IL-15: A NOVEL REGULATOR OF LIPOLYSIS IN HUMANS?

by

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Interactions between and within organ systems such as skeletal muscle (SkM) and adipose tissue (AT), *via* immune cell signaling factors (cytokines), may regulate the development of obesity. The increased expression and concentration of one of these cytokines, interleukin (IL)-15, has resulted in significant decreases in AT mass in animals, through the existence of an endocrine-like (SkM-blood-AT) axis. Studies further indicate that these IL-15-mediated decreases in AT mass can occur through reduced lipid uptake or by increased triglyceride breakdown (lipolysis) and release of degradation products from fat cells. While it is speculated that a similar endocrine axis exists in humans, most studies investigating these IL-15 and AT interactions are in animal and cell models: the impact of IL-15 on human AT remains unknown. Therefore, the global aim of this dissertation was to determine if local SkM and subcutaneous (SC) AT IL-15 sources contribute to circulating IL-15, and to examine if IL-15 induces SCAT lipolysis in humans. A secondary aim was to determine if these sources and lipolytic actions of IL-15 differ between lean (LN) and obese (OB) humans.

Healthy LN (n=10, BMI = 23.1 ± 1.9 $kg \cdot m^{-2}$, age: 24.0 ± 3.7 yr) and OB (n=10, BMI = 34.7 ± 3.5 $kg \cdot m^{-2}$, age: 27.3 ± 9.1) men and women participated in the two studies presented in this dissertation. In the first study, using microdialysis probes (CMA/20: 100 kDa cutoff dialysis membrane) inserted in SkM and SCAT to sample interstitial IL-15, and an I.V. catheter to

sample plasma IL-15, we examined local and systemic IL-15 concentrations and their correlation with SCAT lipolysis. In order to clarify how these correlations might change during exercise, subjects cycled for 1-hr at 60% of their heart rate reserve. In the second study using SCAT microdialysis probes, we perfused SCAT with IL-15 to assess direct IL-15 lipolytic effects, and to assess if this lipolytic response was different between LN and OB humans.

We found that SkM interstitial IL-15 was not different between LN and OB, but that SCAT interstitial IL-15 was higher in OB than in LN subjects. Interestingly, SCAT interstitial IL-15 was positively correlated with SCAT lipolysis. Exercise increased plasma IL-15 in both LN and OB, and this increase was higher in OB than in LN subjects. Despite the expected exercise-induced increase in SCAT lipolysis, this increase was not different between LN and OB, nor correlated with IL-15 changes in plasma or SCAT IL-15. In the second study, we found that IL-15 resulted in increased lipolysis in LN individuals and suppressed lipolysis in OB individuals. Importantly, both of these responses occurred at concentrations likely to occur *in vivo* in AT, and occurred without any marked changes in blood flow.

Our findings do not support the existence of an IL-15 endocrine axis in humans, but rather support the possible existence of an uninvestigated IL-15 autocrine/paracrine axis in human AT. In this paradigm, IL-15 may be produced in, and act on the same subcutaneous adipose tissue depot. Although we have found apparent differential regulation of SCAT lipolysis by IL-15 in lean and obese individuals, the extent to which this local axis regulates human body composition remains to be determined.

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Doctor of Philosophy in Bioenergetics and Exercise Science

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DEDICATION

This dissertation is dedicated to my family and friends who have continually believed in my potential, and have supported me throughout everything I have done and continue to do.

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LIST OF COMMON SYMBOLS AND ABBREVIATIONS

α	alpha; generally used to clarify receptor (R) subunit (e.g. IL-15Rα)
β	beta; generally used to clarify R subunit (e.g. IL-15Rβ)
γ	gamma; generally used to clarify R subunit (e.g. IL-15Rγc)
aa	amino acid; components of polypeptides/proteins
AR	adrenergic receptor
AT	adipose tissue; composed of both fat cells (adipocytes) and other cell types (such as blood cells, vascular tissue, and other infiltrating cells)
ATGL	adipose triglyceride lipase
BMI	body mass index, provided as a ratio of weight to height, kg·m-2
DAG	diacylglycerol or diglyceride molecules
EtOH	ethyl alcohol or ethanol
HSL	hormone sensitive lipase
IL	interleukin; protein involved in immune response and other biological functions
JAK	janus kinase
LN	lean subjects/animals
LPS	lipopolysaccharide
LSP	long signaling peptide associated with IL-15
M	molarity; represents a concentration of moles / liter
MAG	monoacylglycerol or monoglyceride molecules
MD	microdialysis
MGL	monoglycerol lipase

MHC	myosin heavy chain
NHANES	National Health and Nutrition Examination Survey
O:I	outflow : inflow ratio; ratio of molecules in the dialysate relative to the perfusate
OB	obese subjects/animals
OE	overexpression; refers to a genetically modified cell/animal in which a specific protein becomes overexpressed to study the function of a particular protein
PKA	protein kinase A
PKG	protein kinase G
R	cell receptor; generally used for clarifying specific receptor subunit component
SCAT	subcutaneous abdominal adipose tissue
SkM	skeletal muscle
SNP	single nucleotide polymorphism
SSP	short signaling peptide associated with IL-15
STAT	signal transducer and activator of transcription
SVF	stromal vascular fraction
TAG	triacylglycerol or triglyceride molecules
WAT	white adipose tissue

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CHAPTER 1: INTRODUCTION AND REVIEW OF THE LITERATURE

Obesity and Disease Risk

It is increasingly evident that obesity has reached epidemic proportions world-wide (24). In America alone, data from the 2009-2010 National Health and Nutrition Examination Survey (NHANES) assessment indicated that 35.7 % of Americans age 20 and older are obese (OB; defined as having a BMI $\geq$ 30 $kg \cdot m^{-2}$) (76). Obesity also carries an estimated economic burden of $147 billion dollars in related health care costs (27). The reason for such a dramatic cost difference vs. normal weight individuals is that obesity is not just a weight control issue, but rather a disease state that also includes increased morbidity and mortality rates, as well as increased risk for co-morbidities (24), such as insulin resistance (22, 58), cardiovascular disease (106) and even certain forms of cancer (120). Currently, many theories exist that deal with how obesity leads to or at least predisposes individuals to these related diseases. Of particular interest, there are several plausible links between obesity and many other diseases which involve secretion of proteins from adipose tissue (AT) and the immune system (26, 58, 79).

The prevailing thought in obesogenic processes is that imbalances exist between fat deposition, fat mobilization (lipolysis), and oxidation (56); therefore, diet and exercise modifications are employed to offset these imbalances by either reducing caloric intake or increasing caloric expenditure. However, obesity is comprised of a multitude of factors, and thus it is of extreme importance to identify novel targets involved in mitigating obesity trends. Despite known problems in both mobilization and oxidation in obesity (12, 56), this dissertation

will center around the mobilization of fatty acyl units from AT stores, a crucial first step in overall fatty acid metabolism, and therefore a critical point in the overall reduction in fat mass.

Lipolysis and lipolytic cascades

Adipose tissue is the largest organ in the body, and is responsible for storage of the largest pool of potential energy in the form of triglyceride or triacylglycerol (TAG) molecules (56). Lipolysis is the collective series of events that breaks down fat stores (e.g. TAG) into its constituents, glycerol and non-esterified fatty acids (NEFA) (114). Fatty acids are then capable of entering downstream bioenergetic pathways, as the intermediate acetyl CoA, to assist with the energy demand to resynthesize ATP as needed. As can be seen in an overview of the lipolytic cascade in Figure 1.1, beginning on the adipocyte cell surface, stimulation of lipolysis will initiate intracellular cascades ultimately resulting in breakdown of TAG to diacylglycerols (DAG) or monoacylglycerols (MAG) via intracellular intermediates such as cyclic AMP (cAMP)/cyclic GMP (cGMP), protein kinase A (PKA)/protein kinase G (PKG) and varied lipases such as adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL), and monoglycerol lipase (MGL) (20, 56, 114). Factors influencing intracellular lipolytic cascade include growth hormone (GH; +), cortisol (+), glucagon (+), catecholamines [epinephrine and norepinephrine; (+)/(-)], natriuretic peptides (+), and insulin (–) (56, 60). In humans, the most important of these appear to be catecholamines and insulin (15). More recently, additional potential factors have emerged as novel targets in regulating fat metabolism, such as cytokines released from AT or skeletal muscle (SkM). For instance, the cytokine IL-15 and its potential influence on lipolysis will be the focus of this dissertation, and will be discussed in detail below.

