

Representing a Novel Model of Diet-Induced, Inherited Insulin Resistance and Obesity, Atp10c Heterozygous Mice Show Alterations in Glucose Uptake via both Insulin-Dependent and Non-Dependent Pathways

Sarah E Hurst¹, Lisa Amelse², Ling Zhao³ and Madhu S Dhar^{2*}

¹Department of Pharmaceutical Sciences, South College School of Pharmacy, Knoxville, Tennessee USA

²Department of Large Animal Clinical Sciences, College of Veterinary Medicine, University of Tennessee, Knoxville, Tennessee, USA

³Department of Nutrition, University of Tennessee, Knoxville, Tennessee, USA

Abstract

Diet-Induced Obesity (DIO) is a major risk factor for a number of disorders including non-insulin-dependent Type 2 Diabetes Mellitus (T2D). In our novel mouse model, the gene *Atp10c* is a strong candidate for metabolic disorders of Insulin Resistance (IR) and DIO. As such, we hypothesized that *ATP10C* has a key role in glucose metabolism via insulin-dependent and independent signaling pathways. We first examined the expression of *Atp10c* in both a genetic as well as an environmental mouse model of obesity. While our results showed that there were no significant changes in *Atp10c* expression in the genetic and environmental mouse models, our data does show high expression of *Atp10c* in key peripheral tissues namely skeletal muscle and adipose depots. Next, we performed western immunoblot analysis to detect potential targets of ATP10C in both the phosphatidylinositol-3-kinase (PI3K) and the mitogen-Activated Protein Kinase (MAPK) pathways. When accessing MAPK pathway proteins, the mutants showed a significant decrease in the ratio of activated to native forms of p38 and ERK1/2. Additionally, we observed differences in the PI3K pathway as there were significant

results along with data from our investigations of skeletal muscle prove our hypothesis that *Atp10c* must play a role in glucose metabolism, and suggest that the action of *Atp10c* is potentially mediated via both the MAPK pathway as well as the PI3K pathway.

Keywords: Glucose Metabolism; Diet- Induced Obesity; Type 2 Diabetes Mellitus; Insulin Resistance; Atp 10c/ATP10C; PI3K Pathway; MAPK Pathway

***Corresponding Author:** Madhu S Dhar, Department of Large Animal Clinical Sciences, University of Tennessee, 2700 River Drive, Knoxville, Tennessee 37966, USA; E-mail: mdhar@utk.edu

Abbreviations: DIO: Diet-induced obesity; T2D: type 2 diabetes mellitus; IR: Insulin Resistance; PI3K: Phosphatidylinositol-3-Kinase; MAPK: Mitogen-Activated Protein Kinase

Introduction

Diet- induced obesity (DIO) is a foremost risk factor for many common disorders, including non-insulin-dependent type 2 diabetes mellitus (T2D) [1], atherosclerosis [2], and non-alcoholic fatty liver disease (NAFLD) [3]. T2D is characterized

by insulin resistance (IR) in the muscle, fat, and liver, combined with a decreased ability of pancreatic β cells to properly respond to elevated glucose levels. Both DIO and T2D among adults in the United States continue to rise in both sexes. Moreover, in recent years, there has been a dramatic rise in the DIO and T2D rates among children and adolescences [4]. The prevalence of DIO and T2D is also on the rise throughout the world, with the projection that 366 million people will have T2D by 2030 [5]. Based on these increasing statistics, there is a dire need for new approaches to examine the correlation between DIO and T2D, to understand what causes these diseases, and to elucidate unknown players in metabolic pathways related to DIO and T2D. An important link between DIO and T2D is IR, the etiology of which is complex in humans, with both genetic predisposition and environmental factors coming into play [6-8]. A major field of interest is finding genes related to DIO and T2D in humans. This research however is faced with two major challenges. First, the genetic influence on these diseases is based on multiple, polymorphic single genes interacting with other genes that then may be exposed to specific environmental factors. Second, if research focuses on the relationship between a single gene and DIO and/or T2D while failing to control for other involved genes as well as environmental exposures that have not been identified, then both experimental and observational studies become difficult to interpret. Because these factors are difficult to control in human subjects, polygenic diseases can be better studied in animal models, and then deciphered into probable human homologs and phenotypes [9]. In our current mouse model, heterozygous *Atp10c* mice present with the disease states concurrent with pre-diabetes like IR and DIO as well as a host of other related disorders including hyperlipidemia and hyperinsulinemia. Previous research from our laboratory using these mice indicates that the *Atp10c* gene appears to be a strong candidate for DIO and T2D [10, 11]. *Atp10c* is a putative phospholipid translocase that encodes for a type IV P-type ATPase. *Atp10c* maps to the *p*-locus on mouse chromosome 7, to a region of a quantitative trait locus associated with body weight, body fat and diabetic phenotypes. The human ortholog, ATP10A, maps to the syntenic region on

