Investigating IL-15 Metabolic Impact and its Mechanism of Action in Skeletal Muscle Cells

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LIST OF ABBREVIATIONS

Abbreviation	Definition
ACC	Acetyl-CoA Carboxylase
AMP	Adenosine monophosphate
AMPK	AMP-activated Protein Kinase
CaMKKβ	Calmodulin-dependent Protein Kinase Kinase β
CPT	Carnitine palmitoyltransferase
CRTC2	CREB-Regulated Transcription Coactivator 2
EDL	Extensor digitorum longus
ERR	Estrogen-related receptor
FABPpm	fatty acid binding protein
FAT/CD36	Fatty acid translocase
FATP1	fatty acid transport protein
FFA	Free fatty acid
G6P	glucose-6-phosphatase
GAP	GTPase-activating proteins
GLUT	Glucose transporter
HFD	High fat diet
IL	Interleukin
IL-15LSP	IL-15 long signal peptide
IL-15Rα	IL-15 receptor alpha subunit
IL-15SSP	IL-15 short signal peptide
IL-2Rβ	IL-2 receptor β subunit
IRS	Insulin receptor substrate
JAK	Janus kinases
LKB1	Liver Kinase B1
LPS	Lipopolysaccharides
MHC	Myosin Heavy Chain
mTOR	mammalian target of rapamycin
NAD	Nicotinamide adenine dinucleotide
NK	Natural Killer
OXPHOS	Oxidative phosphorylation

Abbreviation	Definition
PEPCK	phosphoenolpyruvate carboxykinase
PI	phosphatidylinositol
PGC1	Peroxisome proliferator-activated receptor gamma coactivator
PKA	Protein kinase A
PPAR	Peroxisome proliferator-activated receptor
Pprc1	PPARγ coactivator-related protein 1
sIL-15Rα	soluble IL-15 receptor alpha subunit
SIRT1	NAD-dependent deacetylase sirtuin-1
STAT	signal transducer and activator of transcription
T2D	Type 2 diabetes
TAK	Transforming growth factor-β activated protein kinase
TBC1D	TBC1 domain
TSC2	tuberous sclerosis complex 2
UCP	Uncoupling protein
UTR	Untranslated region
WAT	White adipose tissue
β-GPA	β-guanidinopropionic acid
γc	γ chain

ABSTRACT

Skeletal muscle secretes many signalisation proteins named myokines. These myokines act as hormones and induce metabolic changes throughout the whole body to facilitate adaptation to physical exercise. Interleukin-15 (IL-15) is highly expressed in skeletal muscle and appears to influence many metabolic parameters that are defective in metabolic pathologies such as insulin resistance. For instance, IL-15 increases glucose uptake in muscle and whole-body fatty acid oxidation and its overexpression in skeletal muscle in mice generates a very lean and active phenotype. However, there are discordant reports throughout scientific literature. The aim of the current study was to 1) characterize the metabolic effects of IL-15 in L6 myotubes to determine whether L6 is a good model to study IL-15 and 2) to determine whether IL-15 activates the AMPK signaling. L6 myotubes were exposed to different concentrations of IL-15 and different metabolic parameters were assayed namely; oxygen consumption, glucose uptake, fatty acid oxidation, Glucose transporter 4 (GLUT4) translocation, oxidative phosphorylation (OXPHOS) complexes protein expression, troponin T expression and Akt, AMPK and Acetyl-CoA Carboxylase (ACC) phosphorylation state. Acute IL-15 treatment increased glucose uptake without activating insulin signaling pathway or GLUT4 translocation. Furthermore, acute IL-15 treatment increased resting oxygen consumption rate (OCR) while chronic IL-15 treatment also increased mitochondrial spare capacity, suggesting an increased mitochondrial biogenesis. IL-15 induced ACC phosphorylation in a dose-dependent manner and tended to increase AMPK phosphorylation but it did not reach statistical significance. Lastly, IL-15 did not influence troponin T state. Altogether, the present study demonstrates that L6 myotubes do not express all the pro-oxidative qualities of IL-15

reported by scientific literature. Nonetheless, IL-15 induces certain pro-oxidative metabolic effect that could help people living with obesity and diabetes.

1 INTRODUCTION

1.1 TYPE 2 DIABETES (T2D) AND INSULIN RESISTANCE

Insulin is a hormone that triggers glucose transport in cells and constitutes a prominent glycemic regulator. Insulin resistance is a physiological condition where tissues and organs have a decreased response to insulin. This condition is an important factor that contributes to type 2 diabetes (T2D) development. T2D is a pathological condition where storage and utilization of energetic substrates are impaired. As Nolan et al. (2011) eloquently described "T2D can be characterised by many factors such as failure of β cells islets to compensate for glucose excess, increased glucagon secretion and reduced incretin response, impaired expansion of subcutaneous adipose tissue, hypoadiponectinaemia and inflammation of adipose tissue, increased endogenous glucose production and development of peripheral insulin resistance. Furthermore, under T2D conditions, excess energy is stored around visceral organs and in ectopic organs such as liver, heart, skeletal muscle and pancreas, which causes tissue damage" (Nolan, Damm, and Prentki 2011)

Hence, T2D is a complex condition with many imminent side effects. It is also widely spread pathology. Nowadays, the Canadian Diabetes Association counts 11 million Canadians living with diabetes or prediabetes (Canadian Diabetes Association (2016)). Understanding metabolic pathways involved in T2D is key for the development of a therapeutic strategy to treat this public health issue.

1.1.1 Skeletal muscle and insulin resistance development

Skeletal muscle is the largest organ in the human body constituting around 40-50% of total body mass of non-obese individuals. It is also an important mediator of glucose homeostasis as it is accountable for 80% of insulin-induced glucose disposal in the post-prandial period (Ferrannini et al. 1985). Because of its major role in glucose homeostasis, impairment of skeletal muscle insulin sensitivity contributes greatly to T2D development and complications (DeFronzo and Tripathy 2009).

There are several mechanisms that alter skeletal muscle insulin sensitivity and all together could engender insulin resistance in skeletal muscle. Many studies demonstrated that high levels of circulating free fatty acids (FFA), a risk factor often found in obese individuals, is an important factor in insulin resistance development (Shulman 2000). Rats infused with lipids develops insulin resistance in skeletal muscle (Griffin et al. 1999). Insulin resistance can be replicated *in vitro* by exposing muscle cells to high concentrations of palmitic and stearic acid (Hirabara, Curi, and Maechler 2010). Randle *et al.* proposed that high circulating FFA levels repress glucose uptake by indirectly inhibiting glycolysis and by prioritizing fatty acid over glucose oxidation as energy fuel. In other words, high lipid supply to skeletal muscle results in higher fatty acid uptake and oxidation in skeletal muscle. Higher fatty acid oxidation raises acetyl-CoA levels which allosterically inhibit pyruvate dehydrogenase and increase citrate levels and ATP/ADP ratio. High citrate level and ATP/ADP ratio allosterically inhibit phosphofructokinase, an important glycolysis regulator, which results in the accumulation of its substrate: glucose-6-phosphate. Glucose-6-phosphate accumulation inhibits hexokinase which converts glucose to glucose-6-phosphate. Accumulation of intramyocellular glucose decreases glucose uptake in skeletal muscle (Randle, P. J., Garland, P. B., Hales, C. N.,

Newsholme 1963). However, Randle's hypothesis that high fatty acid oxidation inhibits glucose utilization by skeletal muscle is not sufficient to explain why high circulating FFA impairs skeletal muscle's insulin sensitivity. For instance, Randle's hypothesis imposes that glucose and glucose-6-phosphate accumulation must happen before glucose transport impairment. However, in humans infused with lipids, Roden *et al.* (1996) observed rather a decrease in glucose-6-phosphate levels in skeletal muscle which is subsequently followed by an insulin resistance-induced depletion of glycogen stock. This observation suggests that high circulating FFA level induce insulin resistance by altering glucose transport or phosphorylation (Roden et al. 1996). Some studies show that FFA directly alters glucose transport pathway without compromising glycolysis pathway (Yu et al. 2002; Cusi, Maezono, Osman, Pendergrass, Patti, Pratipanawatr, DeFronzo, Kahn, and Mandarino 2000; Griffin et al. 1999; Le Marchand-Brustel 1999; Anai et al. 1999). For instance, rats infused with lipids display reduced levels of IRS1 and 2 phosphorylation and lower PI3-kinase activity in muscle (Yu et al. 2002). Decreased tyrosine phosphorylation of IRS1 in muscle was also observed in obese (Cusi, Maezono, Osman, Pendergrass, Patti, Pratipanawatr, DeFronzo, Kahn, and Manadino 2000) and lipid-infused humans (Griffin et al. 1999) and in obese (Le Marchand-Brustel 1999) and high fat diet (HFD) mice (Anai et al. 1999). The inhibition of IRS1 tyrosine phosphorylation may arise from the phosphorylation of other of its residues (serine/threonine residues) by different kinases such as PKCs, IKKβ, JAK and p38 MAP kinase which induce a negative feedback loop and inhibit the insulin signaling pathway (Tirosh et al. 1999).

1.2 PHYSICAL ACTIVITY AND INSULIN RESISTANCE

1.2.1 Physical inactivity and insulin resistance development

Obesity and T2D epidemic can be justified by the combination of a genetic predisposition and behavioral factors that include diet and physical activity. Physical inactivity is closely related to insulin resistance and T2D development (Mcguire et al. 2001; Mikines et al. 1991; Mikines et al. 1989). Bed rest studies demonstrated that physical inactivity deeply reduces whole body insulin response and alters the expression of 54% of oxidative metabolism pathway genes including those involved in mitochondrial biogenesis (Patti et al. 2003). Healthy males that stopped exercising and refrained from performing more than 1,500 steps per day for two weeks showed decreased respiratory capacity, lower glucose infusion rate (17%), displayed lower insulin sensitivity. Furthermore, their muscle presented alteration in proteins involved in insulin signaling pathway. For instance, Akt phosphorylation in response to insulin and lower TBCD1D4 expression in skeletal muscle (Krogh-Madsen et al. 2010). Interestingly, total physical inactivity is also linked to an increase in visceral fat deposition (Belavý et al. 2014). Visceral fat deposition is a source of low-grade systemic inflammation and a contributor to insulin resistance development (Koenen et al. 2011).

1.2.2 Beneficial effect of physical activity for the treatment of insulin resistance

On the other hand, physical exercise is an excellent adjunct treatment for T2D in combination with a hypocaloric diet and medication (Molitch et al. 2003). A lifestyle change intervention decreases diabetes incidence with more efficiency than a metformin prescription (the most common drug used to treat T2D) in persons at high risk to develop the disease (Molitch et al. 2003). It is not surprising that physical activity is beneficial for patients with T2D, since muscle contraction stimulates GLUT4 transcription and translocation to the plasma membrane,

enhancing glucose transport into the cell (Richter and Hargreaves 2013; Kraniou et al. 2006). Also, insulin sensitivity is improved hours following the exercise, but this positive effect of exercise is eventually lost after a long period of physical inactivity (Way et al. 2016). When physical exercise is performed regularly, skeletal muscle undergoes multiple changes that increase its mitochondrial content through the action of multiple transcription factors such as PGC-1α and β, peroxisome proliferator-activated receptors (PPAR)s and estrogen-related receptor (ERR)α (Egan and Zierath 2013). A 6-week aerobic training is enough to increase muscle's mitochondrial density by 50 to 100% (Egan and Zierath 2013). This increase in mitochondrial content consequently increases expression of proteins involved in ATP production (Holloszy 1967) and the Krebs cycle (Egan et al. 2011). Furthermore, physical training increases the activity of enzymes involved in transport and oxidation of fatty acids (Talanian et al. 2010), glycolysis (Tremblay, Simoneau, and Bouchard 1994), antioxidant capacity (Gonchar 2005), glucose transport and glycogen synthesis (Perseghin et al. 1996). The combination of higher metabolic enzyme activities and greater mitochondrial content enhances the capacity of muscle to produce ATP. Furthermore, after an exercise, the trained muscle produces less AMP, ADP, Pi and ammonia than an untrained muscle. Lower levels of AMP and ATP attenuate their inhibition on glycolysis and glycogenolysis, resulting in the reduction of the use of carbohydrates as energetic fuel after exercise (Leblanc et al. 2004). Lipid utilization, however, is increased to compensate (Talanian et al. 2010). Overall, physical training remodels skeletal muscle to be more fatigue resistant by reducing glycogen storage depletion and enhancing ATP production during ATP turnover.

1.2.3 Myokines and their potential role in the beneficial effect of exercise to treat insulin resistance

Regular physical activity has been shown to repress inflammatory response following an exercise and to induce beneficial metabolic effects through the production of small peptides derived from contracting muscle called myokines (Mathur and Pedersen 2008). Myokines can act in a paracrine, endocrine or autocrine fashion and modulate many metabolic parameters (Pedersen and Febbraio 2012). Myokines regroup multiple cytokines families. For instance, myostatin is a cytokine that promotes muscle atrophy (McPherron, Lawler, and Lee 1997) and its expression is repressed by muscle contraction (Louis et al. 2007). Irisin is another cytokine secreted by skeletal muscle in response to muscle contraction that induces browning of white adipose tissue into beige adipose tissue and promotes insulin sensitivity, osteoblasts differentiation and enhances cognitive capacities (Chen et al. 2016). Many interleukins are also secreted during muscle contraction. However, their metabolic effects vary from one interleukin to another and, for many, their mechanism of action remained undetermined. For instance, the most studied myokines, IL-6, acts both in an endocrine and autocrine way. This myokine is secreted by skeletal muscle following muscle contraction and stimulates muscle glucose uptake (Carey et al. 2006), liver glucose production (Febbraio et al. 2004) and adipose tissue lipolysis (Petersen et al. 2005).

1.3 IL-15: A MYOKINE?

Interleukin 15 (IL-15) is a 14 kDa protein composed of 4 alpha helixes folded in a bundle which classifies it as part of the helical cytokine family (Grabstein et al. 1994). This cytokine is highly conserved among species, with 73% of nucleotide and amino acid sequence identity between humans and mice (Anderson et al. 1995). IL-15 is expressed in many tissues such as

placenta, heart, lung, liver, kidney, brain, testis and skeletal muscle (Satoh et al. 1998; Tagaya et al. 1997; Grabstein et al. 1994). IL-15 expression in muscle gained an important attention from the scientific community since skeletal muscle highly expresses IL-15 (Grabstein et al. 1994). Recent research demonstrated that IL-15 has anabolic and oxidative effects on skeletal muscle (Pistilli and Quinn 2013). Different stresses such as muscle contraction, diet and aging influence IL-15 expression in skeletal muscle (Pistilli and Quinn 2013). Therefore, skeletal muscle is thought to be an important source of this cytokine. Nonetheless, there are no *in vivo* studies that directly demonstrate skeletal muscle's contribution to circulating IL-15.

1.3.1 IL-15 expression and genic regulation

IL-15's coding sequence is composed of 9 exons (H. Nishimura et al. 2000; Onu et al. 1997). IL-15 expression is regulated during transcription by its peptide signal sequence. A peptide signal is a short amino acid sequence that drives the protein to its secretion through the endoplasmic reticulum, Golgi apparatus or endosomes. From the primary mRNA transcript, two IL-15 isoforms can be formed through alternative splicing. One is constructed with exons 1 to 8 which produces a protein with a 48 amino acids long signal peptide before upstream of IL-15 sequence (IL-15LSP). The second isoform possesses an extra exon, exon 4a, which introduces a STOP codon followed by a weak Kozak translation initiation site. Consequently, translation is initiated further downstream of IL-15 transcript and results in the production of a shorter signal peptide sequence (IL-15SSP) of 21-aa (Onu et al. 1997). Because of its small signal peptide sequence, IL-15SSP transcript stays in the cytosol and produce a peptide that acts as an intracellular messenger. Meanwhile, because of its longer signal peptide, IL-15 derived from IL-15LSP is localized either at the Golgi apparatus, early endosomes or the endoplasmic reticulum and enters the secretory pathway to be secreted and to act as a cytokine

(Gaggero et al. 1999). Also, to regulate IL-15 intracellular localization, the signal peptide also modulates expression levels and stability of IL-15 protein. Transfection of human embryonic kidney 293 cells with IL-15SSP resulted in IL-15 production 40 times lower than cells transfected with IL-15LSP (Bergamaschi et al. 2009). Also, IL-15SSP has a half-life of 20 min while IL-15LSP half life is 80 min (Bergamaschi et al. 2008). It was also proposed that production of IL-15SSP represses IL-15 production sequestering further IL-15 secretion (Hitoshi Nishimura et al. 2005).

The production and secretion of IL-15 protein is also restrained by three mechanisms. First, the 5'-UTR of IL-15 contains 8 to 10 AUGs upstream to the main translation initiation site. These AUGs handicaps the translational process of IL-15 as the removal of those nucleotides quadruples IL-15 production rate (Bamford et al. 1996; Bamford et al. 1998). Second, the signal peptide of IL-15 is rather inefficient to drive cytokine production. The signal peptide is a small amino acid sequence responsible to localize IL-15 production at the endoplasmic reticulum and thus introduce IL-15 in its secretory pathway. Replacing the signal peptide of IL-15 for IL-2's signal peptide increases IL-15 production by a 20 fold-increase (Bamford et al. 1998). Third, there are evidences of the presence of negative regulatory elements in IL-15 3'-UTR. For instance, replacing the 3'UTR of IL-15 by an artificial epitope increased IL-15 production by 5 to 10 times (Bamford et al. 1998). Altogether, these three regulation mechanisms explains why mRNA levels of Il-15 do not correlate with the protein levels of IL-15 (Waldmann and Tagaya 1999).