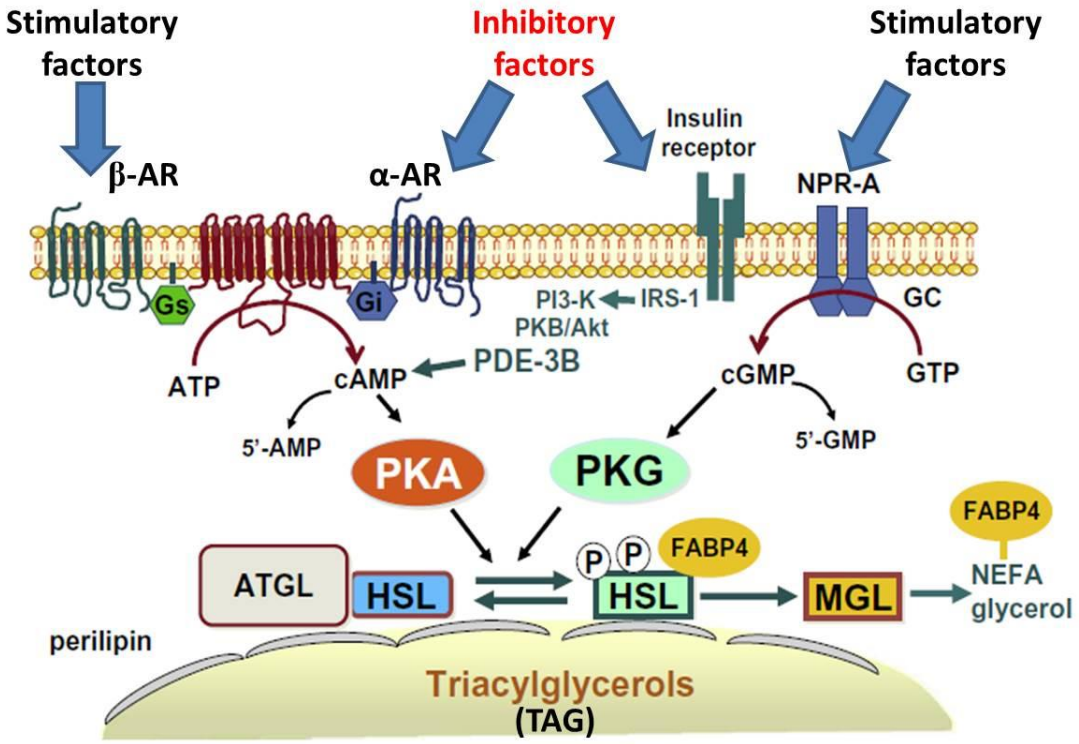

Figure 1.1. Lipolytic cascade overview. The figure illustrates major contributors to adipose tissue lipolysis, including two major pathways converging on the major lipases (ATGL, HSL, and MGL) responsible for the majority of stored fatty acid (Triacylglycerol; TAG) mobilization in the adipocyte. Figure adapted from (56). As shown in the figure, factors that will stimulate G-protein receptors with a G_s subunit will lead to cyclic AMP (cAMP) formation and PKA phosphorylation, ultimately enhancing lipolysis through conformational changes at the lipid droplet (indicated by 'Triacylglycerols' at the bottom of the figure). Alternatively, factors stimulating G_i coupled receptors will inhibit the same pathways leading to suppressed lipolysis. Insulin will likewise suppress lipolysis through increased phosphodiesterase (PDE-3B) activity and cAMP breakdown. Natriuretic peptides will lead to enhanced lipolysis via guanylate cyclase (GC), cyclic GMP (cGMP) production, and protein kinase G (PKG) phosphorylation. Additional lipolytic pathways not shown in figure include MAPK and JAK/STAT pathways.

Major lipolytic pathways

The pathways by which most factors stimulate or inhibit lipolysis are the adrenergic receptor (AR) and natriuretic peptide receptor (NPR) pathways (20, 56), and involve either cAMP and PKA or cGMP and PKG, respectively (refer to Figure 1.1). The main stimulators of the AR pathway are the catecholamines epinephrine and norepinephrine (48, 57). These two catecholamines can stimulate or inhibit lipolysis since they activate both β- and α-ARs, and these

ARs are linked with specific G-protein-coupled stimulatory (G_s) or inhibitory (G_i) second messengers. Moreover, the overall direction and magnitude of lipolysis from adipose stores are under the ultimate control of the α/β stimulatory tone and balance, and this could be one factor explaining regional, yet differential fat deposition (8, 55). Insulin signaling also converges on the AR pathway to suppress lipolysis through phosphodiesterase 3B (PDE-3B) activation. PDE-3B degrades cAMP to 5'AMP, which in turn decreases PKA phosphorylation and downstream lipase phosphorylation (60). The NPR pathway stimulated primarily by atrial and brain natriuretic peptides (ANP and BNP), will alternatively enhance lipolysis through increased cGMP formation and PKG phosphorylation (56). Activation of the NPR pathway, in particular *via* ANP, has been suggested to be important for lipid mobilization observed during both heart failure (14) and exercise (64).

Lipases and related proteins in FA hydrolysis

Regardless of the pathway by which lipolytic induction began, the overall process involves the ultimate phosphorylation of key proteins on the lipid droplet surface. Perilipin A is one of these proteins that plays a critical role in lipid hydrolysis; however, it is not directly involved in the FA hydrolysis reaction as with the other lipase proteins. Instead, phosphorylated perilipin A induces a key physical change in the lipid droplet (15), which allows for subsequent lipase action by increasing the droplet surface area (56). Once the lipid droplet undergoes this conformational change, it is then able to interact with three main lipases: ATGL, HSL, and MGL. While all three participate in FA catabolism, the first two, ATGL and HSL, account for approximately 95% of the FA hydrolysis within the lipid droplet (105). ATGL serves as the primary initiator of TAG hydrolysis, demonstrating nearly 10-fold higher affinity for TAG vs. DAG molecules (122). ATGL's importance in initiating such cascades is highlighted in ATGL-

null animals that demonstrate marked reductions in lipolysis, and therefore a higher prevalence of obesity (40). HSL, on the other hand, was thought for many years to be the rate limiting enzyme for TAG hydrolysis. HSL is capable of hydrolyzing TAG molecules similar to ATGL action; however, it shows a much higher affinity for DAG vs. TAG molecules (28). Once the actions of ATGL and HSL have taken place on TAG and DAG esters, MGL remains a requirement for the complete hydrolysis of the last esterified FA and glycerol molecule, as depleting MGL results in a drastic reduction in glycerol (29).

Exercise and lipolysis

Exercise remains one of the most potent stimulators of lipolysis in humans, most likely through catecholamine release and β-AR pathways (8, 108, 114). The NPR pathway has also been noted to play a partial role in exercise-induced lipolysis (64). As previously mentioned, obese individuals have reduced lipolytic rates vs. LN individuals at rest, when normalized to fat mass (17, 48, 49). However, it is also known that OB individuals can undergo enhanced lipolytic rates from both acute aerobic (109) and resistance (77, 78) exercise, albeit an attenuated response when compared to LN counterparts. To the benefit of OB individuals, short-term (e.g. 10 days) exercise is also capable of improving SkM FA oxidation in OB (12). Therefore, despite any depressed basal lipolytic rates in OB vs. LN individuals, exercise can at least partially "correct" existing imbalances in both FA mobilization and oxidation and thus reducing adiposity and related health consequences.

Cytokines, myokines, and adipokines

Cytokines refer to a family of molecules involved in immune cell signaling. These signals can originate from presence of infection or injury, and typically regulate pro- and anti-

inflammatory actions at both the local and systemic levels. Such actions are needed to correct the insult in affected tissues while concomitantly protecting (e.g. preventing inflammation) in unaffected tissues (39). Therefore, it is of no surprise that many tissues and cells are capable of producing, secreting, and responding to cytokines given the need for this large scale integration. Within a given tissue (e.g. SkM or AT), cytokine production and/or responses can occur between traditional immune cells, such as macrophages and natural killer (NK) cells (39), as well as cells external to the immune core, such as myocytes (96, 111) and adipocytes (1, 2). Interactions between the immune system and cells/tissues external to the immune system via cytokines, once thought to be exclusive to one another, have begun to yield unique perspectives on several linked diseases. The experiments in this dissertation will focus on potential communication between and within SkM and AT via one of these cytokines, Interleukin (IL)-15. The remainder of this literature review will provide the reader with an overview of IL-15 in a physiological context, including a relevant discussion of its signaling pathway, clinical implications, and its role as a myokine and adipokine spanning isolated cell, animal, and human models.

