous system-derived primitive neuroectodermal 2 (PNET2) cells, while inhibition of the kinase significantly stimulated AAH expression as well as cell adhesion and migration [1]. Results from **Chapter 5** indicate that insulin/IGF signaling also regulates AAH through GSK-3 β , as well as through protein kinase C and casein kinase 2, in the hepatoma-derived Huh7 cell line, which promotes motility through activation of Notch signaling networks. It is currently unknown how AAH is regulated by phosphorylation. The purpose of the present work was to determine if AAH is regulated by direct phosphorylation of the protein and the functional consequences of preventing phosphorylation of AAH at predicted phosphorylation sites, as it relates to catalytic activity, activation of Notch signaling, as well as cell adhesion and migration.

6.2. ABSTRACT

Background: Increased insulin and insulin-like growth factor (IGF) signaling in hepatocellular carcinomas (HCCs) is associated with high levels of aspartyl-asparaginyl- β -hydroxylase (AAH) expression and function. AAH has a demonstrated role in cell motility and invasion, and it mediates these effects in part via activation of Notch pathways. Previous studies showed that chemical or molecular inhibition of glycogen synthase kinase-3 β (GSK-3 β) increases AAH protein expression and that AAH has multiple potential phosphorylation sites with consensus sequences corresponding to GSK-3 β , PKA, PKC, or CK2. Herein, we determined if deletion of predicted phosphorylation sites would alter AAH protein expression. **Methods:** Using N-Myc- and C-HA-tagged AAH cDNA constructs, we ablated individual phosphorylation sites by converting S/T to A by site-directed mutagenesis. Wildtype and mutant cDNAs were expressed in transiently transfected human Huh7 cells. Recombinant AAH expression and function were assessed by Western blot analysis, cellular ELISA, motility assays, catalytic activity measurement, and Notch activation. **Results:** Compared with wildtype N-Myc-AAH or C-HA-AAH, individual point-mutated cDNAs had either inhibited or had no significant effect on AAH expression and function. In contrast, AAH cDNAs with mutations involving multiple phosphorylation sites had wildtype or higher levels of catalytic activity. **Conclusion:** Post-translational regulation of AAH expression and function are differentially regulated by the degree and nature of protein phosphorylation. Effects of GSK-3 β phosphorylation of AAH could be governed via priming mechanisms such as by CK2.

Key terms: Hepatocellular carcinoma; insulin-like growth factor; phosphorylation; aspartyl-asparaginyl- β -hydroxylase; hydroxylation; Notch; cell adhesion; cell motility.

6.3. INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide [2,3] and is currently the fastest growing cause of cancer-related mortality among males in the United States [4]. Molecular mechanisms of HCC carcinogenesis include dysregulation of cell cycle checkpoints, apoptosis [5–7], and growth factor signaling [8–10]. Insulin/insulin-like growth factor (IGF) signaling is significantly upregulated in HCC through mechanisms that include gain-of-function mutations in phosphoinositide 3-kinase (PI3K) [11], impaired expression/function of phosphatase and tensin homolog [12], and over-expression of IGF signaling through IRS-1 [10].

Insulin/IGF signaling networks regulate the expression of the molecule aspartyl-asparaginyl- β -hydroxylase (AAH), one of the few HCC biomarkers whose expression correlates with malignant transformation, histological grade, and clinical prognosis [13–15]. AAH is an ~86 kD [16] type 2 transmembrane protein located in the endoplasmic reticulum (ER) [17,18]. AAH is physiologically cleaved into a ~30-34 kD N-terminal fragment that is identical to Humbug (Junctate), a truncated isoform that binds to calcium and promotes adhesion [19,20], and a ~52-56 kD catalytically active C-terminal domain that contains a H675 residue that is essential for hydroxylation [21]. AAH partially mediates cell motility by hydroxylation of β -carbons of specific aspartate (Asp) and asparagine (Asn) residues within consensus sequences of epidermal growth factor (EGF)-like domains of targets [22], such as Notch signaling pathway proteins Notch-1 and Jagged-1 [13,23,24].

Evidence suggests that the C-terminal catalytic domain of AAH is linked to activation of Notch signaling networks in HCC since (1) Notch-1 and, its natural ligand, Jagged-1 contain the consensus sequence for AAH hydroxylation; (2) AAH interacts with Notch-1 and Jagged-1, leading to Notch signaling activation; and (3) AAH, Notch-1, and

Jagged-1 are up-regulated in HCC [13]. Notch signaling is activated by binding of Jagged (or Delta-like family of proteins) to the Notch extracellular domain. This event triggers the cleavage of the extracellular domain from the membrane in a metalloprotease-dependent manner, which is rapidly followed by cleavage of the intracellular domain by the protease presenilin [25]. The Notch intracellular domain (NID) is then able to translocate from the plasma membrane to the nucleus, where it complexes with the transcriptional activator CBF1, suppressor of hairless or lag-1 (CSL), and the transcriptional co-activators of the mastermind-like family of proteins. The complex binds to DNA containing CSL recognition sequences, recruits additional factors, and activates the transcription of downstream targets, such as hairy and enhancer of split-1 (HES-1) and HES-related proteins [25,26]. The AAH-mediated activation of cell migration in HCC has been linked to Notch signaling network upregulation [13].

AAH is regulated by insulin/IGF signaling [10,27,28] at both the transcriptional and post-translational levels [29]. During insulin/IGF signaling, insulin or IGF polypeptide binds to its corresponding receptor, leading to the receptor's auto-phosphorylation at particular tyrosine (pTyr) residues. Insulin receptor substrate-1 (IRS-1) binds to these pTyr residues and activates the mitogen-activated protein kinase (MAPK) (previously known as extracellular-signal-regulated kinases; ERK) and PI3K arms of insulin/IGF signaling. ERK/MAPK promotes the transcriptional expression of AAH, while PI3K stimulates AAH protein through a series of phosphorylation events that inhibit glycogen synthase kinase-3 β (GSK-3 β) activity [29–31]. GSK-3 β was previously shown to regulate AAH expression and function in neuronal cells since (1) chemical and siRNA-mediated inhibition of GSK-3 β increased AAH protein and neuronal cell migration, while (2) GSK-3 β overexpression decreased AAH protein expression and neuronal migration, likely through caspase-mediated degradation [1]. Similar effects were observed in the hepatoma-derived Huh7 cells (**Chapter 5**), in which GSK-3 β inhibition enhanced AAH

protein expression, along with inhibition of protein kinase A (PKA), protein kinase C (PKC), and casein kinase 2 (CK2), which are all predicted to phosphorylate AAH.

Although evidence indicates that kinases regulate AAH protein expression in the liver, it is currently unknown which sites predicted to be phosphorylated on AAH modulate the protein's expression and function. The present study investigated whether deletion of individual predicted phosphorylation sites on AAH by site-directed mutagenesis promoted AAH expression and function. We hypothesized that if AAH was directly phosphorylated by GSK-3 β , PKA, PKC, and CK2, targeting the protein for degradation, then deletion of these sites on AAH would prevent AAH phosphorylation and subsequent targeting for degradation. We found that deletion of individual phosphorylation sites on AAH did not enhance protein expression or function, as it related to catalytic activity, Notch signaling activation, cell adhesion, and migration. The findings suggest AAH needs to be phosphorylated at multiple sites to be targeted for degradation. Nevertheless, since individually mutated phosphorylation sites on AAH significantly impaired cell adhesion and migration, the AAH mutants could potentially serve as a therapeutic treatment to impair HCC tumor infiltration and aggressive growth via antibody gene delivery.

6.4. MATERIALS AND METHODS

6.4.1. Materials. QuickChange Site-directed Mutagenesis Kit from Stratagene (La Jolla, CA, USA). *Escherichia coli* DH5 α cells, Dulbecco's Modified Eagle Medium (DMEM), Lipofectamine 2000 Transfection Reagent, TRIzol, Amplex UltraRed, and 4-methylumbelliferyl phosphate (4-MUP) were purchased from Invitrogen (Carlsbad, CA, USA). pcDNA 3 vector with a 6x Myc-tag was a gift from Dr. Y. Eugene Chin from Brown University (Providence, RI, USA) [32]. QIAquick Gel Extraction Kit, QIAprep Spin Miniprep Kit, RNeasy Lipid Tissue Mini Kit were purchased from Qiagen (Valencia, CA,

USA). MaxiSorb plates, OptiPlates (96-well), BD Falcon culture inserts, and Nunc culture supplies were obtained from Thermo Scientific (Rochester, NY, USA). ATPLite reagents were purchased from Perkin-Elmer (Waltham, MA, USA). Myc antibody purchased from Cell Signaling Technologies (Danvers, MA, USA). Bicinchoninic assay, enhanced chemiluminescence reagents, and Dylight 547 Conjugated to Streptavidin were purchased from Pierce (Rockford, IL, USA). Rabbit polyclonal antibody to large acidic ribosomal protein (RPLPO) was purchased from Proteintech (Chicago, IL, USA). Alkaline Phosphatase Conjugated to Streptavidin was purchased from Vector Laboratories (Burlingame, CA, USA). Positive-charge slides from Fisher Scientific (Pittsburgh, PA, USA). Shandon Cytospin Centrifuge 3 from Thermo Shandon (Pittsburgh, PA, USA). SpectraMax M5 Microplate Reader and Kodak PhosphorImager Screen S0230 with Cassette from Molecular Dynamics (Sunnyvale, CA, USA). Histofix was obtained from Amresco (Solon, Ohio, USA). AMV First Strand cDNA Synthesis Kit, probe-based primer pairs from the Universal ProbeLibrary Assay Design Center, and LightCycler 480 System came from Roche (Indianapolis, IN, USA). MacVector 10 software from MacVector, Inc. (Cary, NC, USA). Boyden well chambers from Neuro Probe (Gaithersburg, MD, USA). α -[^{14}C]-Ketoglutaric acid was purchased from NEN Life Science (Boston, MA). Glass fiber filter paper was purchased from Packard (Meriden, CT). Other fine chemicals were purchased from CalBiochem (Carlsbad, CA, USA) or Sigma-Aldrich (St. Louis, MO, USA).