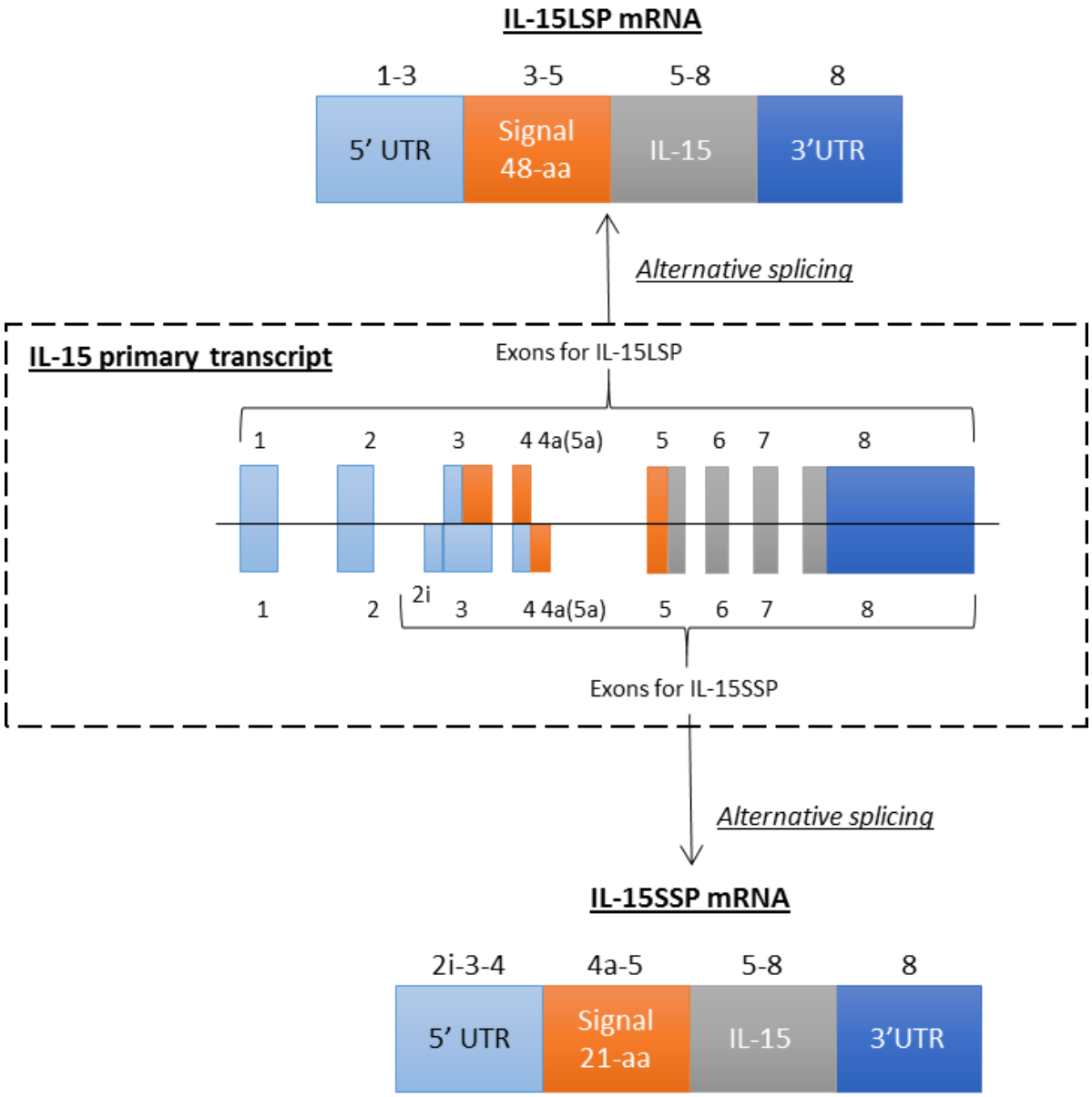

Figure 1: Diagram of IL-15 mRNA regulation. IL-15 gene consists of nine exons and eight introns located on human chromosome 4q31 and mouse chromosome 8. Two mRNA can be produce from the primary IL-15 gene transcript through alternative splicing; IL-15SSP and IL-15LSP. This figure was modified from (Budagian et al. 2006).

1.4 IL-15 AS AN INFLAMMATORY CYTOKINE

IL-15 receptor is made of three subunits:

- **IL-15Rα:** an alpha subunit that binds specifically IL-15
- **IL-2Rβ:** a beta subunit that is common with IL-2
- **γc:** a gamma chain common to several other cytokines such as IL-4, IL-7, IL-9, and IL-21 (Leonard, Shores, and Love 1995).

IL-15Rα has a high affinity for IL-15 ($K_a \geq 10^{11}M^{-1}$), as such that IL-15Rα can bind IL-15 even in the absence of IL-2Rβ/γc complex (Giri et al. 1994). The binding of IL-15Rα to IL-15 happens intracellularly after translation. IL-15Rα helps to stabilize IL-15 and protects its degradation which also enhances IL-15Rα/IL-15 secretion (Bergamaschi et al. 2008). A soluble IL-15Rα isoform (sIL-15Rα) is generated by a proteolytic cleavage with the help of the metalloproteinase ADAM17 (Mortier et al. 2004). IL-15Rα seems to be essential for IL-15 secretion in muscle since isolated muscle cells from IL-15Rα deficient mice lost their ability to secrete IL-15 in response to an LPS treatment (O'Connell and Pistilli 2015).

As opposed to the IL-15Rα, the IL-2Rβ and γc subunits only serves as transducing IL-15 signals across the plasma membrane. Both subunits contain docking sites for signal transducer and activator of transcription (STAT) proteins (Budagian et al. 2006). Activation of IL-2Rβ/γc phosphorylates and activates Janus kinases (JAK)s. IL-2Rβ activates JAK1 while γc activates JAK3 which subsequently activates STAT3 and STAT5 respectively (Johnston et al. 1995). STAT-activated transcription factors either form homo or heterodimers that translocate to the nucleus to modulate transcription of different target genes or DNA regulatory element (Leonard 2001). JAK/STAT activation by IL-15 plays an important role in inflammatory

response and T-cell proliferation and differentiation. Knocking out STAT5 in mice resulted in a deficiency of Natural Killer (NK) cells (Teglund et al. 1998). IL-15 also promotes survival and proliferation of T, CD8+ and NK cells (Budagian et al. 2006). Besides, IL-15 protects B lymphocytes, mast cells, neutrophils and eosinophils from apoptosis and induces productions of other cytokines by neutrophils, dendritic cells, monocytes, and macrophages (Budagian et al. 2006)

1.5 IL-15 AS A MYOKINE

Skeletal muscle display high levels of IL-15 mRNA (Grabstein et al. 1994; Lebris S Quinn, Haugk, and Grabstein 1995) with a preference for the transcription of the secretable IL-15LSP isoform (Tagaya et al. 1997). Despite variability in IL-15 transcription rate based on muscle fiber type, IL-15 protein levels are very similar in all muscle at resting states in humans (Nielsen et al. 2007). Since skeletal muscle possesses a high level of dormant IL-15 mRNA ready for translation, it is reasonable to hypothesize that skeletal muscle may represent an important source of IL-15 in circulation. It has been demonstrated that skeletal muscle is capable of secreting IL-15 in response to different stimuli such as the presence of pro-inflammatory factors (O'Connell and Pistilli 2015). Another potential stimulus that could trigger IL-15 secretion from skeletal muscle is muscle contraction. However, throughout the scientific literature, many studies display contradicting conclusion on whether or not muscle secretes IL-15 in response to exercise. The discrepancy throughout scientific literature may originate from differences in experimental design, the health status of subjects or the type of exercise performed by subjects. The following section will address the different contradicting studies which are also summarized in Table 2 (see Appendice 1).

1.5.1 Effect of physical exercise on IL-15 secretion

1.5.1.1 IL-15 secretion during endurance exercise

Certain studies demonstrated that IL-15 levels in circulation increased 5 to 10 minutes after an endurance exercise in untrained human (Tamura et al. 2011; Pierce, Maples, and Hickner 2015; Lee et al. 2011) and mice (Crane et al. 2015). A similar response was also observed in humans performing a resistance exercise (Bazgir et al. 2014; Riechman et al. 2004). Other research groups observed that circulating IL-15 levels increase only 30 min after an aerobic exercise in humans (Pierce, Maples, and Hickner 2015) and mice (LeBris S. Quinn et al. 2014). Meanwhile, some publications did not observed any increase in circulating IL-15 levels after an endurance exercise in humans (Rinnov et al. 2014; Ostrowski et al. 1998).

This discrepancy in scientific literature might be due to different timing of blood sampling after the endurance exercise. For instance, Rinnov *et al.* (2014) took their first blood sample 1.5h after exercise which could be enough time for IL-15 circulating levels to return to basal levels. Ostrowski *et al.* (1998) not only did not sample blood immediately after the treadmill exercise, potentially missing the peak of increased IL-15, but also analyzed circulating IL-15 levels of only two subjects out of their 32 participants which may not be representative of the overall group. Also, Ostrowski *et al.* (1998) selected endurance-trained subjects for their study whereas in other studies subjects were not very active (Tamura et al. 2011; Riechman et al. 2004; Rinnov et al. 2014; Pierce, Maples, and Hickner 2015; Lee et al. 2011). This difference in research subject's health status raises the question whether adaptation to physical exercise by training could influence skeletal muscle IL-15 secretion in response to exercise.

1.5.1.2 Circulating IL-15 levels in response to endurance training

Some research group looked at the effect of physical training on circulating levels of IL-15 but again, important differences in experimental design prevent establishing clear correlations. For instance, it has been observed that aerobic training in obese (Christiansen et al. 2010) or elderly (Beavers et al. 2010; Crane et al. 2015) subjects increases IL-15 secretion in circulation. At resting state, no difference was observed in IL-15 plasma levels before or after a 12 weeks aerobic training in obese patients (Christiansen et al. 2010). Similar results were also reported in elderly individuals that underwent a 12-month aerobic, strength and balance training program (Beavers et al. 2010). On the contrary, in post-menopausal women, Prestes *et al*. (2009) registered an increase in circulating IL-15 levels 48h after an acute resistance exercise in untrained subjects. After a 4-month resistance training, the increase in IL-15 circulating levels in response to acute exercise was lost in the same subjects, suggesting that training abolished IL-15 secretion in response to an acute exercise. As corroborating evidence, IL-15 plasma remained stable in young elite female soccer players after two soccer games (Andersson et al. 2010).

The different health status of the participants in each of the previously studies are quite different, raising another question; whether age or weight influence IL-15 response to physical exercise.

1.5.2 Effect of physical exercise on skeletal muscle IL-15 mRNA and protein expression

Circulating IL-15 level following exercise constitutes only a circumstantial evidence of IL-15 secretion by muscle as other tissues can also secrete this cytokine. To determine whether

skeletal muscle secretes IL-15 in response to exercise, researchers measured IL-15 expression in skeletal muscle after exercise.

1.5.2.1 Effect of acute endurance and resistance exercise on IL-15 mRNA and protein levels in skeletal muscle

Most studies that focused on IL-15 mRNA expression in muscle in response to an endurance exercise showed no significant increase in IL-15 transcription. For instance, after a 3h-cycling exercise, no increase in IL-15 mRNA level was observed in the next 21h following the exercise in untrained individuals (Rinnov et al. 2014). Similar results were observed by another research group where no change in IL-15 mRNA level in skeletal muscle was observed in young healthy males 60 min after a cycling exercise (Chan et al. 2004). In mice, IL-15 mRNA level decreased in gastrocnemius 30 min after an exhaustive running exercise despite a sharp increase in circulating IL-15, demonstrating that circulating IL-15 levels does not correspond to muscle IL-15 mRNA levels (LeBris S. Quinn et al. 2014). Louis *et al.* (2007) compared muscle IL-15mRNA response to resistance and endurance acute exercise. They showed no change in muscle IL-15 mRNA level in individuals that performed a weight lifting exercise, whereas subjects that performed a running, aerobic exercise displayed an increased IL-15 mRNA levels in skeletal muscle 8h after the run (Louis 2007).

Very few studies measured IL-15 protein level in skeletal muscle after an acute endurance exercise. In untrained individuals, a treadmill endurance exercise did not change muscle IL-15 protein levels (Rinnov et al. 2014).

As for muscle IL-15 expression in response to a resistance exercise, some study did not observed a significant change of IL-15 expression. For instance, Nieman et al. did not observe any change in skeletal muscle IL-15 mRNA in young men performing a weight lifting session

(Nieman et al. 2004) or in marathon runners after a 3h run (Nieman et al. 2003). As observed in humans, acute resistance exercise did not influence skeletal muscle IL-15 mRNA levels in trained rats (Zanchi et al. 2010). On the other hand, Molanouri *et al.* (2014) did register an increase in IL-15 mRNA expression in soleus 12h after a resistance exercise bout in trained rats. In addition, after a weight lifting exercise, muscle IL-15 mRNA increased 24h post-exercise in humans. However, IL-15 protein levels remained unchanged (Nielsen et al. 2007).

1.5.2.2 Effect of endurance and resistance training on IL-15 mRNA and protein levels in skeletal muscle

To my knowledge, no reports showed an effect of physical training on IL-15 mRNA levels in skeletal muscle. No difference was found for IL-15 mRNA between endurance-trained and untrained young men (Rinnov et al. 2014). In rats, endurance training increased resting IL-15 mRNA levels in skeletal muscle compared to untrained rats (H.-T. Yang et al. 2014), whereas IL-15 protein level in muscle was not increased compared to sedentary rats (H. Yang et al. 2013). Interestingly, rats that were fed an HFD showed a reduced IL-15 protein levels in skeletal muscle that was restored following endurance training (H.-T. Yang et al. 2014).

Resistance training did not influence IL-15 mRNA levels in skeletal muscle of rats (Molanouri, Zuhair, and Hassan 2014). Another study showed that, in the resting state, resistance trained rats had lower IL-15 mRNA but higher IL-15 protein levels in skeletal muscle than untrained rats (Molanouri Shamsi et al. 2014).

By comparing these different studies discussed above, one can identify a lack of consistency throughout these studies. Comparing different exercise protocol with subjects that possess different level of fitness may be a source of error that produce conflicting conclusions on IL-15 regulation in response to exercise in skeletal muscle. To summarize, differences observed

in IL-15 expression in skeletal muscle in response to exercise may suggest that IL-15 expression may vary depending on the fitness, gender, species and diet of test subjects.

1.6 IL-15's METABOLIC-ENDOCRINE EFFECTS

1.6.1 Effect of IL-15 at the whole-body level

Many studies investigated IL-15 metabolic endocrine effect by raising IL-15 level in circulation by administrating high IL-15 doses intravenously in rats or by overexpressing IL-15 in transgenic mice. IL-15 concentrations applied in those studies may not represent physiological IL-15 concentrations as they induce circulating IL-15 levels ranging from 12,200 (L. Quinn 2011) to 100,000 pg/ml (Lebris S Quinn, Anderson, Conner, and Wolden-hanson 2013) compared to 1.7 pg/ml at resting state under physiological conditions in humans (Pierce, Maples, and Hickner 2015; Bazgir *et al.* 2014; Nielsen *et al.* 2007; Tamura *et al.* 2011; Riechman *et al.* 2004). Therefore, the phenotypes observed in these experiments may not be representative of IL-15's action physiologically. Nonetheless, these experiments found that IL-15 can change greatly overall body composition.

Rats that were administered high doses of IL-15 (100 µg per kg of body weight) daily for seven days had similar body weight than untreated rats but showed a 33% reduction of WAT mass, a 23% increase in spleen mass and a slight increase in the soleus mass (7%). Interestingly, the reduction in adiposity of IL-15-treated rats was not accompanied by lower food intake, suggesting that IL-15 may have interesting anti-obesity qualities (Carbó et al. 2001; Almendro et al. 2006; Almendro et al. 2008).

Quinn's research group designed mice that overexpress and oversecrete IL-15 specifically from skeletal muscle. However, simply increasing IL-15 transcription rate was found

inefficient to increase IL-15 secretion in circulation as IL-15 signal peptide which regulates IL-15 incorporation to its secretory pathway is inefficient. This research group then tried to substitute IL-15 inefficient signal peptide for IL-2 signal peptide. The resulting mice overexpressed IL-15 protein by 500 to 2500 times and had 350 to 1,000-fold increased circulating IL-15 levels (Lebris S Quinn et al. 2009; Lebris S Quinn, Anderson, Conner, and Wolden-hanson 2013). These transgenic mice displayed lower visceral and retroperitoneal fat pad mass and smaller fast and mixed muscle fibers (namely the extensor digitorum longus (EDL) and gastrocnemius). As reported in the rat studies, food intake was not compromised by high circulating IL-15 levels in these mice, meaning that low adiposity induced by IL-15 was not due to lower energy intake. However, low adiposity of transgenic mice could originate from higher energy expenditure, as their ambulatory activity were two-fold higher than wild-type (WT) mice during the dark phase. In addition, transgenic mice had the capacity to run 2 times longer than WT mice on a treadmill, demonstrating impressive endurance capacities. Under resting state, they also displayed a significantly lower respiratory exchange ratio (RER) than WT mice, indicating a preference for fatty acid instead of glucose as energetic fuel

In addition to its impressive anti-obesity characteristic, IL-15 treatment also induces beneficial effects on glucose regulation. Quinn's transgenic mice that overexpress and oversecrete IL-15 from their skeletal muscle were also resistant to HFD-induced obesity, and were more insulin sensitive and had lower glucose and insulin levels under a normal chow diet (L. Quinn 2011). Non-obese diabetic (NOD) mice are a commonly used model to imitate type 1 diabetes as their insulin production is compromised by leukocytes infiltrating their pancreatic beta cells islet at an early life stage. Twenty-four weeks old NOD mice that were treated with single or multiple doses of 100 μg chimeric human IL-15, a modified version of IL-15 that has a longer half-life

in circulation, showed smaller incidence to develop diabetes at 33 weeks of age compared to untreated mice (Signore et al. 2003). Sun *et al.* (2014) induced temporary IL-15 overexpression in random tissues using an untargeted plasmid gene transfer in mice. These transgenic mice became resistant to obesity induced by an HFD (Sun and Liu 2014). They also exhibited lower blood glucose and insulin levels at resting state compared to WT mice (Sun et al. 2016).

1.6.2 Effect of IL-15 on white adipose tissue

As discussed above, IL-15 treatments induced interesting anti-obesity effects. Certain studies demonstrated that IL-15 plasma level is negatively correlated with fat mass content in humans (total, trunk and percent fat mass) (Nielsen et al. 2008; Barra et al. 2009). In addition, Nielsen and coworkers found that IL-15 mRNA expression in skeletal muscle, similarly to plasma levels, was also negatively correlated with body mass index, percent and total, trunk and limb fat mass (Nielsen et al. 2008). Despite the fact that there was no correlation between IL-15 mRNA in skeletal muscle and IL-15 plasma levels, high IL-15 circulating level was associated with a leaner phenotype. Curiously, in patients with T2D, IL-15 plasma levels did not differ between lean and obese patients (Nielsen et al. 2008). Pierce and coworkers (2015) also did not observe any difference in circulating IL-15 between obese and lean humans. Finally, in contradiction to the previous claim, obese individuals that underwent weight loss had a reduction in circulating IL-15 levels (Christiansen et al. 2010). Therefore, whether there is a relationship between circulating IL-15 levels and adiposity remains debated. Despite a few studies that negates that lean individuals possess higher circulating levels of IL-15, there are mountains of evidence that IL-15 promotes WAT atrophy.