chromosome 15q12 which is also associated with an elevated body mass index (BMI) [12]. Moreover, microarray gene profiling on *Atp10c* heterozygous mice indicated noteworthy changes in the mRNA expression of factors involved in GLUT 4-mediated glucose uptake in addition to changes in other metabolically relevant pathways [11]. Previous experiments from our laboratory have shown that on a high-fat (HF) diet, glucose uptake in the soleus muscle of *Atp10c* heterozygotes is decreased about 35% [10]. Recently, we determined that down regulation of *Atp10c* expression had a similar effect *in vitro* when *Atp10c* expression was transiently silenced in mouse skeletal muscle C2C12 cells [13]. In those *C210c*⁻ cells, insulin stimulation was shown to cause a significant 2.54-fold decrease in 2-DOG uptake ($P < 0.05$). This data thus complement previous *in vivo* findings, suggesting that *Atp10c* is necessary for insulin-stimulated glucose uptake in skeletal muscle and its knockdown renders the myotubes insulin resistant, again supporting the phenotype observed in the heterozygotes. As such, we suggest a direct correlation between *Atp10c* and glucose metabolism, strongly supporting its involvement in insulin signaling.

Although translocases like *Atp10c* have been studied for many years, their character and function remains vague. These proteins are suggested to maintain the asymmetry of the lipid bilayer by translocating specific phospholipids from one leaflet to the other and vice versa [14], but they may also participate in the formation of transport vesicles [15]. Moreover, deficiencies in these proteins have been shown to cause defects in lipid metabolism and have been implicated in the disease states of DIO, T2D and NAFLD [10-12]. Not much is known about the role of *Atp10c*/ATP10C in regulating IR, DIO, and/or T2D, and its possible molecular and cellular targets have not been investigated.

Since ATP10C is a novel and putative phospholipid translocase, in this study, we first wanted to assess whether its expression is influenced by genetic and/or environmental factors. To do so, we analyzed *Atp10c* expression in two mouse models of obesity, one genetic and the other was environmental. Based on previous results, and since, ATP10C is a

transmembrane domain protein, we hypothesized that ATP10C has an important role in glucose metabolism via insulin-dependent and independent signaling pathways. To investigate the biological role of *Atp10c*/ATP10C in these processes, adipose tissues from heterozygous *Atp10c* mice fed a HF diet for 4 and 12 weeks were harvested, and changes in key proteins of Mitogen Activated Protein Kinase (MAPK) and Phosphatidylinositol 3-Kinase (PI3K) pathways were analyzed using western immunoblot analysis. Based on previously published data, a time point of 4 weeks was chosen to simulate an acute or pre-diabetic condition and a time point of 12 weeks was chosen to stimulate a chronic or full T2D condition [10, 11].

Materials

Cell culture reagents and oligonucleotides were obtained from Thermo Fisher Scientific (Waltham, MA) along with Dulbecco's Modified Eagle Medium containing 4.5mg/mL glucose, and 4.5 mM/L L-glutamine (DMEM), antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin), Fetal Bovine Serum (FBS), and Radio Immuno Precipitation Assay (RIPA) buffer. DMEM with 1% antibiotics and 10% FBS is furthermore referred to as the complete growth media. Protease inhibitor cocktail in DMSO solution was obtained from Sigma Aldrich (St. Louis, MO). A Bicinchoninic Acid Kit (BCA) and an Enhanced Chemiluminescence (ECL) Western Blotting Detection Kit were purchased Pierce Biotech Inc. (Rockford, IL) and used in all protein experiments. Primary antibodies (p38, phospho-p38, JNK, phospho-JNK, ERK1/2, phospho-ERK1/2, AS160) as well as the secondary antibody anti-rabbit IgG, Horseradish Peroxidase (HRP)-linked, were obtained from Cell Signaling Technology (Danvers, MA). The antibody, caveolin-1, a Western immunoblotting control, was purchased from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA) along with the secondary antibody, donkey anti-goat IgG, HRP conjugated and the primary antibody, PI3K. An *Atp10c*-specific siRNA construct (Quantitect primer) was obtained from Qiagen (Valencia, CA). The iScript cDNA synthesis kit was acquired from Bio-Rad Laboratories (Hercules, CA). Quantitative RT-

PCR (qPCR) was performed for *Atp10c* mRNAs using ABsolute SYBR Green ROX quantitative PCR mix (Thermo Scientific, Waltham, MA) with *Gapdh* as an internal control. The *Gapdh* primers were commercially obtained from Operon (Huntsville, AL).

