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The Effect of Ethanol on Skeletal Muscle Endocrine Function

and the Novel Myokines Myonectin and Irisin

A thesis

presented to

the faculty of the Department of Biological Sciences

East Tennessee State University

In partial fulfillment

of the requirements for the degree

Master of Science in Biology, concentration in Biomedical Sciences

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by

Kendra Lyndsey Hagood

May 2018

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Keywords: Skeletal Muscle, Ethanol, Myokines, Myonectin, Irisin, FNDC5

ABSTRACT

The Effect of Ethanol on Skeletal Muscle Endocrine Function
and the Novel Myokines Myonectin and Irisin

by

Kendra Lyndsey Hagood

Excessive alcohol consumption is a leading cause of death and disability globally and can lead to diseases such as alcoholic skeletal myopathy. Skeletal muscle is the largest organ in the human body and functions to regulate whole-body energy homeostasis. Additionally, skeletal muscle can function as an endocrine organ via secretion of myokines. Two myokines, myonectin and irisin, present a wide range of effects upon metabolism, inflammation, and tissue survival-signaling. We hypothesized that chronic alcohol consumption will result in reduced circulating myonectin and irisin levels. Mice were fed an ethanol-containing or control diet for 10 days or 6 weeks. Tissues and serum were collected from mice and immunoblotting was used to quantify myonectin and irisin levels. Our data demonstrated that neither a 10 day nor 6-week ethanol diet was effective in altering myonectin levels, whereas irisin was undetectable. Therefore, we conclude that these myokines are not affected by alcohol consumption.

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CHAPTER 1

INTRODUCTION

Excessive alcohol consumption has led to approximately 88,000 deaths per year in the United States from 2006-2010 (Fact Sheets 2018) and is the fifth leading cause of death and disability globally (Lim et al. 2012). Over time, alcohol can impose detrimental health effects such as heart, pancreatic, and liver disease, stroke, digestive disorders, alcoholic induced muscle disease, and various cancers.

Many of the debilitating effects of alcohol are a consequence of harmful byproducts emitted during alcohol consumption. Others are caused by alcohol's ability to disrupt cellular processes, such as fat metabolism (Clot et al. 1997: Cederbaum 2001; Duryee 2007). When alcohol is initially digested, it may be oxidized by enzymes in the stomach, thus reducing levels entering circulation. In a fasted state, alcohol quickly enters the duodenum and the rate of initial oxidation by alcohol dehydrogenase (ADH) in the stomach is minimized. Additionally, initial metabolism in the stomach is reduced in those who excessively consume alcohol (Cederbaum 2012).

In the liver, both the ADH-enzyme system and the cytochrome P450-dependent ethanol-oxidizing system (CYP2E1) are responsible for the metabolism of alcohol. The cofactor nicotinamide adenine dinucleotide (NAD^+) accepts hydrogen ions and electrons from ethanol, resulting in the production of acetaldehyde and nicotinamide adenine dinucleotide plus hydrogen (NADH), the reduced form of NAD. Acetaldehyde is then oxidized via aldehyde dehydrogenase (ALDH), producing acetate and reducing NAD^+ to NADH. The liver can then metabolize acetate, producing acetyl coenzyme A (acetyl CoA) (Berg 2002).

Alcohol and Liver Disease

Excessive alcohol consumption accounts for over 50% of all liver related deaths in the United States (Shah 2005). The progression of alcoholic liver disease (ALD) encompasses simple steatosis or fatty liver, alcoholic steatohepatitis, fibrosis, and cirrhosis (Torruellas 2014). The effect of alcohol on the liver has become an economic burden throughout the world with alcohol serving as a causal factor in 60 types of diseases and injuries and a contributing factor in 200 others (Alcohol 2015). Even though alcohol has been implicated as a major cause of liver disease, research studies have focused mainly on viral hepatitis (El-Serag 2012) and non-alcoholic fatty liver disease (NAFLD) (Zezos 2014). There are no current treatments available for ALD other than abstinence from alcohol. Additionally, ALD is usually asymptomatic and liver function-tests often appear normal. Liver function is effected to various degrees from patient to patient and metabolic derangement may not be evident until the advanced stage of liver disease has been reached. Therefore, no single test accurately measures the overall state of the liver. By investigating the dysregulation of hepatic lipid metabolism induced by alcohol, studies targeting the mechanisms responsible can provide future insights into the pathogenesis of alcoholic fatty liver.

Owing to the increased rate of NADH production in response to ethanol metabolism, both gluconeogenesis and fatty acid (FA) oxidation are inhibited while FA synthesis, hepatic triacylglycerol (TAG) accumulation, and fatty liver disease are promoted. During FA oxidation, NADH is produced and may be used during oxidative phosphorylation to produce adenosine triphosphate (ATP), the main energy source of most cellular processes. However, when NADH begins to accumulate, FA oxidation is hindered (Berg 2002).

Acetaldehyde and reactive oxygen species (ROS), produced during ethanol metabolism,

are harmful hepatotoxins. Acetaldehyde and ROS have the ability to alter the expression of genes involved in lipid metabolism as well as those involved in the fibrogenic response during liver injury (Ceni et al. 2014). This allows acetaldehyde and ROS to influence intracellular signaling pathways, altering transcriptional control of particular genes. Additionally, acetaldehyde and ROS promote a chronic inflammatory state that plays a role in liver disease.

Excess fat deposition in the liver as a result of ethanol consumption results in the earliest stage of alcohol-related liver disease, known as simple steatosis or fatty liver. Simple steatosis results in 90% of heavy drinkers (Ceni et al. 2014), however liver damage is usually reversible over time with abstinence from alcohol consumption. If alcohol consumption is continued, ALD can progress to more advanced stages such as alcoholic hepatitis, fibrosis, or cirrhosis. The progression of lipid accumulation in the liver causes an inflammatory wound-healing response which serves as the driving force for excessive accumulation of extracellular matrix (ECM) components, further exacerbating liver damage (Bataller and Brenner 2005; Wobser et al. 2009; Machado and Diehl 2016). This process is characterized by a reduction in the number of endothelial cell fenestrations, portal hypertension, hepatic stellate cell activation (HSC), and the disintegration of the liver's internal framework (Ceni et al. 2014). HSCs acquire a myofibroblast-like phenotype that functions in heightened cell proliferation, cytokine production, and synthesis of ECM constituents (Lieber 2000; Mello et al. 2007).

Alcohol and Skeletal Muscle Disease

In addition to the liver, excessive alcohol consumption has the capacity to modulate the physiology and function of skeletal muscle. Skeletal myopathy caused by excessive alcohol consumption occurs more frequently than many myopathies that are inherited (Preedy et al.

2003) and is generally presented by a decrease in muscle mass, contractility, and strength in addition to decreased protein synthesis and increased proteolysis. Skeletal muscle is equipped with a large regenerative capacity useful in the event of injury. However, fibrosis, or the excess deposition of ECM components, severely impends skeletal muscle's ability to repair itself. Mediated by chronic alcohol consumption, genes responsible for fibrosis of skeletal muscle are up-regulated (Steiner and Lang 2015), further disrupting skeletal muscle function.

Skeletal muscle is the largest organ in the human body and is an important site for glucose storage and lipid utilization. Skeletal muscle stores triacylglycerol (TAG) in the form of lipid droplets and the balance of lipid storage and breakdown is essential in maintaining normal skeletal muscle metabolism. Due to skeletal muscle's role in glucose uptake and fatty acid oxidation (FAO), excess lipid accumulation may disrupt muscle size, function, and morphology. Further, skeletal myopathy caused by excessive alcohol consumption increases both the morbidity and mortality rate of the consumer (Prado et al. 2012).

The mechanisms by which skeletal muscle modulates glucose and lipid metabolism are well known, however its newly discovered endocrine role is still being investigated. Dysregulation of skeletal muscle fat metabolism is implicated in the development of numerous diseases including insulin resistance, cardiovascular disease, diabetes, and obesity. Skeletal and cardiac muscle as well as the liver serve as primary locations for FAO, therefore, it comes as no surprise that damage to these components may disrupt typical homeostatic parameters. Further, maintenance of blood glucose levels through gluconeogenesis, a process that occurs in the liver, is ultimately related to catabolism of muscle protein. Due to the various mechanisms of inter-organ cross talk that are required for daily homeostatic maintenance, the correlation between skeletal muscle health and disease aversion is evident.

Liver-Skeletal Muscle Axis: The Role of Myokines

Although a considerable amount of research concerning ALD has focused on the liver, the effect of alcohol consumption on organs such as skeletal muscle plays an important role in the progression, outcome, and mortality rate associated with ALD. In patients with alcoholic hepatitis and end-stage decompensated liver disease, for example, mortality is typically due to multi-organ failure rather than a result of isolated liver injury (Moreau et al. 2013). As previously discussed, skeletal muscle participates in a variety of functions aside from locomotion and posture, such as nutrient storage and body temperature regulation. Additionally, skeletal muscle contributes to homeostatic events by secreting a collection of muscle-derived cytokines, termed myokines, which can function in an endocrine, paracrine, or autocrine manner. Myokines are involved in various cellular processes such as tissue repair, cellular signaling, and cellular differentiation, and are typically secreted in response to exercise or nutritional changes. The signaling pathway skeletal muscle implements to communicate with non-muscle tissue has long been recognized, but not fully elucidated. Therefore, due to the association of skeletal myopathy and liver disease, the skeletal muscle-liver axis is believed to play a pivotal role in metabolic perturbations caused by alcohol consumption. Additionally, the wasting of skeletal muscle in response to stimuli such as alcohol consumption is associated with a higher rate of mortality in various disease states, suggesting that alcohol abuse either initiates or hinders inter-organ cross talk, contributing to the progression and exacerbation of disease.

Due to the role of skeletal muscle in whole-body fatty acid metabolism, myokines could potentially serve a therapeutic role in the prevention of metabolic disorders and other maladies resulting from lipid accumulation. In particular, two recently identified myokines, irisin and myonectin, appear to play a role in the activation of hepatic lipid oxidation (Seldin et al. 2012;

Mo et al. 2016; Tang et al. 2016). Reduced circulating irisin and myonectin levels could serve as a potential mechanism for excessive lipid accumulation.