As stated above, high IL-15 doses injections in rats reduced visceral fat but not subcutaneous fat without compromising food intake (Almendro et al. 2006; Carbó et al. 2001; Almendro et al. 2008). Similar results were found in transgenic mice (Alvarez et al. 2002). This decrease in adiposity was correlated with a decrease in lipogenesis, lipoprotein lipase activity and glucose incorporation in WAT (Carbó et al. 2001). Overexpressing IL-15 in mice resulted in a similar phenotype. Whole-body IL-15 overexpressing mice (IL-15Tg mice) weighted almost half of the mass of WT mice under an HFD (Barra et al. 2009). Biopsy of adipose tissue revealed that IL-15Tg adipocytes were smaller and showed higher NK cells content than control adipocytes (Barra et al. 2009). On the other hand, whole-body IL-15 deficient mice (IL-15 KO) showed increased weight gain on an HFD, bigger adipocytes and very low levels of inflammatory NK cells in adipose tissue compared to WT mice (Barra et al. 2009). All three groups, IL-15Tg, IL-15 KO and WT, had similar food intake. Therefore, change in adipose tissue was not due to a change in appetite. Furthermore, treating KO mice with IL-15 induced weight loss confirming that weight modulation was strictly related to IL-15 action (Barra et al. 2009). Similarly, transient IL-15 overexpression in mice by untargeted plasmid gene transfer induced a 200-fold increase in circulating IL-15 compared to WT mice. These mice developed a resistance to obesity when undertaking an HFD and displayed smaller adipocytes compared to their WT counterparts (Sun and Liu 2014). When IL-15 was co-overexpressed with its receptor α subunit using plasmid gene transfer in mice, circulating IL-15/IL-15Rα peaked to 10^5 pg/ml in the circulation and mice started to lose weight. Ten days after the last IL-15/IL-15Rα plasmid injection, mice started to gain back weight associated with a small increase in food intake (Sun et al. 2016). This observation underlines that weight loss induced by IL-15 is temporary and is lost after IL-15 clears out from circulation. The authors proposed that the

increased food intake may originate partially from “changes in peripheral hormones that are involved in appetite regulation following weight loss” (Sun et al. 2016). Injecting again IL-15/IL-15Rα plasmid reinstated weight lost (Sun et al. 2016).

It has not yet been proven that circulating IL-15 originates form skeletal muscle. Interestingly, weight loss and the pro-oxidative shift were also observed when IL-15 overexpression was induced in lymphocyte-deficient or NK-depleted mice (Barra et al. 2012; Barra et al. 2014). Therefore, IL-15-induced weight loss was independent of lymphocytes. Adipose tissue does not express very high levels of IL-15 mRNA compared to skeletal muscle. For instance, in mature C2C12 myotubes, IL-15 mRNA expression was 24 times higher than in 3T3-L1 adipocytes (Lebris S Quinn et al. 2005). Therefore, it is most likely that IL-15 originates from skeletal muscle to act on adipose tissue.

To study how IL-15 acts on WAT, 3T3-L1 preadipocytes were exposed to different IL-15 concentrations (0 to 250 ng/ml for eight days) during their differentiation period. This treatment resulted in lower lipid deposition in mature adipocytes (Fuster et al. 2011; Barra et al. 2014; Lebris S Quinn et al. 2005; Almendro et al. 2009) which seems to be regulated via an upregulation of calcineurin, an inhibitor of adipocyte differentiation (Almendro et al. 2009). On the other hand, exposure of mature adipocytes to IL-15 did not reduce lipid content (Lebris S Quinn et al. 2005). Therefore, Il-15 seems to repress lipid deposition during maturation of adipocytes. Furthermore, IL-15 triggered the secretion of certain cytokines in mature adipocytes but not in pre-adipocytes. More specifically, 5 and 10 ng/ml IL-15 increased secretion of adiponectin, a cytokine that promotes fatty acid breakdown (Lebris S Quinn et al. 2005). At higher concentrations (250 ng/ml), IL-15 also stimulated IL-6 secretion (Barra et al.

2014), another pro-oxidative cytokine. This IL-6 secretion in 3T3-L1 concurred with a higher mitochondrial membrane potential, hence a greater capacity to produce ATP (Barra et al. 2014). Such effects were not observed in preadipocytes treated with IL-15 (Lebris S Quinn et al. 2005; Barra et al. 2014). IL-15 treatment also reduced glucose incorporation into lipid and induced lipolysis through the protein kinase A (PKA) and Janus Kinase (JAK) pathway in adipocytes isolated from pigs (Ajuwon and Spurlock 2004).

In summary, *in vitro* studies demonstrate that IL-15 acts directly on adipose tissue to stimulate secretion of cytokines which promotes lipolysis in mature adipocytes and reduces lipid deposition in preadipocytes. Since IL-15 is believed to be a myokine, it has been suggested that this cytokine establishes a cross-talk between skeletal muscle and adipose tissue and could explain the beneficial effect of exercise/muscle contraction on adipose tissue and weight loss (Argilés et al. 2005).

1.6.3 Effect of IL-15 on liver

The IL-15 experiments performed in rats described above (100 µg/kg body weight IL-15, daily for seven days) decreased lipoprotein lipase (LPL) activity (Alvarez et al. 2002; Carbó et al. 2001; Almendro et al. 2006) and lipogenesis in liver (Carbó et al. 2001). These observations suggest that IL-15 blocks lipid transport into liver and endogenous lipid delivery from the liver. This reduction in lipogenesis was further confirmed by significantly lower levels of VLDL in circulation (Carbó et al. 2001). IL-15 treatment increased mRNA expression of mitochondrial fatty acid transporters carnitine palmitoyltransferase (CPT) 1 and CPT2 suggesting that IL-15 also promotes fatty acid transport into the mitochondria for their oxidation in liver (Almendro et al. 2006).

Temporary untargeted IL-15 overexpression by plasmid gene transfer in mice prevented triglyceride accumulation induced by an HFD in liver. Liver of these mice also expressed lower mRNA levels of lipogenic genes (namely; stearoyl-CoA desaturase-1, fatty acid synthase, acetyl-CoA carboxylase-1 (ACC-1)) and displayed higher mRNA levels of key proteins involved in lipid oxidation (CPT-1α, medium chain acetyl-coenzyme A dehydrogenase and long chain acetyl-coenzyme A dehydrogenase) compared to untreated mice on either chow or HFD (Sun and Liu 2014). These characteristics make IL-15 an interesting target to reduce fatty liver disorders.

By looking at the expression of genes involved in glucose metabolism, Sun *et al.* (2014) observed that high circulating IL-15 levels decreased mRNA expression of two genes involved in gluconeogenesis in liver namely phosphoenolpyruvate carboxykinase and glucose-6-phosphatase. Therefore, IL-15 may reduce circulating basal glucose levels by inhibiting endogenous glucose production in the liver.

1.6.4 Effect of IL-15 on brown adipose tissue

Brown adipose tissue (BAT) is a tissue with elevated energy expenditure. BAT highly expresses uncoupling proteins UCP1 and UCP3 that forces the electron transport chain pathway to oxidize more energetic fuels, mostly lipid substrates (Argyropoulos and Harper 2002), in order to maintain optimal proton gradient through the inner mitochondrial membrane.

The experiments in rats described above (100 µg/kg body weight IL-15, daily for seven days) did not reduce BAT weight. However, IL-15 treatment increased mRNA expression of many genes involved in mitochondrial uncoupling (UCP1 and UCP3) and fatty acid oxidation and transport (fatty acid translocase, fatty acid transport proteins, CPT1, CPT2, acyl-CoA

synthetase 4, PPARα, PPARδ) in BAT (Almendro et al. 2008). Therefore, IL-15 may increase BAT energy expenditure by increasing the expression of uncoupling proteins which in turn contributes to the IL-15-induced fat loss. However, no measurements of energy expenditure were taken during the study. Therefore, there is no tangible evidence that links IL-15 anti-obesity effects with high BAT energy expenditure. Furthermore, mice that overexpress and oversecrete IL-15 specifically in skeletal muscle, despite being leaner and having a highly active phenotype with high energy expenditure, did not show more BAT mass than WT mice (Lebris S Quinn, Anderson, Conner, and Wolden-hanson 2013). If BAT truly participates to the increase in energy expenditure induced by IL-15, it is therefore not by the formation of new brown adipocytes but rather by an increase in UCP1 and UCP3 activity.

1.6.5 Effect of IL-15 on skin

Human primary dermal fibroblasts exposed to media conditioned with serum isolated from physically active humans showed increased expression of complex IV of the electron transport chain. Inhibiting IL-15 action by adding an anti-IL-15 antibody abolished physical exercise-induced complex IV increase (Crane et al. 2015). Furthermore, primary dermal fibroblasts exposed to 10 pg/ml IL-15 had increased oxygen consumption rate and OXPHOS complex IV activity (Crane et al. 2015). Furthermore, 1h following a treadmill exercise in mice, mRNA levels of PPARγ coactivator-related protein 1 (Pprc1), an activator of mitochondrial biogenesis, was decreased by two-fold and cytochrome b, an important player in electron transport chain, increased by a factor of 1.3 in skin. These changes in gene expression induced by exercise were lost after neutralizing IL-15 by injecting anti-IL-15 antibodies. Taken together, these observations demonstrate that IL-15 stimulates mitochondrial activity in skin cells and may constitute a therapeutic target for skin thinning associated with many diseases

or could promote skin healing process. Further studies are needed to confirm these metabolic changes.

1.6.6 IL-15 autocrine/ paracrine effects on skeletal muscle

1.6.6.1 IL-15 anabolic effect on skeletal muscle

In vitro studies demonstrated that IL-15 has an anabolic effect on muscle cells. 72h treatment at 10ng/ml IL-15 increased myosin heavy chain (MHC) expression and induced hypertrophy of C2C12 muscle cells and bovine primary myotubes (Lebris S Quinn, Haugk, and Grabstein 1995). IL-15 overexpression in muscle cells repressed protein degradation and stimulated global protein synthesis accompanied by an accumulation of contractile proteins such as MHC and alpha-actin (Lebris S Quinn et al. 2002). IL-15 anabolic effects were shown to suppress muscle wasting and DNA breakdown in muscles of cachectic tumour-bearing rats by inhibiting TNFα signaling (Figueras et al. 2004). Furthermore, IL-15 mRNA expression in skeletal muscle increased with aging and muscle unloading, two processes that induce muscle atrophy (Pistilli, Siu, and Alway 2007). Pistilli *et al.* proposed that the increased in IL-15 secretion by skeletal muscle is a way to cope with atrophic stimuli linked to the aging process. In contrast, Marzetti *et al.* observed that aging decreased IL-15 protein expression in gastrocnemius muscle in rats (Marzetti et al. 2009). Therefore, it is not clear whether IL-15 expression in muscle increases or decreases with age. Nonetheless, IL-15 may be involved in muscle loss due to aging since caloric restriction induced a stress that increased IL-15 protein expression in muscle and reduced its apoptosis index (Marzetti et al. 2009).

1.6.6.2 Effect of IL-15 on skeletal muscle oxidative shift

Daily IL-15 administration (100 µg/kg body weight) for 7 days in rats increased mRNA expression of the pro-oxidative transcription factor PPARδ in skeletal muscle (Almendro et al.

2006), a nuclear receptors that, once activated, modulates different gene expression which switches fuel preference from glucose to lipids (Brunmair et al. 2006). PPARδ upregulation was correlated with an increase in total body and EDL muscle fatty acid oxidation (Almendro et al. 2006).

As described above, Quinn's research group designed transgenic mice that overexpress and oversecrete IL-15 specifically from the skeletal muscle (Lebris S Quinn, Anderson, Conner, and Wolden-Hanson 2013). These mice display a very lean phenotype with a lower RER implying a preference for fatty acid as energetic fuel than glucose. The authors suggested that the RER reduction could originate from the reduction of glycolytic fiber content and the promotion of pro-oxidative fibers in fast-twitch muscles. The authors also suggested that RER reduction could come from higher lipid oxidation rate which could be driven by higher mitochondrial uncoupling activity as uncoupling protein (UCP)-2 mRNA expression was increased in liver, EDL, and WAT of muscle-specific IL-15 transgenic mice (Lebris S Quinn, Anderson, Conner, and Wolden-Hanson 2013). However, liver and skeletal do not usually express uncoupling protein UCP-2 at the protein level. Therefore, it is doubtful that UCP2 mRNA upregulation induced by IL-15 in those tissues would impact mitochondrial uncoupling (Rupprecht et al. 2012). These mice show high endurance as they can run two times longer than the WT group. This high endurance may be explained by an increase in pro-oxidative muscle fibers content and a decrease of glycolytic muscle fibers content. In EDL, a muscle rich in fast/glycolytic fibers, along with gastrocnemius (mix muscle) and soleus (oxidative muscle), troponin I slow-twitch mRNA and MHC-1 mRNA were higher in muscle-specific IL-15 Tg mice compared to WT mice. Furthermore, mRNA expression of troponin I fast-twitch isoform and MHC-IIa were decreased in gastrocnemius and soleus of muscle-specific

IL-15 Tg mice compared to WT mice (Lebris S Quinn, Anderson, Conner, and Wolden-hanson 2013). Furthermore, IL-15 oversecretion by skeletal muscle resulted in the upregulation of several markers that promote oxidative metabolism, namely NAD-dependent deacetylase sirtuin-1 (SIRT1), PPARδ, PGC 1α, and CPT 1 and 2 in skeletal muscle (Lebris S Quinn, Anderson, Conner, and Wolden-hanson 2013). However, considering that IL-15 overexpression in skeletal muscle results in a high ambulatory activity (Lebris S Quinn, Anderson, Conner, and Wolden-Hanson 2013), this increase in pro-oxidative contractile proteins may be a consequence of this highly active behavior.

Curiously, IL-15Rα global knock-out recreates a phenotype similar to mice oversecreting IL-15 in skeletal muscle. IL-15Rα deficient mice were smaller, had higher ambulatory activity, and longer running distance. Furthermore, their muscles expressed higher levels of prooxidative markers PPARδ and PGC1α mRNA and had higher mitochondrial content (Pistilli et al. 2011; Pistilli, Guo, and Stauber 2013). Also, their fast-twitch EDL muscle displayed a slower contractile phenotype. That is to say; EDL demonstrated lower twitch and tetanus force as would a slow twitch muscle (Pistilli et al. 2011). IL-15 mRNA expression in muscle was upregulated in the IL-15RαKO mice and circulating IL-15 levels were also higher than in the control group (Pistilli et al. 2011). This increase in IL-15 may be a response to compensate for the lack of IL-15Rα KO and therefore render a similar phenotype as overexpressing and oversecreting IL-15. (Pistilli et al. 2011; Pistilli and Quinn 2013).

1.6.6.3 Effect of IL-15 on skeletal muscle glucose metabolism

A single IL-15 injection (100 µg/kg body weight) in rats administered intravenously increased glucose uptake by 32% in skeletal muscle (Busquets et al. 2006). *In vitro* assays also showed that C2C12 myotubes exposed to 10 ng/ml of IL-15 for 3h increased basal glucose uptake by

23% and GLUT4 mRNA expression by 25% (Busquets et al. 2006). In the same study, EDL muscles isolated from rats incubated with 100 ng/ml IL-15 for 2h showed a 30% increase in glucose uptake, a 14% increase in glucose oxidation and a 9% increase in glucose incorporation into lipid while no change was observed in glycogen storage. Furthermore, C2C12 myotubes exposed to 1 ng/ml IL-15 for 2h increased insulin-stimulated glucose uptake by 29% (Gray and Kamolrat 2011).

In skeletal muscle, temporary IL-15 overexpression through untargeted plasmid gene transfer increased GLUT4 mRNA expression. Since skeletal muscle is the main player in insulin-induced glucose disposal, higher GLUT4 expression could increase skeletal muscle ability for glucose transport (Sun and Liu 2014). IL-15 induced GLUT4 upregulation could explain the low blood glucose and insulin under resting state in IL-15 overexpressing mice (Sun and Liu 2014). In light of these novel findings, IL-15 effect could constitute an interesting regulator of glucose homeostasis. As a myokine, IL-15 may, under muscle contraction, be secreted from skeletal muscle and enhance muscle insulin sensitivity by increasing GLUT4 transcription and translocation.

1.7 AMPK PATHWAY: A POTENTIAL TARGET FOR IL-15

The mechanism of action by which IL-15 promotes a pro-oxidative metabolism and increases glucose uptake in skeletal muscle remains undetermined. A possible target is the AMP-activated protein kinase (AMPK) pathway. AMPK is a protein highly conserved in eukaryotes and is an important mediator of intracellular energy. This protein is composed of 3 subunits (α, β, and γ) which also have multiple isoforms (α1, α2, β1, β2, γ1, γ2 and γ3) (Viollet et al. 2009). The alpha subunit contains the activation and catalytic site of the enzyme. The β subunit

possesses mainly a structural function and contains binding sites for α and γ subunit. The γ subunit possesses a cystathionine-β-synthase motif that binds either two AMP or two ATP molecules (Viollet et al. 2009). Skeletal muscle co-expresses subunits α1 and α2. Both subunits are activated by upstream kinases, namely liver kinase B1 (LKB1) complex, transforming growth factor-β activated protein kinase-1 (TAK1) and calmodulin-dependent protein kinase kinase (CaMKKβ) by phosphorylating Thr172. AMP is an allosteric activator of AMPK that binds to the γ subunit and induces a conformation change that promotes Thr172 phosphorylation. In addition, AMP represses Thr172 dephosphorylation by inhibiting phosphatase protein 2A and 2C. Under low energetic conditions, AMP levels rise and promote AMPK activation by Thr172 phosphorylation. On the other hand, under high energetic conditions, AMP levels are low, and AMPK remains inactivated (Viollet et al. 2009). This modulation of AMPK activity by AMP characterizes AMPK as a prominent energetic sensor.

Skeletal muscle contraction increases AMP/ATP ratio and activates AMPK (Winder and Hardie 1996). Hence, AMPK plays a crucial role in metabolic adaptation to physical exercise. Knocking down AMPK-α2 decreased voluntary activity (Mu et al. 2001) in mice and inactivating AMPK-α2 by mutagenesis reduced exercise tolerance (Jørgensen et al. 2007). Chronic activation of AMPK increased the proportion of mixed type fibers IIa/x and the activity of mitochondrial markers in sedentary animals compared to their WT counterparts (Rockl et al. 2007). Muscle AMPK activation by AICAR or β-guanidinopropionic acid (β-GPA) treatments increased PGC-1α mRNA expression which promotes mitochondrial biogenesis in muscle (Terada et al. 2002). Furthermore, AMPK-α2 knock down mice treated with β-GPA did not display an increase in mitochondrial content or an increase in PGC1α,

showing that the effect of β-GPA on mitochondrial biogenesis was AMPK-dependent (Zong et al. 2002).