DIO mice as well as *ob/ob* and their control littermates were acquired from the Jackson Laboratory (Bar Harbor, ME). For DIO mice, C57BL/6J males were fed a HF diet (60% energy) (Research Diets, New Brunswick, NJ) or Regular Chow (RC) diet (Laboratory Rodent Diet, Checkers PMI Nutritional International, Brentwood, MO) for a period of 20 weeks before being sacrificed at age 26 weeks. Male *ob/ob* and their controls were fed the RC diet for a period of 8 weeks before being sacrificed at age 14 weeks.

Female mice containing the radiation-induced chromosomal deletion p^{23F1OD} located at the pink-eyed dilution (p) locus on mouse chromosome 7 were previously created at Oak Ridge National Laboratory (ORNL) [16, 17]. These mice are currently housed and maintained at the University of Tennessee as previously described [18]. These female mice and their littermate controls were used as *Atp10c* heterozygotes for experiments performed in this study. All animal procedures were approved by and in agreement with the University of Tennessee Institutional Animal Care and Use Committee (IACUC) (protocol #1309-1206).

Methods

The following procedures were performed as described elsewhere [19, 20]. Total RNA from skeletal muscle, liver, and adipose tissues was isolated using the TRIZOL method as described in Roshwalb, *et al.* [20] followed by purification using the RNeasy kit. Single-stranded cDNA was made using the iScript cDNA synthesis kit (Bio-Rad). The cDNA was then amplified using gene-specific primers (Quantitect) by qPCR; the internal control is mouse *Gapdh*. All mRNA expressions were achieved by qPCR using ABsolute SYBR Green ROX quantitative PCR mix reagents. The comparative abundance of each target gene expression was calculated using the $2^{-\Delta\Delta CT}$

and standard curve method; $\Delta\Delta CT$ is the difference between CT of the target gene normalized with respect to the internal control CT. All qPCR data is presented as the ratio of *Atp10c* expression to that of an internal control, *Gapdh* expression. Total cell lysates were collected using RIPA buffer [19-21]. Briefly, cells were sonicated for 5 seconds and lysed in RIPA buffer (with protease inhibitor cocktail) at 4°C for 30 minutes. Cell lysates were centrifuged at 16,000 g for 10 minutes at 4°C. Protein concentration was assessed using the Bicinchoninic Acid Kit (BCA) (Pierce Biotech Inc.). Western immunoblotting analysis was performed using the quantitated cell lysates [13, 20-21]. Equal concentrations ranging from 25 to 100 µg of protein were resolved on 10% SDS-PAGE, using 5X Laemmli sample buffer. Cell lysates were heated before application onto the SDS-PAGE gel. Afterwards, proteins were transferred to nitrocellulose membranes, blocked for 1 hour in 1-5% BSA in TBST buffer, and incubated with specific primary antibodies overnight at 4°C. The following day, primary antibody detection was conducted using HRP-linked secondary antibodies and an ECL Western Blotting Detection Kit (Pierce Biotech Inc.). Results here are expressed as the ratio of target protein expression to that of an internal control, caveolin-1.

Relative densitometry analyses of the Western immunoblots were determined using the software analysis program, Image J (<http://rsb.info.nih.gov/ij/index.html>). An arbitrary value of 1.0 was assigned with respect to the control sample (caveolin-1) of each experiment, and a ratio of relative density was calculated for each protein of interest. The resulting data are expressed as mean $\pm$ SE. For comparison of two groups (control versus mutant), p-values were calculated using the standard Student's t test (a two-tailed, two-sample with equal variance). In all cases, a p-value of $p \leq 0.05$ was considered to be statistically significant and a p-value of $p \leq 0.10$ were suggestive of a trend pattern.

Results

Based on previous results from our laboratory, *Atp10c* heterozygotes represent a novel model of DIO and T2D. To further validate this model, we examined the expression of

Atp10c in two standard mice models of obesity, genetic and environmental. Since we hypothesize that *Atp10c*/ATP10C plays a role in DIO and T2D, we wanted to examine its expression in key tissues (skeletal muscle, adipose and liver tissues) responsible for glucose homeostasis. As a putative trans-membrane domain protein, ATP10C protein analysis has proved challenging and thus far, the generation of a good antibody against ATP10C has not been successful. Therefore, for our experiments, alterations in the expression of *Atp10c* were determined solely at the mRNA level.