6.4.2. Recombinant AAH plasmid constructs. The coding region of human AAH was amplified from a 293T cell cDNA library by the polymerase chain reaction (PCR) using the forward primer 5'-CGGAATTCATGGCCCAGCGTAAGAATGCCA-3', reverse primer 5'-CCGCTCGAGCTAAATTGCTGGAAGGCTGC-3' and *Pfu* DNA polymerase [16]. The AAH PCR product was digested with EcoRI and XhoI restriction enzymes and gel purified with the QIAquick Gel Extraction Kit, cloned into the pcDNA 3 vector with a 5'-

end 6x Myc-tag insert (pN-Myc-AAH), and transformed into *Escherichia coli* DH5 α competent cells. Ampicillin-resistant clones were cultured in *Luria Broth* media and the plasmids were purified with the QIAprep Spin Miniprep Kit. The correct sequence and orientation of pN-Myc-AAH was verified by DNA sequencing and protein expression by Western blot. Since AAH is predicted to be phosphorylated at specific serine (S)/threonine (T) residues by GSK-3 β [1], the residues were point-mutated to Alanine (A) using the QuickChange Site-directed Mutagenesis kit, according to the manufacturer's protocol using the primer sequences in **Table 6-1**. The PCR products were transformed into *E. coli* DH5 α cells and correct mutant clone sequences confirmed by DNA sequencing.

6.4.3. Cell culture. Huh7 cells were maintained in DMEM supplemented with heat-inactivated 5% fetal bovine serum (FBS), 2 mM L-glutamine, and 1x non-essential amino acids in 5% CO₂ at 37°C. To examine the effects of deleting GSK-3 β sites on AAH, Huh7 cells were transiently transfected with a plasmid that encoded for wild-type (WT) or point-mutated (S/T→A) AAH (pN-Myc-AAH) at semi-confluency with Lipofectamine 2000 Transfection Reagent. Cells were co-transfected with plasmid containing green fluorescent protein (GFP) to monitor transfection efficiency; AAH and GFP plasmids were under the control of cytomegalovirus (CMV) promoter sequence to overexpress the proteins.

6.4.4. Duplex Enzyme-linked immunosorbent assay (ELISA). Huh7 protein homogenates were prepared in radio-immunoprecipitation assay buffer in the presence of protease and phosphatase inhibitors. Protein concentration was measured by the bicinchoninic acid (BCA) assay. Duplex ELISA measured target protein immunoreactivity that was normalized to large acidic ribosomal protein (RPLPO) directly measured in the wells [33,34]. Protein homogenates (100 ng/50 μ l) were adsorbed onto the bottom surfaces of FluoroNunc MaxiSorp 96-well plates by overnight incubation on a rotating

platform. Non-specific protein binding to the wells was prevented by incubation with 1% bovine serum albumin (BSA) in TRIS buffered saline (TBS). After overnight incubation with myc antibody (1:1000), immunoreactivity was detected with horseradish peroxidase (HRP)-conjugated secondary antibodies and Amplex UltraRed soluble fluorophore (*Ex* 530 nm/*Em* 590 nm). After rinsing, the wells were incubated with biotinylated RPLPO primary antibody and immunoreactivity detected with streptavidin-conjugated alkaline phosphatase (1:1000) and the 4-methylumbelliferyl phosphate (4-MUP) fluorophore (*Ex* 360 nm/*Em* 460 nm). The SpectraMax M5 microplate reader directed levels of fluorescence. Non-specific binding was assessed by parallel incubations in which the primary or secondary antibodies were omitted. The ratios of Myc/RPLPO fluorescence were calculated to allow inter-well comparisons. At least six replicates were analyzed for each experiment.

6.4.5. Immunofluorescence staining. 50,000 cells were suspended in 10% FBS culture medium and cytocentrifuged in Shandon Cytospin Centrifuge 3 onto positively-charged slides, fixed in Histofix, and permeabilized with 0.05% saponin in TBS for 2 min. Sites of non-specific binding were blocked with SuperBlock in TBS. After overnight incubation with Myc antibody (1:1000), immunofluorescence was detected with biotinylated secondary antibody and DyLight 547 fluorophore conjugated to streptavidin (*Ex* 557 nm/*Em* 574 nm). Cells were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) and imaged by fluorescence or confocal microscopy.

6.4.6. $^{14}\text{CO}_2$ capture assay. The $^{14}\text{CO}_2$ capture assay measures ^{14}C -labeled CO_2 produced by CO_2 releasing enzymes, such as AAH, as a byproduct of its hydroxylase reaction, as previously described [35]. Briefly, MaxiSorp plate was coated with 400 ng of A85G6+A85E6+FB50 antibodies per well on a gentle rotating platform at 4°C overnight. The samples were incubated with 1% bovine serum albumin (BSA) in PBS and 0.05% Tween-20 (PBST) for 3 hours to prevent non-specific protein binding. After rinsing, 25 μg

of protein homogenates in PBS were captured by bound antibodies inside the wells. 200 ng BSA and 200 ng rAAH per well were used as negative and positive controls, respectively. After rinsing, 60 μ M epidermal growth factor (EGF)-like peptide (60 μ M), 40 μ M [14 C]- α -ketoglutarate acid in 50 mM PIPES pH 7.0, 100 mM NaCl, 100 μ M FeCl₂, 0.2 mg/ml casein and H₂O were added for a total reaction volume of 40 μ L per well. Plates were then covered with glass filter paper soaked in Ca(OH)₂ (30 mM) and incubated for 1 hour at 25°C. Glass filter paper was dried under ultraviolet light. Signal was detected with a phosphor imager cassette and the STORM 820 Phosphor Imager.

6.4.7. Quantitative real-time PCR (qRT-PCR). Huh7 expressing WT or S/T→A point-mutated N-Myc-AAH were harvested in TRIzol and RNA extracted with the RNeasy Lipid Tissue Mini Kit, following manufacturer's guidelines. Purified RNA was converted to cDNA by reverse transcription PCR using random oligodeoxynucleotide primers and the AMV First Strand cDNA synthesis kit. cDNA was used as a template for qRT-PCR with gene-specific primer pairs to detect Notch signaling gene expression (**Table 6-2**). Primer pairs were obtained from the Universal ProbeLibrary Assay Design Center, optimized with MacVector 10 software, and specificity verified with the Basic Local Alignment Search Tool from the National Center for Biotechnology Information. The gene-specific amplified signals were continuously detected by the LightCycler 480 System and equal cDNA amounts per well were determined by hypoxanthinephosphoribosyltransferase (HPRT) amplification. The amplification protocol consisted on an initial 10 min denaturation step at 95°C, followed by 45 cycles of amplification, 15 sec denaturation at 95°C, and 1 min annealing and extension at 60°C.

6.4.8. Directional motility assay. Directional motility was measured using the ATP luminescence-based motility invasion (ALMI) assay [3]. Briefly, 1% FBS culture media was placed in the bottoms of blind well chambers and 8- μ m pore polycarbonate membranes were placed in-between the upper and lower chambers. 100,000 viable cells

were seeded into the upper chambers in 0.1% FBS culture media and allowed to migrate for 30 min at 37°C. The ATPlite luminescence assay system quantified motile adherent cells collected from the undersurfaces of the membranes and motile non-adherent cells collected from bottoms of the wells. The percentages of total motile (adherent+non-adherent) were calculated relative to the total population of cells. Six replicates were used for statistical analysis.

6.4.9. Statistical analysis. Data depicted in the graphs represent the means $\pm$ standard error of the mean (S.E.M.) for each group. Inter-group comparisons were made using repeated measures of one-way analysis of variance (ANOVA) with the post hoc Fisher's Least Significant Difference test. Statistical analyses were performed using GraphPad Prism 5 and significant P-values are indicated over the bars or time-points (*P<0.05; ξ P<0.05; **P<0.01; $\xi\xi$ P<0.01; ***P<0.001; $\xi\xi\xi$ P<0.001; ****P<0.0001; $\xi\xi\xi\xi$ P<0.0001).