Upon activation, AMPK promotes energy production by activating catalytic pathways, inhibiting high-energy consuming pathways and energy storage. For instance, protein synthesis requires as much as 30-50% of the cell energy expenditure for skeletal muscle growth (Viollet et al. 2009). The mammalian target of rapamycin (mTOR) plays a crucial role in protein synthesis and is essential for muscle hypertrophy. AMPK directly phosphorylates and inactivates mTOR (Inoki, Zhu, and Guan 2003). AMPK can further inhibit mTOR by phosphorylating and activating a target further downstream in mTOR signaling pathway, namely tuberous sclerosis complex 2 (TSC2) which in turn inactivates G-protein Rheb (Inoki, Zhu, and Guan 2003). In addition, AMPK reduces initiation and the elongation of ribosomal peptide synthesis (Kimura et al. 2003). AMPK phosphorylates and inhibits eEF-2 kinase which promotes peptide elongation (Horman et al. 2002). Altogether, AMPK activation inhibits protein synthesis in skeletal muscle (Bolster et al. 2002) which helps to spare ATP under low energetic condition. Furthermore, AMPK regulates important parameters in glucose and lipid metabolism, making it an interesting target for treating insulin resistance and T2D.

1.7.1 Regulation of glucose metabolism by AMPK

Stimulating AMPK activity by AICAR results in an increased glucose uptake in skeletal muscle (Merrill et al. 1997). Besides, deleting AMPK-α2 or γ3 subunit completely abolished AICAR-induced glucose uptake in skeletal muscle (Fujii et al. 2005; Mu et al. 2001). It was later found that AICAR stimulates GLUT4 translocation to the cell membrane independently from insulin, enhancing glucose uptake in skeletal muscle (Koistinen et al. 2003). Since

AMPK is activated by muscle contraction, it was hypothesized that AMPK is responsible for the increase in glucose transport in skeletal muscle during physical exercise. This hypothesis was revealed to be partially true since mice that overexpressed a kinase-dead AMPK-α2 show only partial impairment of muscle contraction-induced glucose uptake in skeletal muscle (Mu et al. 2001; Fujii et al. 2005). However, in those studies, there might be a residual AMPK-α1 activity that can compensate the lack of the α2 subunit activity.

The mechanism by which AMPK stimulates GLUT4 translocation remains unclear. Since AICAR induces Thr642 phosphorylation of TBC1D4 in human (Thr649 in mouse), it is thought that AMPK activates GLUT4 translocation by directly phosphorylating TBC1D4 (Treebak et al. 2010). As mentioned above, TBC1D4 is part of the insulin signaling pathway and act as a regulator of Rab-GAP proteins that allows GLUT4 translocation when phosphorylated. Replacing Thr649 for an alanine in a knock-in mouse model decreased insulin sensitivity and GLUT4 trafficking but surprisingly did not alter contraction-induced glucose transport (Ducommun et al. 2012). Therefore, further research is required to understand how AMPK impact GLUT4 translocation.

Besides stimulating glucose transport in skeletal muscle, AMPK prevents anabolic glucose pathways. First, AMPK phosphorylates cAMP response element-binding protein (CREB)-Regulated Transcription Coactivator 2 (CRTC2) on Ser171 which prevents the latter to translocate to the nucleus. In consequence, transcription of many genes upregulated by CRTC2 is suppressed. Among those genes, there are phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6P): two key proteins of gluconeogenesis. Second, AMPKβ subunit contains a highly conserved domain that binds to glycogen. Once stimulated by

AICAR treatment, AMPK phosphorylates and inhibits directly glycogen synthase (Carling et al. 1989), the limiting enzyme in glycogen synthesis. Since AICAR stimulation of AMPK also increases glucose uptake in skeletal muscle, AMPK inhibition of glycogen synthesis forces the intramyocellular glucose-6-phosphate to undertake glycolysis for ATP production (Bouskila et al. 2010).

1.7.2 Regulation of fatty acid metabolism by AMPK

AMPK stimulation increases fatty acid oxidation (Merrill et al. 1997). In response to AICAR treatment, activation of AMPK inhibits ACC2 by phosphorylating its Ser221 residue (Ser212 in mouse) (Munday, Carling, and Hardie 1988). ACC regulates the carboxylation of acetyl-CoA to malonyl-CoA, the rate-limiting step of fatty acid biosynthesis. Furthermore, malonyl-CoA allosterically inhibits CPT1 which controls fatty acid transport into the mitochondria for β-oxidation. Therefore, by inhibiting malonyl-CoA formation, AMPK simultaneously represses storage of energy by lipogenesis and increases mitochondrial fatty acid oxidation (Dzamko and Steinberg 2009). AMPK plays quite an important role in promoting fatty acid oxidation as inducing a gain-of-function mutation in AMPKγ3 prevented obesity in mice by increasing fatty acid oxidation in skeletal muscle (Barnes et al. 2004). However, many studies point that there are probably other signaling pathways that stimulate fatty acid oxidation in parallel to AMPK. For instance, knocking out AMPK-β2 subunit in mice decreases ACC2 Ser221 phosphorylation, however, there was no significant change in fatty acid oxidation and intramyocellular triacylglycerol levels (Steinberg et al. 2010). Furthermore, completely knocking out LKB1 decimated AMPKα2 activity and ACC phosphorylation but did not influence fatty acid oxidation in skeletal muscle (Thomson et al. 2007). Lastly, O'Neill *et al.* (2015) changed Ser212 for Ala212 in ACC2 in mice, preventing ACC2

phosphorylation/inhibition. Nonetheless, skeletal muscle fatty acid oxidation levels remained unchanged in these mice, even after a treadmill exercise (O'Neill et al. 2015). Therefore, while AICAR-induced AMPK activation greatly stimulates fatty acid oxidation by phosphorylating/inhibiting ACC2, there are possibly AMPK-independent pathways that contribute to fatty acid oxidation during physical exercise.

Fatty acid uptake in skeletal muscle is regulated by transport proteins. So far, there are three well documented type of fatty acid transporters in skeletal muscle: $FABP_{pm}$ located on the outer leaflet of the plasma membrane (Stremmel et al. 1985), FATP1-6 that possesses 6 transmembrane domains and FAT/CD36 that possesses 2 transmembrane domains and is known to be the most efficient transporter for long chain fatty acid uptake (Nickerson et al. 2009). Stimulation of AMPK by AICAR treatment induced the translocation of long-chain fatty acid transporters FAT/CD36, FABPpm and FATP 1 and 4 to the plasma membrane (Chabowski et al. 2005). Furthermore, muscle contraction is dependent on calcium influx in the cytosol (Berchtold, Brinkmeier, and Müntener 2000). Stimulation of cytosolic calcium influx by caffeine treatment activates CAMKK (Abbott, Edelman, and Turcotte 2009) which stimulates fatty acid transport (Abbott, Edelman, and Turcotte 2009; Balu et al. 2016), and FAT/CD36 translocation to the plasma membrane (Abbott, Edelman, and Turcotte 2009). Therefore, AMPK may enhance fatty acid transport into tissues via the activation of CaMKK. However, Raney *et al.* observed that low-intensity exercise increased fatty acid uptake in skeletal muscle without any increase in AMPK activity (Raney et al. 2005). Therefore, while fatty acid uptake in skeletal muscle is significantly increased by pharmaceutical stimulation of AMPK, fatty acid transport during physical exercise is regulated by AMPK-independent pathways.

Chronic activation of AMPK increases mitochondrial content by upregulating PGC1α expression and by directly phosphorylating/activating PGC1α on Thr1722 and Ser538 residues (Jäger et al. 2007). In addition, AMPK also phosphorylates CREB (Thomson et al. 2008) and class IIA histone deacetylase (HDAC) (McGee et al. 2008), two factors involved in PGC1α-mediated transcription. Knocking out PGC1α in mice diminishes mitochondrial content with, demonstrating that AMPK effect on mitochondrial content is PGC1α-dependent (Adhihetty et al. 2009).

Despite the fact that the metabolic role of AMPK during exercise is not well characterized, its activation using drugs such as AICAR and metformin shows important metabolic effects that are used today as therapeutic treatments for metabolic pathologies. Furthermore, there are many similarities between IL-15 metabolic impact observed *in vivo* and *in vitro* and AMPK effect on metabolism (Table 1). Both pathways stimulate glucose uptake, repress lipogenesis, increase mitochondrial content and PGC1α expression in skeletal muscle. Therefore, AMPK may represent an interesting target for IL-15 and explain the cytokine's impact on skeletal muscle metabolism.

Table 1: Comparison between AMPK pathway and IL-15 metabolic effects

AMPK	IL-15
↑ GLUT4 translocation	↑ Glucose uptake in muscle
↑ insulin sensitivity	↑ insulin sensitivity
↓ Neoglucogenesis	
↑ Fatty acid oxidation	↓ lipid deposition
	↑ pro-oxidative capacities
↓ Lipogenesis	↓ lipogenesis
↑ PGC1α	↑ PGC1α, SIRT1, PPARδ
↑ mitochondrial content	↑ mitochondrial DNA
↑ FAT/CD36 translocation	

2 OBJECTIVES

In vivo studies demonstrate that IL-15 induces many metabolic effects on skeletal muscle. Nonetheless, little is known about its mechanism of action in skeletal muscle. Furthermore, because IL-15 can act in synergy with other cytokines on muscle cells (Gray and Kamolrat 2011), observations made using *in vivo* models may reflect indirect effects of high circulating IL-15 levels. Thus, the research objectives of the current studies are:

1. Characterize the effect of IL-15 in L6 myotubes in terms of glucose uptake, oxygen consumption rate, fatty acid oxidation, ADP/ATP levels and OXPHOS protein concentration, testing whether L6 is a good model to study IL-15.
2. To determine whether IL-15 activates the AMPK signaling pathway

3 METHODOLOGY

3.1 CELL CULTURE

L6 myoblasts cell line was chosen as model to imitate muscle tissue as it has similar GLUT4 translocation machinery and insulin sensitivity as muscle fibers and is considered a great model to study muscle glucose metabolism (Ueyama et al. 1999). L6 and L6-GLUT4myc myoblasts were kindly provided by Dr. Amira Klip (Sick Kid Hospital, Toronto). Myoblasts were seeded at a density of 50,000 cells/ml and were cultured in alpha-minimal essential medium (αMEM) (Wisent). Myoblasts were grown in medium supplemented with 10% Fetal Bovine Serum (FBS) (Life Technologies), 1X antibiotic/antimycotic (Wisent) and 3 µg/ml gentamicin (Life Technologies). When cells reached ~80% confluence, growth medium was changed for differentiation medium (α-MEM supplemented with 2% FBS, 1X

antibiotic/antimycotic, 3 µg/ml gentamycin). The experiments were performed on day 7 of differentiation.

3.2 IL-15 TREATMENTS

3.2.1 IL-15 doses

Myotubes were exposed to different concentrations of IL-15 in serum free media for different incubation time. Plasma level of IL-15 at resting states in humans is around 1.7 pg/ml (Pierce, Maples, and Hickner 2015; Bazgir *et al.* 2014; Nielsen *et al.* 2007; Tamura *et al.* 2011; Riechman *et al.* 2004) and increases slightly ranging levels between 2 and 5.57 pg/ml after exercise (Bazgir et al. 2014; Tamura et al. 2011; Riechman et al. 2004; Crane et al. 2015). Pierce et al (2015) also demonstrated that IL-15 levels in the insterstitial space surrounding skeletal muscle are 10 to 15 times higher than plasma levels. In some experiments myotubes were exposed to concentrations representative of physiological levels of IL-15 (i.e., from 1 to 100 pg/ml).

3.2.2 Incubation periods

Myotubes were exposed to IL-15 for 3h or 48h. The aim of the 3h incubations at high doses of IL-15 (1,000 to 20,000 pg/ml) was to mimic the acute effect of exercise, while the 48h incubation at physiological condition was to reflect the chronic effect of exercise.

The 3h incubations were performed in serum-free media in order to isolate the effect of IL-15 from the effects from the other cytokines present in serum. The 48h incubations were performed in differentiation media (2% serum) as we observed that L6 myotubes cannot survive serum starvation more than 10h. IL-15 doses were renewed every 24h.

3.3 FATTY ACID OXIDATION

Fatty acid oxidation was determined as previously described in (Aguer, Fiehn, et al. 2013). Myoblasts were seeded in 12-well plates. Prior to experiment, myotubes were pre-incubated for 60 min in serum-free, glucose-free DMEM supplemented with (in ng/ml): 0, 1, 10 or 20 IL-15. Glucose and serum deprivation is an important step to encourage the myotubes to oxidize fatty acid. Three wells were treated with 2 mM AICAR as a positive control. Subsequently, myotubes were incubated for 2 hours in DMEM without glucose/pyruvate to which was added 0.05 µCi/ml [1-^{14}C] palmitate (0.1 µCi/well), 20 µM cold palmitate, 1 mM carnitine, 0.25% fatty acid-free bovine serum albumin (BSA) adjusted with the corresponding concentration of IL-15. Cells were kept at 37°C incubator with 5% CO_2 throughout glucose deprivation and palmitate incubation. Complete fatty acid oxidation was determined by measuring ^{14}C-labelled CO_2 production. Radioactivity remaining in cells and in media constitute incomplete oxidation of the [1-^{14}C] palmitate into Acid Soluble Product (ASP)s. ASPs could be palmitoyl-CoA, palmitoyl-carnitine or any intermediate products of beta-oxidation if beta-oxidation was incomplete for some palmitoyl-carnitined that entered mitochondria; acetyl-CoA or acetyl-carnitine if the Krebs cycle was unable to deal with every acyl-CoA produce by beta-oxidation; any intermediate from the Krebs cycle if this later was incomplete.

3.3.1 Complete fatty acid oxidation

Cell media was transferred into airtight vials containing a microcentrifuge tube filled with 0.3 ml of 1 M benzethonium hydroxide to capture $^{14}CO_2$, and palmitate oxidation was quenched by injecting 70% perchloric acid through the rubber cap using a syringe. 0.5 ml of each sample was taken by syringe for ^{14}C-labeled ASPs measurement in medium. Radioactively-labelled

CO_2 was measured by liquid scintillation counting by mixing the benzethonium hydroxide to 10 ml of scintillation cocktail (CytoScint, #882453).

3.3.2 Incomplete fatty acid oxidation

^{14}C-labelled ASPs in medium were measured as previously described (Veerkamp et al. 1983; Jacobs, Veerkamp, and Publishers 1984). Briefly, 0.5 ml of media from each sample was added to 3 ml chloroform: methanol (2:1). ASPs were isolated in the aqueous phase by adding 1.2 ml KCl: HCl (2M) and centrifuging at 4000g for 10 min. Radioactively labeled ASPs were measured by liquid scintillation by mixing 1 ml of the aqueous layer with 5 ml of scintillation cocktail (CytoScint, #882453).

^{14}C-labelled ASPs in cells was measured as previously described (Kitzmann et al. 2011). Briefly, after the [1-^{14}C] palmitate incubation, cells were washed with PBS and incubated in 10% perchloric acid for 15 min. Cells were subsequently scraped, and radioactivity of cell lysate was determined by liquid scintillation by combining 0.5 ml of cell lysate to 5 ml of scintillation cocktail (CytoScint, #882453).

Radioactive samples were counted once for 3 min using the Tri-Carb2910TR scintillation counter (Perkin Elmer) with tSIE/AEC as quench indicator.

Protein content was measured in parallel wells, with an identical number of cells and incubations by Bradford assay (Biorad). Fatty acid oxidation rates are expressed as nmol of [1-^{14}C] palmitate oxidized/min/mg of total protein.

3.4 GLUCOSE UPTAKE

Glucose uptake was determined as previously described (Koivisto et al. 1991). Briefly, myoblasts were seeded in 24-well plates and differentiated in myotubes. Myotubes were serum

starved for 3 hours prior to experiment to obtain optimal response to insulin as growth hormones present in media is known to stimulate insulin signaling pathway (Klip, Li, and Logan 1984). *For the 3h IL-15 treatments, IL-15 incubation was performed during the serum starvation step.*

During the last 20 minutes of the 3h serum starvation, half of the wells were treated with 100 nM insulin, which triggers glucose uptake in L6 at near maximal capacity (Ueyama et al. 1999). To determine glucose uptake by facilitated diffusion in cells, 3 wells were treated with Cytochalasin B (5 μM), a GLUT1, 2, 3 and 4 inhibitor (Jung and Rampal 1977). Wells were then washed 3 times with Hepes Buffered Saline (HBS) (140 mM NaCl, 20 mM Hepes-Na, 5 mM KCl, 2.5 mM $MgSO_4$, 1.0 mM $CaCl_2$, pH 7.4) to subsequently receive 0.2 ml of Transport Solution (HBS with 10 μM 2-Deoxy-Glucose and 0.5 μCi/ml [^{3}H] 2-Deoxyglucose) adjusted with the corresponding IL-15 concentration for 10 minutes. Transport solution was quickly removed, and myotubes were washed 3 times with ice-cold Phosphate Buffered Saline (PBS). Myotubes were lysed in 0.5 ml of 0.05 M NaOH. 0.4 ml of myotube lysate was mixed with 5 ml Scintillation Cocktail (CytoScint, #882453) and counted twice for 1 minute using a Tri-Carb2910TR scintillation counter (PerkinElmer). 0.1 ml of the remaining cell lysate was used to measure protein concentration using a Bradford assay (Biorad). Glucose transport activity is expressed as pmol of 2-deoxy-glucose taken/min/μg of total protein.

3.5 GLUT4 TRANSLOCATION

L6 expressing GLUT4 tagged with Myc epitope (L6-GLUT4myc) were seeded in 48-well plates and differentiated myotubes were serum deprived for 3h prior to experiment as described above. Cells were treated with ±100 nM insulin during the last 20 min of the 3h

serum starvation step. Cells were subsequently fixed on ice with 3% paraformaldehyde in PBS for 10 min and washed twice with PBS. Paraformaldehyde reaction was quenched by exposing cells to 0.1 M glycine for 10 min. After 3 more PBS washes, cells were blocked with 5% Goat Serum in PBS for 15 min. Myc epitope was targeted by 1 µg/ml anti-Myc polyclonal antibody (Santa Cruz, #sc-789). Surface-bound Myc epitope was revealed by exposing myotubes to *o*-Phenylenediamine dihydrochloride reagent (Sigma, #P9187) for 30 min. Reaction was stopped using 3 M HCl and GLUT4 translocation was quantified by measuring absorbance at 462nm.

3.6 OXYGEN CONSUMPTION RATE

L6 myoblasts were seeded on XF24-well or XF96-well plates (Seahorse Biosciences Inc.). Prior to oxygen consumption rate measurement, myotubes were serum-deprived in HCO_3-free assay buffer (Seahorse medium) (4 mM glutamine, 1 mM pyruvate and 5.5 mM glucose, no IL-15 (pH 7.4)) at 37°C for 40 min. Myotubes oxygen consumption rates (OCR) were measured using a Seahorse extracellular flux analyzer as described in (Aguer, Pasqua, et al. 2013). After basal OCR measurement, myotubes were treated with 600 ng/ml oligomycin to measure OCR due to mitochondrial proton leak (state 4). Subsequently, uncoupled and non-mitochondrial OCR were determined by treating myotubes with 1 µM FCCP and 4 µM antimycin A, respectively. Myotubes were then lysed in 0.05 M NaOH, and cellular protein content was measured using a Bradford protein assay. Antimycin OCR values (non-mitochondrial OCR) were subtracted from total OCR values (basal, oligomycin, and FCCP OCR) to present mitochondrial OCR.