As shown in Figures 1 and 2, while not significant, *Atp10c* expression is up-regulated in the skeletal muscle (3.78 fold) as compared to adipose and liver tissues in the environmental model of obesity. Similarly, while not significant, *Atp10c* expression is up-regulated in both adipose tissue, (2.94 fold), and in the skeletal muscle (2.44 fold) in *ob/ob* mice when compared to their age and gender-matched wild type controls.

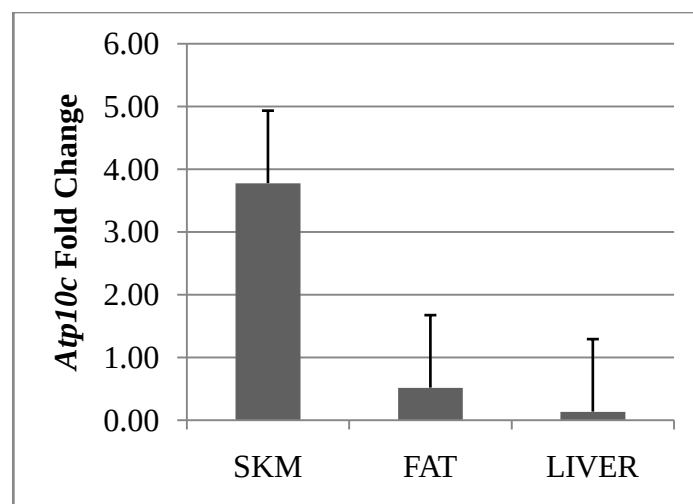


Figure 1: *Atp10c* expression was examined by qPCR in a DIO mouse model. *Atp10c* mRNA expression is highest in skeletal muscle and lowest in the liver for the DIO mice (n=6) compared to chow-fed controls (n=6). The expression of *Atp10c* is denoted as fold change and is represented normalized to *Gapdh*. Each sample was run in triplicate.

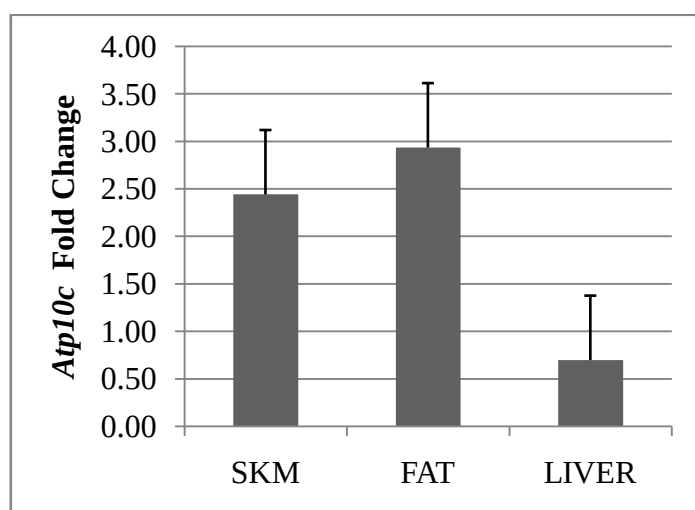


Figure 2: *Atp10c* expression was examined by qPCR in the *ob/ob* mice model. *Atp10c* mRNA expression is highest in fat tissue and lowest in the liver for the *ob/ob* mice (n=6) compared to wild-type controls (n=6). The expression of *Atp10c* is denoted as fold change and is represented normalized to *Gapdh*.

Data from cell culture studies using peripheral tissue models for skeletal muscle and adipose tissues show contrasting results. In C2C12 skeletal muscle cells, 85% knockdown of *Atp10c* resulted in a 2.54 fold significant decrease in glucose uptake, which might be of consequence to impaired insulin signalling in skeletal muscle [13]. Comparatively, in 3T3-L1 adipocytes, 88% knockdown of *Atp10c* renders the cells insulin responsive and resulted in a 4 fold increase in glucose uptake [22]. The differences we believe may involve the prominent signalling pathways for each tissue and as such, we sought to investigate whether the key proteins of MAPK and PI3K pathways are the targets of *Atp10c* in the adipose tissues collected from the *Atp10c* heterozygotes. Insulin-induced glucose uptake into key peripheral tissues like skeletal muscle and adipose tissue are known to involve a complex series of intracellular signalling cascades terminating in glucose disposal and metabolism [23-28]. Probable mechanisms include insulin-mediated activation of the insulin receptor and its various isoforms in addition to downstream molecules, namely PI3K pathway proteins; this mechanism ultimately effects GLUT4 expression and translocation. Alternatively, the non-insulin dependent pathway, MAPK, may also be effected thus altering