6.5. RESULTS

6.5.1. Effects of AAH S/T→A mutations of GSK-3 β sites on AAH protein expression. AAH is predicted to contain consensus sequences for phosphorylation by various kinases, most of which correspond to GSK-3 β , although there are also sequences for PKA, PKC, and CK2 phosphorylation. S/T residues predicted to be phosphorylated were mutated to A to examine the consequences of preventing phosphorylation at these sites on AAH protein expression. Duplex ELISA demonstrated that Huh7 cells overexpressing wildtype (WT) N-Myc-AAH had significantly higher levels of Myc expression (P<0.0001) indicating, as expected, N-Myc-AAH was overexpressed relative to EV control (**Figure 6-2A**). Significant decreased levels of Myc were observed with S/T→A mutants located near the N-terminus (P<0.0001-P<0.00001) and M19 (H675Q; P<0.01), in which the H675 residue responsible for AAH's catalytic activity was rendered inactive relative to EV control. Myc expression from M6 (S20A) and M12

(T198A) was similar to EV. In cells overexpressing constructs with S/T→A mutations located within the C-terminal catalytic domain (M14-M18), levels of Myc immunoreactivity were not significantly different from EV. Further analysis revealed that Myc expression significantly increased in cells overexpressing AAH M1 (S9A) ($P<0.001$), while it decreased in cells overexpressing M5 (S18A; $P<0.01$), M6 (S20A; $P<0.00001$), M12 (T198A; $P<0.0001$), and M14-M19 ($P<0.0001$) relative to WT. Myc expression from the remaining constructs was similar to Myc expression from WT AAH. Taken together, except for cells overexpressing M1 (S9A), S/T→A mutations at predicted phosphorylation sites on AAH either decreased Myc expression or caused it to remain similar to WT levels.

6.5.2. Effects of AAH S/T→A mutations of GSK-3 β sites on AAH subcellular localization. In addition to examining total AAH protein expression, we studied the effects of S/T→A mutations on the subcellular distribution of selected AAH mutant constructs. Cells overexpressing WT N-Myc-AAH displayed bright red Myc immunoreactivity distributed throughout the cytoplasm and cellular processes (**Figure 6-2B**). Overexpression of M7 (S24A) (**Figure 6-2C**) caused Myc immunoreactivity to lose its cytoplasmic distribution and become perinuclear, similar to cells overexpressing M19 (H675Q) (**Figure 6-2E**). In cells overexpressing M18 (T748A) acquired a more particulate distribution corresponding to ER or microsomal localizations (**Figure 6-2D**). Cells overexpressing EV control were used as a negative control (**Figure 6-2F**).

6.5.3. Effects of AAH S/T→A mutations of GSK-3 β sites on AAH catalytic (hydroxylase) activity. AAH promotes cell migration and adhesion through hydroxylation of its target molecules. As such, we investigated the effects of preventing phosphorylation of AAH by S/T→A mutations of GSK-3 β sites on AAH's catalytic activity. The catalytic activity of AAH was measured by the $^{14}\text{CO}_2$ capture assay, which quantifies ^{14}C -labeled CO_2 release as a byproduct of hydroxylase activity [35]. Huh7

overexpressing WT N-Myc-AAH released significantly higher levels of $^{14}\text{CO}_2$ ($P<0.001$) relative to non-transfected (NT) or EV control cells (**Figure 6-3**). Cells overexpressing S/T→ mutant constructs had significantly lower levels of released $^{14}\text{CO}_2$ ($P<0.01$ - $P<0.001$), corresponding to decreased catalytic activity, relative to WT. However, S/T→A mutations at more than one site restored levels of released $^{14}\text{CO}_2$ close to WT.

6.5.4. Effects of AAH S/T→A mutations of GSK-3 β sites on expression of Notch signaling genes. Several lines of evidence indicate that AAH hydroxylation of Notch signaling proteins leads to the activation of Notch signaling networks. For this reason, we investigated the effects of deleting GSK-3 β sites by S/T→A mutations on the protein's ability to mediate the transcriptional activation of Notch signaling targets. qRT-PCR showed that overexpression of WT N-Myc-AAH significantly increased HES-1 gene expression ($P<0.0001$), a target of Notch signaling, relative to EV control (**Figure 6-4C**). In contrast, overexpression of selected AAH S/T→A mutant constructs significantly reduced Notch-1 ($<0.01<P<0.00001$) (**Figure 6-4A**), Jagged-1 ($P<0.01$ - $P<0.0001$) (**Figure 6-4B**), except for M11, and HES-1 ($P<0.0001$) (**Figure 6-4C**). S/T→A mutations decreased HEY-1 expression for M1 (S9A) ($P<0.01$), M7 (S24A) ($P<0.00001$), M11 (S115A) $P<0.01$, and M12 (T198A) ($P<0.01$) (**Figure 6-4D**) relative to WT. Overexpression of M19 (H675Q), in which AAH catalytic activity is rendered inactive, decreased expression of the four Notch signaling genes and served as a negative control. To summarize, S/T→A mutations on AAH reduced Notch signaling gene expression relative to WT AAH.

6.5.5. Phosphorylation of AAH at particular GSK-3 β sites modulates cell adhesion and motility. AAH hydroxylation of Notch signaling proteins activates cell adhesion and migration. Cell adhesion and migration can be measured by the ALMI-based directional motility assay using a blind-well chamber [36]. Overexpression of WT N-Myc-AAH increased the percentages of total motile ($P<0.0001$) **Figure 6-5A**) motile adherent cells

($P < 0.0001$) (**Figure 6-5B**), while it did not change the percentage of motile non-adherent cells (**Figure 6-5C**) relative to EV control. Overexpression of S/T→A mutant AAH constructs significantly reduced the percentages of total motile ($P < 0.00001$) and motile adherent ($0.001 < P < 0.00001$) cells, rendering them similar to or lower than EV control ($P < 0.01$). Overexpression of S/T→A mutants either decreased the percentage of motile non-adherent cells ($0.01 < P < 0.0001$) or rendered them similar to WT or EV control. M3 (S14A) was the exception since overexpression significantly increased the percentage of motile non-adherent cells ($P < 0.00001$), relative to WT or EV control.

6.6. DISCUSSION

6.6.1. Study rationale and objectives. Insulin/IGF signaling promotes AAH expression and function through GSK-3 β inhibition. Prior to stimulation, when basal GSK-3 β activity is relatively high, AAH protein decreases. This project was initiated to investigate whether AAH is regulated by direct mechanisms of phosphorylation (*i.e.* phosphorylation of AAH to target the protein for caspase-mediated degradation) and determine the functional consequences of preventing AAH phosphorylation at predicted phosphorylation sites on AAH expression and function, as it related to the activation of cell adhesion and motility through hydroxylation and activation of Notch signaling networks. To this end, we mutated S/T residues predicted to be phosphorylated by GSK-3 β to A to delete these sites and prevent their possible phosphorylation.

6.6.2. Deletion of GSK-3 β sites on AAH decreased AAH protein expression and altered its subcellular localization. Several lines of evidence indicate that insulin/IGF regulate AAH protein expression, as well as its function, as it relates to activation of cell adhesion and migration, through GSK-3 β activity. More specifically, GSK-3 β was previously reported to be a negative regulator of AAH in central nervous system-derived primitive neuroectodermal tumor 2 (PNET2) cells since overexpression of the kinase

inhibits AAH expression and function through caspase-mediated degradation of the protein, while GSK-3 β inhibition promotes AAH expression as well as cell adhesion and migration. Similar results were observed in Huh7 cells (**Chapter 5**), in which GSK-3 β inhibition enhanced the positive effects of IGF-1 stimulation on AAH protein expression. Although GSK-3 β regulates AAH protein expression and function, the mechanism is currently unknown. Individual GSK-3 β sites were mutated to Ala and we found that deletion of individual phosphorylation sites on AAH did not increase AAH protein expression. Rather, it decreased AAH protein levels and altered its subcellular distribution, particularly altering its localization on the ER and cellular processes. The subcellular localization of AAH is particularly important to its function. In **Chapter 5**, I described that upon insulin/IGF activation, AAH translocates from the ER to the plasma membrane to interact with Notch-1 and Jagged-1 and activate expression of important Notch signaling targets that mediate adhesion and motility. Overall, our findings suggest that AAH may need to be phosphorylated at more than one site in order to target it for degradation.

6.6.3. GSK-3 β phosphorylation at particular sites on AAH regulates catalytic activity, Notch activation, and migration. As previously mentioned, AAH promotes cell motility, at least in part, by hydroxylation of targets molecules [37] and the residue H675 is essential to catalyze this reaction [22]. Our findings demonstrate that deletion of individual phosphorylation sites on AAH did not enhance catalytic activity. However, deletion of multiple sites of phosphorylation restored the catalytic activity of AAH. The results indicate that AAH may be phosphorylated at multiple sites to be targeted for degradation. AAH hydroxylates Notch-1 and Jagged-1, activating Notch signaling networks, as a mechanism to promote cell adhesion and migration [38] [23] [39] [13]. Since AAH is linked to Notch signaling, we determined the consequences of deleting

individual sites on the activation of Notch signaling. We found that deletion of individual phosphorylation sites on AAH does not enhance Notch signaling. Rather, preventing phosphorylation of individual sites downregulates the expression of Notch signaling target genes. AAH promotes cell adhesion and motility, at least in part, through hydroxylation of Notch and Jagged proteins, which activates downstream signaling events that promote cell adhesion and migration. AAH may also regulate the transcriptional expression of Notch, Jagged, and HES. Reduced mRNA expression of Notch-1, its ligand Jagged-1, and the Notch signaling downstream target gene HES-1 in cells expressing the single point AAH mutants suggest the transcriptional activity of Notch and Jagged is regulated by AAH, but the mechanism is currently unknown. The regulation could be through direct or indirect mechanisms. For instance, si-RNAs directed against AAH and hypoxia-inducible factor-1 α (HIF-1 α) were previously shown to decrease Jagged-1 mRNA. Therefore, AAH may regulate Notch signaling protein Jagged at the mRNA through mechanisms possibly involving HIF-1 α and/or AAH. This is possible since HIF-1 α , AAH, and Notch signaling were previously demonstrated to cross-talk [38]. As observed with catalytic activity and Notch signaling, deletion of individual sites predicted to be phosphorylated on AAH did not improve adhesion or migration.