For the 0 to 3h treatments, after 6 days of differentiation, myotubes were serum-deprived in Seahorse medium for 40 min. Myotube OCR was then assayed under resting state. IL-15 was

injected in wells for a final IL-15 concentration of (in pg/ml): 0, 1, 10, 100, 1 000, 10 000 or 20 000. OCR was measured every 15 min for 3h. Uncoupled, maximal and non-mitochondrial OCR were then measured as described above.

3.7 WESTERN BLOTS

L6 myoblasts were seeded in 6-well plates. After 6 days of differentiation and an overnight serum deprivation, myotubes received serum-free differentiation medium supplemented with (in ng/ml): 0, 0.5, 0.75, 1, 10 or 20 of IL-15 for a given incubation time. For 48h incubations, IL-15 dose was renewed every 24h. As positive controls for pACC and pAMPK analysis, L6 myotubes were treated either with 1 µg/ml oligomycin or 2 mM AICAR for 10 min and 3h respectively. For pAkt analysis, myotubes were treated ±insulin (100 nM). Myotubes were lysed in ice-cold RIPA buffer (Thermo Scientific) supplemented with phosphatase inhibitors (1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM EGTA) and protease inhibitors (ThermoScientific, PI88666). Cell lysates were analyzed by Western blotting. Briefly, proteins were separated by 8% or 12% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. Membranes were then blocked for 1 h at room temperature in blocking buffer (TBST-Milk: Tris buffer saline, 0.1% Tween20, 5% powdered milk).

The following primary antibodies were used: pAMPKα (#2535), AMPKα total (#5831), pACC (#11818), ACC total (#3676), pAkt (#2965), Akt (#C67E7), troponin T (#5593) and α-Tubulin (#3873), all purchased at Cell Signaling Technologies, diluted to 1:1000 in TBST-BSA (5%). OXPHOS primary antibodies were purchased at Abcam (#ab110413) and were diluted to 1:2000 in TBST-milk. The secondary antibodies were anti-rabbit and anti-mouse coupled to horseradish peroxidase diluted 1:5000 in TBST-Milk (Santa Cruz Biotechnologies Inc.).

Proteins were visualized using enhance chemiluminescent kit and exposed to UV light in a ChemiDoc system. Protein bands were quantified by density analysis using Image J Launcher Software (NIH, USA).

3.8 ADP/ATP RATIO

ADP/ATP ratio was measured using EnzyLight™ ADP/ATP Ratio Assay Kit (ELDT-100) (Bioassay Systems). Briefly, cells were lysed, and ATP levels were determined by measuring the luminescence produced by luciferase activity.

$$ATP + Dluciferin + O_2 \xrightarrow{Luciferase} oxyluciferin + AMP + PP_i + CO_2 + light$$

Subsequently, ADP levels were determined by converting ADP to ATP by enzymatic reaction and luminescence was measured again.

3.9 STATISTIC

Data are means ± standard error of the mean (SEM). Statistical analyses were performed using StatView 5.0 (SAS Institute Inc. Cary, NC, USA). One and 2-ways ANOVAs with Fisher's protected least significant difference (PLSD) posthoc test were used to assess statistical differences. A P value inferior to 0.05 was the threshold to reject the null hypothesis that IL-15 treatment did not influence the metabolic parameter of interest.

4 RESULTS

4.1 ACUTE IL-15 TREATMENT DID NOT INFLUENCE EXPRESSION OF CONTRACTILE PROTEIN TROPONIN T IN L6 MYOTUBES

First, we determined whether IL-15 treatment could influence L6 differentiation. L6 myotubes were exposed to different IL-15 concentrations for 3h and slow/fast troponin T protein expression was assayed by western blotting as a marker that represents the differentiation of myoblasts into myotubes (Branco et al. 2015). There was no difference in troponin T expression between conditions (Figure 2) indicating that acute IL-15 exposure did not influence differentiation of L6 myotubes.

4.2 ACUTE IL-15 TREATMENT DID NOT INFLUENCE FATTY ACID OXIDATION RATE IN L6 MYOTUBES

Many studies showed that high circulating levels of IL-15 are associated with low adiposity (Almendro *et al.* 2006; Carbó *et al.* 2001; Lebris S Quinn, Anderson, Conner, and Wolden-Hanson 2013). Nonetheless, *in vivo* studies cannot determine whether this lost in adipose tissue is caused by higher fatty acid oxidation in skeletal muscle. Only one study measured a 40% increase in fatty acid oxidation in EDL muscle from rats in response to IL-15 treatment. However, in this study, EDL was exposed to a very supraphysiological dose of IL-15 (i.e., 100 ng/ml) (Almendro et al. 2006). Thus, to measure fatty acid oxidation rate in L6 myotubes, oxidation of radioactively labeled [1-^{14}C]-palmitate was followed while L6 myotubes were exposed to IL-15 at concentrations varying from 1 to 20000 pg/ml. IL-15 had no significant effect (Figure 3) implying that it does not modulate fatty acid oxidation in skeletal muscle cells. However, AMPK activation by AICAR treatment did not increase palmitate oxidation either as previously observed using this cell line (M. Kelly et al. 2009).

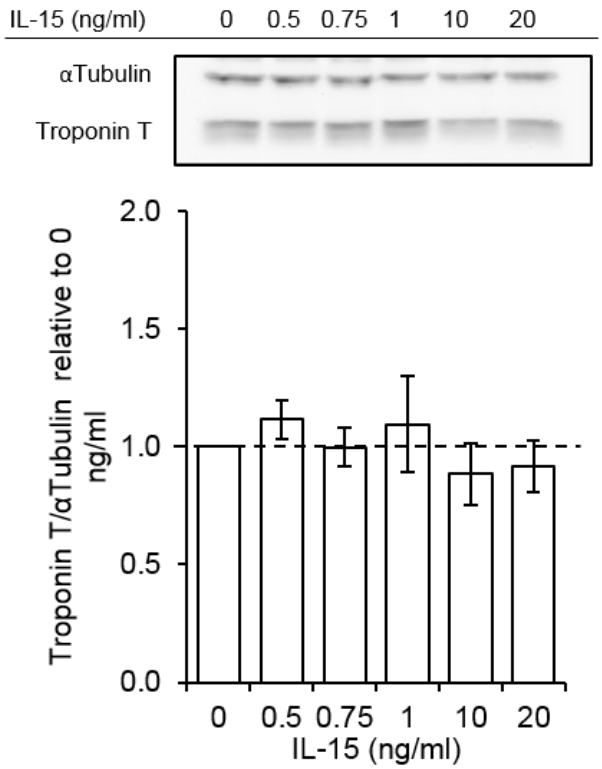

Figure 2: Troponin T expression in response to IL-15 treatment. L6 myotubes were exposed to 0, 0.5, 0.75, 1, 10 or 20 ng/ml IL-15 for 3h in serum free media. Troponin/αTubulin ratio was measured by western blotting. Data represents mean ±SEM * = P<0.05 vs 0 ng/ml

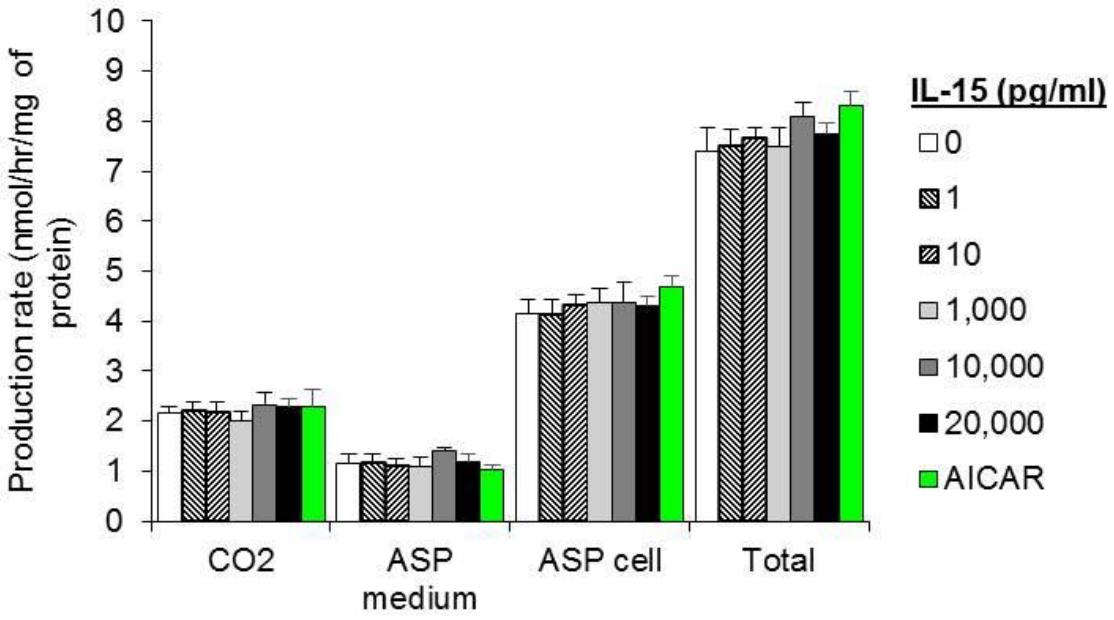

Figure 3: Palmitate oxidation in L6 myotubes is not influenced by IL-15 or by AICAR treatments. L6 myotubes were exposed to different IL-15 concentrations for 3h. CO_2 production represents complete oxidation of palmitate and acid soluble products (ASP) represent incomplete oxidation of palmitate. ASP may either stay inside muscle cells (ASP cell) or exit in the medium (ASP medium). Data represent mean ±SEM (n=3)

4.3 ACUTE IL-15 TREATMENT STIMULATED GLUCOSE UPTAKE IN L6 SKELETAL MUSCLE CELLS

High circulating IL-15 levels are associated with lower blood glucose and insulin levels under basal condition and an overall higher insulin sensitivity (L. Quinn 2011; Sun and Liu 2014; Sun *et al.* 2016). *In vitro*, IL-15 is known to stimulate glucose transport in C2C12 muscle cells (Gray and Kamolrat 2011; Busquets *et al.* 2006) and to increase GLUT4 mRNA expression (Busquets *et al.* 2006). We thus estimated necessary to confirm the effect of IL-15 on glucose transport in L6 myotubes to insure that our models replicates what has been previously observed in scientific literature. Glucose uptake was measured by following the uptake of radioactively labeled [1-^{3}H] 2-Deoxyglycose in response to different IL-15 concentrations. The 2-Deoxyglycose uptake absolute value under resting state was between 8 to 12 pMol/min/µg protein with a 1.5-2 fold increase in response to insulin. These values correspond to what has been previously published in L6 myotubes and thus we consider our myotubes to be healthy and our assay valid (Somwar, Sweeney, and Ramlul 1998). For the 3h IL-15 exposure, 2-Deoxyglucose uptake under basal condition increased in response to IL-15 doses higher than 0.75 ng/ml but show statistical difference only at 20 ng/ml (Figure 4A). Deoxyglucose uptake fold-increase in response to insulin was however not increased in any IL-15 treatments (Figure 4B). In order to verify whether longer IL-15 exposition stimulates glucose uptake in muscle cells in a similar fashion, L6 myotubes were exposed to identical IL-15 concentration for 48 hours. IL-15 treatments did not influence glucose transport under basal condition or enhance insulin effect on glucose uptake (Figure 4C and 4D).

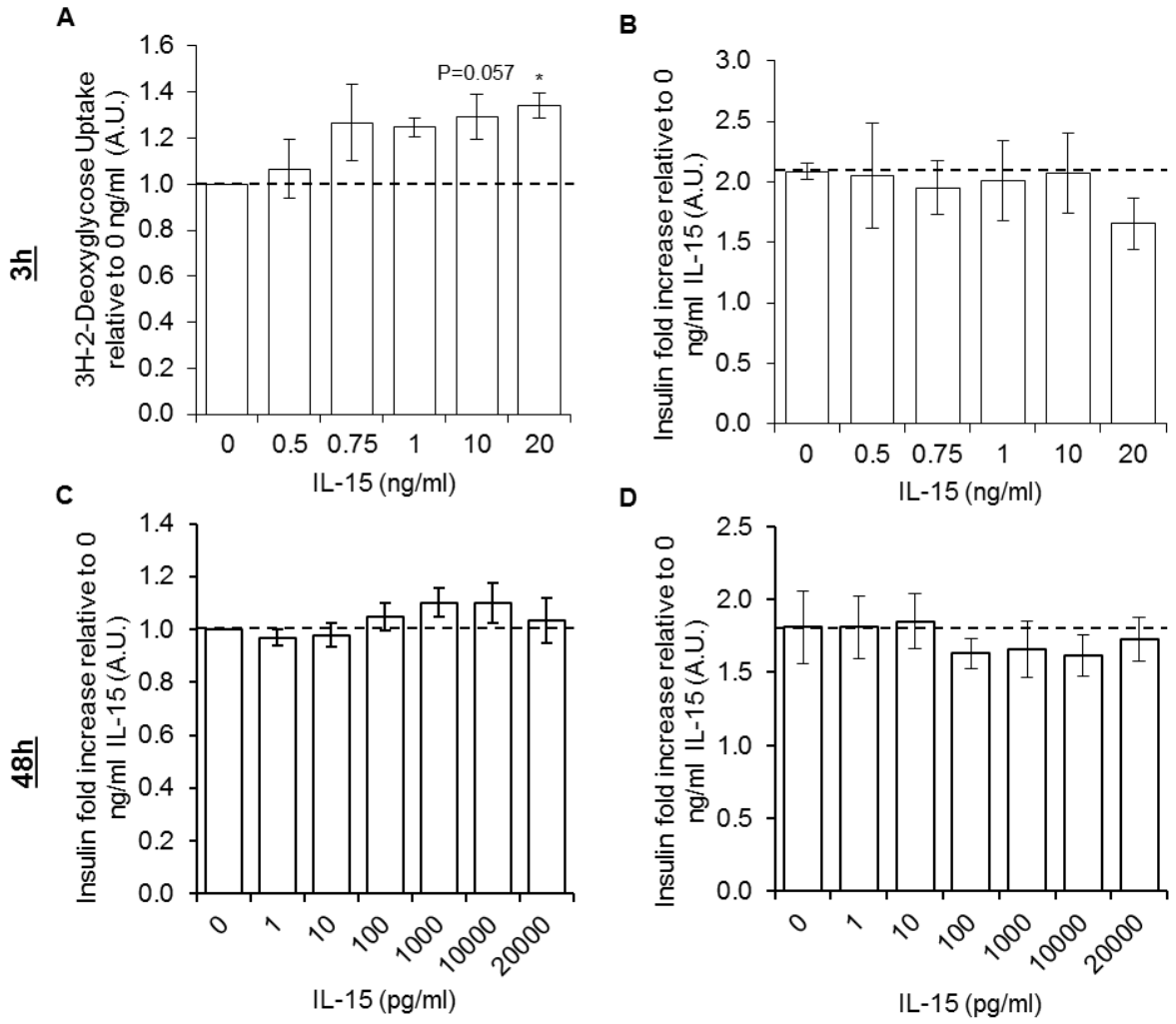

Figure 4 : **IL-15 increases ^{3}H-2-Deoxyglucose uptake in L6 myotubes under basal conditions but does not influence insulin sensitivity**. L6 myotubes were exposed to different IL-15 concentrations for 3h **(A and B)** or 48 hr **(C and D)** and subsequently treated with no insulin **(A and C)** or with 100 nM insulin **(B and D)** for 20 min and were exposed to 10 µM 2-Deoxy-Glucose and 0.5 µCi/ml [^{3}H] 2-Deoxyglucose for 10 min. Data represents mean ±SEM (experiment A and B, n=3; experiment C and D, n=5) * = P<0.05 vs 0 ng/ml

Since IL-15 stimulates glucose uptake, at least on an acute basis, we determine whether IL-15 stimulates insulin signaling pathway by assaying Akt phosphorylation state, a key protein that stimulates GLUT4 translocation once phosphorylated. Insulin treatment induced Akt phosphorylation in L6 myotubes. Three hours IL-15 treatment increased Akt phosphorylation state in L6 myotubes under basal condition but did not meet statistical significance (Figure 5A). IL-15 did not influence Akt phosphorylation when insulin-stimulated (Figure 5A and B). Furthermore, longer IL-15 exposure did not induce Akt phosphorylation (Figure 5C and D). Therefore, based on our results, IL-15 does not significantly activate the insulin signaling pathway in L6 myotubes.

Since 20 ng/ml IL-15 increased basal glucose uptake in L6 myotubes, we tested whether GLUT4 translocation was activated in response to 20 ng/ml IL-15 for different incubation periods. GLUT4 translocation to cell membrane was followed using an L6 cell line with GLUT4 tagged with a Myc epitope. GLUT4 translocation in response to insulin followed a 1.5 fold increase which matches what has been previously observed in scientific literature (Roher et al. 2008). Acute or chronic IL-15 treatments did not influence GLUT4 translocation to cell membrane under basal condition (Figure 6A) nor in response to insulin stimulation (Figure 6B).