glucose uptake without any GLUT4 translocation. As these metabolic processes involve an intricate relationship with a variety of proteins and as their alterations have not been studied in *Atp10c*-silenced adipose tissue, we next sought to identify changes in the key players of these two pathways (PI3K and MAPK) in the absence of any insulin stimulation. Specifically, in the present study, the effect of *Atp10c*-silencing was studied on essential MAPKs (p38, JNK, and ERK1/2) and their activated or phosphorylated versions. For MAPK pathway analysis, results indicate a significant decrease in the ratio of phospho-p38 to native p38 protein expression (p-value = 0.0008) (Figure 3) at 12 weeks only and phospho-ERK1/2 to native ERK1/2 protein expression (p-value = 0.0003 and 8.9×10^{-6}) (Figure 4) at both 4 and 12 weeks. For phospho-JNK to native JNK protein expression (p-value = 9.1×10^{-5} and 0.0002) (Figure 5), there was a significant decrease at 4 weeks (p-value = 9.1×10^{-5}) and a significant increase at 12 weeks (p-value = 0.0002).

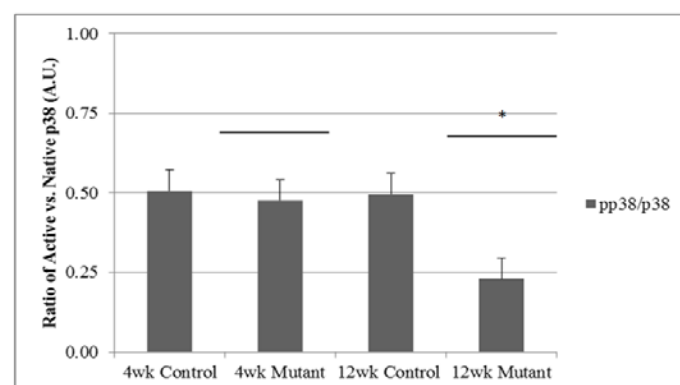


Figure 3: *Atp10c* heterozygous mice (n = 6 to 8) were fed a high-fat diet for 4 and 12 weeks, and then sacrificed. Proteins were collected from the adipose tissue of these mice and subjected to immunoblot analysis. Data shown are representative of multiple independent experiments (n = 2 to 4), all analyzed in triplicate. The expression of p38 and phospho-p38 is normalized to Caveolin-1, and the ratio of pp38/p38 is calculated; * p-value = 0.0008.

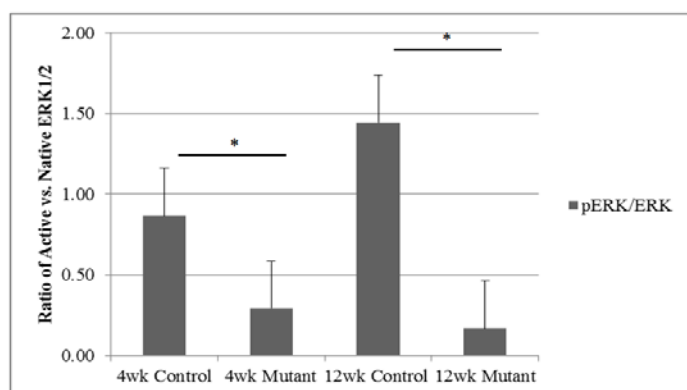


Figure 4: *Atp10c* heterozygous mice (n = 6 to 8) were fed a high-fat diet for 4 and 12 weeks, and then sacrificed. Proteins were collected from the adipose tissue of these mice and subjected to immunoblotting analysis. Data shown are representative of multiple independent experiments (n = 2 to 4), all analyzed in triplicate. The expression of ERK1/2 and phospho-ERK1/2 is normalized to Caveolin-1, and the ratio of pERK1/2/ERK1/2 is calculated; * p-value = 0.0003 and 8.9×10^{-6} .

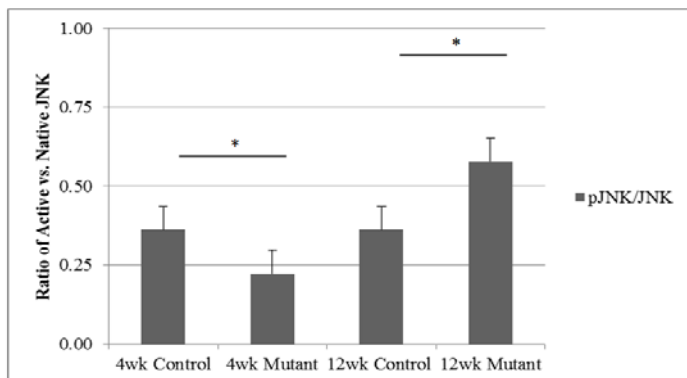


Figure 5: *Atp10c* heterozygous mice (n = 6 to 8) were fed a high-fat diet for 4 and 12 weeks, and then sacrificed. Proteins were collected from the adipose tissue of these mice and subjected to immuno blotting analysis. Data shown are representative of multiple independent experiments (n = 2 to 4), all analyzed in triplicate. The expression of JNK and phospho-JNK is normalized to Caveolin-1 and the ratio of pJNK/JNK is calculated; * p-value = 9.1×10^{-6} and 0.0002.