The myokine hypothesis

The idea that SkM secretes substances, such as cytokines ('myokines' in the case of SkM), particularly in the face of contraction/exercise, is in line with the 'exercise factor hypothesis.' This hypothesis partially explains how contracting muscle can lead to positive effects in tissues and organs outside of SkM (84, 86, 87), with these effects made possible through the secretion of myokines (83, 84, 88). In the absence of physical activity, the same processes can at least partially explain why chronic diseases could result from physical inactivity (84). Thus, there is a network of related diseases, termed "the diseasome of physical inactivity," which highlights how secreted substances, or the lack thereof, from skeletal muscle can result in

robust consequences in other organs. In this model, Pedersen, et al., suggest that contracting SkM secrete myokines, which in turn communicate with visceral and/or other fat stores, ultimately resulting in altered fuel metabolism or specific disease-related outcomes (84).

There has been a multitude of myokines identified so far, with the number continually growing. Each represents alternate influences that skeletal muscle can have on other organs, collectively representing the muscle "secretome" (46). More specifically, IL-6, IL-15, IL-8, brain derived neurotrophic factor (BDNF), fibroblast growth factor (FGF-21), and myostatin have been implicated in serving disparate signaling roles as myokines (46, 83). Of these, IL-6 has served as the prototype muscle-derived cytokine since it is the best characterized of the myokines to date. When released from SkM, IL-6 is purported to not only support anti-inflammatory cascades following exercise (84, 85), but also lead to fatty acid mobilization and oxidation (89, 121), and glucose homeostasis (3, 36). IL-15, also identified as potent myokine, is capable of modulating adipocyte metabolism in cell (1, 2), animal (4, 94), and human (10, 69) models, as well as SkM growth and regulation (70, 90, 98).

Adipokines

Much like myokines refer to cytokines expressed and secreted from muscle, similar cytokine products produced in and secreted by AT are called adipocytokines or adipokines. A plethora of research has substantiated that secreted factors from AT qualifies AT as a dynamic endocrine organ (106), as opposed to a once-thought-of dormant lipid sink. While some of these adipokine signals are thought to positively influence adipocyte biology, more often than not, increasing adiposity is commonly associated with an increased secretion of inflammatory biomarkers, such as IL-6, TNF-α, IL-1, MCP-1, resistin, etc. (26, 58, 101), particularly within the AT space. These pro-inflammatory signals are thought to subsequently attract inflammatory-

related cells to the area (e.g. macrophages), and may play substantial roles in the etiology of obesity and related co-morbidities (53). Furthermore, these signals and recruited cells are often more strongly associated with visceral fat vs. subcutaneous fat (26, 75). Interestingly enough, another cell population closely integrated with adipocytes, the stromal vascular fraction (SVF), which includes fibroblasts, vascular endothelial cells, and other inflammatory cells, may likely be the source of such cascades (16, 26, 75, 103). However, that does not discount the fact that adipocytes themselves secrete adipokines as well. It is also common to observe an increased systemic inflammatory profile in obese individuals (16, 51). Thus, it is plausible that the systemic circulation is a reflection of local AT inflammation. Regardless of the exact AT source (e.g. adipocytes or SVF cells), secreted endocrine factors that communicate with cells/tissues within and external to AT potentially represent important links between obesity, inflammation, and metabolic disorders (24, 53, 75, 79, 103, 106).

Interleukin (IL)-15

IL-15 structure and function overview

IL-15 is a 14-15 kDa cytokine was first identified as a T-cell differentiation factor sharing some biological function with IL-2 (38), both containing 4 α-helices and similar cell surface receptor (R) subunit constituents (33). More specifically, while they share a common Rβ subunit (IL-2Rβ) with each other, and the common γ (γc) chain with several cytokines (33), each has a distinct α (Rα) chain (6, 35, 112). Given this, there is strong affinity for each respective receptor complex; however, IL-15 actively competes for the IL-2 receptor given the similar β and γc subunits (33, 38). Additionally, IL-15 is expressed more ubiquitously (38, 112, 113) than IL-2, with a particular high expression in placenta and skeletal muscle (38). Cellular production of this

cytokine is quite complex, involves multi-level regulation (e.g. transcription, translation, and cellular trafficking), and produces two known isoforms from the same gene (73, 113). Each isoform and its relevance to IL-15 function will be discussed briefly below.

IL-15 cDNA encodes a 162 amino acid (aa) peptide, and depending on post-transcriptional alternative splicing, two known isoforms are either 114 aa (48 aa leader sequence) or 141 aa (21 aa leader). The main function of this type of expression appears to direct its intracellular trafficking (113) and ultimate target tissue specificity (94). The 21 aa leader sequence, known as the short signaling peptide (SSP) will maintain IL-15 protein intracellularly, sequestered mostly in cytoplasm or nuclear compartments, whereas the 48 aa leader sequence, or the long signaling peptide (LSP), is targeted to the golgi apparatus for secretion (113). In addition to the intracellular trafficking, evidence also suggests that the signal peptides may also alter expression levels (112) and/or biological half-life (13) of IL-15 protein. For example, SSP IL-15 transfection into the mammalian HEK-293 cell line resulted in approximately 40-fold lower IL-15 protein when compared with LSP transfection (13). Evidence at both transcriptional and translational levels (13, 73) has demonstrated that alternative splicing yielding the non-secretable/SSP IL-15 is capable of suppressing secretable/LSP IL-15 expression through negative feedback mechanisms. Furthermore, SSP half-life is estimated to be ~20 min in contrast to LSP's approximate 80 min half-life (13). With the LSP isoform thought to be more biologically relevant for immune cell development and expansion vs. SSP IL-15, alternative splicing therefore represents an additional method for the cell to regulate IL-15 bioactivity (73, 74). While additional postulations are currently being investigated surrounding IL-15's dual isoform expression, it demonstrates an exquisite level of self-regulation appearing to be biologically significant, as this expression remains highly conserved across several species (13).

IL-15 receptor components and signaling

Beyond the SSP/LSP signaling peptide, the mature IL-15 protein interacts with other cells via binding to a heterotrimer complex (IL-15Rα, IL-2/15Rβ, γc) expressed on the target cell membrane. The high affinity of IL-15 for IL-15Rα, with an approximate Kd = 0.05 nM (65), is thought to primarily confer binding to the other two subunits, either as a trimer receptor on the target cell membrane, or via transpresentation to adjacent target cells expressing β and γc subunits (25, 104), although it does not appear requisite for IL-15 signaling (6). Despite a lack of signaling capabilities, the Rα can substantially modify IL-15 bioactivity through several mechanisms. For instance, intracellular dimerization of Rα and IL-15 increases stabilization, reduces degradation, and even extends the biological half-life of IL-15 (13), much like the LSP provides compared to the SSP leader sequence. It has also been shown that association of IL-15 with its cell surface Rα can sustain in vitro biological activity up to 48 hours after exogenous IL-15 administration has ceased, as evidenced through endosomal internalization, subsequent recycling, and trans-presentation to nearby cells (25). Proteolytic cleavage of the membrane-bound alpha subunit also leads to a soluble receptor (sIL-15Rα) (65), which can further modify extracellular IL-15 function, most likely dependent on the specific biological context. As an antagonist, sIL-15Rα binds free IL-15 preventing binding to a membrane-bound trimer complex (65). In rheumatoid arthritis and other inflammatory pathological conditions (11, 123), antagonistic quenching of these signaling pathways could prove beneficial to patients due to IL-15's pro-inflammatory nature. Agonistic actions, such as enhancement of IL-15 anti-tumor function may prove just as beneficial in chemotherapeutic applications (68, 107, 110, 117).

In contrast to the Rα, the Rβ and γc components serve as the primary signal transduction subunits for IL-15 (33), functionally integrated with intracellular janus kinase (JAK) members 1

and 3, and signal transducer and activator of transcription (STAT) proteins 3 and 5 (50, 102). As evidenced through cell culture transfection experiments, both the Rβ and γc subunit are required for proper IL-15 function. For instance, murine BAF/B03 cells, a cell line which constitutively expresses adequate IL-2Rα and γc receptor components and not the Rβ, only becomes responsive to both IL-2 and IL-15 treatment after transfection with the Rβ. Administration of the Mikβ1 (anti-IL-2Rβ) antibody after transfection abrogates this response (33). Giri and colleagues further demonstrated the importance of these two subunits on COS cells not inherently expressing Rβ or γc components. Significant amounts of IL-15 ligand binding only occurred on COS cells with co-transfection of IL-2/15Rβ and γc, while the individual transfection (e.g. Rβ or γc alone) resulted in minimal binding (33). Work that followed demonstrated that while the βγ heterodimer can induce signaling, it only occurred at very high IL-15 concentrations (450 ng/mL) when not in the presence of IL-15 Rα (6). It has been suggested that among a wide variety of cell lines and tissues that express the βγ heterodimer, the presence/absence of the Rα is the limiting factor in IL-15 responsiveness, not through signaling capabilities, rather via establishing adequate binding to the other subunits (6). Therefore, while signaling can occur without the Rα subunit, it is unlikely in most physiological conditions. Moreover, under normal conditions (e.g. rather low IL-15 concentrations), the highest affinity and signaling occurs when all three subunits work in concert to mediate pro-inflammatory IL-15 effects.