6.6.4. Implications for the treatment of HCC. Although we did not find the appropriate combination of sites that may target the protein for degradation, the AAH mutants provide a therapeutic option to prevent infiltrative growth of HCC using antibody-based gene delivery. To minimize potential damage to non-tumorous tissues, a high level of tumor specificity is required and it may be achieved by specific delivery of nucleic acids to tumor cells. Gene delivery vectors conjugated to ligands, such as antibodies, for cell surface receptors were previously demonstrated to deliver and express foreign genes in cells carrying the respective receptor. The AF-20 monoclonal antibody is a good

candidate since it binds to a 180 kD homodimeric cell surface glycoprotein expressed uniformly in several HCC cell lines. Studies previously showed that following binding of AF-20 mAb to its antigen, the antigen-antibody complex is rapidly internalized. Therefore, this gene delivery method provides a treatment option to deliver dominant negative mutant constructs of AAH that may deter the and infiltrative growth and intra-hepatic metastatic behavior of HCCs [40].

6.7. CONCLUSION

Deletion of individual GSK-3 β sites impaired catalytic activity, cell migration, adhesion, and signaling through Notch. This is likely due to the constitutive inhibition of GSK-3 β activity in HCC. The AAH mutant constructs discussed herein could be used to ameliorate the infiltrative growth and intra-hepatic metastatic potential of HCC tumors.

6.8. FUTURE DIRECTIONS

Future investigations include the regulation of AAH by phosphorylation at multiple sites, possibly through priming mechanisms of phosphorylation. Therefore, further investigations will include examining the expression and function of AAH when phosphorylation is prevented at multiple sites.

Table 6-1

Site-Directed Mutagenesis of N-Myc-AAH

Primer	Sequence (5'→3')	Primer	Sequence (5'→3')
S9A(M1)-F	GCGTAAGAATGCCAAGGC	S115A(M11)-F	CTTAAAGAGAGATCTACTG
S9A(M1)-R	CAGCGGCAACAGCAGCAG CTGCTGCTGTTGCCGCTGG CCTTGGCATTCTTACGC	S115A(M11)-R	CAGAGCCAGCAGTCC GGACTGCTGGCTCTGCAG TAGATCTCTCTTTAAG
S10A(M2)-F	GTAAGAATGCCAAGAGCG CCGGCAACAGCAGCAGCA G	S198A(M12)-F	GTAGATGATAGATTTGAGG CCCTGGAACCTGAAG
S10A(M2)-R	CTGCTGCTGCTGTTGCCGG CGCTCTTGGCATTCTTAC	S198A(M12)-R	CTTCAGGTTCCAGGGCCTC AAATCTATCATCTAC
S14A(M3)-F	GAGCAGCGGCAACAGCGC CAGCAGCGGCTCCGGCAG	T340A(M13)-F	TAAATAAATTTGATAAGGC TATTAAAGCTGAACCTTG
S14A(M3)-R	CTGCCGGAGCCGCTGCTG GCGCTGTTGCCGCTGCTC	T340A(M13)-R	CAAGTTCAGCTTTAATAGC CTTATCAAATTTATTTA
S16A(M4)-F	GGCAACAGCAGCAGCGCC GGCTCCGGCAGCGG	S374A(M14)-F	GCAAATACCCTCAGGCTC CACGAGCAAGATATG
S16A(M4)-R	CCGCTGCCGGAGCCGGCG CTGCTGCTGTTGCC	S374A(M14)-R	CATATCTTGCTCGTGAGC CTGAGGGTATTTGC
S18A(M5)-F	CAGCAGCAGCGGCGCCGG CAGCGGTAGCAC	S427A(M15)-F	GTTTGAAGCGTCGCGCAG ACAGGCAACAATTTTC
S18A(M5)-R	GTGCTACCGCTGCCGGCG CCGCTGCTGCTG	S427A(M15)-R	GAAATTGTTGCCTGTCTGC GCGACGCTTCAAAC
S20A(M6)-F	GCAGCGGCTCCGGCGCCG GTAGCACGAGTGC	T484A(M16)-F	GAGGTGCTGAGTGTGGCA CCTAATGATGGCTTTG
S20A(M6)-R	GCACTCGTGCTACCGGCG CCGGAGCCGCTGC	T484A(M16)-R	CAAAGCCATCATTAGGTGC CACACTCAGCACCTC
S24A(M7)-F	GGCAGCGGTAGCGCGAGT GCGGGCAGCAG	T577A(M17)-F	CACAGCCTTGGTGGGCCC CAAAAGAAACGG
S24A(M7)-R	CTGCTGCCC GCACTCGCG CTACCGCTGCC	T577A(M17)-R	CCGTTTCTTTTGGGGCCCA CCAAGGCTGTG
T23A(M8)-F	GCAGCGGTAGCACGGCTG CGGGCAGCAGCAG	T748A(M18)-F	GCATCCGGAAGTGGCACC ACAGCAGAGAC
T23A(M8)-R	CTGCTGCTGCCCCGAGCC GTGCTACCGCTGC	T748A(M18)-R	GTCTCTGCTGTGGTGCCAG TTCCGGATGC
S29A(M9)-F	GGGCAGCAGCGCCCCGG GGCCCG	H675Q(M19)-F	CATGCACCCCGGACTCA GGTGTGGCCGCACACAGG
S29A(M9)-R	CGGGCCCCGGGGGCGCTG CTGCC	H675Q(M19)-R	CCTGTGTGCGGCCACACC TGAGTCCCGGGGTGCATG
T36A(M10)-F	GCCCGGAGAGAGGCAAAG CATGGAGGAC	H675R(M20)-F	CATGCACCCCGGACTCG CGTGTGGCCGCACACAGG
T36A(M10)-R	GTCCTCCATGCTTTGCCTC TCTCCGGGC	H675R(M20)-R	CCTGTGTGCGGCCACACG CGAGTCCCGGGGTGCATG

Figure 6-1. Site-directed mutagenesis of N-Myc-AAH. Ser/Thr residues on N-Myc-AAH predicted to be phosphorylated were mutated to Ala by site-directed mutagenesis using (A) forward and reverse primer pairs containing mutated nucleotide sequence (underlined).

Table 6-2

Primers and Probes for qRT-PCR of Notch Signaling Gene Targets

Primer*	Sequence (5'→3')	Position (mRNA)	Amplicon (bp)	UPL probe #
hNotch1-F	ATGCTGGCAGACCCACAC	3219	72	28
hNotch1-R	GGCACGTCGCAGTAAAGG	3290		
hJagged1-F	AACACCTTCAACCTCAAGGC	826	66	28
hJagged1-R	ACTGAAAGGCAGCACGATG	891		
hHES1-F	CTTACGGCGGACTCCATG	1041	85	63
hHES1-R	GAGAGGTGGGTTGGGGAG	1125		
hHEY1-F	TTCCATGTCCCCAACTACATC	311	92	55
hHEY1-R	TGTTATTGATCCGGTCTCGTC	402		

Table 6-2. Primers and probes for qRT-PCR of Notch signaling gene targets.

Abbreviations: *h*, human; *HES1*, hairy and enhancer of split 1; *HEY1*, hairy/enhancer-of-split related with YRPW motif protein 1; *bp*, base pairs; *UPL*, universal probe library; *F*, forward; *R*, reverse.