More extensively studied, insulin-dependent glucose uptake is mediated via the PI3K pathway. Results from PI3K and AS160

protein analysis show that the expression of PI3K is elevated significantly (p-value = 0.0007) (Figure 6) at 4 weeks in mutant mice. At the same time point, AS160 expression is also increased (p-value = 0.03) (Figure 7). At 12 weeks, however, the expression of both PI3K and AS160 decrease significantly (p-value = 0.0002 and 0.01 respectively) (Figures 6 and 7).

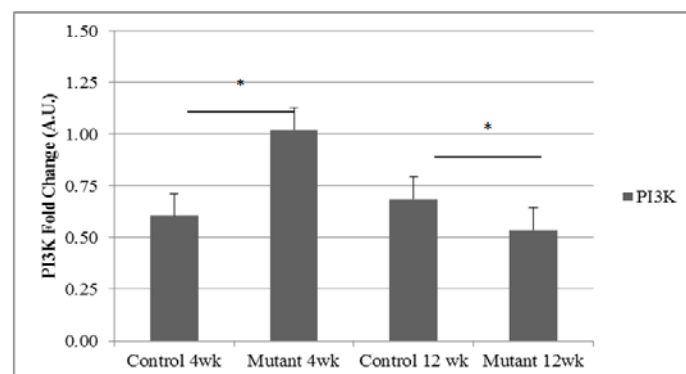


Figure 6: *Atp10c* heterozygous mice (n = 6 to 8) were fed a high-fat diet for 4 and 12 weeks, and then sacrificed. Proteins were collected from the adipose tissue of these mice and subjected to immunoblotting analysis. Data shown are representative of multiple independent experiments (n = 2 to 4), all analyzed in triplicate. The expression of PI3K is represented as the fold change normalized to Caveolin-1; * p-value = 0.0007 and 0.0002.

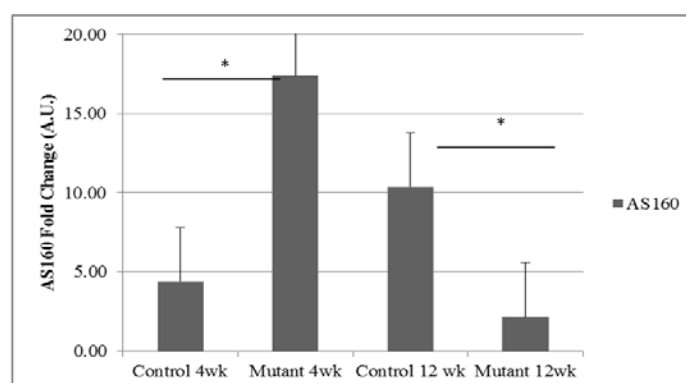


Figure 7: *Atp10c* heterozygous mice (n = 6 to 8) were fed a high-fat diet for 4 and 12 weeks, and then sacrificed. Proteins were collected from the adipose tissue of these mice and subjected to immunoblot analysis. Data shown are representative of multiple independent experiments (n = 2 to 4),

all analyzed in triplicate. The expression of AS160 is represented as the fold change normalized to Caveolin-1; * p-value = 0.03 and 0.01.

Discussion

The structure and function of many cell channels, ion transporters, and signal transducing proteins are affected by phospholipid randomization, and this effect has been implicated in numerous pathophysiology processes. As such, maintaining the organization and activity of the lipid bilayer must be maintained as it is essential for normal cell function. One class of proteins that perform this action is the type-IV ATPases [14, 15]. These proteins act by retaining the asymmetry of the lipid bilayer via the translocation of specific phospholipids. Although these ATPases have been studied for over twenty years, their exact biological nature remains unclear. While playing a vital part in membrane stability, they may also serve another function as a participant in the formation of transport vesicles such as glucose storage vesicles (GSVs) [14, 15]. Abundant yeast studies have revealed that these proteins cause the translocation of glycerophospholipids, and that this movement is crucial for both intracellular membrane and protein trafficking. Moreover, insufficiencies in proteins like ATP10C have been shown to cause defects in both lipid and glucose metabolism and as such are implicated in the disease states of DIO, T2D, and NAFLD [10-12]. Our laboratory has repeatedly demonstrated that *Atp10c* heterozygous mice are insulin resistant and have an altered insulin-stimulated response in peripheral tissues (skeletal muscle, adipose tissue) [11]. Moreover, another group of independent researchers recently cited ATP10C as a potential biomarker for DIO and other related metabolic disorders [9].