As discussed in the next sections, myonectin expression and circulating levels are decreased in a fasted state as well as in DIO (diet-induced obese) and insulin resistant mice. Further, the addition of free fatty acids (FFA) or glucose to cultured myotubes upregulates the expression of myonectin. Similar to myonectin, irisin expression is reduced in the serum of patients with insulin resistance (Yang et al. 2015). Chronic ethanol consumption inhibits glucose use by skeletal muscle (Garriga et al. 2005; Spolarics et al. 2013) and induces whole-body insulin resistance. (Lindtner et al. 2013; Ramirez et al. 2013). Additionally, chronic alcohol use contributes to decreased skeletal muscle mass (Thapaliya et al. 2014; Steiner et al. 2015). Therefore, we hypothesized that alcohol consumption would result in reduced circulating irisin and myonectin levels.

FNDC5/Irisin

FNDC5 is a recently identified transmembrane protein first described as receptor functioning in myoblast and neural differentiation (Ferrer-Martinez et al. 2002; Teufel et al. 2002; Hashemi et al. 2013). Since its discovery, FNDC5's potential role as a receptor has not been elucidated, however its physiological role in whole body metabolism has been the subject of various studies (Huh et al. 2012; Tanisawa et al. 2014; Xiong et al. 2015).

The beneficial effects of exercise have been well characterized and are known to play a role in the prevention and treatment of morbidities such as cardiovascular disease and metabolic dysregulation. Much of the improvements exerted through exercise rely on skeletal muscle's ability to regulate glucose and lipid levels. Peroxisome-proliferator activated receptor gamma

coactivator-1 α (PGC1α) is a key regulator of energy metabolism and is involved in processes such as mitochondrial biogenesis and muscle fiber type determination. Therefore, during an attempt to analyze the skeletal muscle secretome in response to PGC1α expression, Boström et al. identified Fndc5 as a PGC1α target gene in 2012. In Boström's study, increased PGC1α expression resulted in increased FNDC5 expression (Boström et al. 2012). Due to the regulation of FNDC5 expression via PGC1α, FNDC5 is purportedly induced in response to exercise. This discovery highlighted a new understanding of the skeletal muscle-adipose tissue axis, which could be important in the regulation of whole body energy metabolism. Further, discovery of FNDC5 as metabolic energy regulator led to the identification of a novel myokine, irisin. Irisin is a soluble peptide produced and secreted following proteolytic cleavage of the FNDC5 ectodomain (Boström et al. 2012).

The identification of FNDC5's structure has been pivotal in determining its physiological role. FNDC5 is composed of a signal peptide, a fibronectin type III domain, a linking peptide, and a hydrophobic carboxyl-terminal. FNDC5 is highly conserved in vertebrates and three *Fndc5* gene variants exist in humans, with mRNA expression highest in the heart, followed by the liver, skeletal muscle, and ovaries (Kim et al. 2017). CpG islands, which are regions of DNA involved in both the activation and inhibition of gene promoters, were identified in the promoter region of *FNDC5* variants 2 and 3 in humans. Further, histone modifications within the CpG island were found to contribute to the transcriptional regulation of *FNDC5* (Kim et al. 2017).

In a previous study in which primary adipocytes derived from subcutaneous tissue were treated with FNDC5, the expression of uncoupling protein 1 (UCP1) mRNA was highly up-regulated (Boström et al. 2012). UCP1 is a protein that induces the "browning" of white adipocytes by decreasing the proton gradient of the inner mitochondrial membrane, causing a

release of heat rather than the synthesis of ATP. Therefore, FNDC5 is believed to induce the browning of white adipocytes abetted by overexpression of UCP1, which in turn increases energy expenditure and fat oxidation (Boström et al. 2012). Additionally, FNDC5 could potentially be implicated in the removal of lipids from the bloodstream.

Various studies have reported the effect of exercise on FNDC5/irisin expression in both human (Lecker et al. 2012; Tanisawa et al. 2014) and rodent models (Dun et al. 2013; Roberts et al. 2013) and irisin has been identified in both human and rodent serum. Additionally, FNDC5/irisin has been the subject of various projects such as those focusing on diabetes and cardiovascular disease. Further, a positive association has been reported between FNDC5 skeletal muscle expression and insulin resistance (Huh et al. 2012). In a separate study, insulin resistant DIO mice exhibited no alteration in FNDC5/irisin skeletal muscle levels while irisin serum levels and FNDC5/irisin levels secreted from adipose tissue were decreased. The addition of irisin to skeletal muscle cells was then shown to reverse insulin resistance (Yang et al. 2015).

The cleavage site of FNDC5 has been debated, but when cleaved the 112 amino acid myokine, irisin, is secreted. While irisin retains similar folds to those seen in other FNIII structures, its secondary structure is unique in that it is optimal for dimerization. The structure of irisin is a β-sandwich with β-sheets combining to form an antiparallel 8-stranded β-sheet. Within the irisin core, 10 inter-subunit hydrogen bonds enhance protein stability. In addition to inter-subunit interactions, neighboring subunits are connected via two salt bridges and a tryptophan-like-zipper, securing the ends of the dimer and locking the subunits together, respectively (Schumacher et al. 2013). The irisin dimer may be either glycosylated or non-glycosylated at two asparagine residue locations.

In addition to an adipo-myokine axis, there also exists a hepatic-myokine axis in which FNDC5 is known to play a role. In response to the metabolism of alcohol and various other stimuli, FAO is often impaired which may result in hepatic lipid accumulation. Additionally, autophagic impairment is thought to occur as a result of alcohol consumption which could contribute an additional hit, further exacerbating the development of fatty liver. Peroxisome proliferator activated receptor-α (PPARα), a regulator of hepatic lipid metabolism, is modulated by FNDC5 expression, exposing a possible mechanism in which FNDC5 could play a role in the prevention of fatty liver. Irisin inhibits hepatic gluconeogenesis while promoting glycogen synthesis, a reversal of the typical abnormalities associated with metabolic dysregulation, as observed in both hepatocytes and type 2 diabetic mice (Liu et al. 2015). Additionally, serum irisin levels of obese Chinese adults diagnosed with NAFLD were observed to be inversely correlated with hepatic triglyceride levels, while patients with decreased serum irisin levels displayed increased levels of the hepatic biomarkers alanine aminotransferase (ALT) and asparagine aminotransferase (AST) (Zhang et al. 2013). Further, male C57BL/6 FNDC5$^{-/-}$ mice exhibited hepatic steatosis and blunted FAO, while overexpression of FNDC5 prevented hyperlipidemia and hepatic lipid accumulation (Liu et al. 2016). Further, PPARα is known to up-regulate the fibroblast growth factor 21 (*Fgf21*) gene, resulting in improved hepatic steatosis and insulin sensitivity (Xu et al. 2008) raising the possibility that irisin effects lipid metabolism via regulation of *Fgf21*. Therefore, these findings suggest FNDC5/irisin could possibly play a protective role against hepatic liver disease.

FNDC5/Irisin Controversy

Limitations of various studies analyzing the function of FNDC5/irisin arise from non-validated antibodies. Antibody validation is the process in which the specificity and affinity of an antibody is proven as well as the specificity of an antibody when using particular analytical techniques (Bordeaux et al. 2010). Antibody validation is not required, however, and specific guidelines concerning validation have not been established. Due to lack of effective antibody validation guidelines, a large number of reports regarding variations in irisin levels have frequently arisen. In some instances, models under the same physiological conditions have displayed altered FNDC5/irisin levels, even when antibodies from the same company were used.

In 2012, Boström's lab used antibodies that targeted the transmembrane region of FNDC5; this created controversy over the existence of irisin considering that the ectodomain of FNDC5 is cleaved to produce irisin. Boström et al. then verified the presence of the secreted peptide, irisin, using mass spectrometry. In 2013, Raschke et al. implemented a multi-species alignment to determine the start codon of *Fndc5* in humans is a non-canonical, ATA codon, rather than the typical ATG start codon. Raschke then argued that *Fndc5* is only a pseudogene and the benefits of irisin previously described could not be translated to humans (Raschke and Eckel 2013). The claims by Raschke have several limitations; first, describing *Fndc5* as a pseudogene is incorrect, as pseudogenes are defined as genes that have lost their protein-coding ability. In Raschke's study, FNDC5 protein made from the ATA-*Fndc5* sequence was clearly identified and non-canonical start codons are typically indicative of a complex mode of translation (Chang and Wang 2004; Starck et al. 2012). Moreover, the high-degree of conservation of the amino acid sequence of irisin has been very clearly determined. Additionally, Raschke et al. observed a small amount of protein production from a cytomegalovirus (CMV)-

promoter driven plasmid in human embryonic kidney cells 293 (HEK293). This finding is in no way evidence for inefficient FNDC5 translation, contrary to Raschke et al.'s opinion. Further, the study did not analyze endogenous regulation of FNDC5 in its native state in humans.

As reported by Albrecht et al. in 2015 (Albrecht et al. 2015), the available FNDC5/irisin antibodies are prone to cross-reactivity in both human and animal sera. Additionally, a ~20 kDa band was visible using liquid-chromatography mass spectrometry following SDS-PAGE of human serum, however this band was not detected following immunoblotting using available commercial antibodies. In this study, FNDC5 was not completely deglycosylated and therefore the molecular weight would not be as expected. Additionally, Albrecht et al. used shotgun proteomics for their method of mass spectrometry and this technique is not ideal because it commonly fails at identifying low abundance proteins. Targeted proteomics would yield more reliable information.

In 2015, Spiegelman's lab employed a tandem mass spectrometry analysis of human plasma irisin using two different peptides (Jedrychowski et al. 2015). The first peptide was located at the amino-terminus of irisin, immediately following the signal peptide. This peptide was downstream from the non-conical ATA codon, but upstream from the first ATG codon. The second peptide analyzed using tandem mass spectrometry was located in the middle of irisin, three amino acids away from the typical ATG start codon. Samples were taken from four sedentary and four aerobically trained individuals. The analysis provided by Spiegelman's lab presented an unbiased and precise identification of irisin and confirmed that the irisin sequence is 100% conserved between humans and mice. Further, in 2013, the crystal structure of irisin was elucidated (Schumacher et al. 2013). Taking into consideration the various studies targeting

FNDC5/irisin, novel, validated antibodies need to be generated in order to efficiently study FNDC5/irisin.