Figure 6-1

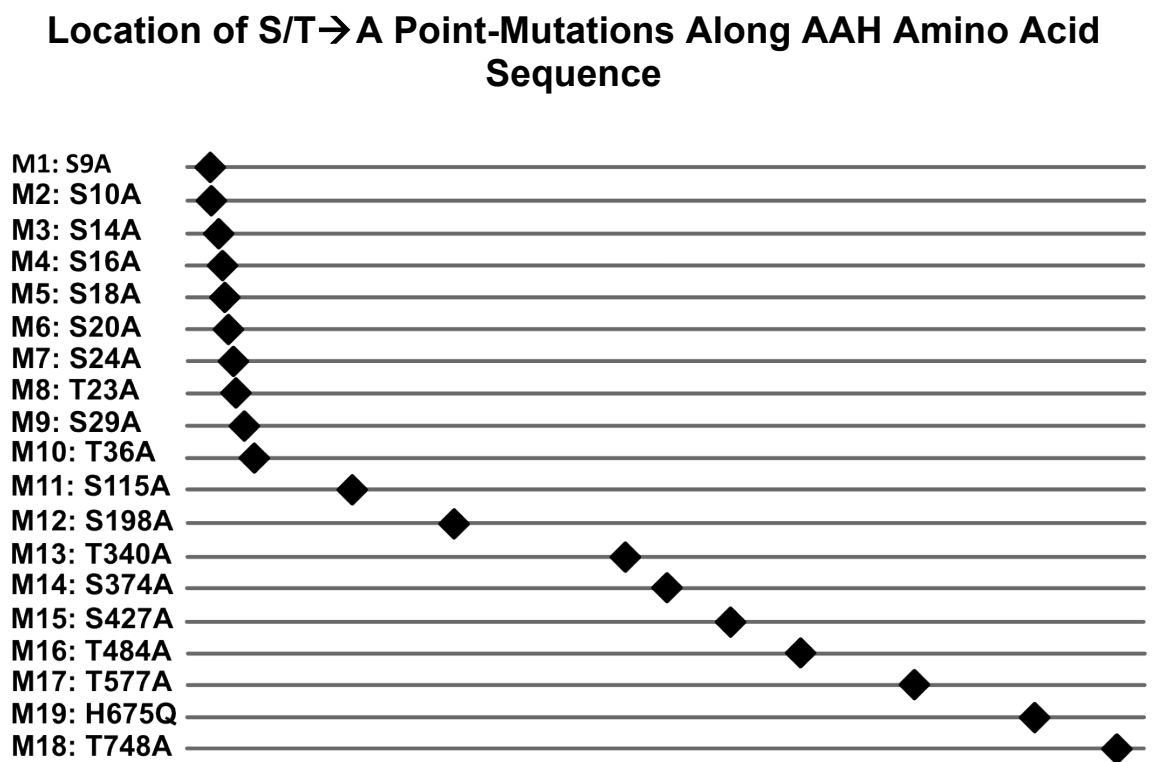


Figure 6-1. Locations of S/T→A point-mutations along AAH amino acid sequence.

Figure 6-2

Effects of AAH S/T→A Mutations of GSK-3β Sites on AAH Protein Expression and Subcellular Localization

A

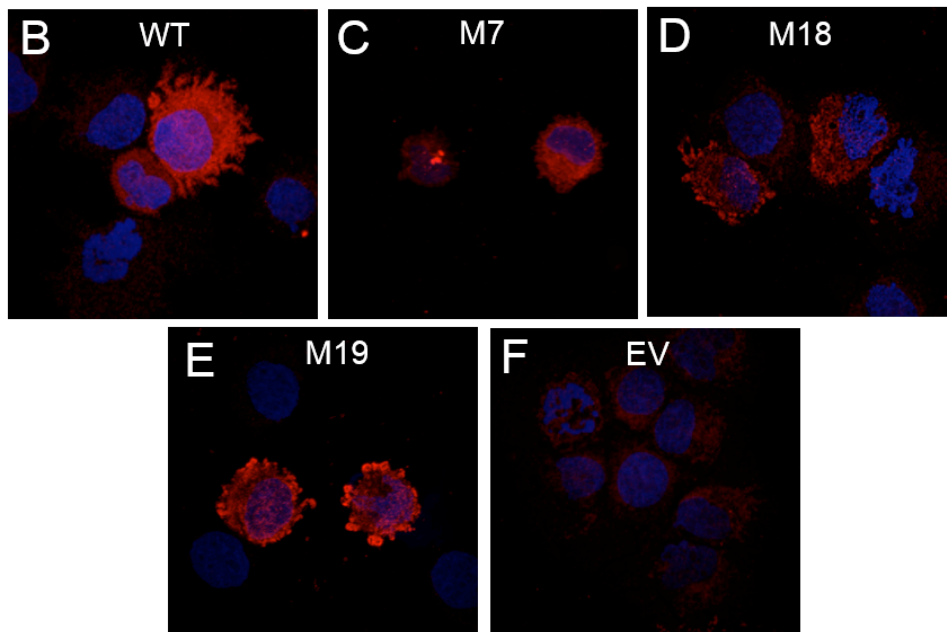
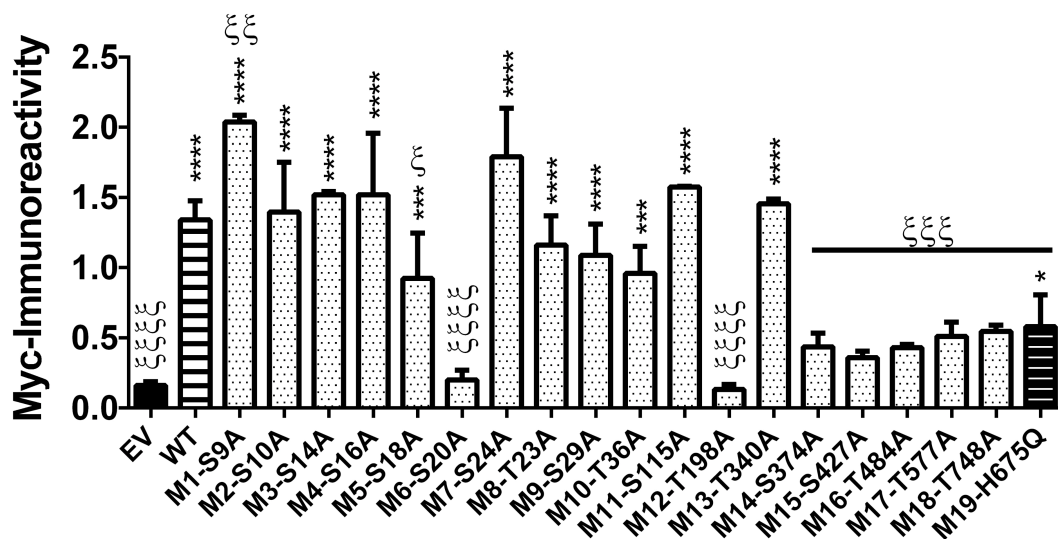


Figure 6-2. Effects of AAH S/T→A mutations on AAH protein expression. Huh7 cells were transiently transfected with wildtype (WT) or point-mutated (M:S/T→A) N-Myc-AAH cDNA. As control, empty vector (EV) and a cDNA carrying a point mutation in the critical His-675 (H→Q) residue needed for catalytic activity were studied in parallel (M19). (A) 24 hours after transfection, homogenates prepared in weak lysis buffer were used in ELISAs to measure N-Myc-AAH expression. Immunoreactivity was detected with horseradish peroxidase-conjugated secondary antibody and Amplex UltraRed soluble fluorophore (M5 SpectraMax microplate reader). The graph depicts the calculated mean $\pm$ S.E.M of Myc immunoreactivity. Inter-group comparisons were made by one-way ANOVA with post-hoc Fisher LSD tests for comparison with EV (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001) or WT (ξ P<0.05; $\xi\xi$ P<0.01; $\xi\xi\xi$ P<0.001; $\xi\xi\xi\xi$ P<0.0001). (B-F) Cells transfected with (B) WT N-Myc-AAH, (C) M7 (S24A) N-Myc-AAH, (D) M18 (T748A) N-Myc-AAH, (E) H675Q N-Myc-AAH, or (F) EV were used to generate cytospin preparations on glass slides. After fixation in Histofix, the cells were permeabilized and used to detect Myc immunofluorescence. Immunoreactivity was revealed with biotinylated secondary antibody and Streptavidin-conjugated Dylight 547 (red). Cells were counterstained with DAPI (blue). Images were captured by confocal microscopy (600x magnification, 2x digital zoom) and merged in Photoshop.

Figure 6-3

Effects of AAH S/T→A Mutations of GSK-3β Sites on AAH Catalytic Activity

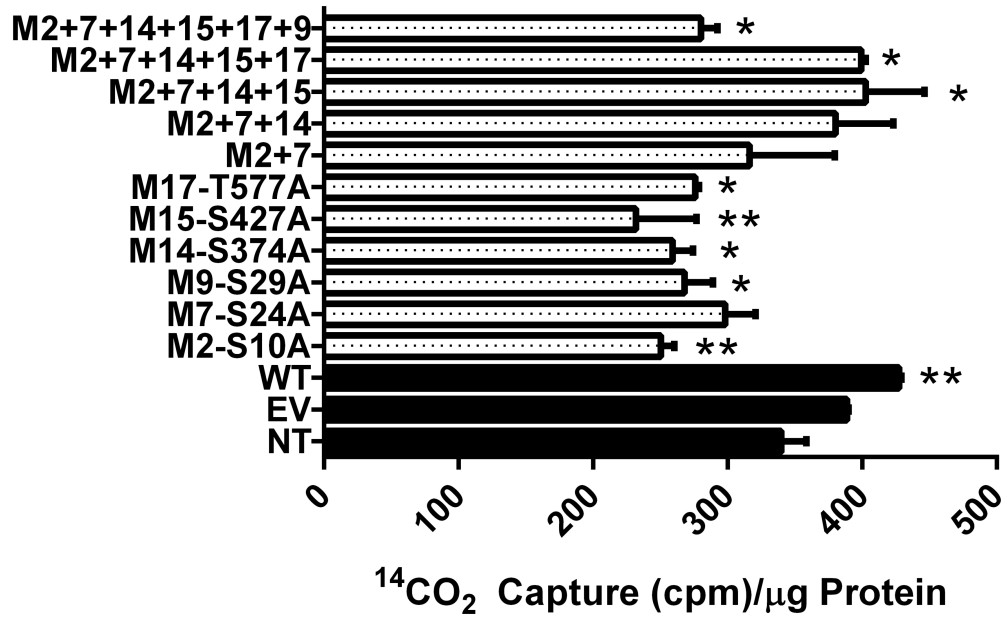


Figure 6-3. Effects of AAH S/T→A mutations on AAH catalytic activity. Huh7 cells were transiently transfected with wildtype (WT) or mutated (M:S/T→A) N-Myc-AAH cDNAs carrying one or more point mutations in phosphorylation motifs. As control, empty vector (EV) and mock transfected (NT) cells were studied in parallel. 24 hours after transfection, the cell lysates were used to measure hydroxylase activity with the $^{14}\text{CO}_2$ capture assay with recombinant EGF peptide as the substrate (see Methods). The graph depicts the mean $\pm$ S.E.M. of released $^{14}\text{CO}_2$ (cpm)/ μg protein. Inter-group comparisons were made using one-way repeated measures of ANOVA and the post hoc Fisher's Least Significance Difference test relative to EV (*P<0.05; **P<0.01).