Prior research using *Atp10c* heterozygous mice indicates that the *Atp10c* gene appears to be a strong candidate for DIO and T2D. Our mouse model is diet-induced and shows IR characterized by hyperinsulinemia, hyperglycemia, hyperlipidemia and obesity-associated glucose intolerance [10-12]. While none of the tissues from the *ob/ob* or DIO mice show significant expression of *Atp10c* when compared to their age

and sex-matched controls, the results are trending towards an up-regulation of *Atp10c* mRNA in skeletal muscle (in DIO mice) and in adipose tissue (in *ob/ob* mice). As reported by Keightley *et al.* [29] and confirmed by a previously published paper from our laboratory, *Atp10c* maps to a region of quantitative trait locus on mouse chromosome 7, and its contribution towards the DIO and T2D phenotypes is relatively low (roughly 3%). Despite this low percentage, *Atp10c* has shown to be important in both DIO and T2D making functional investigations involving these disease processes not only warranted, but necessary [12, 29]. Overall, in the genetic model of obesity (*ob/ob*), *Atp10c* expression was highest in adipose tissue (2.94 fold), thus advocating that *Atp10c* plays an important part in adiposity, and such a pronounced effect can then, in turn, effect tissues in the body like skeletal muscle and liver. Studies in our laboratory have further demonstrated that *Atp10c* encodes an aminophospholipid translocase and is a type 4 P-type ATPase involved in several processes including, but not limited to, lipid trafficking, maintenance of the phospholipid asymmetry, and fluidity of the plasma membrane. Furthermore, as results would indicate, *Atp10c* appears to be involved in body fat regulation. Evidence continues as mice inheriting a maternal deletion of *Atp10c* are considered a unique model of DIO and T2D as they have more severe hyperinsulinemia, IR, and NAFLD when compared to mice inheriting the same deletion paternally. Likewise, simulations of maternal deletion of *Atp10c* point to an anabolic metabolism consistent with the known clinical phenotypes of DIO [9]. For the DIO mice, the expression of *Atp10c* was higher in the skeletal muscle (3.78 fold), suggesting that changes in diet appear to affect the expression of *Atp10c* validating its role in DIO and other related disorders like IR and T2D. Moreover, results here demonstrate that diet induces a greater response in skeletal muscle than it did in adipose tissue. In a 2004 study, Dhar *et al.* [10] showed that when these mutant mice were placed on a HF diet (45% energy), factors such as insulin, leptin and triglyceride plasma levels as well as body weight and adiposity index were significantly greater in the mutants when compared with their age and sex-matched controls fed the same HF diet.

Additionally, glucose and insulin tolerance tests were performed on these mice, and the *Atp10c* mutants showed an altered tolerance/response to both glucose and insulin challenges compared with control mice. Moreover, standard gross and histological evaluations of key organs (liver, pancreas, adipose tissue, and heart) were performed and histological evaluation of the liver showed micro and macrovesicular lipid deposition within the hepatocytes which was more severe in the *Atp10c* mutant mice than in age-matched controls. Important to note, the 2004 Dhar *et al.* study [10] focused primarily on the DIO issues and therefore, studies were exhaustive for adipose tissue.

Insulin stimulation results in the activation of two known and distinct pathways; the PI3K pathway and the MAPK pathway, both of which are crucial for glucose uptake [23-28, 30-32]. In order to study the effect *ATP10C* has on the MAPK signalling pathway, we performed Western immunoblotting analysis on specific pathway proteins. As illustrated in Figures 3, 4, and 5, there is a significant decrease in active p38 and ERK1/2 proteins in *Atp10c* heterozygotes after they are fed the HF diet for 12 weeks. Our study additionally showed that the expression of active JNK increased at the same time point. Data clearly shows that mis-expression of *Atp10c* in the heterozygotes significantly decreases the activation of these proteins as demonstrated by comparing the phosphorylated forms to the native ones. Given the data presented, it appears that at 4 weeks, the activity of the PI3K pathway is up-regulated as there is a significant increase in PI3K and AS160 expression, while there is abnormal MAPK pathway activity as the activated forms of key proteins (p38, ERK1/2, and JNK) are significantly down-regulated. As such, the PI3K pathway appears in control of normal glucose uptake, resulting in normal glucose clearance from the body. Conversely, at 12 weeks, the PI3K pathway activity decreases along with activity in the MAPK pathway. This then, puts more stress and strain on both pathways as validated by the significant increase in active JNK expression (p-value = 0.0002) and as such, glucose metabolism. Multiple independent studies have reported JNK activity increasing in response to T2D. As such, elevated JNK levels and subsequent activity interferes with insulin action both *in vitro* and *in vivo*