Due to the potential functional role of myonectin and irisin in the regulation of whole body energy metabolism, our research attempted to analyze the expression of these two novel myokines in response to a NIAAA, ten-day ethanol diet as well as a chronic, six-week ethanol diet.

Myonectin

The second myokine of interest, myonectin (CTRP15), has only been recently discovered and characterized. Myonectin is a member of the highly conserved C1q/TNF-related protein (CTRP) family and is characterized by trimer formation, which can fold further to assume multimeric complexes (Seldin et al. 2014) assembled through cysteine residues at the amino-terminus (Seldin et al. 2012). Myonectin is composed of five domains including a signal peptide domain, two amino-terminal domains, a collagen domain, and a C1q/TNF-like domain, as deduced by Seldin et al. (Seldin et al. 2012). Expression of myonectin occurs predominantly in skeletal muscle, where expression is higher in oxidative fibers as compared to glycolytic fibers (Seldin et al. 2012). Additionally, myonectin expression and secretion is regulated by both exercise and the intake of glucose and FAs. Therefore, myonectin is presumed to link skeletal muscle and systemic lipid homeostasis.

In a study using mouse models, the expression of myonectin was induced by re-feeding following an overnight fast (Seldin et al. 2012), highlighting a possible postprandial role. Moreover, recombinant myonectin has been shown to lower circulating FFA levels by acting on lipid uptake genes, thereby increasing FFA uptake in hepatocytes (Seldin et al. 2012).

Furthermore, recombinant myonectin has been shown to increase phosphorylation of AMP-activated protein kinase (AMPK), triggering an increase in glucose transporter 4 (GLUT4) expression and subsequently glucose uptake and FA oxidation (Park et al. 2009). Myonectin is also believed to induce the mammalian target of rapamycin (mTOR) pathway, resulting in the suppression of hepatic autophagy, which could serve either a pro- or anti-protective role (Seldin et al. 2013) and may be a contributing factor to metabolic disruption.

Skeletal Muscle Injury and Related Serum Biomarkers

As previously mentioned, chronic alcohol consumption has the ability to cause severe morphological and biochemical derangements to skeletal muscle. Additionally, damage to skeletal muscle caused by alcohol may prime the tissue for deferred or incomplete repair, which may result in increased morbidity and mortality, especially when coupled with damage to other tissues. Specific cytokines, myokines, and adipokines have shown to be involved in myopathic states and they may play a role in either disease progression or inhibition (Krahenbuhl 2001; Moran and Mastaglia 2014).

For example, the cytokine tumor necrosis factor-α (TNF-α) is an inflammatory mediator that is highly expressed in injured myofibers and functions in fiber regeneration (Collins and Grounds 2001). Interleukin-6 (IL-6), a myokine released into the serum following exercise, functions to promote skeletal muscle hypertrophy (Serrano et al. 2008). In addition, disruption of myokine, cytokine, and adipokine signaling in response to ethanol may contribute to deregulated inter-organ cross talk.

The quantity and duration of ethanol consumption and its effects on various circulating factors may play a significant role in the formation of skeletal myopathy. Therefore, specific

cytokines, myokines, and adipokines that play a pro-inflammatory or anti-inflammatory role in skeletal muscle were of interest. Thus, we aimed to analyze the effect of ethanol on TNF-α, IL-6, and plasminogen activator inhibitor-1 (PAI-1) serum levels of NIAAA and chronic ethanol fed mice.

CHAPTER 2

EXPERIMENTAL PROCEDURES

Animals

C57BL/6 mice (females at 12 weeks of age, males at 8 weeks of age) were housed in pathogen free conditions, 2-4 per cage, at 20-22°C with a reversed 12:12-h light-dark cycle, and fed either a Lieber-DeCarli diet (Bioserve #F1258SP) or a calorically-matched control diet (Bioserve #F1259SP). Following the feeding protocol, mice were anesthetized with isoflurane and euthanized via exsanguination. All animal procedures were conducted in accordance with institutional guidelines, and ethical approval was obtained from the University Committee on Animal Care (East Tennessee State University, Animal Welfare Assurance #A3203-01).

Feeding Protocol

Two independent feeding protocols were used: First, in the chronic plus binge model, hereby referred to as the NIAAA model, male and female mice at 12 weeks of age were acclimatized to a liquid diet without the addition of alcohol (Bioserve #F1259SP) for a period of 4 days, followed by 10 days on the Lieber-DeCarli ethanol diet (Bioserve #F1258SP) (5% ETOH/volume) *ad libitum*. On the 11th day of feeding, food was removed and replaced with water and a single gavage of either maltose dextrin or ethanol (5g kg^{-1}). Nine hours post gavage, mice were euthanized and tissues and serum were collected. This model mimics hepatic steatosis, liver injury, and inflammation as seen in alcoholic hepatitis patients (Bertola et al. 2013).

In the second model, hereby referred to as the chronic model, 12 week old male and female mice were acclimatized to the liquid diet *ad libitum,* without the addition of alcohol for 1-week, and gradually transitioned from a 1% to a 5% Lieber-DeCarli ethanol diet (v/v ethanol) over the course of two weeks. For the remaining 4 weeks, a 5% ethanol diet (v/v ethanol) was maintained. This protocol is presumed to reflect chronic alcohol abuse in which low volumes are initially consumed, but increase over time (Breitkopf et al. 2009). This model results in hepatic steatosis with only moderate inflammation (Bertola et al. 2013). For both models, body weight and food intake was measured daily. Food intake for mice on the control diet was limited to match the daily intake for the previous day of the corresponding ethanol-fed mice.

Tissue Collection

Immediately following euthanasia, the hind-limb skeletal muscles (gastrocnemius, plantaris, and soleus muscles) from each limb were immediately collected, snap-frozen, and stored at -80°C in LabServ 1.5mL microcentrifuge tubes (Cat#111558), following euthanization. Serum was prepared according to manufacturer's instructions (Sarstedt, Cat#41.1500.005).

Immunoblot Analysis

In order to isolate proteins, RIPA assay buffer plus protease and phosphatase inhibitors (Bimake Cat# B14001 & B15001) were added to the muscle tissues, followed by homogenization with an electric homogenizer (Tissue Tearor, model #985370) Protein concentrations were determined by Thermo Scientific™ Pierce™ Coomassie Plus (Bradford) Protein Assay (ThermoFisher #PI23236). An equal amount of protein was diluted with loading buffer and RIPA, and samples were boiled at 100 °C for 5 minutes, cooled on ice, vortexed, and

then centrifuged. 30μg per sample of skeletal muscle or 1 μl of serum were separated by SDS-PAGE gel (Cat#456-1086) with Precision Plus Protein™ Dual Color Standards used as the ladder (Cat#161-0374). The gel was then transferred to a nitrocellulose membrane (BioRad, Cat#162-0115) and equal loading of the proteins was confirmed using Ponceau S (0.1% Ponceau 5, 5% acetic acid). Membranes were blocked with 2% non-fat milk (0.5g NFM, 25mL TBST) and incubated with either myonectin (rabbit polyclonal anti-FAM102B antibody-C-terminal, 1:500, ab177994, lot# GR265682-3; secondary antibody goat anti-rabbit 1: 10,000, Ab31460) or irisin (rabbit monoclonal anti-FNDC5 antibody, 1: 10,000, ab174833; secondary antibody goat anti-rabbit 1: 10,000, Ab31460) antibodies using established procedures.

RNA Isolation

RNA was isolated according to manufacturer's instructions (Direct-zol RNA MiniPrep Plus, Cat# R2070). Muscle tissue was placed on ice in an RNase free tube with 2ml of Trizol reagent (Life Technologies Lot# 147206) and homogenized for 1s using either an electric homogenizer or sonicator. 750ul of homogenate was placed in RNAse free tubes with an equal amount of ethanol (95-100%) and mixed thoroughly. RNA was isolated according to manufacturer's directions. All samples were also treated during the isolation with the optional included DNaseI treatment step, as per manufacturer's directions. RNA concentrations and RNA integrity were determined by agilent analysis (Agilent 2100 Bioanalyzer, Agilent Technologies, Agilent RNA 6000 Nano Kit #5067-1511). Samples with an RNA integrity number below 7.0 were excluded and an RNA isolation step was repeated with stored aliquot for those samples. 1μg RNA was reverse transcribed to complementary DNA (cDNA) with commercial assay (ThermoFisher Scientific, Cat# 170-8896).

Real-Time Quantitative Reverse Transcriptase PCR Analysis

Primers for myonectin (forward: 5'-GCAGGCTCTTACCCTTACCA-3' and reverse: 5'-GCCAAGCAGGGCTTAGGATA-3'), FNDC5 (forward: 5'-GGGCAGGTGTTATAGCTCTCTT-3' and reverse: 5'-GTCATCATATCTTGCTGCGGA-3'), and Hprt1 (forward: 5'-CAAACTTTGCTTTCCCTGGT-3' and reverse: 5'-TCTGGCCTGTATCCAACACTTC-3') were purchased from Integrated DNA Technologies and validated. A 10-fold dilution series was generated for each gene as the standard curve. cDNA from the reverse transcription reaction was incubated in the appropriate mix (SABbiosciences) for an initial denaturation at 94°C for 30s, followed by 40 PCR cycles each consisting of 95°C for 0s, 61°C for 7s, and 72°C for 10s. Specificity of amplification products was further confirmed by analyzing melting curve profiles for primers and products and subjecting the amplification products to agarose gel electrophoresis.

Biochemical Serum Analysis

Tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and plasminogen activator inhibitor-1 (PAI-1) were determined with commercially available kits using the Bio-Plex® Multiplex Immunoassay System (Bio-rad Cat# 171F7001M, 171G5023M, 171I50001, 171G5007M). The intra- and inter-assay coefficients of variation for all assays were ≤8% and ≤12%, respectively. Serum glucose and triglyceride concentrations were determined using commercially available assays according to manufacturer's directions (Fisher Diagnostics, Cat# TR22421).