Figure 6-4

Effects of AAH S/T→A Mutations of GSK-3β Sites on Notch Signaling Gene Expression

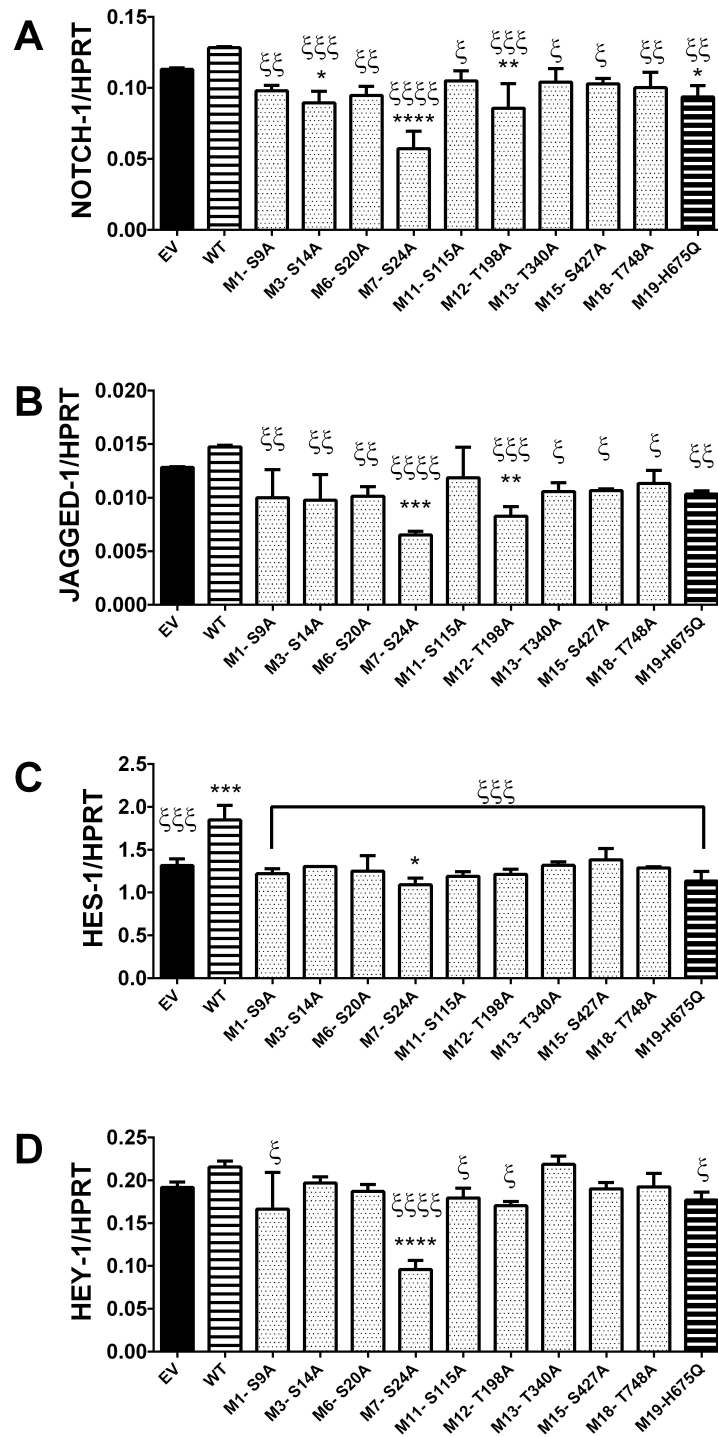


Figure 6-4. Effects of AAH phosphorylation site point mutations on Notch signaling proteins. Huh7 cells were transiently transfected with wildtype (WT) or point-mutated (M: S/T→A) N-Myc-AAH cDNAs. As control, empty vector (EV) and a cDNA carrying a point mutation of the critical His-675 (H→Q) residue needed for catalytic activity were studied in parallel (M19). 24 hours after transfection, RNA was isolated from culture homogenates to measure (A) Notch-1, (B) Jagged-1, (C) hairy and enhancer of split-1 (HES-1), and (D) hairy/enhancer-of-split related with YRPW motif protein-1 (HEY-1) expression by duplex qRT-PCR analysis using a probe hydrolysis-based assay (see Methods). Gene expression was normalized to hypoxanthinephosphoribosyltransferase (HPRT) transcript levels detected in the same reactions. The graph depicts the calculated mean $\pm$ S.E.M ratios of target gene/HPRT. Inter-group comparisons were made by one-way ANOVA with post-hoc Fisher LSD tests for comparison with EV (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001) or WT (ξ P<0.05; $\xi\xi$ P<0.01; $\xi\xi\xi$ P<0.001; $\xi\xi\xi\xi$ P<0.0001).

Figure 6-5

Effects of AAH S/T→A Mutations of GSK-3β Sites on Cell Adhesion and Motility

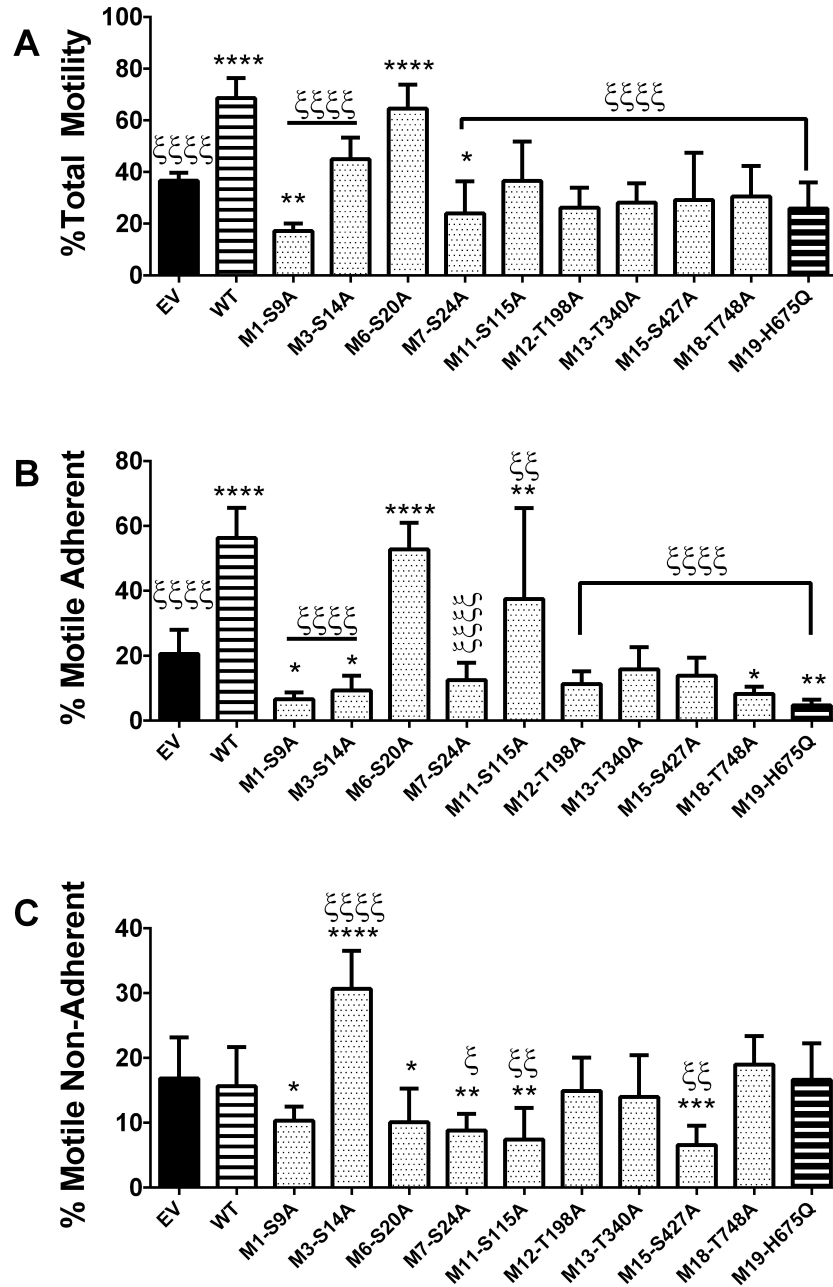


Figure 6-5. Effects of AAH phosphorylation site mutations on directional cell adhesion and motility. Huh7 cells were transiently transfected with wildtype (WT) or point-mutated (M: S/T→A) N-Myc-AAH cDNAs. As control, empty vector (EV) and a cDNA carrying a point mutation of the critical His-675 (H→Q) residue needed for catalytic activity were studied in parallel (M19). 24 hours after transfection, cells were harvested to measure directional motility and adhesion using the ATP-Lite Motility and Invasion (ALMI) luminescence-based assay with 1% FBS supplied as the trophic factor (see Methods). Graphs depict the calculated mean $\pm$ S.E.M percentages of (A) total cell motility, (B) motile adherent cells, and (C) motile non-adherent cells per assay chamber (see Methods). Inter-group comparisons were made by one-way ANOVA with post-hoc Fisher LSD tests for comparison with EV (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001) or WT (ξ P<0.05; $\xi\xi$ P<0.01; $\xi\xi\xi$ P<0.001; $\xi\xi\xi\xi$ P<0.0001).