Once the IL-15 receptor components trimerize, signaling is made possible through the β and γ coupling to JAK proteins 1 and 3. Activation of the JAK-STAT pathway, a pathway common to other cytokine and peptide hormones, ultimately results in nuclear transcription factor binding (e.g. NFκB) and protein synthesis. With regard to IL-15's pro-inflammatory role, this downstream protein synthesis aids in growth, development, and proliferation of relevant

immune cells (e.g. NK cells) responding to an immune challenge (23). Activation of this pathway may alternatively involve phosphorylation of various intracellular intermediates, partially explaining this cytokine's pleiotropic actions, such as lipolytic induction and energy provisioning (2) following the same challenge. This should be of no surprise with the ubiquitous nature of this pathway and its ability to influence immune responses, growth, energy expenditure, and cell differentiation mediated from a whole host of signaling molecules (97).

Disruptions in the JAK-STAT pathway, mediated by cytokines such as IL-15, manifest themselves in clinically relevant functional outcomes. Selected JAK-3 deletions (82) or JAK-1 pathway blockade (23) in animals has been shown to result in drastic reductions in lymphoid cells. Specific reductions of up to 90% reduction in NK cell populations were demonstrated in one study where animals were administered antibodies directed against IL-2/15Rβ (coupled to JAK-1) (23). Mutations in the γc subunit linked to JAK3, leads to X-linked severe combined immunodeficiency (XSCID) in humans, resulting in a diminished T-cell population as well as T-cell responses to immune challenges (102). Dysfunction along this pathway can therefore compromise the ability of both animal and human innate immune systems to mount successful responses to immune challenges. Yet, blockade of these same pathways may benefit patients with autoimmune disorders. Ongoing later-stage clinical trials are investigating the effects of JAK inhibitors in the treatment of rheumatoid arthritis (RA) with promising results thus far (54). In one trial, patients demonstrated significant reductions in RA scores (>70% symptom resolution with all doses of JAK inhibitor vs. 26% resolution in placebo) with minimal side effects. Even more promising, these outcomes occurred in patients for whom other biologic treatments, such as TNF-α antagonists, were ineffective or potentially harmful (54). Taken together, IL-15's interaction with its receptor components/intracellular targets highlights not only

the pleiotropic nature of this cytokine, but also its clinical and therapeutic potential. It also represents a complex axis in need of further exploration, in particular with its potential effects external to the immune system.

IL-15 as a myokine

As previously mentioned, IL-15 and IL-2 ligands and receptors share some structural homology. It is therefore not surprising that IL-2 and IL-15 possess similar properties in terms of T-cell differentiation (38, 112, 113). While IL-2 seems rather exclusive to the immune system, IL-15 has been noted to regulate various non-immune actions, such as the case of SkM IL-15, exerting effects within (autocrine/paracrine) and external (endocrine) to SkM. These spatially-distinct actions are first made possible through SkM dual isoform expression, where muscle-derived IL-15 exists as both 21-aa SSP (remains in SkM) and 48-aa LSP isoforms (able to exit the SkM space) (113). Further, SkM (91) along with tissues external to SkM (e.g. adipocytes) (4) express the full IL-15 trimeric receptor necessary for signal transduction. SkM IL-15 acting as a myokine, both within and external to SkM will be briefly discussed below.

IL-15 effects within SkM

Similar to well-known anabolic factors, such as insulin-like growth factor I (IGF-I), IL-15 has been implicated as a potential regulator of muscle mass, supporting contractile protein accrual in animal and human skeletal muscle cultures (31, 95) and potentially preserving muscle in atrophic animal models (91). One study revealed that IL-15 at doses of 10 or 100 ng/mL increased myosin heavy chain (MHC) expression 5-fold in murine C2C12 cells and 2.5-fold in primary fetal bovine cultures. In the bovine cultures, IL-15 served to supplement IGF-I-induced MHC accumulation, with their synergistic effects nearly doubled that of either factor alone (95). A follow-up study employing human primary SkM cultures (31) demonstrated similar effects on

human muscle at even lower doses of 1-5 ng/mL; yet, this same investigation also revealed that IL-15 and IGF-I have different temporal resolution in SkM anabolism. Specifically, IL-15 appears to increase MHC protein accretion after differentiation (e.g. myotube hypertrophy), as opposed to influencing myoblast proliferation or myotube differentiation (31, 95). IL-15 may also influence SkM mass through regulating (mitigating) atrophic processes. Pistilli and colleagues (91) demonstrated rat soleus IL-15 mRNA expression increased in both aging (20% increase) and unloading (81% increase) conditions. While the observed increase in IL-15 did not prevent muscle atrophy from occurring, the authors suggested muscle IL-15 may increase in such situations to counteract atrophic stimuli, likely through anti-apoptotic pathways (91). Although more research needs to evaluate the effectiveness of IL-15 on human muscle regulation *in vivo*, IL-15 appears to be a novel regulator of SkM anabolic and catabolic processes (7, 31). While we will not examine IL-15 effects in/on SkM in this dissertation, we will in part examine how SkM IL-15 can potentially influence SCAT lipolysis in human subjects.

IL-15 effects external to SkM

IL-15 can also leave SkM through a secretory pathway. Evidence suggests that SkM can influence AT metabolism through this IL-15 secretion, acting as an endocrine organ. In this paradigm, and opposite to the anabolic paradigm in SkM presented above, secreted IL-15 is associated with a decrease in AT mass. Foundations of this axis stem from high muscle IL-15 expression, compared to that of the adipocyte at all stages of differentiation (96), coupled with robust adipose tissue metabolic regulation (2, 4, 9, 10, 18, 92, 94). The most compelling work in support of a unidirectional muscle-to-adipose axis lies in several transgenic (TG) and knockout (KO) animal models (9, 10, 94). Albeit speculation exists of a similar muscle-to-fat relationship in humans, more work is needed to support these hypotheses.

The earliest work on SkM IL-15 effecting adjacent/external tissues aimed to describe the metabolic effects of IL-15 treatment in animals once it was known that muscle highly expressed this cytokine (4, 18). It was first reported that 7 days of IL-15 administration (100 μg/kg body weight) resulted in a 33 % reduction in white AT (WAT) mass in male Wistar rats (18). A follow-up study confirmed these findings in several animal strains (lean [C57BL/6] and obese [ob/ob] mice, and lean Zucker rats), demonstrating loss of WAT fat mass ranging from 14 - 44 % (4). Subsequently, several investigations altered endogenous IL-15 production in several transgenic (TG) and knockout (KO) animal models to better understand the *in vivo* metabolic role of IL-15. Barra, et al. (10), demonstrated that altering IL-15 expression drastically influenced body weight and fat mass. When compared to control C57BL/6 mice, 6 month old IL-15 KO mice (IL-$15^{-/-}$) gained approximately 23% body weight and nearly doubled their visceral fat mass, while IL-15 TG (IL-15tg) mice overexpressing IL-15, lost nearly 36% body mass as well as 42% visceral fat mass. A partial recovery of the control phenotype in IL-$15^{-/-}$ mice treated with daily i.p. injections of 500 ng IL-15 was also observed, leading to a 12% body mass reduction compared to a PBS control (10). Most importantly, the body composition changes occurred without altering food intake. It was not determined, however, if activity levels were modified in these mice. In addition, diet-induced obese C57BL/6 mice on a 60% high fat diet also responded to IL-15, having lost ~11% body weight with adenoviral delivery optimizing IL-15 secretion to a concentration of 4.8 ng/mL (10).

Although important to understanding the metabolic effects of IL-15, the above findings only highlighted a global reduction or increase in IL-15 on body composition. However, a seminal study from Quinn, et al. (94), demonstrated a direct link between SkM IL-15 and changes in body composition by modeling intracellular vs. secreted IL-15, similar to SSP vs.

LSP IL-15 previously discussed. Toward this end, two SkM IL-15 OE constructs in male and female mice were created: one an inefficiently secreted (intracellular) isoform, and the other an efficiently secreted isoform. Both transgene constructs resulted in an increased SkM IL-15 protein expression as expected. The most salient finding, however, was that the overexpressed secreted isoform characterized by an elevated circulating IL-15 (concentrations mostly near assay sensitivity <10 pg/mL increased up through ranges between 300-650 pg/mL), was also accompanied by significant reductions in percent fat mass while on a 22% moderate-fat diet. These investigators noted that the male mice lost a greater amount of fat vs. their female counterparts (50 vs 25% reduction, respectively), although these losses were both different from control mice. IL-15 over-secretion also prevented increased adiposity during high-fat (60% of kcal) feeding, while this effect occurred in male mice only. Conversely, intramuscular OE without over-secretion to the circulation did not result in body composition alterations in either gender (94). It was concluded that although there were some slight gender differences in the absolute fat mass lost, IL-15 can modulate AT mass accumulation through endocrine mechanisms (e.g. secreted from SkM, traveling through the circulation to a terminal target tissue such as AT), once efficiently secreted from muscle.