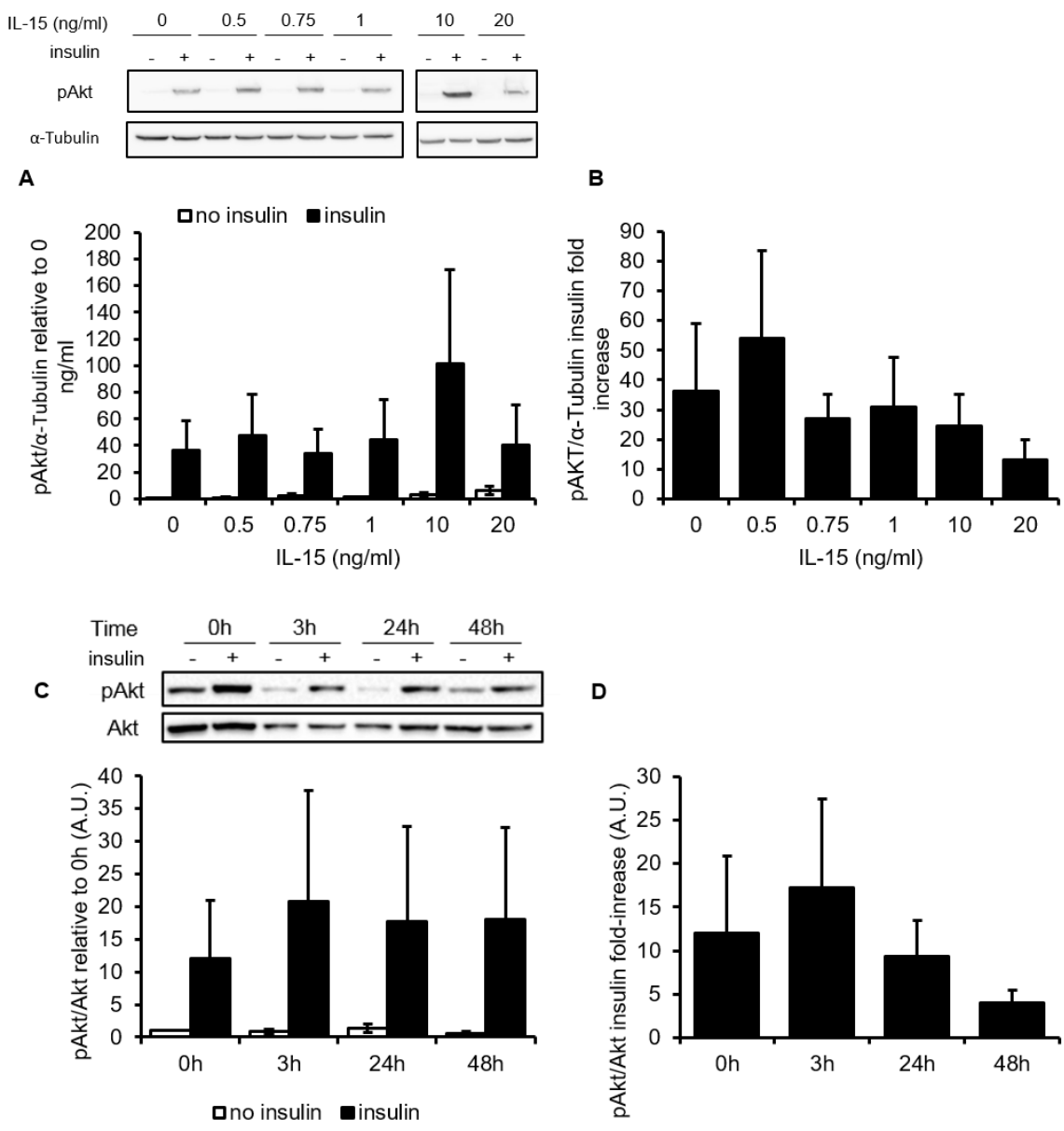

Figure 5 : Acute IL-15 treatment does not induce Akt phosphorylation or insulin-induced Akt phosphorylation in L6 myotubes. L6 myotubes were exposed to different IL-15 concentrations for 3h **(A and B)** or exposed to 20 ng/ml IL-15 for different incubation time **(C and D)**. Cells were treated subsequently with 100 nM insulin for 10 min. pAkt/αTubulin **(A)** and pAkt/Akt **(C)** ratio were determined by Western blotting. **B and D** represent pAkt/ αTubulin and pAkt/Akt fold increase in response to insulin. Data is represented as mean ±SEM (n=3)

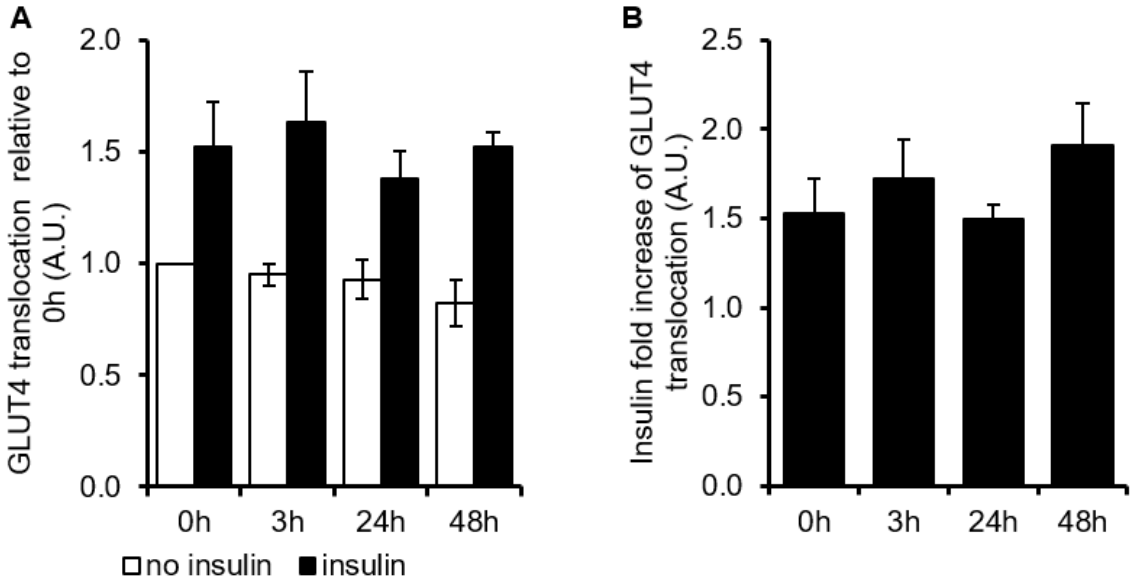

Figure 6 : IL-15 does not influence GLUT4 translocation in L6 myotubes. L6-GLUT4myc myotubes were exposed to 20 ng/ml IL-15 for 0, 3, 24 or 48h. Cells were treated with ± 100nM insulin. Cells were subsequently fixed, and GLUT4myc located at cell membrane was quantified using an *o*-Phenylenediamine dihydrochloride activity assay **(A)**. GLUT4 translocation fold-increase in response to insulin is represented in **B.** Data is represented as mean ±SEM (n=3)

4.4 ACUTE IL-15 INCREASED RESTING OXYGEN CONSUMPTION RATE (OCR) WHEREAS CHRONIC IL-15 EXPOSURE INCREASED MITOCHONDRIAL SPARE CAPACITY

It has been recently demonstrated that IL-15 stimulates oxygen consumption *in vitro* in fibroblasts (Crane *et al.* 2015). OCR of L6 myotubes was measured throughout a 3h IL-15 exposure. Resting OCR was significantly increased when L6 myotubes were treated for 3h with 20 ng/ml IL-15 compared to untreated myotubes (Figure 7B). Furthermore, resting OCR tended to be higher in L6 myotubes treated with 10 and 100 pg/ml despite no significant difference. OCR related to proton leak, or maximal and spare respiratory capacities were not significantly affected by acute IL-15 exposure (Figure 7). Therefore, acute exposure to high levels of IL-15 increases mitochondrial activity without influencing mitochondrial uncoupling activity or capacity to produce ATP potentially.

L6 myotubes were then exposed for 48h to different IL-15 concentrations to investigate how chronic exposure to IL-15 might affect mitochondrial activity. Under resting state, OCR was significantly increased when L6 myotubes were exposed to concentrations of IL-15 as small as 1 and 10 pg/ml. Resting OCR returned to basal levels when L6 myotubes were exposed from 100 to 750 pg/ml IL-15. At higher concentrations (>1,000 pg/ml IL-15), resting OCR was again significantly increased (Figure 8). This biphasic profile was also observed in OCR related to proton leaking and maximal mitochondrial capacity (Figure 8). The difference between OCR at maximal capacity and resting states represents the mitochondrial spare capacity to use oxygen to potentially produce ATP. The biphasic dose response curve was also observed in spare capacity, implying that 48h IL-15 exposure enhanced the mitochondrial capacity of L6 myotubes to produce ATP.

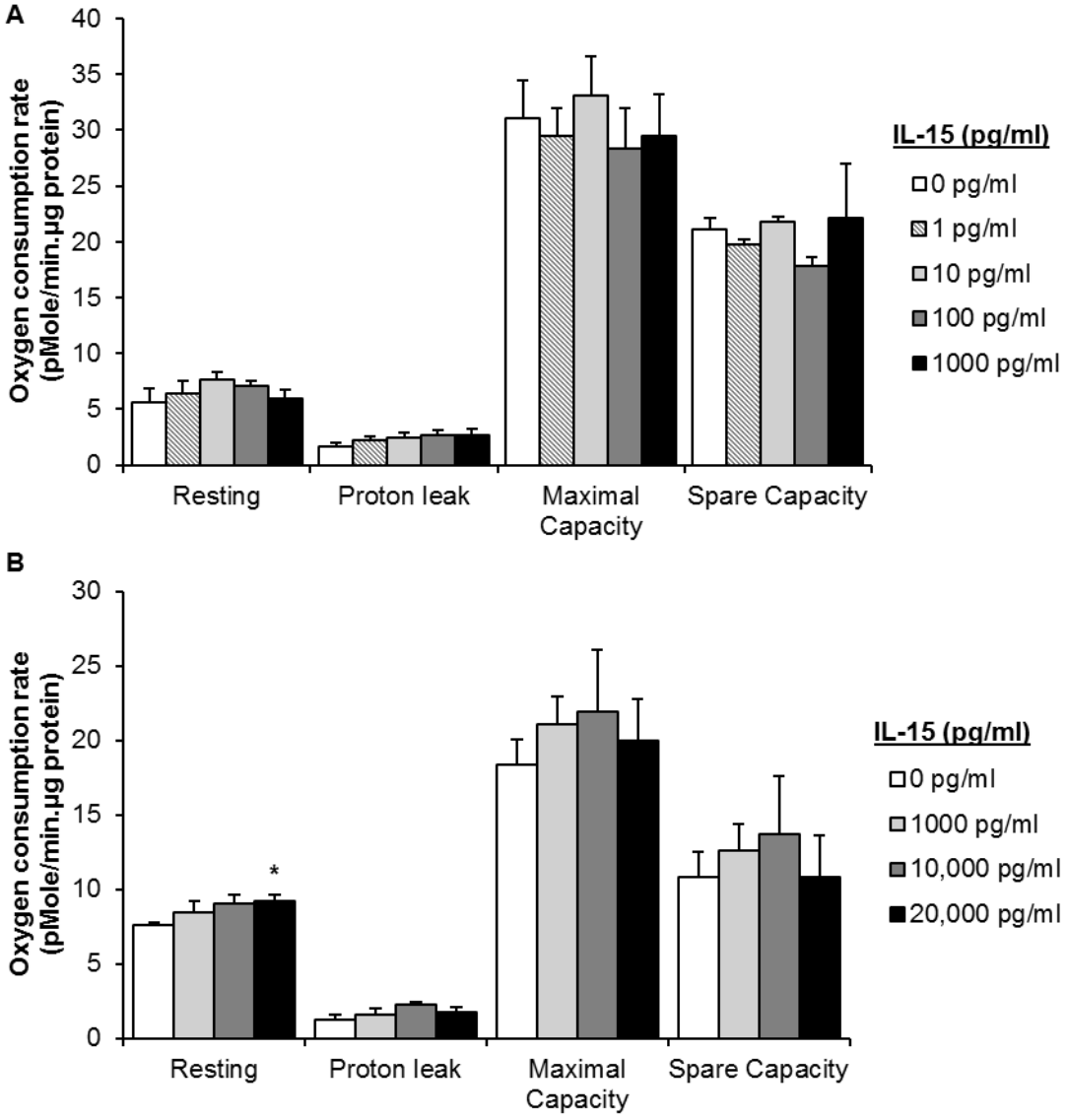

Figure 7 : Acute IL-15 treatment increases Oxygen Consumption Rate (OCR) in L6 myotubes. L6 myotubes were exposed to different IL-15 concentrations for 3 hours. Cells were treated subsequently with 600 ng/ml oligomycin, 1 µM FCCP, and 4 µM antimycin A to determine OCR due to proton leak, uncoupled, and non-mitochondrial respiration, respectively. **A)** OCR of L6 myotubes treated with 0 to 1000 pg/ml IL-15. **B)** OCR in L6 myotubes treated with 0 to 20,000 pg/ml at different states. Data is represented as mean ±SEM (A, n=4; B, n=3) *= $P<0.05$

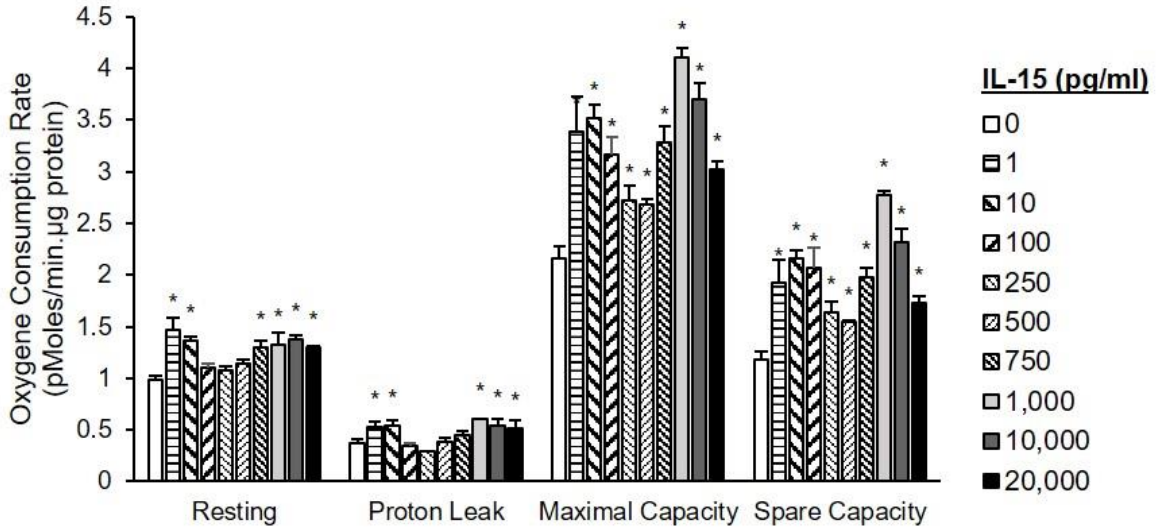

Figure 8 : Chronic IL-15 increases resting oxygen consumption rate and spare capacity in L6 myotubes. L6 myotubes were exposed to different concentrations of IL-15 for 48h. After measuring basal OCR, cells were treated subsequently with 600 ng/ml oligomycin, 1 µM FCCP and 4 µM antimycin A to measure OCR due to proton leak, uncoupled and non-mitochondrial respiration, respectively. Data is represented as mean ±SEM (n=3) *=P<0.05 vs. 0 ng/ml

4.5 Effect of chronic IL-15 treatment on OXPHOS protein expression

The increased mitochondrial spare and maximal capacity in response to chronic IL-15 treatment (Figure 8) may reflect higher mitochondrial content. To determine whether 48h IL-15 exposure increased mitochondrial content, oxidative phosphorylation (OXPHOS) protein expression was measured in response to 48h IL-15 treatment as a marker for mitochondrial content. While a trend toward an increase in protein expression of mitochondrial complexes I, II, III and IV could be observed, the increases did not reach significant difference, especially because of large variations (Figure 9).

4.6 IL-15 treatment in skeletal muscle induces ACC phosphorylation

The second objective of the present study was to determine whether IL-15 activates AMPK pathway. L6 myotubes were exposed to different IL-15 concentrations for 3h. 20 ng/ml IL-15 increased pACC/ACC ratio (Figure 10B). Similarly, pAMPK/AMPK ratio showed a trend towards an IL-15 dose-dependent increase (Figure 10A), but it did not reach statistical significance. The change in pAMPK/AMPK ratio did not match pACC/ACC ratio. L6 myotubes exposed to 20 ng/ml IL-15 for different incubation periods displayed a trend for an increase in pAMPK/AMPK and pACC/ACC ratios (Figure 11). However, no IL-15 treatment was statistically different from myotubes exposed to 0 ng/ml IL-15.

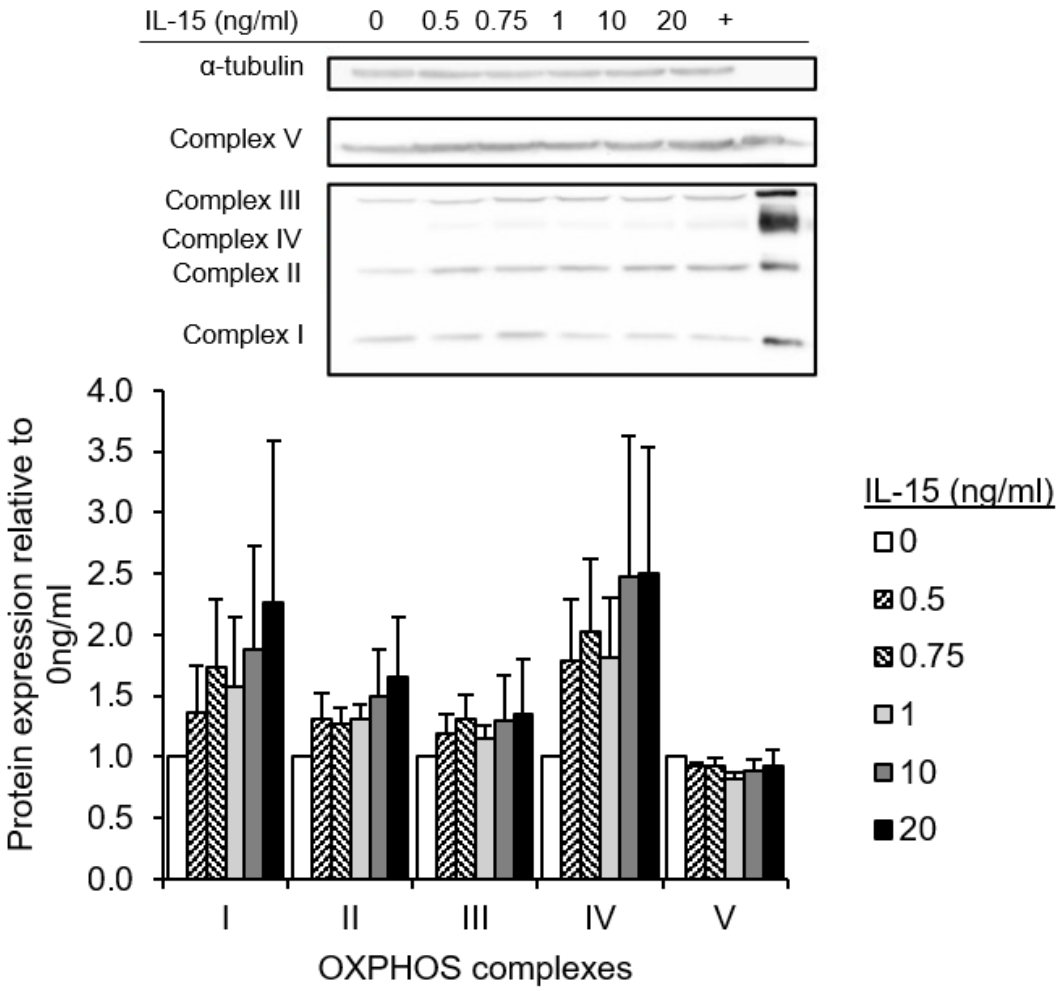

Figure 9: OXPHOS complex expression in response to chronic IL-15 treatment. L6 myotubes were exposed to 0, 0.5, 0.75, 1, 10 or 20 ng/ml IL-15 during the last 48h of differentiation. OXPHOS complex expression was assayed by Western blotting. Data represents mean ±SEM (n=4)