Skeletal Muscle Lipid Analysis

Lipids were extracted as described by Bligh and Dyer (Bligh and Dyer 1959). Skeletal muscle samples were weighed, homogenized in phosphate-buffered saline, and 3.75 mL/mL of sample homogenate 1:2 sample homogenate (vol/vol) of cholorform-methanol was added. Chloroform and methanol prohibit lipid clustering. This was followed by the addition of 1.25 mL of distilled water. Samples were then vortexed between each addition for 30 seconds and centrifuged at 1,100 x *g* for 10 minutes at room temperature. This resulted in a two-phase solution, with an aqueous phase plus methanol and polar molecules constituting the top phase and an organic phase containing chloroform and lipids on the bottom. The lower phase was collected using a glass pipette and samples were dried under a nitrogen bath. One aliquot of each sample was dissolved in t-butyl alcohol Triton X-100 (3:2 vol/vol) and samples were prepared for fatty acid methyl ester analysis.

Statistical Analysis

Descriptive statistics (mean and standard deviations) were calculated for all measured variables. As the feeding models were not performed or analyzed concurrently, each model was analyzed as an independent experiment. Body weight and food intake were analyzed by two-way repeated measures ANOVA followed by Tukey's multiple comparisons test. An unpaired t-test was used to compare immunoblot blot and serum analyte data between control and ethanol fed groups. Lipid data was compared using a 1-way ANOVA between groups. All statistical analysis was performed by Graphpad Prism 6.

CHAPTER 3

RESULTS

Effect of Ethanol on Body Weight and Food Intake

Body weight and food intake was measured daily, with no statistical differences observed between groups in either the NIAAA or chronic model (Fig. 1). Body weight for mice on the NIAAA diet are reported as daily values while values for the chronic model were taken daily and averaged for each week.

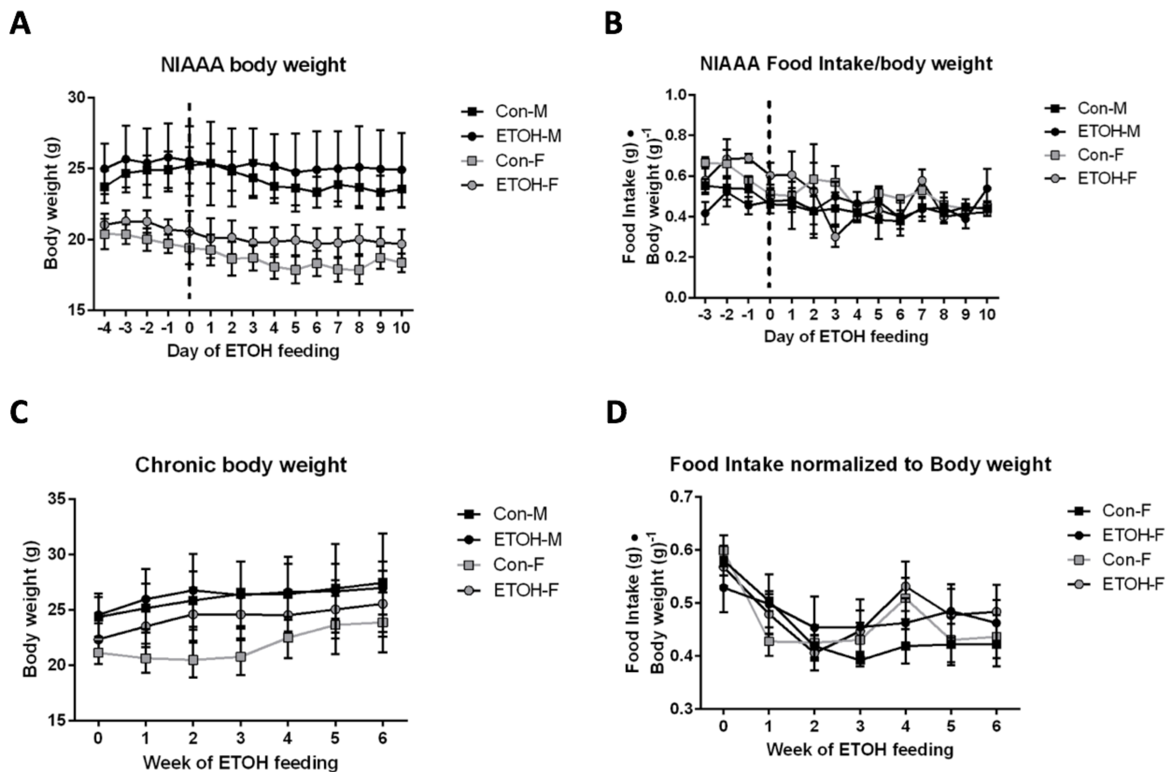

FIGURE 1—*Effect of Ethanol on Body Weight and Food Intake*. Control versus ethanol diet did not affect body weight or food intake in either the NIAAA or Chronic model. Body weight and food intake were measured daily. For the NIAAA model, data was reported as grams of diet consumed/mouse each day (Fig 1B) and daily body weights were reported as raw values normalized to day 0 (Fig. 1A). Day 0 measurements (also indicated by the vertical dashed bar) were taken before mice started the ethanol containing diet. For the chronic model, daily food intake for each mouse was measured and the average daily intake per mouse were recorded over the course of the week (data not shown). Daily body weights were obtained and averaged for each mouse over the week time period and reported in absolute grams (Fig. 1C) or normalized to week 0 (Fig. 1D). Data was analyzed using a 2-way ANOVA and is reported as mean ± standard deviation. n=27.

Expression of FNDC5 in Skeletal Muscle in Response to the NIAAA Diet

Western blot analysis was used to determine FNDC5 skeletal muscle protein levels in both male and female ethanol versus control fed animals of the NIAAA model. Values were normalized using β-tubulin. FNDC5 levels were reduced in male (Fig. 2A) and increased in female (Fig. 2B) ethanol fed mice, however these results were not significantly different from

control fed mice. FNDC5/irisin was undetectable in the serum of the NIAAA model (data not shown) when using western blot analysis.

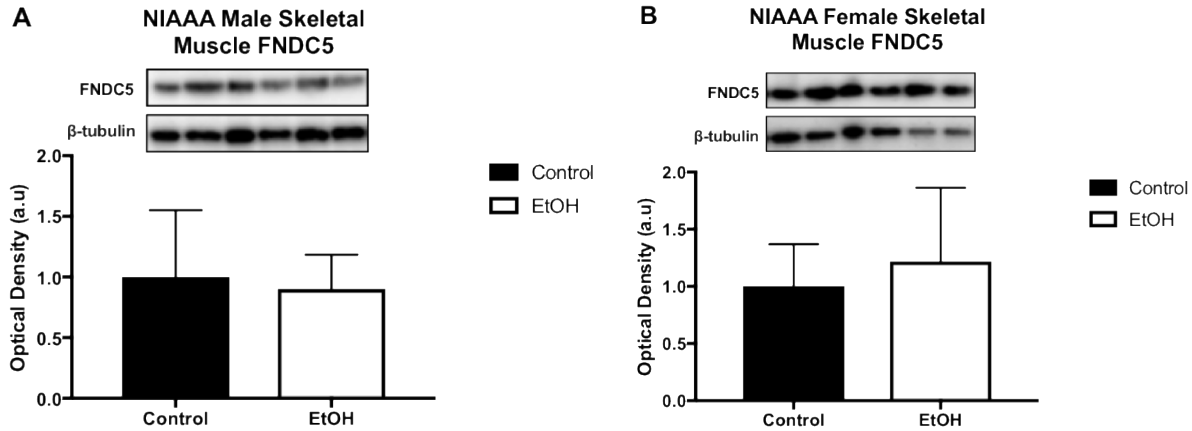

FIGURE 2—*NIAAA Skeletal Muscle FNDC5 Content.* Immunoblot analysis of skeletal muscle in male (Fig. 2A) and female (Fig. 2B) C57BL/6 mice fed with either a control or ethanol diet over a 10 day period, with either a maltose-dextrin (control) or ethanol gavage given on the 11th day. Rabbit monoclonal anti-FNDC5 antibody was used to detect the presence of FNDC5 and band intensity was analyzed and normalized to β-tubulin. No significant difference was found between control and ethanol fed groups in either male or female mice. Data was analyzed using an unpaired t-test and is represented by mean + S.D. n=6 per group for males, n= 4 control, 5 ethanol fed for females.

Expression of Myonectin in Skeletal Muscle and Serum in Response to the NIAAA Diet

Using western blot analysis, myonectin levels of skeletal muscle were determined and normalized to β-tubulin. In male ethanol fed mice, levels of myonectin were essentially unaltered as compared to control fed mice (Fig. 3A). In female ethanol fed mice, skeletal muscle myonectin content was increased (Fig. 3B) when compared to control, albeit this increase was not statistically significant. Myonectin was undetectable in the serum of the NIAAA model (data not shown) when using western blot analysis.