In contrast to available animal data discussed above, human studies surrounding IL-15's role in potential AT regulation are clearly lacking. Furthermore, the most promising data in humans with muscle IL-15 and adiposity has been largely associative and speculative. It has been demonstrated that OB subjects have lower circulating IL-15 (10, 69), and that plasma IL-15 negatively associated with total fat mass, trunk fat mass, and percent fat mass (69), even when adjusted for age, sex, fitness level, and smoking status using multivariate analysis. From the relationships that exist in humans (69), and reports of increased circulating IL-15 in LN vs. OB

(10, 69), researchers to date have only speculated that OB SkM secretes less IL-15 into the systemic circulation. Conversely, prior published data also indicated that SkM IL-15 mRNA expression in OB was not correlated with plasma IL-15 (69). While this doesn't prove causality, it might rather suggest that SkM is not a major contributing source of circulating IL-15 in humans. Furthermore, exercise/muscle contraction is thought to be a potential stimulus leading to IL-15 secretion, yet both increases (98, 115) and no changes (70, 99) in IL-15 following exercise have been observed. Therefore, although the assumption that SkM is a major contributing source to circulating IL-15 and that circulating IL-15 leads to a decreased fat mass in humans seems to be within reason given existing reports, the source of circulating IL-15 and/or the stimuli leading to its secretion in humans remain unknown (10, 93).

Collectively, the studies discussed above suggest that whether exogenously delivered or endogenously produced, IL-15 appears to play a substantial role in the regulation of AT, at least more definitively in animals. IL-15 also potentially links SkM and AT in metabolic reciprocity, such that their inter-organ communication can elucidate important obesogenic mechanisms not yet fully understood. Although of interest to understanding novel control points in obesity, most of the questions answered to date are limited to these artificially created systems and are still in need of further substantiation within more physiologically-relevant IL-15 ranges, especially in humans. The experiments in this dissertation were designed to simultaneously examine SkM, systemic, and AT IL-15, providing a comprehensive look at this potential axis in lean and obese humans. Further, we aimed to determine if IL-15 could induce lipolysis at physiologically-relevant up through pharmacological concentrations in human subjects. If there is evidence of this axis existing in humans, this could enhance our understanding of *in vivo* inter-organ communication, and also represent important therapeutic targets in the treatment of obesity.

IL-15 as an adipokine

As opposed to many cytokines found in AT depots, that are primarily the products of nonfat AT-infiltrating cells (26), IL-15 is both found in the AT space and expressed by adipocytes. Two separate investigations have reported that adipocytes express IL-15 mRNA (1, 96), although a much lower expression compared to that of SkM (96). However, Ajuwon, et al., demonstrated in isolated porcine adipocytes that a relatively low IL-15 expression was significantly increased with interferon-γ (IFN-γ) exposure for 2-hr, and further increased at 3-hr (1). When exposed to a pro-inflammatory mediator such as IFN-γ, an increased IL-15 expression suggests that the adipocyte could serve an important role in the innate immune response by augmenting IL-15 concentrations. Alternatively, given that the adipocyte expresses all 3 IL-15 R subunits (4), and that IL-15 has robust effects on lipid metabolism (2, 9, 10, 18, 94), a potentially important autocrine/paracrine loop may exist in which lipids are redirected from the adipocyte toward other energetic and signaling needs under the above conditions (2). IL-15 incubation has also been noted to increase adiponectin secretion in fully differentiated 3T3-L1 cells (96). Thus, in addition to its established myokine properties, IL-15 may also operate as a potent adipokine, produced by and remaining in AT to mediate local actions under various conditions.

Previous studies have reported that obese AT has increased pro-inflammatory marker expression and secretion (66, 67). Confirmation that adipocytes are capable of producing IL-15 (1) and that obesity is generally viewed as a low-grade inflammatory condition (26), it is possible that the pro-inflammatory IL-15 cytokine could also be higher in obese vs. lean AT. Since almost every IL-15 study to date has neglected AT IL-15 concentrations, IL-15's *in vivo* adipokine role in obesity remains to be clarified. Furthermore, it would be of particular interest to examine how the previously mentioned autocrine/paracrine loop may influence lipid metabolism in the context

of human obesity, if indeed there is a differential expression/secretion from obese vs. lean AT. Detailed in Chapters 2 and 3, we directly assessed AT IL-15 concentrations as well as perfused IL-15 directly into the subcutaneous AT via microdialysis probes to further clarify the potential role of IL-15 in lipid metabolism (specifically lipolysis) in lean and obese individuals.

Possible mechanisms explaining IL-15 actions in AT

Explaining decreased fat mass with IL-15 exposure has validation on both sides of the lipid balance equation, as demonstrated through decreased lipid uptake and increased lipid mobilization. Further, the direct involvement of IL-15R and IL-15's intracellular signaling targets have also been highlighted in such responses. In one of the first papers to highlight the metabolic effects of exogenous IL-15 treatment, Carbo, et al. (18), demonstrated that a 33% decrease in WAT mass following 7 days of IL-15 exposure was likely explained by reductions in lipid uptake and storage. Specifically, they reported both a 31% reduction in WAT lipoprotein lipase (LPL) activity as well as a 37% reduction of labeled glucose incorporation into WAT lipid (18). In an important follow-up study, Alvarez, et al. (4), demonstrated that in 3 of 4 animal strains which lost WAT mass with short term IL-15 treatment, the fat loss was paralleled with a reduction in WAT LPL activity in the absence of alterations in food intake. Accordingly, the one strain that did not lose WAT mass with IL-15 treatment, the fatty Zucker (fa/fa) rat, did not display the accompanying reduction in LPL activity, despite a similar food intake compared to the other strains. The most important finding of this study was that the effect of IL-15 in reducing LPL-mediated FA uptake appeared a direct consequence of the IL-15 receptor. When compared to their genetic (lean Zucker) counterpart, the fa/fa Zucker rats demonstrated an 84% decrease in IL-15Rγc subunit expression, without differences in the other two IL-15 R subunits, Rα or Rβ. Results in both cultured porcine (2) and human derived adipose cells (10) establishing

that IL-15 can reduce lipid accumulation corroborate the animal data above, although one study reported that IL-15 did not alter lipid deposition in fully differentiated 3T3-L1 cells (96). Therefore, overwhelming evidence suggests that declines in fat mass with IL-15 exposure were likely the result of a reduced FA uptake and incorporation, and that the adipocyte IL-15 cell surface receptor also plays a direct and critical role in this mechanism (4).

On the other side of the lipid balance equation, there is also verification that IL-15 may regulate the loss of AT through lipolytic induction. In favor of this mechanism, Ajuwon, et al. (2), established that IL-15 stimulated lipolysis in isolated porcine adipocytes in a time-sensitive but transient manner, demonstrating the maximal increase in lipolysis observed at 2-hr, thereafter declining towards baseline. During an additional 2-hr incubation, this response to IL-15 was also shown to be dose-dependent, with slight lipolytic induction at 25 ng/mL, and subsequent 2- and 3-fold higher lipolytic responses with 50 and 100 ng/mL, respectively (2). Using inhibitors directed at IL-15 signaling mediators, IL-15-induced lipolysis in the porcine adipocytes was depressed with PKA and JAK inhibitors by ~ 19% and 50%, respectively. Inhibitors directed at MEK, PKC, and PI3K, which mediated IL-15 signaling in other cell types, did not inhibit IL-15-driven lipolytic cascades in these cells (2). In opposition to IL-15 stimulating lipolysis, another study reported that daily administration of IL-15 for 1 week did not enhance basal or isoproterenol stimulated lipolytic rates from extracted WAT sections of Wistar rats (18). However, it was not clear how long after the IL-15 exposure lipolytic assessments were made in this investigation. Given the temporal lipolytic effects observed in isolated porcine adipocytes noted above (robust increase at 2-hr followed by a decline), it is possible that this latter group may not have made their observation within an optimal window of time. It is also possible that species-specific differences drive divergent adipocyte responses to IL-15. Despite some

opposition as noted above, IL-15 appears to be capable of directly effecting lipid mobilization, in isolated cells. It also suggests that the well-known signaling pathway linked to IL-15 intracellular kinases (e.g. JAK proteins), likely mediates mobilization of lipids from the adipocyte (2). It remains to be determined if a similar lipolytic induction occurs in human adipocytes when exposed to IL-15.