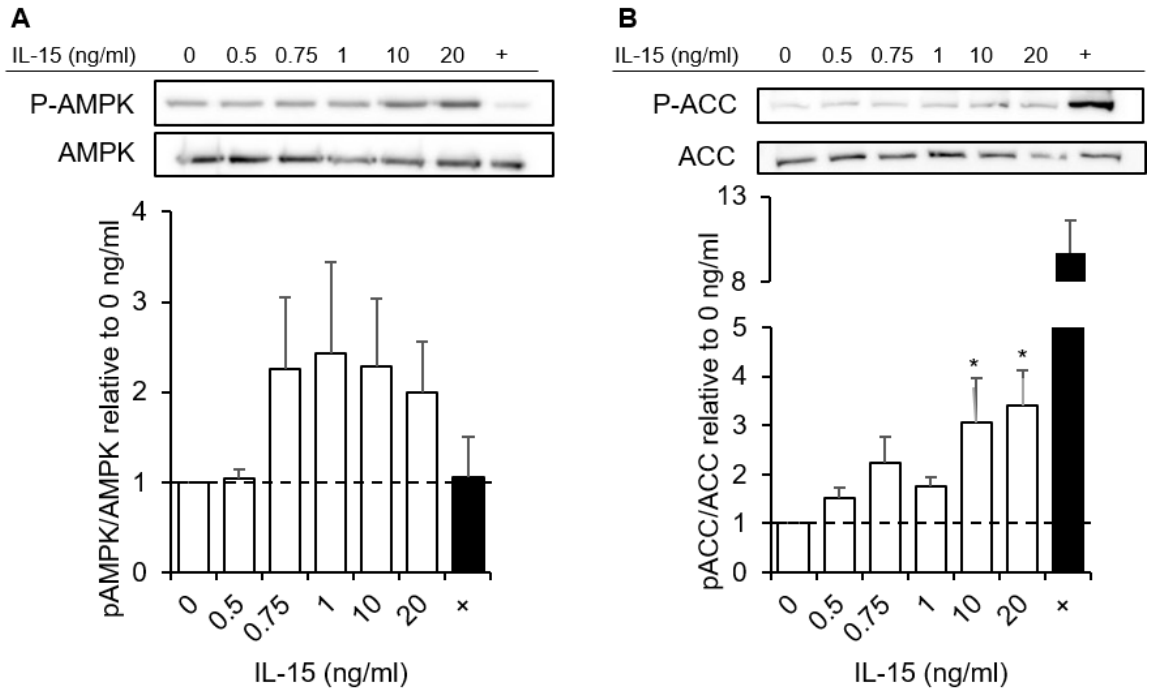

Figure 10: IL-15 treatment increases ACC phosphorylation in L6 myotubes. L6 myotubes were exposed to 0, 0.5, 0.75, 1, 10 or 20 ng/ml IL-15 for 3h in serum free media. 1 μM oligomycin was used as positive control. A) pAMPK/AMPK (n=8) and B) pACC/ACC (n=9) ratios were measured by Western blotting. Data represents mean ±SEM * = P<0.05 vs 0 ng/ml

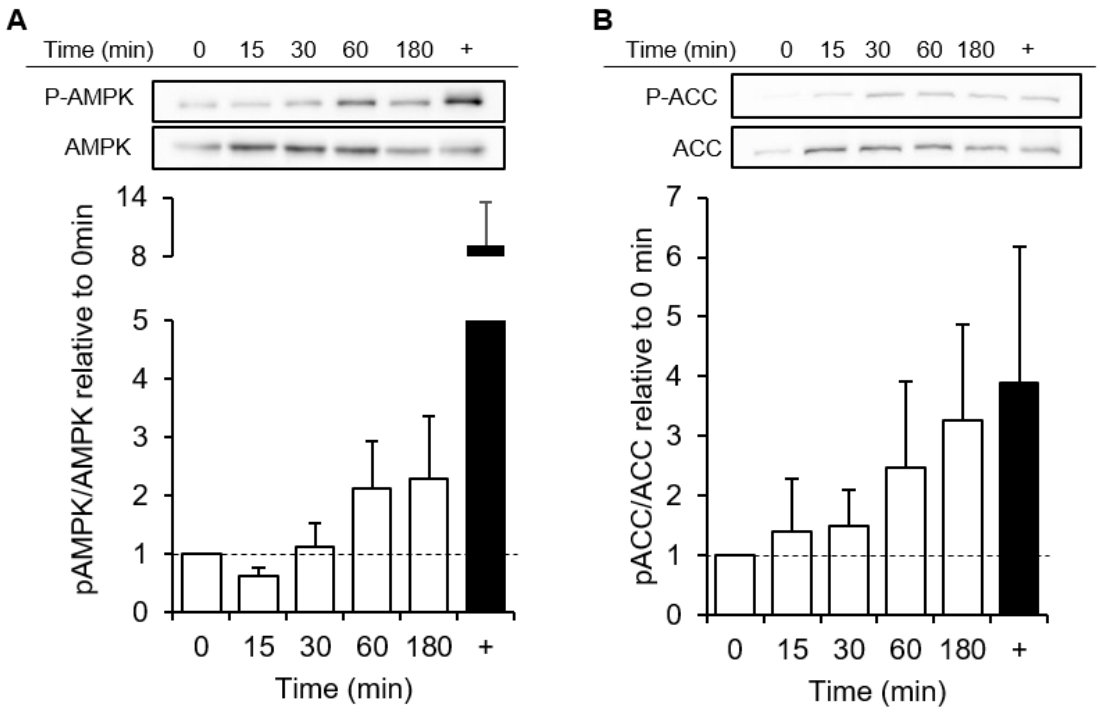

Figure 11 : AMPK and ACC phosphorylation states in response to 20 ng/ml IL-15 throughout time. L6 myotubes were exposed to 20 ng/ml IL-15 for 0, 15, 30, 60 or 180 min in serum free media. 2 mM AICAR (+) was used as a positive control. A) pAMPK/AMPK (n=4) and B) pACC/ACC (n=4) ratios were measured by Western blotting. Data represents mean ±SEM.

4.7 NEITHER ACUTE NOR CHRONIC IL-15 TREATMENT INFLUENCED ADP/ATP RATIO

Since AMPK can be stimulated by a depletion of intracellular ATP levels, ATP and ADP levels were measured in L6 myotubes in response to 3h or 48h IL-15 treatment at different concentrations (Figure 12). Under control conditions, L6 myotubes display ADP/ATP ratios values around 0.1 which corresponds to what has been observed in literature in healthy L6 myotubes (Liu et al. 2012). No significant change was observed in ATP levels or ADP/ATP ratio in response to IL-15 exposure for either 3 or 48h suggesting that IL-15 does not activate the AMPK pathway by increasing ATP depletion.

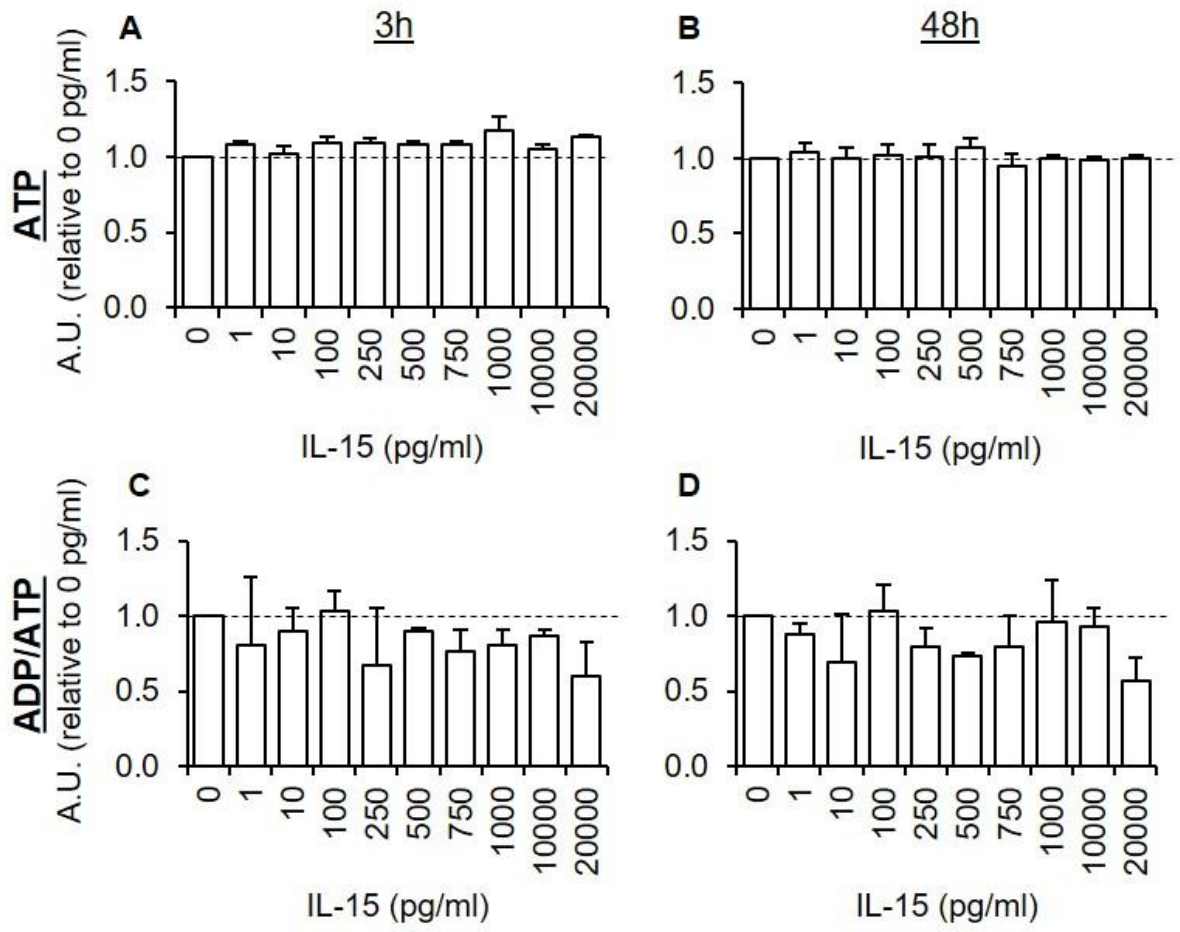

Figure 1 : Acute and chronic IL-15 treatments do not influence ATP content and ADP/ATP ratio in L6 myotubes. L6 myotubes were exposed to 0, 1, 10, 100, 250, 500, 750, 1000, 10000 or 20000 pg/ml IL-15 for 3h **(A and C)** or 48h **(B and D)**. ATP levels **(A and B)** and ADP/ATP ratio **(C and D)** were assayed by luminescence using EnzyLight™ ADP/ATP Ratio Assay Kit (Bioassay Systems)

5 DISCUSSION

The secretory capacity of skeletal muscle has been discovered for many years (Goldstein 1961). However, muscle secretome is still not well characterized. IL-15 has been classified as a novel myokine and under high concentrations, triggers anti-obesity and anti-diabetic effects (Ye 2015). Nonetheless, other research groups argue the opposite showing that IL-15 triggers inflammation which could contribute to the development of metabolic pathologies (Cepero-Donates *et al.* 2016; Lacraz *et al.* 2016). In order to study IL-15 effect on skeletal muscle cells, we used L6 myotubes as a model to mimic muscle tissue. We exposed L6 myotubes to both physiological and supraphysiological doses of IL-15 for 3 (mimicking acute exposure) or 48hr (mimicking chronic exposure). We tested supraphysiological concentrations of IL-15 (500 to 20000 pg/ml) since *in vitro* settings can sometime require higher doses of a cytokine to register a significant response. For example, it was previously reported that IL-15 acts in synergy with IL-6 and IL-8 to improve glucose uptake at small concentrations. However, higher concentrations were needed in order to induce glucose uptake using IL-15 alone (Gray and Kamolrat 2011).

The objective of the present study was 1) to determine if L6 myotubes can be a good model to study the role of IL-15 in skeletal muscle; i.e., to determine if we can replicate the reported metabolic effects of IL-15 in L6 myotubes and 2) to determine whether IL-15 activates the AMPK pathway.

5.1 ACUTE IL-15 TREATMENT IS NOT SUFFICIENT TO INCREASE FATTY ACID OXIDATION RATE IN L6 MYOTUBES

There is a negative association between circulating IL-15 levels and body adiposity (Lebris S Quinn, Anderson, Conner, and Wolden-hanson 2013; Carbó *et al.* 2001; Sun and Liu 2014; Sun *et al.* 2016; Almendro *et al.* 2006). Furthermore, 100 ng/ml IL-15 treatment increased fatty acid oxidation rate in rat skeletal muscle *ex vivo* (Almendro *et al.* 2006). Contrary to expectation, IL-15 did not influence palmitate oxidation in our *in vitro* L6 myotube model (Figure 3). Even an AMPK activation by AICAR did not increase fatty acid oxidation. TOFA, Metformin and C13, three known pro-oxidative drugs, also failed to stimulate ASP production from 14C-palmitate (Appendix 2). We investigated whether the lack of response of L6 myotubes to different pro-oxidative drugs was caused by their glucose-starved state. As expected, the presence of glucose in the media significantly reduced palmitate oxidation rate (Appendix 3). The decrease in palmitate oxidation induced by glucose indicates that the assay is sensitive enough to recognize fluctuations in palmitate oxidation rate and that L6 myotubes have the ability to choose their energetic substrates, which is also called metabolic flexibility. AICAR treatment remained inefficient despite the presence of glucose. Taking all these results and studies together, fatty acid oxidation in L6 myotubes i) can be modulated by the presence of glucose, ii) the effects of AICAR is very small or non-present while iii) it is not responsive to pro-oxidative drugs treatment, including IL-15 treatment. Only one research group has been able to measure a 20% increase in CO_2 production in L6 myotubes in response to 2 mM AICAR for 60 min in MEM+ (K. R. Kelly, Abbott, and Turcotte 2010). The use of MEM+ compared to DMEM may constitute a significant factor that could influence myotube's behavior. For instance, the number of straight and contracting muscle fibers following

dispersion of single fibers after a collagenase digestion were much higher when MEM was used compared to DMEM (Selvin, Hesse, and Renaud 2015). Therefore, the choice of medium may be an influencing factor in fatty oxidation in L6 myotubes.

5.2 IL-15 INCREASES GLUCOSE UPTAKE UNDER BASAL CONDITION BUT DOES NOT ACTIVATE THE INSULIN SIGNALING PATHWAY

A negative correlation between circulating IL-15 levels and resting blood glucose and insulin levels has been reported (Sun *et al.* 2016; Sun and Liu 2014). High circulating IL-15 also promotes higher whole-body insulin sensitivity (Sun *et al.* 2016; Sun and Liu 2014), and increased skeletal muscle glucose uptake (Busquets *et al.* 2006; Gray and Kamolrat 2011) and oxidation (Busquets *et al.* 2006) in C2C12. In our *in vitro* model, we observed a 20% increase in basal glucose uptake (Figure 4) as previously observed in C2C12 by Busquets et al. (2006). IL-15 treatment did not however influence insulin-stimulated glucose uptake of L6 myotubes. The insulin concentration used in this study was 100 nM, a concentration that induces glucose uptake in L6 myotubes near maximal capacity (Ueyama et al. 1999) and 10 nM did not induced a significant increase in glucose uptake (Appendix 4). In future studies, it will be necessary to test the effect of IL-15 on insulin, for which the dosage will have to be between 10 nM and 100 nM.

IL-15 did not stimulate Akt phosphorylation or GLUT4 translocation, implying that IL-15 did not activate the insulin signaling pathway. While this observation is contrary to other studies that reported an IL-15 induction of Akt phosphorylation in many tissues (Ellery and Nicholls 2002; Marzec *et al.* 2008; Jakobisiak, Golab, and Lasek 2011) including skeletal muscle (Zhao and Huang 2012), it is in agreement with the lack of any effect in C2C12 cell line (Huang *et al.* 2015). Therefore, C2C12 and L6 cell lines may lack certain factors that are crucial for IL-

15 to influence Akt signaling pathway. It also implies that perhaps IL-15 does not influence the insulin effect on glucose uptake as reported in this study.

5.3 ACUTE IL-15 TREATMENT INCREASES RESTING OCR IN MUSCLE CELLS WHILE CHRONIC IL-15 EXPOSITION IMPROVES MITOCHONDRIAL CAPACITIES

Exposure to 20 ng/ml IL-15 levels acutely, i.e. over 3h, increased resting OCR (Figure 7). The OCR increase under resting state was not accompanied by an increase of OCR related to proton leaking or mitochondrial maximal or spare capacity. This observation suggests that acute IL-15 exposure does not influence mitochondrial uncoupling or mitochondrial capacity to produce ATP. There was a slight increase of ADP/ATP ratio in L6 myotubes (Figure 12) which is in agreement with the increased OCR (Figure7). The OCR increase induced by IL-15 in myotube could be explained as a way to increase mitochondrial capacity and consequently enhance the cell's capacity to react to any stress-induced energy demand. Whatever the reason, a single IL-15 secretion seems to improve OCR which could enhance muscle oxidative capacity to increase ATP production.

Longer myotube exposure to IL-15 induced a biphasic dose-response curve similar to what Crane *et al.* (2015) observed in fibroblasts. This biphasic trend is also observed in OCR related to proton leaking, mitochondrial maximal, and spare capacity. This biphasic profile suggests that IL-15 activates a certain pathway at concentrations less than 100 pg/ml which increases OCR in skeletal muscle cells. However, this signaling pathway is neutralized when muscle cells are exposed to concentrations ranging from 100 to 750 ng/ml. When muscle cells are exposed to IL-15 concentrations higher than 1,000 pg/ml, IL-15 may stimulate a second signaling pathway that increases OCR. However, this second signaling pathway may not be

specific to IL-15. Most of published literature used very high doses of IL-15, sometimes 10,000 folds higher than reported interstitial concentrations (Lebris S Quinn, Anderson, Conner, and Wolden-Hanson 2013). Excessively high IL-15 concentrations may stimulate an unspecific pathway perhaps by binding non-specifically to another receptor raising the question as to whether the IL-15 effects reported by many published studies are a result of the activation of a pathway unrelated to IL-15. Nonetheless, physiological IL-15 doses stimulate mitochondrial activity and promote mitochondrial capacity to produce ATP.