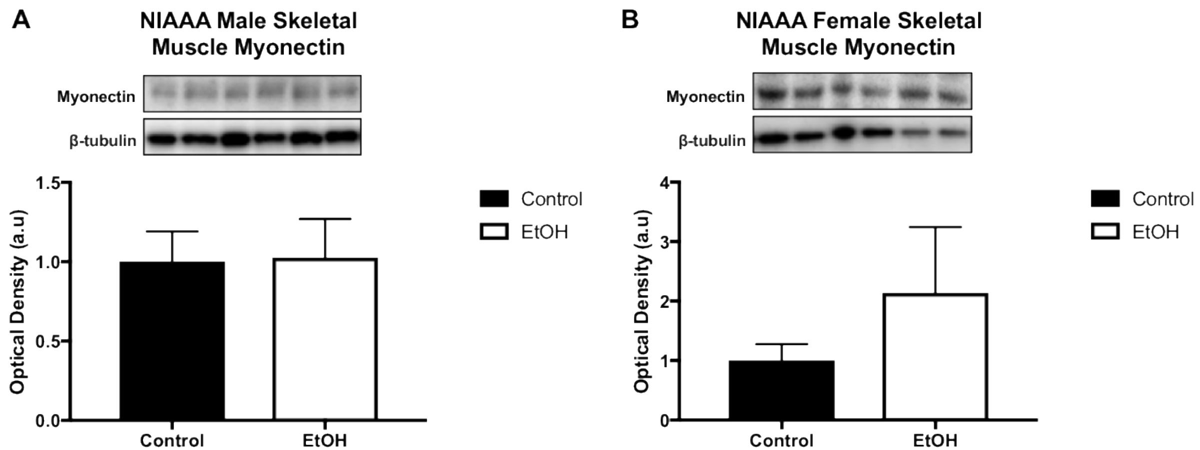

FIGURE 3—*NIAAA Skeletal Muscle Myonectin Content*. Immunoblot analysis of skeletal muscle in male (Fig. 3A) and female (Fig. 3B) C57BL/6 mice fed fed with either a control or ethanol diet over a 10 day period, with either a maltose-dextrin (control) or ethanol gavage given on the 11th day. Rabbit polyclonal anti-FAM102B was used to detect myonectin content and band intensity was analyzed and normalized to β-tubulin. No significant difference was found between control and ethanol fed groups in either male or female mice. Data was manalyzed using an unpaired t-test and is represented by mean + S.D. n=6 per group for males, n= 4 control, 5 ethanol fed for females.

Expression of FNDC5 in Skeletal Muscle in Response to the Chronic Diet

Using western blot analysis of skeletal muscle from mice fed the 6-week, chronic ethanol diet, it was determined that FNDC5 levels were not significantly different between control and ethanol fed mice in either male (Fig. 4A) or female (Fig. 4B) skeletal muscles. Irisin was undetectable in the serum of both male and female mice, whether they were control or ethanol fed (data not shown). We did detect a clear band in the serum around 50 kDa with 2 different irisin antibodies, however, this is double the predicted size for circulating irisin and determining the identity of this band was beyond the scope of this study. Further, no differences in optical density were noted in this off-target band.

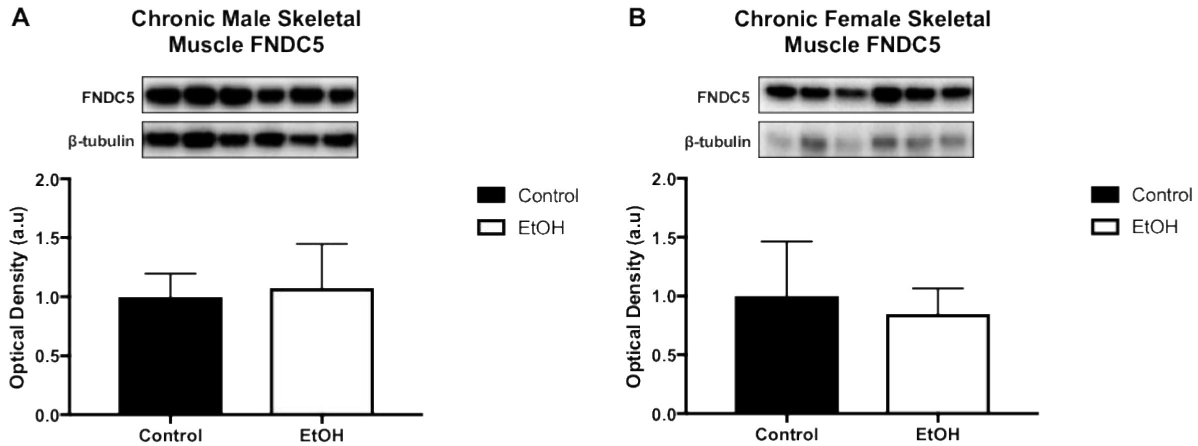

FIGURE 4—*Chronic Skeletal Muscle FNDC5 Content.* Immunoblot analysis of skeletal muscle in male (Fig. 4A) and female (Fig. 4B) C57BL/6 mice fed with either a control or ethanol diet over a 6-week period. Rabbit monoclonal anti-FNDC5 antibody was used to detect the presence of FNDC5 and band intensity was analyzed and normalized to the average intensity of control fed mice. No significant difference was found between control and ethanol fed groups in either male or female mice. Data was analyzed using an unpaired t-test and is represented by mean + S.D. n=6 per group for males, n= 4 control, 5 ethanol fed for females.

<u>Expression of Myonectin in Skeletal Muscle and Serum in Response to the Chronic Diet</u>

Using western blot analysis of skeletal muscle from mice fed the 6-week, chronic control or ethanol diet, it was determined that male and female (Fig. 5A, 5B) mice exhibited no alterations in myonectin levels. Myonectin was detected in serum via western blot analysis in both male (Fig. 6A) and female (Fig. 6B) mice, however, no significant difference was observed between control and ethanol fed mice.

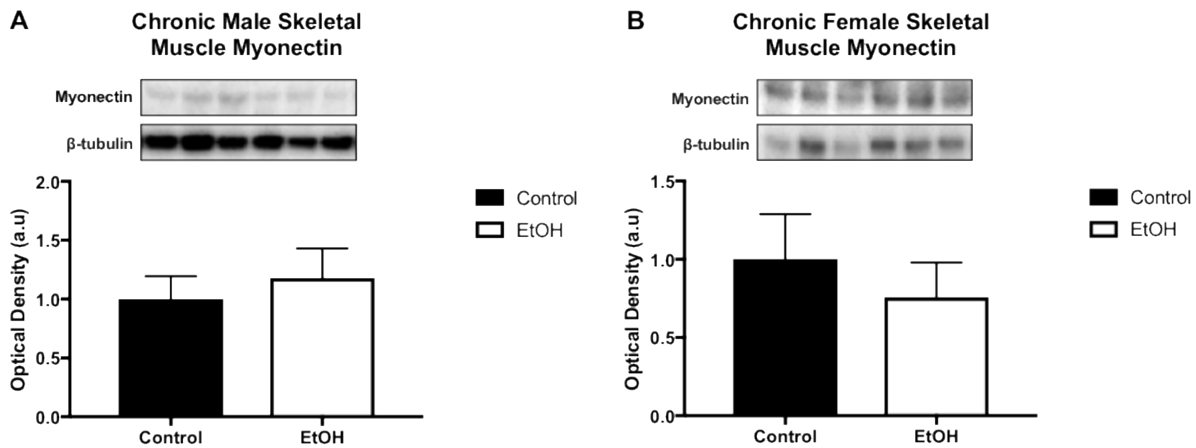

FIGURE 5—*Chronic Skeletal Muscle Myonectin Content.* Immunoblot analysis of skeletal muscle in male (Fig. 5A) and female (Fig. 5B) C57BL/6 mice fed either control or ethanol diet over a 6-week period. Myonectin antibody was used to detect myonectin content and band intensity was analyzed and normalized to β-tubulin. No significant difference was found between control and ethanol fed groups in either male or female mice. Data was analyzed using an unpaired t-test and is represented by mean + S.D.

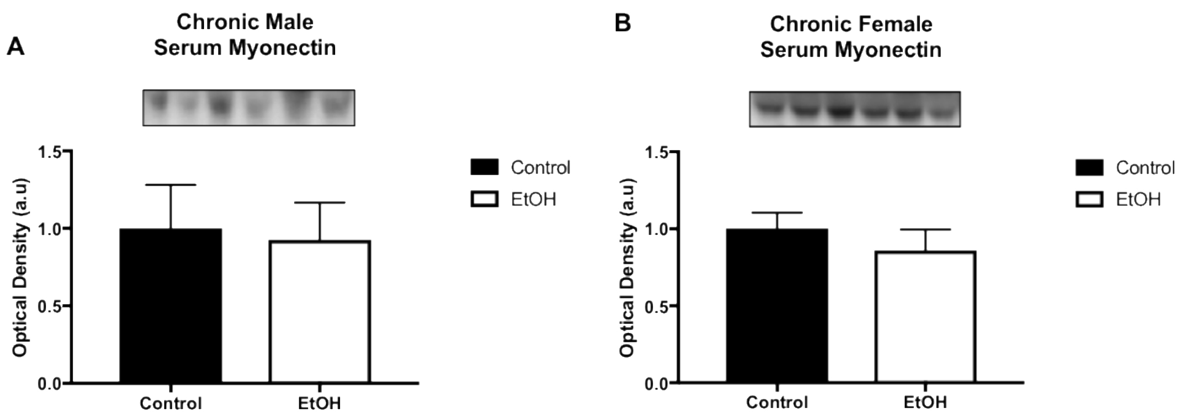

FIGURE 6—*Chronic Serum Myonectin Content.* Immunoblot analysis of C57BL/6 male, *A* and female *B*, mice serum fed either control or ethanol diet. Serum levels of myonectin showed a slight decrease in both male and female ethanol fed mice, however these results were not significant. Data was analyzed using an unpaired t-test and is represented by mean + S.D. n= 5 per group for males, n=6 per group for females.

Quantitative Real-Time PCR Analysis of FNDC5 and Myonectin in Skeletal Muscle

Quantitative RT-PCR analysis was used to quantify FNDC5 (Fig. 7A, 7B) and myonectin (Fig. 7 C, 7D) expression levels with hypoxanthine-guanine phosphoribosyltransferase-1 (Hprt1) used as a house-keeping gene. FNDC5 mRNA levels of male ethanol fed mice was significantly decreased as compared to control fed mice (Fig. 7A) ($P<0.0012$) while myonectin expression was not altered between groups. FNDC5 expression of female skeletal muscle was not altered between groups (Fig. 7B) while myonectin expression was up-regulated in ethanol fed females (Fig. 7D) ($P<0.0378$).