Cell culture experiments using cells from several species along with the fact that all 3 IL-15R subunits are present on adipocytes (4), suggest that the effects of IL-15 on decreasing fat deposition (2, 4, 10, 96) and mobilization (2) are direct. As with previous points regarding IL-15 actions in AT, studies conducted thus far with the most concrete mechanistic evidence have exposed cultured cells and/or animals to doses rarely seen by the adipocyte *in vivo* and therefore need to be rectified. Although we will not address FA deposition in this dissertation, we will attempt to clarify the physiological role IL-15 may have in human obesity through lipolytic induction by measuring IL-15 concentration in the interstitial space of AT and exposing human AT to physiologically-relevant IL-15 concentrations using *in situ* microdialysis.

Microdialysis as a technique to monitor local biochemical events

Microdialysis sampling of interstitial fluid (ISF) contents

Microdialysis (MD) is a technique used to glean information at the local tissue level through the use of an inserted probe in a tissue of interest. Perfusing a physiological solution, termed the 'perfusate,' through this MD probe allows molecules from the interstitial fluid (ISF) to exchange across a porous membrane, subsequently collected in a dialyzed sample called the 'dialysate.' Refer to Figures 1.2 and 1.3 for illustrations of the MD technique. Factors such as membrane length, perfusion flow rate, and molecular weight of the molecule(s) of interest will

dictate the recovery across the membrane, and thus the molecule dialysate concentration (41). Increasing the membrane length and flow rate will increase the overall volume of ISF and associated molecules with access to the probe, in turn increasing the absolute recovery. However, as flow rate increases, the relative recovery of the molecule of interest will decrease accordingly (41). In this dissertation, we used MD probes to examine SkM and SCAT IL-15, but also SCAT lipolytic responses to direct IL-15 perfusion. Details for probes placed in SkM and SCAT as well as considerations for the collection of larger macromolecules, such as IL-15, in the dialysate will be discussed further below.

In Chapter 2, we assessed dialysate IL-15 concentrations from SkM and SCAT to determine their contribution to the circulating IL-15 pool. While we are unaware of other investigations sampling IL-15 using MD probes, multiple cytokines and macromolecules of similar molecular size have been successfully dialyzed from the AT interstitial space (66, 67, 71) as well as *in vitro* solutions (119). IL-15 is a relatively small molecule in general terms (~14-15 kDa) (38); yet, a 'large' pore size membrane must be used in order to recover IL-15 due to the functional molecular weight cut off (MWCO) of a given probe. We used a 100 kDa MWCO membrane (Harvard Apparatus/CMA Microdialysis, Holliston, MA) for the studies in this dissertation, as these probes are reported to have a functional MWCO of 30 kDa (personal communications with the probe manufacturer). When sampling for macromolecules, such as cytokines and other peptide hormones through larger pore sizes (67, 71, 72), ultrafiltration (loss of fluid) is a concern along the implanted membrane. To account for and prevent this loss, large molecular weight substances such as BSA (116), heparin (119), and dextrans of varying molecular weight (72, 118) have been added to the perfusate to maintain osmotic pressure. Through multiple *in vitro* and *in vivo* pilot experiments using the 100 kDa membrane, we

determined that Dextran 40 (40 kDa molecular weight) added to the perfusate at a concentration of 30g/L is sufficient to maintain osmotic pressure within the MD probe, thus preserving adequate dialysate collection volumes.

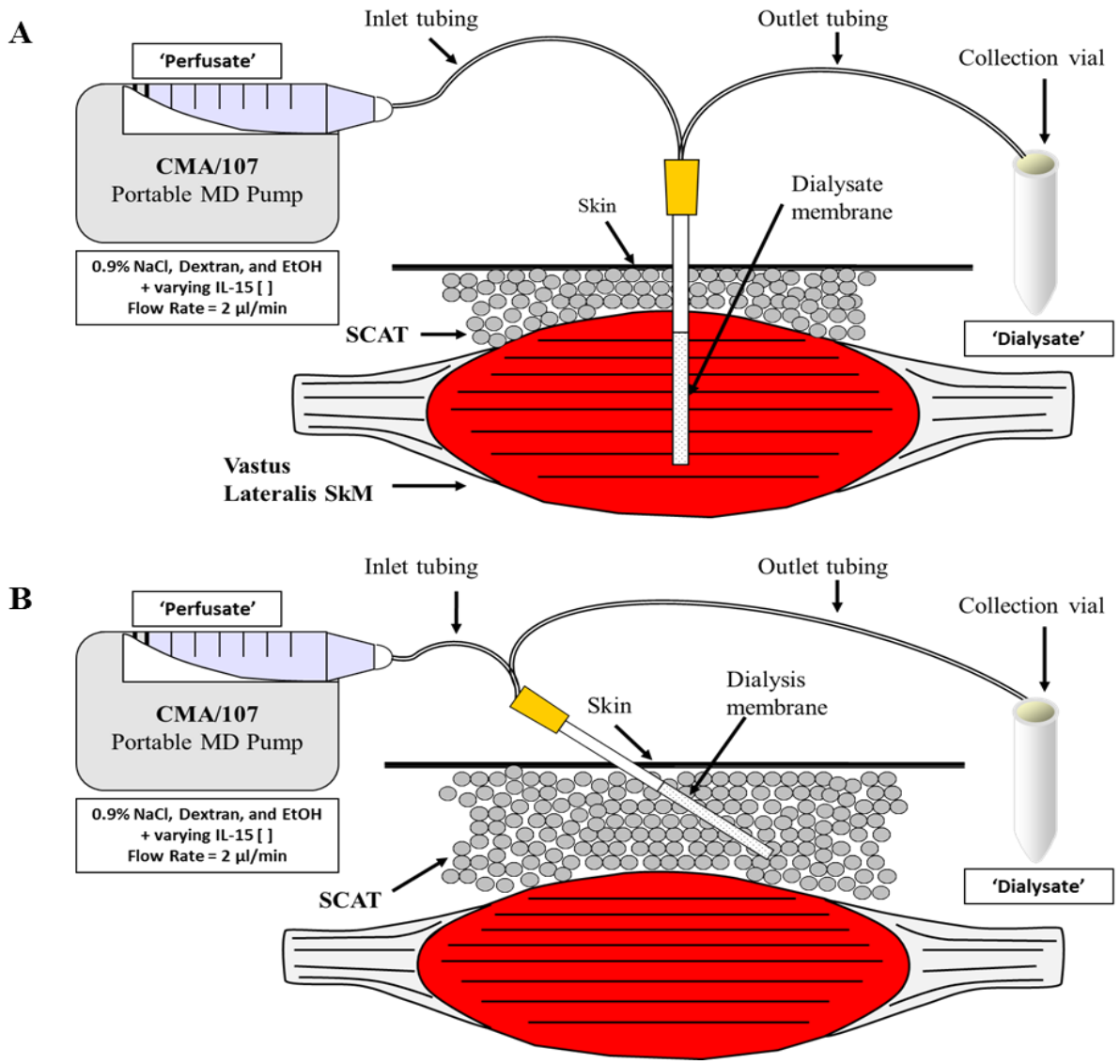

Figure 1.2. MD probe placement in SkM (**A**) or in SCAT (**B**). In Chapter 2 (Study 1), we placed SkM probes in the distal vastus lateralis thigh muscle and SCAT probes in the abdominal SCAT region adjacent to the umbilicus to estimate interstitial IL-15 concentrations as well as relationships between endogenous IL-15 and SCAT lipolysis. In Chapter 3 (Study 2), we inserted probes to perfuse recombinant human (rh)IL-15 into SCAT to assess the direct lipolytic response to this cytokine by sampling glycerol in dialysate. As noted, the perfusate was 0.9% saline (NaCl) to which we added Dextran at [30g/L], ethanol (EtOH) at [10 mM], and varying IL-15 concentrations [0, 20, 650, 6500, 65000 pg/mL].

In both SkM and SCAT MD, probes are inserted under the skin such that the porous membrane remains in the muscle of interest beyond the subcutaneous AT, or contained within the SCAT itself, respectively (Figure 1.2 Panels A and B). Probe insertion (and the overall MD technique) is considered to be minimally invasive, yet the insertion alone can induce some local microtrauma. Therefore, it is recommended to allow an equilibration period of at least 60 min prior to the collection of metabolites (41). This is especially important when examining certain cytokines, particularly those of a pro-inflammatory nature, as research has demonstrated local IL-6 elevation from the probe insertion (100).