Increased mitochondrial spare capacity suggests a higher mitochondrial content. However, the increase in spare capacity did not concur with a significant increase in OXPHOS complex expression (Figure 9). Only a trend was observed where the expression of complex I, II, III and IV seems to increase with IL-15 concentrations. This trend supports the fact that high IL-15 levels stimulate mitochondrial biogenesis in skeletal muscle (Pistilli, Guo, and Stauber 2013; O'Connell *et al.* 2015) and increase complex IV activity (Crane *et al.* 2015). It also supports the fact that IL-15 upregulates pro-oxidative transcriptions factors that promote mitochondrial biogenesis such as Pprc1 (Crane *et al.* 2015), PGC1α and β, PPARδ and SIRT1 (Lebris S Quinn, Anderson, Conner, and Wolden-Hanson 2013; Thornton, Krolopp, and Abbott 2016).

Otherwise, IL-15 could also enhance mitochondrial pro-oxidative capacities through the formation of mitochondrial supercomplexes. Mitochondrial supercomplexes are different assemblies of OXPHOS complexes I, III, IV and V. Supercomplex formation improves mitochondrial spare capacity by optimizing electron and substrate transfer during OXPHOS (Genova and Lenaz 2014). Therefore, as a future experiment, we could analyze mitochondrial

supercomplexes in IL-15-treated L6 myotubes using blue-native polyacrylamide gel electrophoresis.

5.4 IL-15 INDUCES ACC PHOSPHORYLATION WITHOUT SIGNIFICANTLY COMPROMISING ATP STOCK AND ACTIVATING AMPK

IL-15 mechanism of action in skeletal muscle remains undetermined. By assessing phosphorylation state of different key proteins, we observed that IL-15 induces phosphorylation of ACC in a dose-dependent manner. IL-15-induced phosphorylation of ACC indicates that IL-15 may inhibit fatty acid synthesis in skeletal muscle. To our knowledge, we are the first to show a link between IL-15 treatment and ACC activity. ACC is the rate-limiting enzyme in fatty acid synthesis pathway. IL-15-induced ACC inhibition supports *in vivo* studies in rodents where high circulating IL-15 levels decreased lipogenesis in liver and WAT (Carbó *et al.* 2001).

Normally increases in pACC matches increases in pAMPK as the latter is a major target of the former. Interestingly, only a non-significant trend was observed for a greater phosphorylation of AMPK. Considering that AICAR was able to induce AMPK phosphorylation, then it is suggested that AMPK is not a major component in the mechanism of action for IL-15. Gray and Kamolrat (2011) observed an AMPK activation in response to a co-treatment of insulin and IL-15 in C2C12. Therefore, maybe insulin is an important factor in the effect of IL-15 on the AMPK pathway. Furthermore, it is also possible that IL-15 induces ACC phosphorylation through an AMPK-independent pathway. For instance, ACC inhibition can also be induced by the inhibition of phosphatase 2A (Krakower and Kim 1981).

IL-15 does not alter ADP/ATP ratio in L6 myotubes. However, ADP/ATP ratio does not reveal the absolute quantify of ATP stock. The only way to determine whether IL-15 influence intramyocellular ATP content is to energetically challenge the myotube.

6 CONCLUSION

In summary in L6 myotubes, IL-15 is a myokine that slightly increases glucose transport on an acute basis (as chronic exposure has no effect) and mitochondrial function in skeletal muscle cells, especially during chronic IL-15 exposure. There was no evidence that IL-15 recruits the insulin signaling pathway for its effect on glucose uptake. Furthermore, IL-15 inhibits ACC by increasing its phosphorylation, but no strong evidence was obtained that this effect involves a phosphorylation of AMPK. Despite the increase in pACC, no evidence was obtained for any increases in FA oxidation but is expected to result in the repression of fatty acid synthesis. Overall, chronic IL-15 exposure induces certain positive effects on skeletal muscle metabolism that could help people living with obesity and diabetes.

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APPENDIX 1

Table 2: IL-15 expression in skeletal muscle and secretion in response to physical exercise in literature.

Ref	Subjects characteristics	Type of exercise	Timing of measuremens after last exercise	Effect of exercise on IL-15 level		
				mRNA	Protein	circulation
(Nielsen *et al.* 2007)	Physically active men Age: 25±1yr BMI: 25±1	Acute resistance exercise (leg press)	0h, 6h, 24h, 48h	↑	-	N/A
(H.-T. Yang *et al.* 2014)	Rats	Endurance training (treadmill)	0h, 12h	↑soleus ↑gastroc	↑soleus -gastroc	N/A
	Rats on HFD	Endurance training (treadmill)	0h, 12h	↑soleus ↑gastroc	↑soleus ↑gastroc	N/A
(H. Yang *et al.* 2013)	Rats on HFD	Endurance training (treadmill)	0h, 12h	↑soleus ↑gastroc	N/A	↑
(Tamura *et al.* 2011)	Untrained physically active men Age: 28.1±1.3yr BMI:22.4±0.7	Acute endurance exercise (treadmill)	-30m, 0m, 10m, 60m, 180m	N/A	N/A	↑ at 10m
(Riechman *et al.* 2004)	Men and women Age: ~20yr	Acute resistance exercise (weight)	5m	N/A	N/A	↑
	Men and women Age: ~20yr	Resistance Training (weight)	5m	N/A	N/A	-
(Rinnov *et al.* 2014)	Young physically active men BMI <25.1	Endurance training (treadmill)	10-14 days before training, 3-5 days after training	-	↑	-
	Young physically active men	Acute endurance	Pre, 0m, 1.5h, 3h, 6h, 21h	-	-	↓

	BMI <24.4	exercise (treadmill)				
(Bazgir *et al.* 2014)	Young men Non athlete vs athlete	Acute resistance exercise (weight)	Before exercise, 1-5m after exercise	N/A	N/A	↑ in both groups
(Pierce, Maples, and Hickner 2015)	Lean men Age: 24.0±3.7 BMI: 23.1±1.9	Acute endurance exercise (cycling)	0m, 30m, 60m, 120m	N/A	N/A	↑ at 30m and 60m
	Obese men Age:27.3±9.1 BMI: 34.7±3.5	Acute endurance exercise (cycling)	0m, 30m, 60m, 120m	N/A	N/A	↑ at 30m and 60m
(Crane *et al.* 2015)	Mice	Acute endurance exercise (treadmill)	0h, 1h, 3h, 24h,	N/A	N/A	↑ at 0h and 1h
	Sedentary (:<1h exercise/wk) vs. active elderly humans (act: ≥4h exercise/wk)	Endurance training (cycling)	-	N/A	N/A	↑
(Ostrowski *et al.* 1998)	Male trained in endurance, n=2	Acute endurance exercise (treadmill)	0h. 0.5h, 1h, 1.5h, 2h, 2.5h, 1h, 2h, 3h, 4h, 5h, 6h, 2d, 6d	N/A	N/A	-
(Lee *et al.* 2011)	Healthy male Age: 22±1yr	Resistance training (weight, 10 week program)	Pre, 6week, post 0d, 1d, 2d, 3d, 5d, 7d	N/A	N/A	↑ at 6week and post 0d
(Louis *et al.* 2007)	Trained in resistance 4 men 2 women Age:25±4yr	Acute resistance exercise (leg press)	Pre, immediately post, 1h, 2h, 4h, 8h, 12h, 24h	-	N/A	N/A
	Trained in endurance 1 woman 5 men	Acute endurance exercise (running)	Pre, immediately post, 1h, 2h,	↑ at 8h	N/A	N/A

	Age:25±4yr		4h, 8h, 12h, 24h			
(Molanouri, Zuhair, and Hassan 2014)	Rats Sedentary vs. trained	Resistance training (weight lifting)	24h post exercise	↓	↑	↑
(Molanouri Shamsi *et al.* 2014)	Rats Sedentary vs. trained (n=2-4)	Resistance training (weight lifting)	Immediately post, 4h, 8h, 12h	-	↑ at anytime	N/A
	Diabetic rats Sedentary vs. trained (n=2-4)	Resistance training (weight lifting)	Immediately post, 4h, 8h, 12h	-	-	N/A
(LeBris S. Quinn *et al.* 2014)	Mice (C57BL/6)	Acute endurance exercise (running)	Pre, 0m, 30m, 180m	↓	N/A	↑
(Kim *et al.* 2013)	Zucker diabetic rats	Endurance training (running)	2d after exercise	N/A	↑soleus - gastroc	N/A
(Zanchi *et al.* 2010)	Rats	resistance training (weight)	Pre, 24h	-	N/A	N/A
(Nieman *et al.* 2004)	Humans 19-27yr male Resistance-trained	Acute resistance exercise (weight)	Pre and post exercise	-	N/A	N/A
(Nieman *et al.* 2003)	Human marathoners	Acute endurance exercise (running)	Pre and post exercise	↓	N/A	N/A
(Chan *et al.* 2004)	Healthy male Age: 24±2yr	Acute endurance exercise (cycling)	Pre and 60min post exercise	-	N/A	N/A
(Christiansen *et al.* 2010)	Healthy, obese, elderly men and women	12 weeks, aeorbic training	Pre, post training at baseline	N/A	N/A	-
(Prestes *et al.* 2009)	Elderly post-menopause women	Acute resistance exercise	Pre, post, 24h, 48h exericse	N/A	N/A	↑ at 48h

		Resistance training	Pre, post, 24h, 48h of last exericse	N/A	N/A	-
(Andersson *et al.* 2010)	Elite female soccer players	2 soccer games (90m) spaced with 72h recovery	**1st game** : 1h pre, 15-20m, 21h, 45h, 69h **2nd game;** 15,20m after	N/A	N/A	-
(Beavers *et al.* 2010)	Elderly sedentary men and women	12 months of aerobic, strength and balance training	0,6, 12 months	N/A	N/A	-

Arrows represents change in IL-15 level, "-" represents no change in IL-15 level, N/A means that the study did not measured this parameter

APPENDIX 2

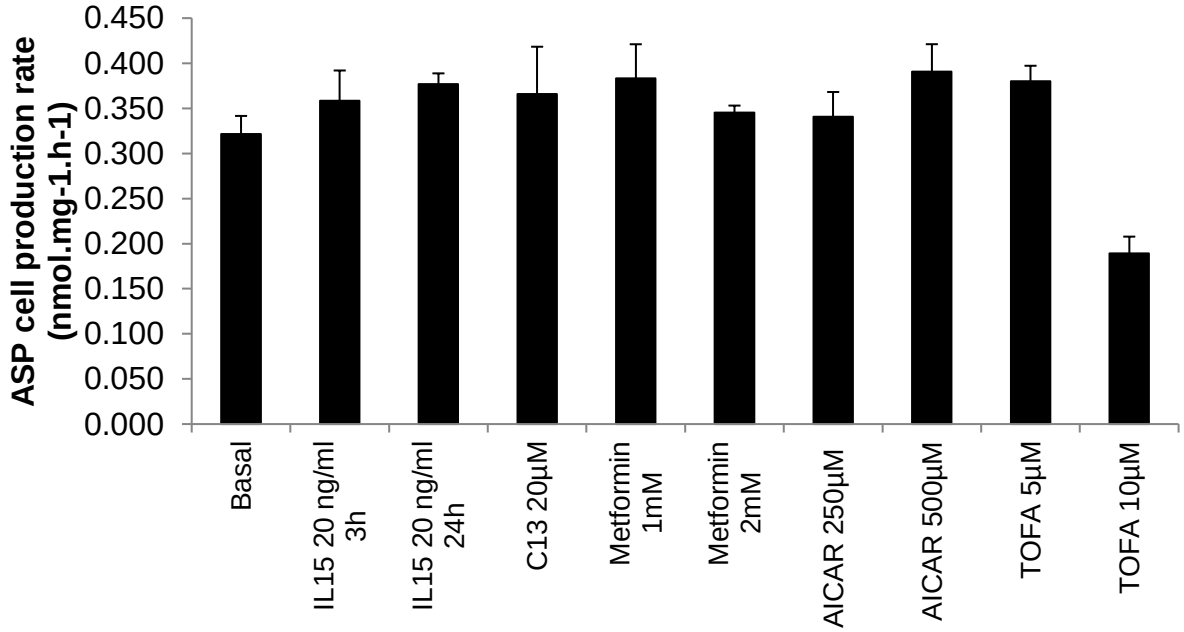

Figure 13: Intracellular ASPs production by palmitate oxidation in L6 myotubes is not influenced by many pro-oxidative drugs. L6 myotubes were exposed to different pro-oxidative drugs, namely C13, Metformin, AICAR and TOFA, for 3h. Data represent mean ±SEM of 3 technical replicates (n=1)

APPENDIX 3

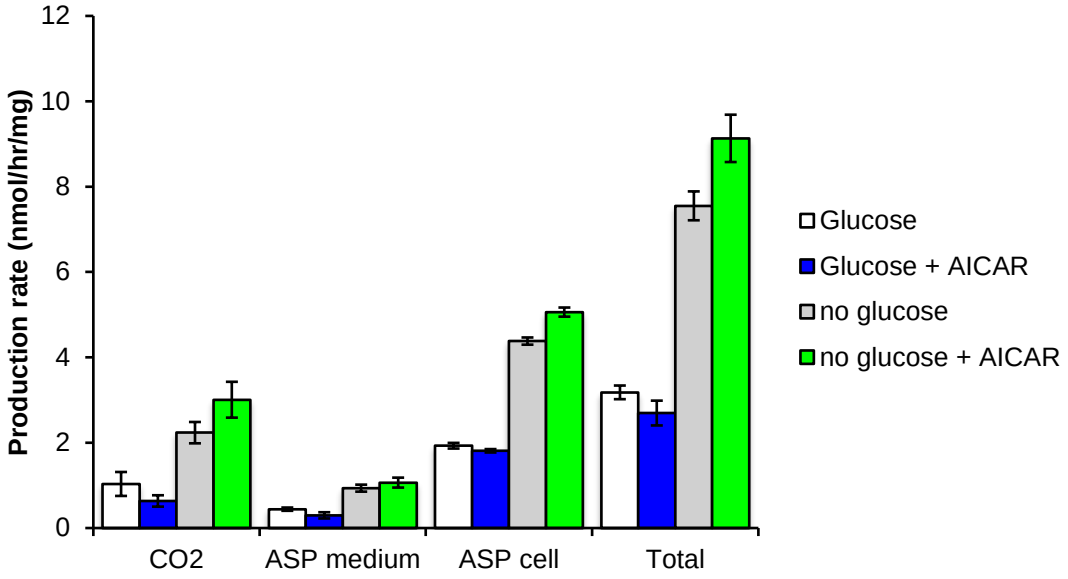

Figure 14: cL6 myotubes were exposed to ± 1000 mg/L Glucose and ± 2 mM AICAR for 3h. CO_2 production represents complete oxidation of palmitate and acid soluble products (ASP) represent incomplete oxidation of palmitate. ASP may either stay inside muscle cells (ASP cell) or exit in the medium (ASP medium). Data represent mean ±SEM of 3 technical replicates (n=1)

APPENDIX 4

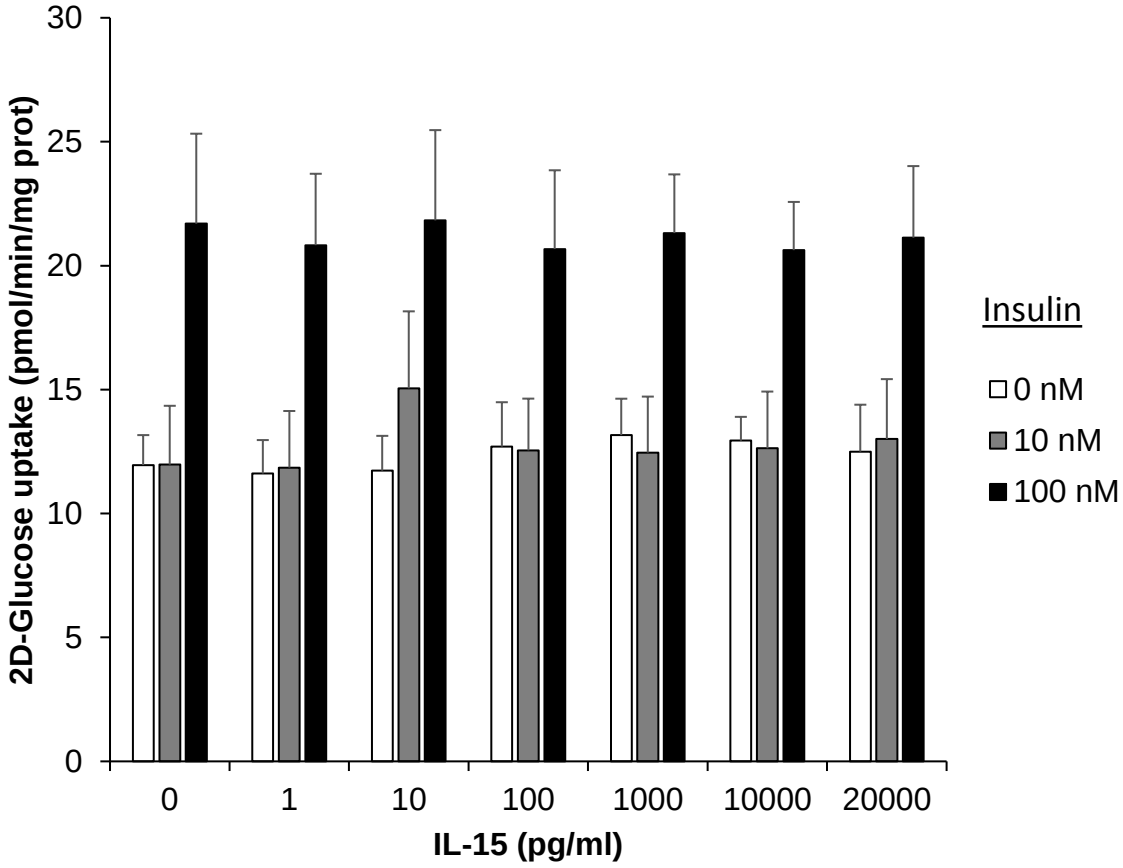

Figure 15: ^{3}H-2-Deoxyglucose uptake in L6 myotubes in response to different insulin concentrations. L6 myotubes were treated with 0, 10 or 100 nM insulin for 20 min and were exposed to 10 μM 2-Deoxy-Glucose and 0.5 μCi/ml [^{3}H] 2-Deoxyglucose for 10 min. Data represents mean ±SEM of 3 technical replicates (n=1) * = P<0.05 vs 0 ng/ml