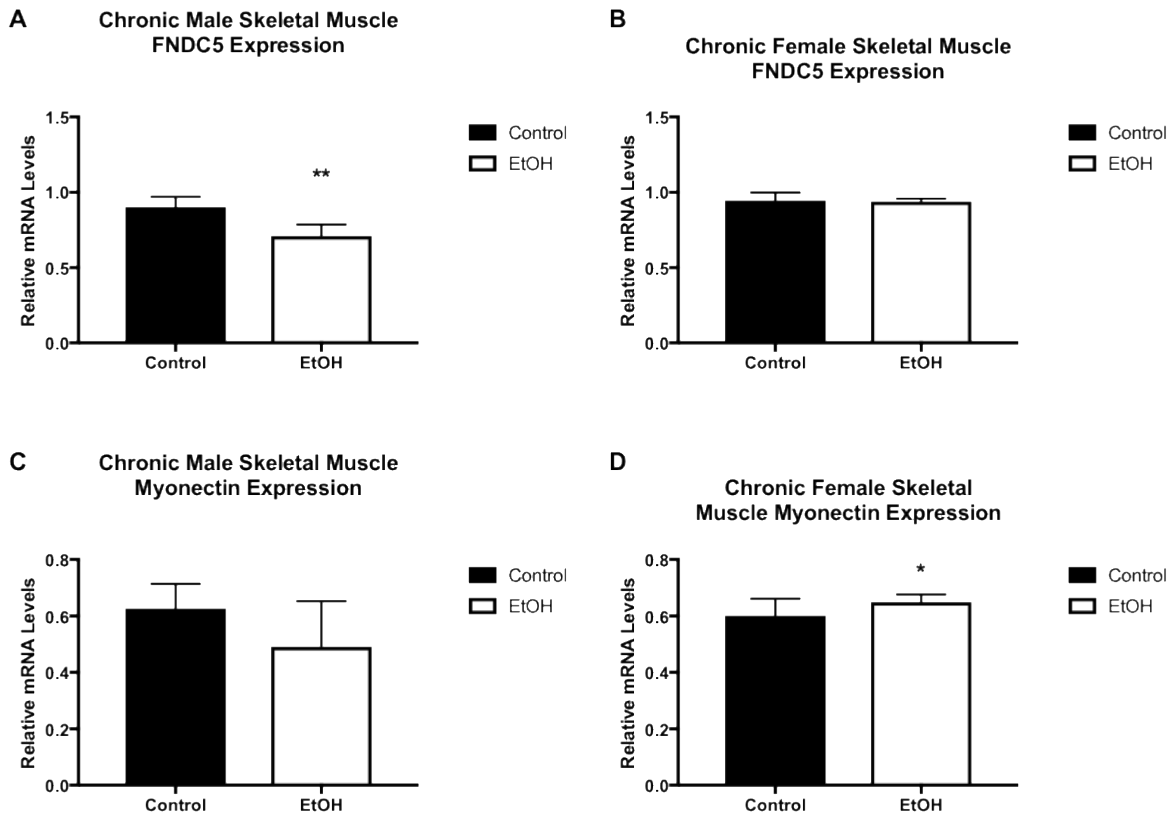

FIGURE 7—*Chronic RNA Expression of FNDC5 and Myonectin.* Real-time quantitative reverse transcriptase PCR analysis of skeletal muscle was used to determine FNDC5 and myonectin expression of male and female mice treated with a control or ethanol diet for 6 weeks. FNDC5 expression of male ethanol fed mice was down-regulated ($P<0.0012$) (Fig. 7A), while myonectin expression was not altered (Fig. 7C) between groups. FNDC5 expression was not altered in female mice (Fig. 7B) while myonectin expression was up-regulated in ethanol fed females (Fig. 7D) ($P<0.0378$). Data was normalized to Hprt-1. For FNDC5 and myonectin analysis, n= 11 males (5 control, 6 EtOH) and n=17 females (6 control, 11 EtOH). Data was analyzed using an unpaired t-test and is represented by mean + S.D. (*, $p<0.05$; **, $p<0.01$).

NIAAA and Chronic Serum Analyte Analysis

Using serum from the NIAAA and chronic model, levels of various biochemical parameters were assessed. NIAAA male ethanol fed mice displayed significantly increased levels of PAI-1 (Fig. 8C) ($P<0.0097$) while female ethanol fed mice displayed significantly increased levels of IL-6 (Fig. 9B) ($P<0.0170$). In the chronic model, male ethanol fed mice displayed significantly increased levels of PAI-1 (Fig. 10C) ($P<0.0296$) while female ethanol fed mice

displayed significantly increased levels of TNF-α (Fig. 11A) (P<0.0190), IL-6 (Fig. 11B) (P<0.0360) (Fig. 11B), and PAI-1 (Fig. 11C) (P<0.0101).

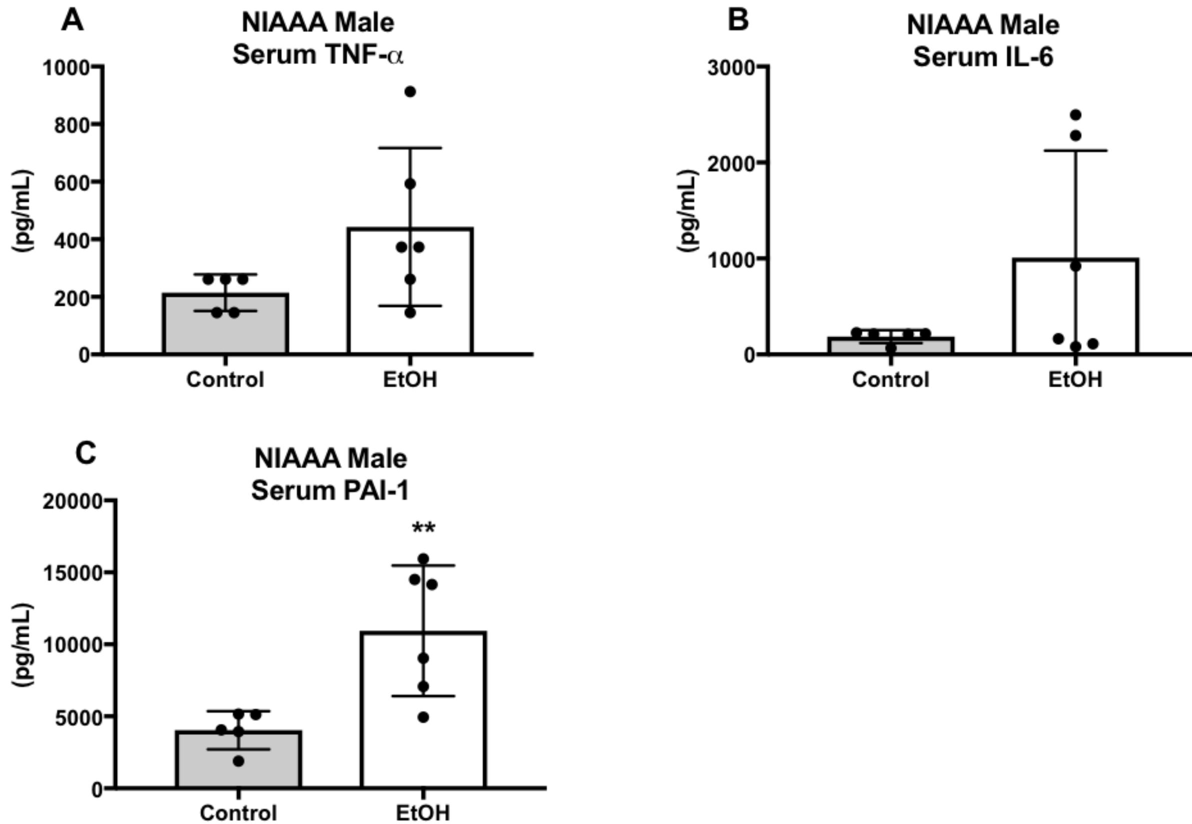

FIGURE 8—*Male NIAAA Serum Analyte Analysis.* Concentrations of circulating analytes (TNF-α, IL-6, PAI-1) of male NIAAA mice were measured using Bio-Plex Multiplex. PAI-1 levels of ethanol fed mice were significantly elevated as compared to control (P<0.0097) (Fig. 8C). Data was analyzed using an unpaired t-test and is represented by mean + S.D. (**, p<0.01). n=5 control, 6 ethanol.

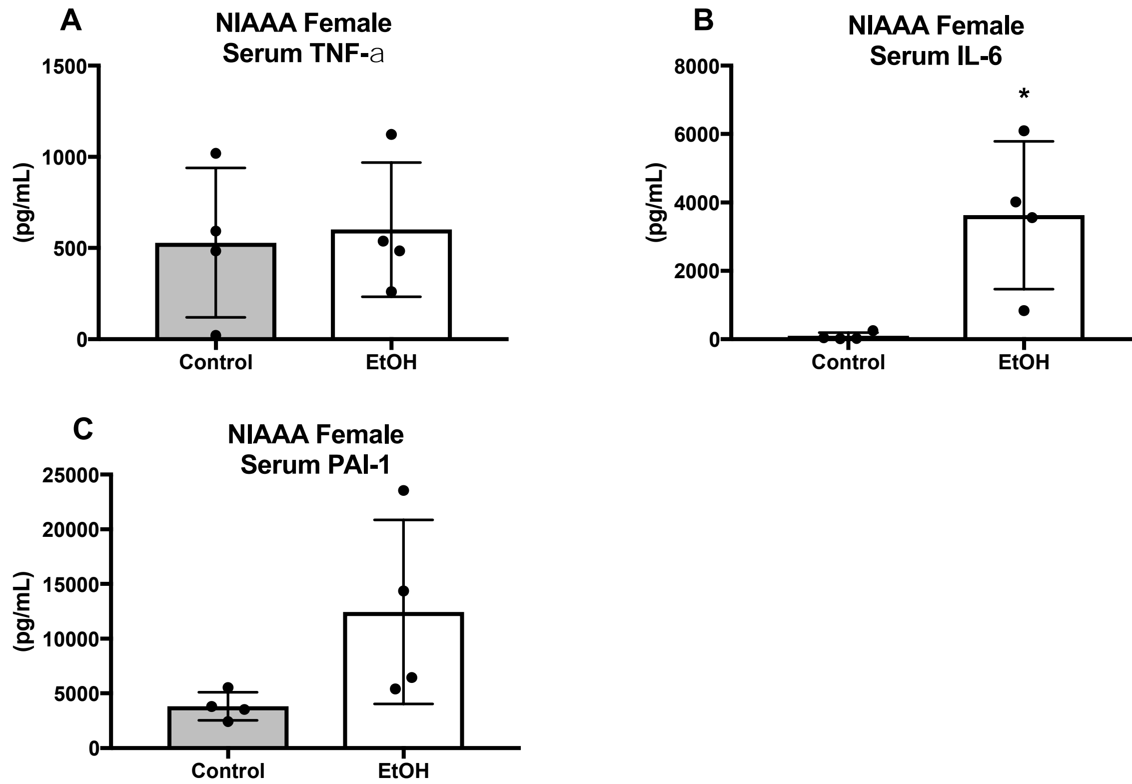

FIGURE 9—*Female NIAAA Serum Analyte Analysis*. Concentrations of circulating analytes (TNF-α, IL-6, PAI-1) of female NIAAA mice were measured using Bio-Plex Multiplex. Levels of IL-6 were significantly elevated (P<0.0170) in ethanol fed mice as compared to control (Fig. 9C). Data was analyzed using an unpaired t-test and is represented by mean + S.D. (*, p<0.05). n= 4 per group.