Ethanol in perfusate for estimation of blood flow and interstitial concentrations of molecules

Ethanol (EtOH) is used as an indicator of local blood flow through the tissue (42-45), and also analyte relative recovery when estimating interstitial concentrations from the collected dialysate. EtOH included in the perfusate diffuses out over the membrane and into the local interstitial space, removed from the site by the nutritive blood flow, and not metabolized locally to a large extent in either SkM or SCAT (41). Thus, the EtOH outflow:inflow ratio (O:I ratio = $[EtOH]_{dialysate}$ / $[EtOH]_{perfusate}$) is inversely related to blood flow in the area of the dialysis membrane (44, 45). Determination of EtOH concentration in both collected dialysate relative to the perfusate, and calculating the O:I ratio, allows for simultaneous monitoring of nutritive blood flow and metabolism. In our lab, we add 10 mM EtOH to the perfusate for these purposes. Figure 1.3 below illustrates the dialysis of EtOH used in MD. More information regarding calculation of interstitial analyte concentration (e.g. IL-15, glycerol, etc.) from the dialysate analyte concentration is provided in Chapters 2 and 3.

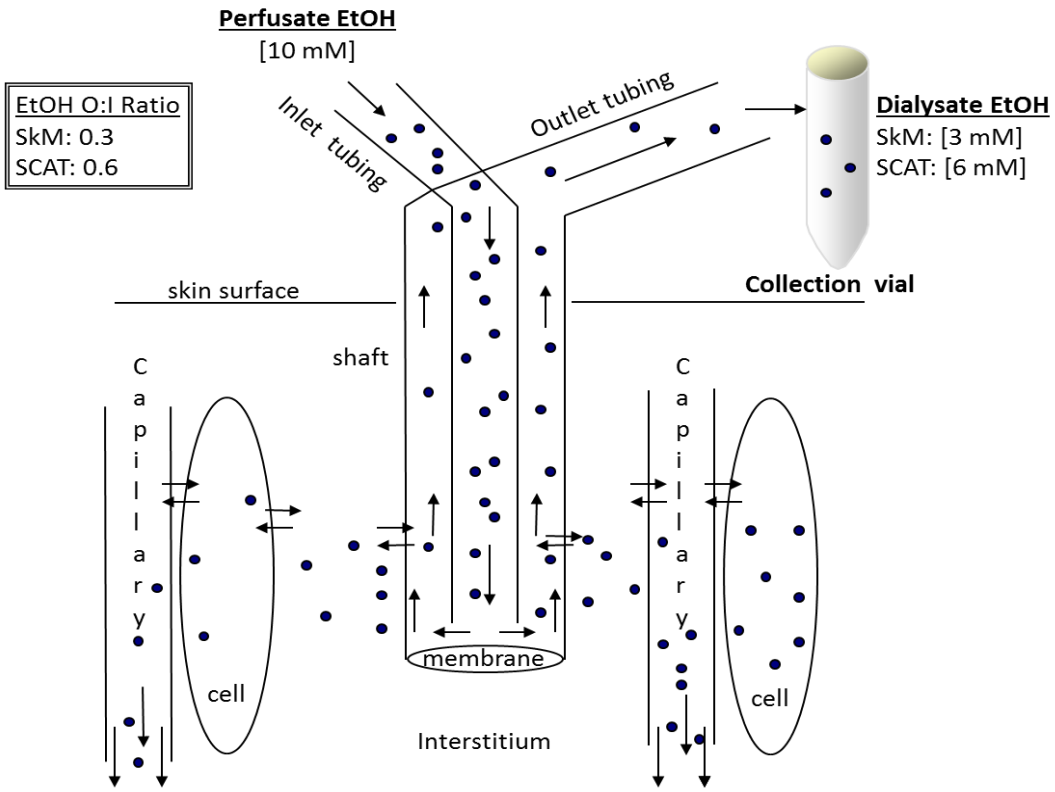

Figure 1.3. Illustration of EtOH (circles) added to the MD perfusate. From the EtOH outflow/inflow ratio (O:I), both regional blood flow and analyte interstitial concentration can be estimated. Concentrations in the figure are representative given for EtOH added to the perfusate and EtOH collected from SkM and SCAT dialysates. SkM = skeletal muscle; SCAT = subcutaneous adipose tissue; EtOH = ethanol. Figure adapted from R.C. Hickner (41).

Microdialysis perfusion of various agents

Many agents are capable of being added to the perfusate depending on the desired experimental conditions. Ethanol is added to account for blood flow and estimate interstitial concentrations, and Dextran is added to maintain the osmotic pressure and prevent ultrafiltration. Other perfusate agents, such as stimulators (isoproterenol, β-AR stimulator) and inhibitors (propranolol, β-AR blocker) of lipolysis can be added to examine lipid mobilization in various AT depots (32). In the study detailed in Chapter 3, we added the cytokine, IL-15, at physiologically-relevant up through pharmacological concentrations to assess its effects on SCAT lipolysis. This allowed us to study potential *in vivo* adipokine and myokine roles in

humans, which would have been very challenging otherwise. In exploiting a great benefit of MD, agents added to the perfusate allow the local delivery of drugs, pharmacological substances, etc. that can yield unique perspectives into tissue physiology and metabolism without the exposing the rest of the body to potentially harmful doses and undesired side effects. As a promising clinical application, MD perfusion of chemotherapy agents may allow better targeting of tumors with the proper drugs, while also avoiding the systemic side effects commonly associated with many chemotherapeutic drugs currently available (62).

Conclusion of literature review

Many factors leading to obesity have been identified and investigated. However, it remains important to identify novel factors potentially involved in obesity, aiming to more completely understand obesogenic processes and possibly improve strategies for treating obesity. Newly investigated factors which could positively impact treatment approaches include traditional immune signaling molecules, or cytokines, released from cells both within and external to the immune system. IL-15, a potent cytokine for proper immune cell development and signaling, is also expressed and released from SkM and AT. Exogenous IL-15 delivery and strategies aimed at increasing endogenous IL-15 secretion have led to substantial decreases in fat mass in animal models. Evidence that adipocytes express the full IL-15 receptor suggests that IL-15 can directly mediate fat reductions, most likely through decreased lipid deposition and increased lipolysis. However, most questions regarding IL-15's role in adipocyte regulation have been answered in animal models and isolated cell systems with supraphysiological doses. Thus, IL-15's *in vivo* roles as a myokine and adipokine, specifically regarding the potential to impact human obesity, remain poorly understood.

Statement of the problem, aims, and hypotheses

Researchers have demonstrated that IL-15 has potent anti-obesogenic properties across several animal and cell culture models. Furthermore, it appears that SkM can reduce AT mass through secretion of IL-15 in a unidirectional muscle-to-fat endocrine axis, at least in animal models. It has been speculated that a similar axis exists in humans, although the existing human data is largely associative with little direct evidence to support these claims. Therefore, the **principle aim** of this dissertation is to determine if local SkM and SCAT IL-15 sources contribute to circulating IL-15, and to examine if IL-15 induces SCAT lipolysis in humans. A **secondary aim** is to determine if these sources and lipolytic actions of IL-15 differ between lean and obese human subjects, intending to better understand inter-organ communication and its potential involvement in human obesity. It is the main hypothesis of this study that endogenous SkM and SCAT IL-15 concentrations are related to lipolysis. Reported differences in circulating IL-15 between lean and obese subjects has led us to further expect that local sources of IL-15 and their relationships with lipolysis will be altered in obese subjects. In order to answer questions surrounding our central hypotheses, we developed the following specific aims:

Specific Aim 1. **To determine if SkM and SCAT sources of IL-15 are related to SCAT lipolysis in humans at rest as well as during and after exercise. A secondary aim was to determine if resting and/or exercise local IL-15 and SCAT lipolysis relationships were different between lean and obese individuals.**

Previous research has demonstrated that circulating IL-15 is higher in lean vs. obese individuals (10, 69). Researchers have speculated that SkM is the source of this elevated IL-15; however, it is not clear if resting SkM secretion is actually higher in lean subjects, or if secreted

SkM IL-15 is related to increased lipolysis. Further, no studies have taken into account SCAT IL-15 as a contributor to circulating IL-15 or its relationship with AT lipolysis in obesity. Finally, it would be important to know if exercise, a potent stimulator of lipolysis, alters SCAT IL-15 or the SCAT IL-15/lipolysis relationship, and if this relationship is different between lean and obese humans.