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CHAPTER 7
DISCUSSION AND FUTURE DIRECTIONS

7.1. OVERVIEW

My graduate research focused on investigating insulin and insulinlike growth factor-1 (IGF-1) signaling-mediated regulation of AAH and downstream Notch signaling activation as a mechanism to promote hepatocyte migration. The investigations were performed in the contexts of alcoholic liver disease (ALD) and hepatocellular carcinoma (HCC). The two models, which used *in vivo* and *in vitro* approaches, provide environments in which AAH is naturally downregulated (*i.e.* ALD) or upregulated (*i.e.* HCC). In ALD, insulin/IGF-1 networks are impaired by alcohol, resulting in disrupted AAH expression and activation of Notch signaling. Impairment of these mechanisms disrupts cell adhesion and migration, which is essential for liver remodeling. On the other hand, in HCC, several signaling pathways are dysregulated that result in increased activation of insulin/IGF-1 signaling, AAH, and Notch signaling networks, promoting cell migration, tumor infiltration and aggressiveness. Overall, I employed various pharmacological tools that affect insulin/IGF-1 signaling and AAH protein expression including ethanol exposure, an inhibitor of *de novo* ceramide synthesis (*i.e.* myriocin), insulin sensitizers treatments (*i.e.* peroxisome proliferator activated receptor (PPAR) agonists), and kinase inhibitors. The studies expanded our understanding of insulin/IGF-1 regulation of AAH-Notch signaling activation, as a mechanism to promote adhesion and migration in the liver.

The overarching hypothesis is that insulin/IGF-1 signaling regulates AAH and downstream Notch signaling activation through phosphorylation in the contexts of ALD and HCC (**Chapter 1**). We initiated our investigations by (1) examining the ability of an inhibitor of ceramide synthesis (**Chapters 2 and 3**) and PPAR agonist treatments (**Chapter 4**) to restore insulin/IGF-1 signaling, AAH, and Notch signaling protein expression in the context of chronic ethanol exposure. We extended our investigations to (2) the post-translation regulation of AAH and its consequences on Notch signaling

(**Chapter 5**) and the (3) consequences of preventing phosphorylation at individual sites on AAH predicted to be phosphorylated on the protein's expression and function, as it related to catalytic activity, activation of Notch signaling, cell adhesion and migration (**Chapter 6**).

7.2. DISCUSSION

AAH has a demonstrated role in promoting cell adhesion and migration through the activation of Notch signaling networks [1–4]. The purpose of my graduate research was to investigate insulin/IGF-mediated regulation of AAH-Notch signaling networks in two disease contexts, ALD and HCC (**Chapter 1**).

In **Chapter 2**, we used ethanol and myriocin as pharmacological tools that modulate insulin/IGF signaling and investigated the downstream effects on AAH expression. Alcohol impairs insulin signaling at various points of the cascade [5–8], resulting in downregulation of AAH protein and cell migration [1,9,10]. In an ex vivo model of chronic alcohol-induced steatohepatitis, alcohol induced aberrant lipid accumulation, particularly of ceramides, partly impaired insulin signaling, reduced AAH protein, and induced ER stress. Treatment with the inhibitor of ceramide synthesis myriocin, restored the aforementioned detrimental effects of ethanol and induced an early ER stress response likely to restore ER homeostasis [11]. Myriocin restored ethanol-induced insulin resistance likely by reducing the ceramide-mediated activation of negative regulators of insulin receptor substrate-1 (*i.e.* c-Jun N-terminal kinase) and Akt (*i.e.* protein kinase C ζ and protein phosphatase 2), leading to increased GSK-3 β inhibition and enhanced AAH protein expression. Taken together, the results suggest ceramides are partly responsible for inducing insulin resistance and impairing AAH expression in ALD and these effects can be ameliorated by reducing ceramide load. The

results from this chapter were published in the Journal of Biochemical and Pharmacological Research [11].

In **Chapter 3**, we went a step further and investigated the role of alcohol and ceramides on the expression of AAH and proteins of the Notch signaling pathway. Using an in vivo model of chronic ethanol exposure [12], we observed that ethanol increased steatosis, impaired insulin signaling, and expression of AAH as well of Notch signaling proteins. Myriocin significantly restored the detrimental effects of ethanol on the expression of AAH and Notch signaling proteins, resulting in activation of Notch signaling networks that mediate cell adhesion, migration and remodeling of the liver. Taken together, the studies with myriocin suggest inhibitors of ceramide synthesis could aid in restoring hepatic architecture and function in patients suffering from ALD. The results from this chapter were published in the Journal of Gastroenterology and Hepatology [12].

In **Chapter 4**, we investigated the effects of insulin sensitizer treatments, agonists of PPAR- α , PPAR- δ , and PPAR- γ on restoring ethanol-impaired AAH and Notch signaling protein expression. Previous reports indicated treatment with a PPAR- δ agonist remarkably restored hepatic architecture [13] despite chronic alcohol exposure through improved insulin/IGF signaling [14]. PPAR- δ agonist likely enhanced insulin/IGF signaling activation by replenishing the plasma membrane with lipids that promote appropriate insulin receptor binding. We used the same protein samples to examine the effects of PPAR agonist treatments on the expression of Notch signaling proteins. We found that treatment with PPAR- δ restored Notch-1 protein expression in livers chronically exposed to ethanol. However, the treatments did not restore AAH protein expression as previously reported possibly due to the age of the samples. Although PPAR- δ agonist treatment was previously demonstrated to remarkably restore hepatic architecture, including ER structure, despite continued exposure to ethanol, other

aspects of ALD, including aberrant lipid or ceramide accumulation and ER stress, were not resolved. Therefore, additional therapies to PPAR- δ agonist treatment are needed to fully resolve the aspects contributing to ALD development and liver injury. Since myriocin reduces ceramide load, contributes to restoring ER homeostasis, insulin/IGF signaling, and activation of AAH-Notch signaling networks, PPAR- δ agonist and myriocin treatments provide a feasible therapeutic option to promote hepatic remodeling in ALD.

The evidence presented in **Chapter 5** demonstrated insulin/IGF signaling promoted AAH protein expression and subcellular trafficking using an in vitro model. In light of the previous reports [1–4] and the findings herein, we propose insulin/IGF signaling promotes trafficking of the AAH catalytic domain from the endoplasmic (ER) to the plasma membrane, where it interacts with and hydroxylates Notch-1 and, its ligand, Jagged-1. AAH hydroxylation may promote activation of Notch signaling because hydroxylation of Asn/Asp in the epidermal growth factor (EGF)-like domains of Notch-1 and/or Jagged-1 may neutralize the positive charges at the EGF-like domains and facilitate the complex formation between Notch-1 and Jagged-1. Interaction of Notch-1 and Jagged-1 promotes cleavage and the nuclear translocation of the Notch-1 intracellular domain (NID). At this point, AAH may also act as a chaperone for NID until it reaches the nucleus, where it activates the transcriptional expression of target genes involved in cell adhesion and motility [15,16]. An alternative explanation could be that AAH hydroxylation of Notch-1 and Jagged-1 may stabilize protein folding, as previously demonstrated with hydroxylation of ankyrin repeats in which β -hydroxylation at Asn residue stabilized the canonical folding of ankyrin repeat domain [17]. Furthermore, the results presented in the chapter indicate AAH is post-translationally regulated by phosphorylation since inhibition of kinases glycogen synthase kinase-3 β (GSK-3 β), protein kinase A (PKC), and casein kinase 2 (CK2), which are predicted to phosphorylate AAH promoted AAH expression and trafficking. The results suggest that

AAH may be regulated through waves of phosphorylation in that GSK-3 β may phosphorylate AAH prior to activation of insulin/IGF-1 signaling. GSK-3 β is inhibited upon stimulation with IGF-1, promoting AAH protein expression through direct mechanisms (i.e. AAH is not phosphorylated and targeted for degradation) or indirect mechanisms (i.e. GSK-3 β does not activate another negative regulator of AAH). There is also the latent possibility that AAH may be regulated by GSK-3 β through priming mechanisms of phosphorylation since AAH may need to be phosphorylated by another kinase, such as CK2, prior to being phosphorylated by GSK-3 β .