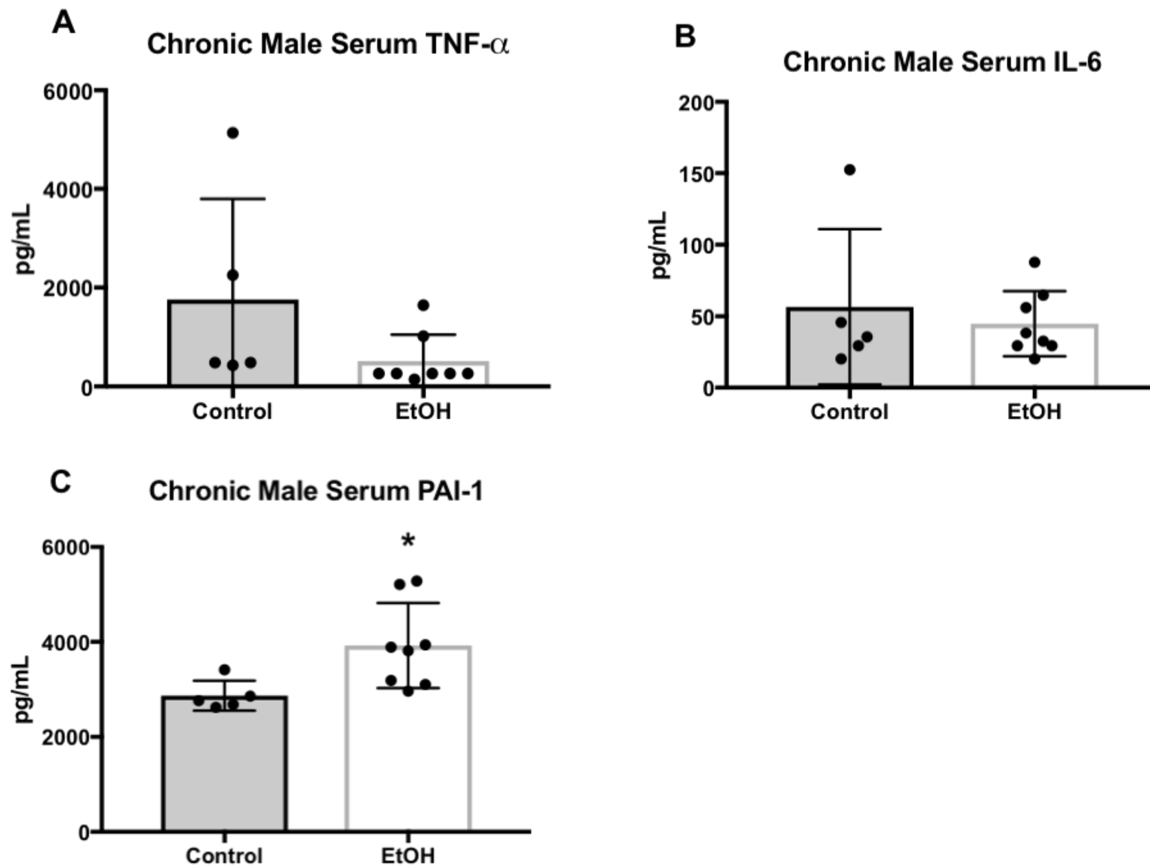

FIGURE 10—*Male Chronic Serum Analyte Analysis.* Concentrations of circulating analytes (TNF-α, IL-6, PAI-1) of male chronic mice were measured using Bio-Plex Multiplex. Levels of PAI-1 were elevated in ethanol fed mice ($P<0.0296$) as compared to their control counter-parts (Fig. 10C). Data was analyzed using an unpaired t-test and is represented by mean + S.D. (*, $p<0.05$). n= 5 control, 8 ethanol fed.

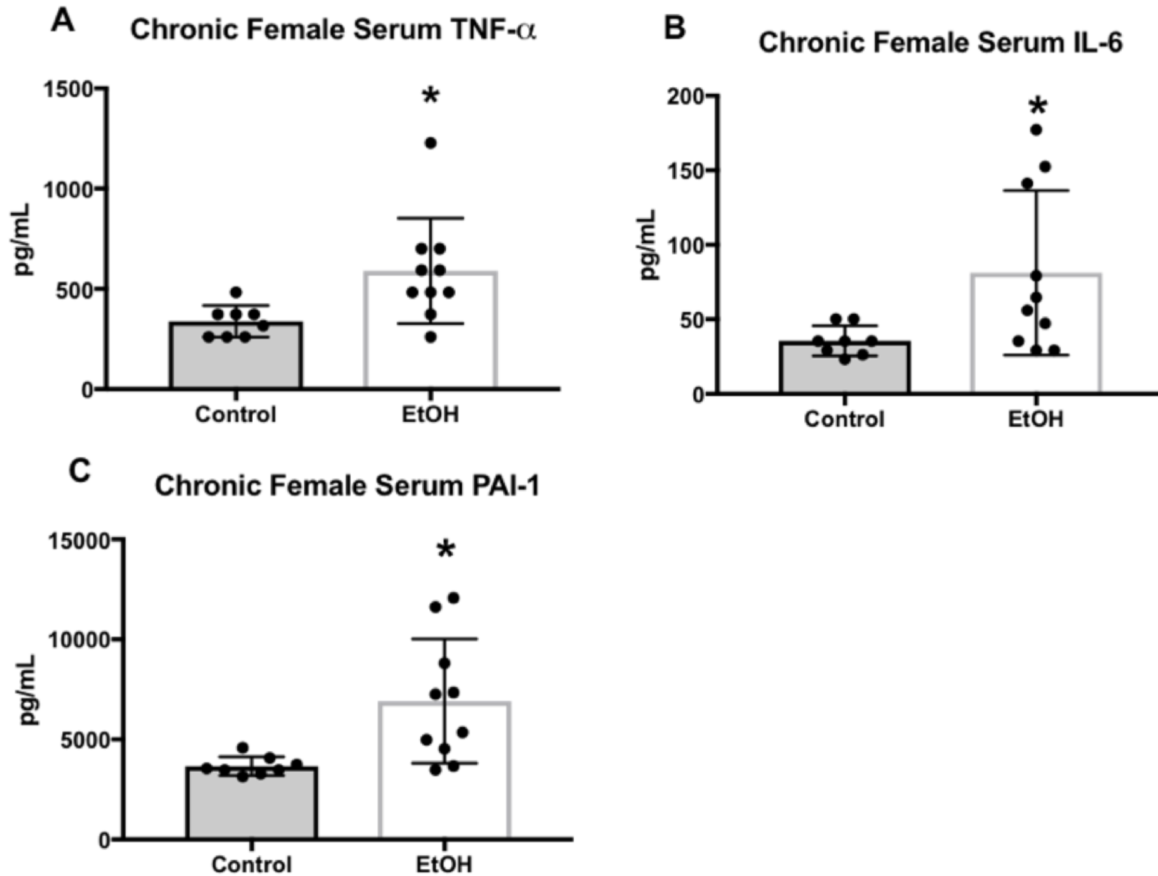

FIGURE 11—*Female Chronic Serum Analyte Analysis.* Concentrations of circulating analytes (TNF-α, IL-6, PAI-1) of female chronic mice were measured using Bio-Plex Multiplex. Ethanol fed mice displayed significantly increased levels of TNF-α (P<0.0190), IL-6 (P<0.0360), and PAI-1 (P<0.0101). Data was analyzed using an unpaired t-test and is represented by mean + S.D. (*, p<0.05). n=8 control, 10 ethanol fed.

Chronic Model Skeletal Muscle Lipid Analysis

Skeletal muscle is a main regulator of carbohydrate and lipid metabolism and conditions such as insulin resistance or excess alcohol consumption are characterized by ectopic lipid deposition in skeletal muscle (Sunnasy et al. 1983; Corocoran et al. 2007). Therefore, skeletal muscle lipid content of the chronic model was analyzed. Male and female mice fed ethanol displayed no significant alterations in skeletal muscle levels of palmitic (16:0), palmitoleic

($16{:}1^{\Delta 9}$), linoleic ($18{:}2^{\Delta 9,12}$), γ-linoleic ($18{:}3^{\Delta 9,12,15}$), arachidonic ($20{:}4^{\Delta 5,8,11,14}$), or nervonic acid ($24{:}1^{\Delta 9}$) as compared to control (Fig. 12A, 12B).