models [33]. Numerous studies have concluded that glucose uptake is stimulated by insulin which when bound to its receptor causes the rapid recruitment of GLUT4 from intracellular compartments to the cell surface of skeletal muscle and adipose tissue. However, many reports show that the extent of this GLUT4 recruitment in response to an acute insulin challenge is less than the extent of the increase in glucose uptake [27, 30]. This discrepancy between glucose uptake and GLUT4 translocation may be reconciled if the intrinsic activity of GLUT4 were also elevated by insulin. Taken together, these observations suggest that GLUT4 at the cell surface may require activation by some critical mechanism before it can transport glucose to its maximum capacity [30-32]. It has been suggested by various researchers that insulin increases glucose uptake by first increasing GLUT4 translocation and then further activating the additional transporters in a separate pathway from the PI3K pathway. It is hypothesized that after translocation toward the plasma membrane, there is yet another MAPK-dependent step, which leads to insulin induced glucose uptake progress [34]. Researchers have recently discovered that the MAPK protein, p38, is in fact a necessary component for insulin-stimulated glucose uptake. These studies went on to show that inhibition of p38 actually prevented insulin-stimulated glucose uptake, but not GLUT4 translocation, suggesting that p38 works to regulate this unknown activation step. For that reason, in acute situations, p38 has shown to be beneficial to GLUT4-mediated glucose uptake. However, when chronic insulin exposure occurs as in the case of T2D, p38 was shown to contribute to the development of IR, whereas inhibition of p38 improved GLUT4 protein levels improving insulin-stimulated glucose uptake. Moreover, the unfavourable effects of p38 activation are further supported by the observation that constant activation of the p38 pathway in fact decreases GLUT4 mRNA and protein levels resulting in a decrease in insulin-stimulated glucose uptake as well [35-37]. In addition, microarray and qPCR profiles of gene expression from *Atp10c* heterozygotes show significant variations in the expression of both *Glut4* and *p38* (also referred to as *Mapk14*) [11]. In that study, the expression of *Atp10c* is also significantly changed in the adipose tissue and the soleus

muscle of the mice after eating the HF diet for a period of 12 weeks. It can be assumed that expression of important PI3K pathway proteins should increase at 12 weeks in order to counteract the initial effects of DIO and *Atp10c* deletion. Early results from PI3K and AS160 protein analysis show that the expression of both PI3K and AS160 are significantly elevated at 4 weeks in the mutant mice. However, at 12 weeks, these proteins can no longer keep their expression levels elevated and decrease significantly. As demonstrated by our results, *Atp10c* may play a role in the activation of these proteins further downstream in the PI3K pathway, a portion of which is still largely unknown. For future experiments, we would like to analyze various insulin receptors/substrates and perform GLUT4 translocation in both types of tissues using adipose tissue and skeletal muscle cell culture models to investigate changes in glucose transporter expression.

Data from our investigations of skeletal muscle suggest that *Atp10c* is effecting the non-insulin dependent MAPK pathway as well as the insulin-dependent pathway, PI3K [13], but that for each pathway, the roles in the peripheral tissues differ. There is no “one-size fits all” when it comes to these complex metabolic issues. Hence, to understand the pathogenesis of IR, DIO and T2D, a study of ATP10C at the cellular as well as the molecular level is critical.

As we have seen from our own studies, not all tissues are alike in their responses as adipose tissue and skeletal muscle do not behave the same in relation to IR and T2D. In order to elucidate the differences, experiments specifically targeted to the generation of adipose tissue-specific and skeletal muscle-specific transgenic mice are necessary. Tissue-specific expression of *Atp10c* transgenes will determine if *Atp10c* expressed solely in skeletal muscle or in adipocytes will complement the mutant phenotypes. These experiments will give us information about defects associated with the heterozygous deletion of *Atp10c* in a target tissue without any interference from its expression/mis-expression in the other. Moreover, future work on these *Atp10c*-mutant mice needs to be conducted in order to focus on the skeletal muscle consequences of this genetic aberration. Results from this study as well as

numerous others show that skeletal muscle is a vital player in both glucose and lipid metabolism. Likewise, all these results give direct evidence of the significance of both genetics and environmental conditions in the disease states of DIO and T2D.

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