In **Chapter 6**, we set out to investigate the role that each residue predicted to be phosphorylated on AAH had on AAH protein expression and function, as it related to activation of Notch signaling networks and directional motility. We found that preventing phosphorylation at individual sites on AAH either inhibited or did not have any significant positive effects on AAH protein expression and function; however, inhibiting the phosphorylation of AAH at multiple sites promoted AAH activity. The results suggest AAH expression and function are differentially regulated by the degree and nature of protein phosphorylation. In other words, AAH may need to be phosphorylated at multiple sites in order to be targeted for degradation. One possibility is that AAH may need to be phosphorylated by CK2, prior to phosphorylation by GSK-3 β that could target the protein for degradation. Therefore, we may need to prevent phosphorylation at multiple CK2 and GSK-3 β sites in order to prevent AAH phosphorylation and subsequent targeting for degradation. An alternative interpretation to the findings that preventing phosphorylation at individual sites on AAH did not restore AAH protein expression could be that AAH is regulated by phosphorylation through indirect mechanisms (i.e. AAH is not directly phosphorylated).

Taken together, the results suggest that alcohol promotes aberrant accumulation of toxic ceramide species that promote insulin resistance, disrupts AAH protein

expression and Notch signaling activation, impairing hepatocyte migration. In the context of HCC, aberrant expression of AAH-Notch network mediate increased HCC tumor cell adhesion, migration, and invasion. The model presented in **Figure 7-1** represents how the findings included in this dissertation, using ex vivo, in vivo, and in vitro models contribute to our understanding of the activation of AAH-Notch signaling networks in promoting cell adhesion and migration in the liver and how these mechanisms are dysregulated in ALD and HCC.

7.3. FUTURE DIRECTIONS

Mass spectrometry should be used to confirm that the sites on AAH predicted to be phosphorylated by GSK-3 β , PKA, PKC, and CK2 are accessible to catalytic sites of these kinases and are phosphorylated. To do so, recombinantly expressed Myc-tagged AAH can be purified and subjected to mass spectrometry analysis, particularly taking advantage of its high level of sensitivity given that fact that it can detect 80 Da of difference in molecular weight that corresponds to a phosphate group added to Ser/Thr, or even Tyr residues as a form of post-translational modification. Furthermore, it will be important to prevent phosphorylation of AAH at multiple sites to obtain a more complete understanding of the role of phosphorylation on the protein's expression and function, as it relates to activation of Notch signaling networks and cell migration.

As mentioned in **Chapter 1**, in addition to AAH, the A β H-J-J gene locus gives rise to two additional protein isoforms, Humbug (Junctate) and Junctin (**Figure 1-3**) [18,19]. The N-terminal region of AAH, located outside the catalytic domain, is nearly identical to Humbug. Humbug is involved in the regulation of calcium flux from intracellular stores through the ryanodine receptors. Several lines of evidence indicate that AAH is physiologically cleaved into its C-terminal catalytic domain and the N-terminal region (*i.e.* outside the C-terminal catalytic domain), which may promote

adhesion and migration by two separate mechanisms. As discussed in this dissertation, and based on previous reports, the catalytic domain of AAH interacts with and/or hydroxylates Notch-1 and Jagged-1 to promote cell adhesion and migration [1–4]. This may represent a mechanism by which AAH promotes migration through the transcriptional activation of genes involved in adhesion and motility. On the other hand, the N-terminal region of AAH may promote a faster response to insulin/IGF stimuli that activates cell adhesion and motility, through the regulation of intracellular calcium stores. For this reason, it is important to investigate the role AAH may play in the regulation of intracellular calcium as a fast response to the activation of cell adhesion and motility. Furthermore, it would be interesting to investigate the role that phosphorylation of AAH at individual and multiple sites may play in the regulation of calcium flux from intracellular stores.

Figure 7-1

Proposed Insulin/IGF-1 Regulation of AAH-Notch Networks that Modulate Migration

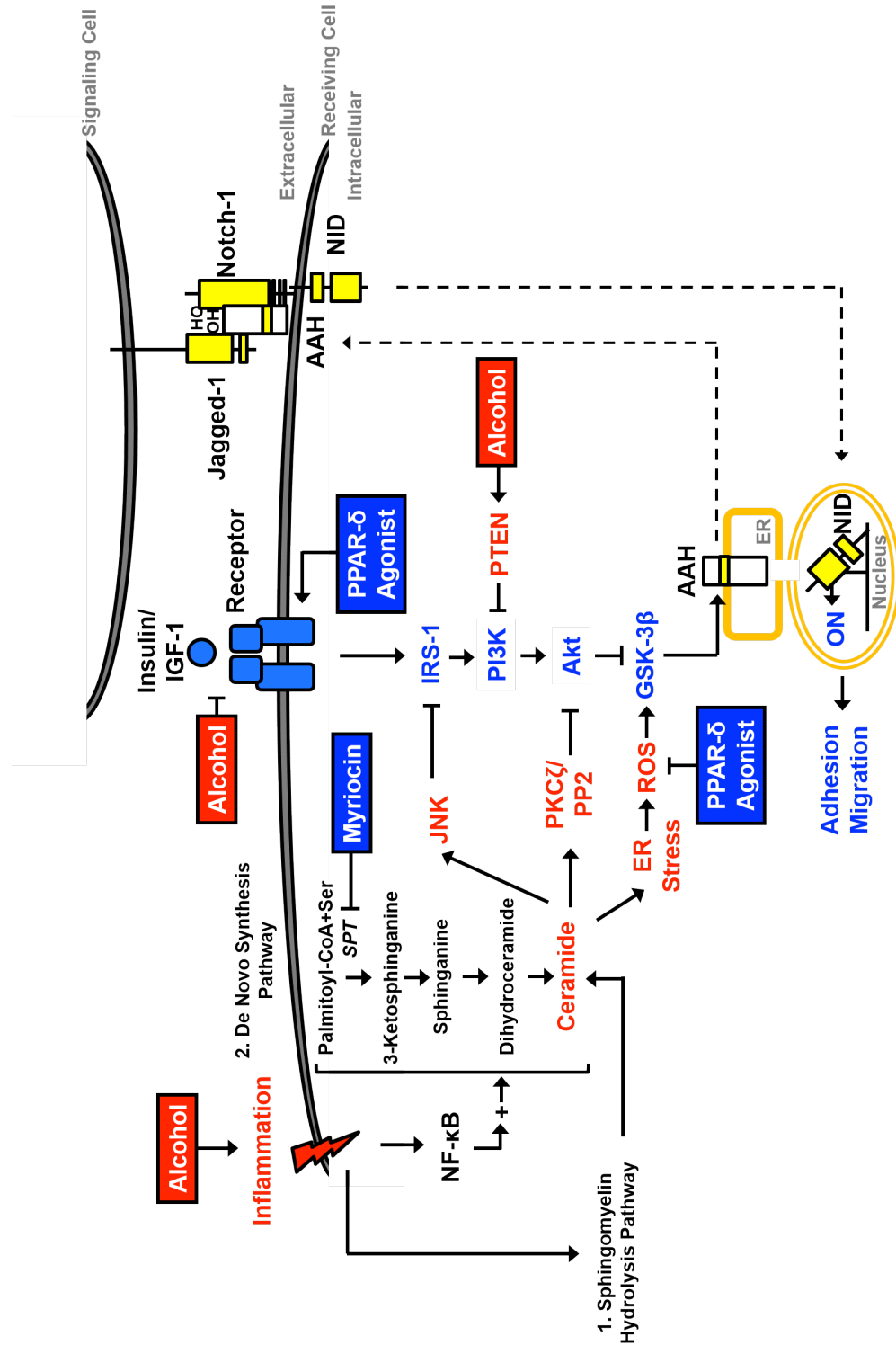


Figure 7-1. Proposed insulin/IGF regulation of AAH-Notch signaling networks that modulate migration. Binding of insulin/IGF-1 polypeptide to corresponding receptor starts a series of phosphorylation events that activate insulin receptor substrate-1 (IRS-1), phosphoinositide-3 kinase (PI3K), and Akt, and inhibit glycogen synthase kinase-3 β (GSK-3 β). In HCC, inhibition of GSK-3 β promotes AAH protein direct or possibly indirect mechanisms of phosphorylation. AAH translocates to the plasma membrane where it hydroxylates Notch-1 and, its ligand, Jagged-1. Hydroxylation may neutralize the charges of the epidermal growth factor-like domains of Notch-1 and Jagged-1, facilitating their interaction and complex formation. Complex formation triggers the cleavage and nuclear translocation of the Notch-1 intracellular domain (NID) to activate transcription of genes involved in adhesion and migration. Alcohol disrupts insulin/IGF signaling by disrupting receptor binding and PI3K activity through activation of phosphatase and tensin homolog (PTEN). Alcohol indirectly disrupts signaling by activating (1) sphingomyelin hydrolysis and (2) de novo synthesis pathways, causing aberrant ceramide accumulation. Ceramides activate c-Jun N-terminal Kinases (JNK), protein kinase C ζ (PKC ζ), and protein phosphatase 2 (PP2) that negatively regulate IRS-1 and Akt. Ceramides induce ER stress and promote formation of reactive oxygen species (ROS) that activate GSK-3 β , exacerbating insulin resistance. Consequences include impaired migration, liver regeneration and remodeling, and progression to ALD. Myriocin inhibits de novo ceramide synthesis by inhibiting serine palmitoyltransferase (SPT), reducing ceramide load, ER stress, restoring insulin sensitivity, and activation of AAH-Notch networks necessary for hepatocyte migration. PPAR- δ agonist treatment also improves signaling through Notch to restore ethanol-impaired hepatocyte migration. In the case of HCC, increased signaling through PI3K/Akt, inhibits GSK-3 β , promoting AAH expression and activation of Notch signaling networks.

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