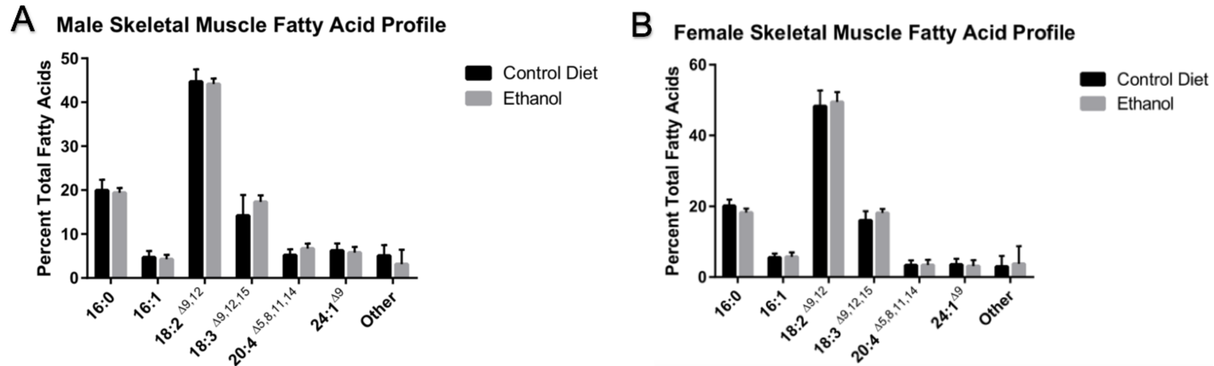

FIGURE 12—*Chronic Skeletal Muscle FAME Analysis.* Skeletal muscle lipids were isolated, extracted using the FAME method, and measured with gas-chromatography mass spectometry. Male mice (Fig. 12A) and female mice (Fig. 12B) displayed no alterations in fatty acid composition between groups. Data was analyzed using a 1-way ANOVA and is represented by mean + S.D. n=5-8 per group in males, n=8-11 per group for females.

CHAPTER 4

DISCUSSION

The goal of this study was to analyze the secretion of the myokines FNDC5/irisin and myonectin in response to both an acute, NIAAA ethanol diet as well as a chronic ethanol diet. We hypothesized that alcohol consumption would result in reduced circulating levels of the myokines myonectin and irisin. Our data did not support our hypothesis. In both models, skeletal muscle protein content of FNDC5 and myonectin was not significantly altered between groups in either male or female mice. Similarly, myonectin serum levels were not altered between groups in male or female mice of the NIAAA or chronic model. Irisin, on the other hand, was undetectable in the serum of both control and ethanol fed mice of the NIAAA and chronic model (data not shown).

Prior studies have shown serum irisin levels to be decreased with type 2 diabetes mellitus (T2DM) (Liu et al. 2013), NAFLD (Shanaki et al. 2017), and various other metabolic conditions (Provatopoulou et al. 2015; Zhao et al. 2015). Therefore, it was initially hypothesized that we would expect to see a decrease in serum irisin levels of ethanol fed mice. During our study, irisin was undetectable in the serum when using immunoblot analysis on both male and female mice. Unfortunately, as previously mentioned, there has been conflicting reports on the accuracy of irisin antibodies that are currently available. Various antibodies have been developed, targeting different portions of irisin or FNDC5. Dun et al developed antibodies targeting FNDC5's C-terminal domain (Dun et al. 2013), Boström et al. (Boström et al. 2012) used an antibody targeting the FNDC5 transmembrane domain, and the antibody used in this study targeted amino

acids 50-150. Therefore, validated antibodies must be generated in order to accurately and efficiently analyze FNDC5/irisin.

Further, the inability to detect irisin in the serum could be a result of extremely low irisin levels, possibly due to a correlation between alcohol consumption and hepatic lipid accumulation. As previously mentioned, an inverse relationship between intrahepatic triglyceride content and irisin levels has been reported by Zhang et al. (Zhang et al. 2013). This could indicate that excess alcohol consumption seen in this study has resulted in large amounts of triglyceride deposits within the livers of the mice. Additionally, it may be inferred that blunted expression of irisin would affect the regulation of PPARα, impairing β-oxidation in the liver. In contrast, the inability to detect irisin in the serum creates a number of different possibilities. FNDC5 expression in the muscle of male mice fed ethanol for 6-weeks was shown to be reduced, possibly due to a high level of irisin cleavage and secretion. However, as previously mentioned, we were unable to detect irisin in the serum.

When analyzing skeletal muscle RNA expression of the chronic model, male ethanol fed mice displayed decreased expression of FNDC5 with no significant alterations of myonectin expression in ethanol versus control mice. In ethanol fed females, RNA expression of myonectin was significantly increased as compared to control while FNDC5 expression was unaltered. In previous studies, changes in myonectin protein, mRNA, and serum levels have been contradictory. For example, serum myonectin levels were unchanged in rats following a restricted-calorie diet (Sharma et al. 2012). In a different study, HFD-fed mice displayed decreased skeletal muscle myonectin mRNA and protein levels (Seldin et al. 2012). Myonectin is also modulated by exercise, however, myonectin regulation has been shown to be both positively (Seldin et al. 2012) and negatively regulated (Peterson et al. 2014) by exercise. Further, if

myonectin plays a primary role in autophagy inhibition due to the induction of mTOR, it may be hypothesized that increased myonectin mRNA expression, such as that seen in the females of the chronic ethanol model, could lead to fatty acid accumulation in the liver in part by defective autophagic signaling.

Several possibilities arise for the results garnered by our study. First off, due to the lack of access to a running wheel, it may be surmised that changes in FNDC5 and myonectin expression were not due to exercise. Therefore, studies analyzing the relationship between FNDC5 or myonectin expression and exercise are of minute importance in relation to the results of this study. Additionally, as mentioned prior, the ultimate goal of this study was to analyze skeletal muscle as an endocrine organ. The endocrine system functions by delivering messenger molecules to various target organs. Therefore, the effects of ethanol regarding organ-to-organ communication bare a great deal of weight on these results. For example, the satiety hormone that inhibits hunger, known as leptin, is thought to act upon myocytes in order to induce myonectin expression (Rodriguez et al. 2014). In previous studies analyzing leptin levels, ethanol consumption has correlated with reduced leptin serum levels (Calissendorff et al. 2001; Otaka et al. 2007). Interestingly, a decrease in leptin serum levels were thought to be a result of suppressed leptin secretion from adipose tissue (Otaka et al. 2007). In the current study, female mice of the NIAAA model exhibited a significant increase in leptin serum levels (data not shown).

RNA expression analysis was not conducted on the skeletal muscle of the NIAAA model, therefore, it would be interesting to determine the association, if any, between the NIAAA diet and myonectin expression. Needless to say, the skeletal muscle-homeostatic axis is an exceptionally intricate network. Therefore, further investigation is required to unravel the various

mechanisms in which skeletal muscle contributes to inter-organ cross talk. Additionally, male NIAAA ethanol fed mice displayed a significant increase in PAI-1 levels while female ethanol fed NIAAA mice displayed a significant increase in IL-6 levels. Further studies analyzing the relationship of these parameters with myokines such as irisin and myonectin need to be completed. In the chronic model, male ethanol fed mice displayed increased levels of PAI-1 while female ethanol fed mice displayed increased levels of TNF-α, IL-6, and PAI-1. Although these cytokines have been shown to be implicated in skeletal muscle disease, it cannot be ruled out that these cytokines were regulated by other networks aside from those present in skeletal muscle. Additionally, it is yet to be determined the role that gender plays on the expression of various myokines, such as myonectin and irisin. However, it is important to note that the mortality rate of females fed an ethanol diet was much higher than their male counterparts (data not shown).

Analysis of RNA expression was not performed on skeletal muscle of the NIAAA model due to the fact that the effects of ethanol were much more pronounced when administered for six weeks. As previously mentioned, the NIAAA model used in this study presented no mortalities while in the chronic model, ~15% of males and ~50% of females succumbed to the effects of an ethanol diet (data not shown). One way in which ethanol consumption promotes morbidity is via excess ectopic lipid deposition. Therefore, skeletal muscle lipid analysis was performed for both males and females of the chronic ethanol diet. FAME analysis of the dietary regimen confirmed no differences between the fatty acid composition of both the control and ethanol diet (data not shown). When analyzing the lipid composition of skeletal muscle, male and female mice displayed no alterations between groups. Further analysis is required to determine the association, if any, of fatty acid parameters with duration and quantity of ethanol consumption.

CHAPTER 5

CONCLUSION

The results from this study are summarized in Table 1 (for the NIAAA model) and Table 2 (the Chronic Model).The main finding of this study was that both the NIAAA and chronic ethanol diet were not effective in altering the expression or protein content of the myokines FNDC5/irisin or myonectin. This finding may result from skeletal muscle's extensive regenerative capacity, as previously mentioned. Further, FNDC5/irisin and myonectin exert many of their effects in non-skeletal muscle tissue, such as hepatic and adipose tissue. Therefore, it would be of interest to examine these myokines and their relationship to parameters such as hepatic autophagy and adipose tissue browning in response to ethanol. Additionally, irisin was undetectable in the serum. In order to properly examine the functional role of irisin, novel, validated antibodies need to be both developed and utilized in future studies. Further, the receptor for irisin has yet to be elucidated; identification of this receptor will be beneficial for future studies and may highlight unknown functional roles of irisin. Lastly, the relationship between ethanol consumption and skeletal muscle as an endocrine organ has not been widely studied. Further elucidation of the molecular mechanisms implemented by skeletal muscle concerning inter-organ cross talk may highlight a wide variety of novel therapeutic treatments.

TABLE 1: Summary of Results for the NIAAA Model

Analysis	Males	Females
Skeletal Muscle FNDC5	No change	No change
Skeletal Muscle Myonectin	No change	No change
Serum Irisin	Undetectable	Undetectable
Serum Myonectin	Undetectable	Undetectable
Serum TNF- α	No change	No change
Serum IL-6	No change	Increased in ethanol fed mice
Serum PAI-1	Increased in ethanol fed mice	No change

TABLE 2: Summary of Results for the Chronic Model

Analysis	Males	Females
Skeletal Muscle FNDC5	No change	No change
Skeletal Muscle Myonectin	No change	No change
Serum Irisin	Undetectable	No change
Serum Myonectin	No change	No change
Skeletal Muscle FNDC5 Expression	Lower in ethanol fed mice	No change
Skeletal Muscle Myonectin Expression	No change	Higher in ethanol fed mice
Serum TNF- α	No change	Higher in ethanol fed mice
Serum IL-6	No change	Higher in ethanol fed mice
Serum PAI-1	Higher in ethanol fed mice	Higher in ethanol fed mice
Percent Total Fatty Acids	No change	No change

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