Completion of Aim 1 will yield a comprehensive look at the proposed SkM-AT IL-15 axis in humans, allowing us to determine if IL-15 serves an important *in vivo* role in human obesity. Further, it will allow us to determine if exercise can serve to increase SCAT IL-15, and if IL-15 is important for exercise-induced SCAT lipolysis. We hypothesize that resting SkM IL-15 will be elevated in lean vs. obese subjects, matching reports of elevated circulating IL-15 in lean individuals. However, we do expect that the SkM IL-15/lipolysis relationship is the same in lean and obese humans. That is, as SkM IL-15 increases, we expect SCAT lipolysis to increase. On the other hand, no one has reported SCAT IL-15 concentrations. Therefore, we are unsure of about the SCAT IL-15/lipolysis relationship, and how this relationship is potentially modified in obesity. It is anticipated that exercise will increase SCAT IL-15, suggesting that exercise-induced IL-15 has a role in driving AT lipolysis; however, we are exploring whether or not exercise modifies the IL-15/lipolysis relationships differently in lean and obese humans.

Specific Aim 2. **To determine if IL-15 perfusion can alter SCAT lipolysis in human subjects. A secondary aim was to determine if the SCAT lipolytic response to IL-15 perfusion was different between lean and obese humans.**

Multiple studies have infused IL-15 in various animal strains, and have demonstrated that IL-15 substantially decreases fat mass in most cases, pending adequate expression of IL-15

receptor subunits. As discussed earlier, one possible mechanism for a loss of fat mass includes an increased lipid mobilization from adipose stores. However, most of the studies examining IL-15 actions on AT have been in animals or animal-derived cells, or involve supra-physiological doses. It remains to be determined: 1) if IL-15 induces lipolysis in human AT, 2) if physiologically-relevant doses of IL-15 can accomplish similar actions as the case with much higher concentrations previously reported, and 3) if these IL-15 lipolytic actions are different in lean and obese subjects.

Completion of Specific Aim 2 will allow us to better understand actions of IL-15 on human AT across a wide range of IL-15 concentrations. Moreover, this should clarify IL-15 *in vivo* role in mediating AT lipolysis (at the lower physiological doses), while at the same time elucidate a potential therapeutic application (at higher pharmacological doses) of IL-15 in decreasing fat mass in obese individuals. We expect that IL-15 will increase lipolysis in both lean and obese individuals. However, we also anticipate that lipolytic induction will be blunted in obese subjects, as previously shown with other lipolytic stimulating agents.

CHAPTER 2: DOES HUMAN IL-15 REGULATE LIPOLYSIS AND IS THE REGULATION MODIFIED WITH EXERCISE OR OBESITY?

Abstract

The hypothesis that IL-15 is secreted from skeletal muscle (SkM), and leads to decreased adipose tissue (AT) mass through a SkM-blood-AT endocrine axis is supported from experiments performed in animal models. It has been speculated that SkM is the source of higher plasma IL-15 in lean (LN) than obese (OB) humans, and that a similar axis exists in humans. Exercise is also thought to increase IL-15 secretion, which could also be related to reductions in fat mass though increased fat mobilization (lipolysis). **PURPOSE**: To examine if SkM and/or subcutaneous adipose tissue (SCAT) sources of IL-15 are related to SCAT lipolysis in humans at rest and during exercise. A secondary aim was to determine if resting and/or exercise IL-15/SCAT lipolysis relationships were different between LN and OB humans. **METHODS**: Twenty LN (n=10, BMI: 23.1 ± 1.9 $kg \cdot m^{-2}$, age: 24.0 ± 3.7 yr) and OB (n=10, BMI: 34.7 ± 3.5 $kg \cdot m^{-2}$, age: 27.3 ± 9.1) men and women volunteered for this study. We inserted microdialysis probes (CMA/20: 100 kDa cutoff dialysis membrane) into the vastus lateralis and abdominal SCAT to measure local IL-15, and sampled plasma to gauge the contributions of local IL-15 to the circulating IL-15 pool. We measured SCAT interstitial glycerol to assess potential relationships between SCAT lipolysis and SkM/SCAT/plasma IL-15. To determine how exercise potentially modifies IL-15/SCAT lipolysis relationships, subjects cycled for 1-hr at 60% heart rate reserve and had plasma IL-15, SCAT IL-15, and SCAT glycerol sampled throughout the exercise bout. **RESULTS**: SkM IL-15 concentration was not different between LN and OB subjects (p=0.45), nor was it related to plasma IL-15 concentration (p=0.64). Resting SCAT IL-

15 was not related to plasma IL-15 (p=0.17); however, resting SCAT IL-15 was higher in OB than in LN subjects (p=0.02) and was correlated with the amount of SCAT lipolysis (r=0.46, p=0.04). Exercise increased plasma IL-15 in both LN and OB, with a higher total amount of circulating IL-15 over the 60-min bout in OB than LN (9.5 ± 4.1 vs. 4.5 ± 2.4 pg/mL*60 min, respectively; p<0.01). Exercise increased SCAT lipolysis in both LN and OB (p<0.01) with no difference between the two groups (p=0.53). Neither the exercise-induced plasma IL-15 change nor SCAT IL-15 change was related to exercise-induced SCAT lipolysis changes. **CONCLUSION**: While we found no support for a human SkM-blood-AT IL-15 axis, there appears to be more support for a local autocrine/paracrine axis (SCAT IL-15 acting within SCAT) that could potentially impact human obesity. Reasons for the augmented plasma IL-15 concentrations during exercise in OB as compared to LN humans remain to be clarified, as IL-15 most likely does not significantly contribute to exercise-induced lipolysis in humans.

Introduction

Immune cell signaling molecules (cytokines) and their communication within and between organ systems, such as skeletal muscle (SkM) and adipose tissue (AT), appear to be among factors potentially involved in human obesity given their anti-obesogenic effects reported in animal and cell models. Interleukin (IL-15), with a particularly high SkM expression (38) and role in regulating muscle mass (91, 95), is one such cytokine shown to have additional non-immune metabolic roles in animals. Indeed, exogenous IL-15 exposure has been reported to decrease white AT (WAT) mass 14-44 % in several animal strains (4, 18). Furthermore, *in vivo* metabolic roles have been noted through genetic knock out (KO) and overexpression (OE) manipulations of IL-15. Compared to wild-type counterparts, IL-15 KO animals gained body

weight and fat mass, while IL-15 OE animals demonstrated lower body mass and fat mass (9, 10): both independent of food intake.

The most concrete evidence of IL-15's *in vivo* regulation of AT mass was presented by Quinn, et al. (94), who confirmed earlier hypotheses of inter-organ communication between SkM and AT (18, 96). Modeling IL-15's *in vivo* two-isoform expression demonstrated that OE of intracellular SkM IL-15 did not produce any changes in body composition, while OE of SkM IL-15 that was over-secreted into the circulation significantly decreased fat mass in male and female mice (94). Human studies indicate that: 1) obese (OB) individuals have lower circulating IL-15 than lean (LN) counterparts (10, 69), and 2) circulating IL-15 is negatively correlated with trunk fat mass and percent body fat (69). From this limited human evidence, it has been speculated that human SkM IL-15 secretion accounts for differences in plasma IL-15. Conversely, evidence that OB SkM IL-15 mRNA expression was not related to plasma IL-15 (69) indicates that SkM may not contribute substantially to systemic IL-15. Moreover, evidence that adipocytes express IL-15 (1, 96), and that obesity is viewed as a low-grade inflammatory condition (26), suggests that AT in OB humans may express/secrete more IL-15 than AT in LN individuals. Since adipocytes also express all 3 IL-15R capable of signal transduction (4), questions regarding a potential autocrine/paracrine axis in human AT also remain unanswered. To our knowledge, no studies have assessed human AT IL-15 concentrations. Therefore, IL-15's *in vivo* myokine (SkM to AT) and adipokine (within AT) metabolic roles in the context of human obesity remain to be clarified.

Losses in fat mass with IL-15 are suggested to result from decreased lipid uptake and incorporation (2, 4, 10, 18) as well as increased lipid mobilization (lipolysis) (2). Lipolysis represents a crucial first step in overall fatty acid catabolism, and thus a critical point in the

overall reduction in fat mass. Normalized to fat mass, lipolysis is depressed in OB vs. LN humans (17, 48, 49), representing a (potentially protective) defect in mobilizing lipids from obese AT. With specific regard to IL-15's role in this process, Ajuwon, et al., (2), demonstrated that exposing porcine adipocytes to IL-15 increases lipolysis in both a time- and dose-dependent manner, and that this response was blunted by blocking an IL-15R cell signaling intermediate, janus kinase (JAK). While it appears that IL-15 may directly modulate lipolysis in the above porcine cells, it remains to be determined if human SkM and AT IL-15 could induce lipolysis in human AT.

Although the SkM-blood-AT endocrine axis appears to be important in the control of obesity, the strongest evidence for this axis exists in animal and isolated cell culture models. The assumption that SkM is a major contributing source to circulating IL-15 and that circulating IL-15 aids in decreasing fat mass in humans seems to be within reason given existing reports. However, the sources of circulating IL-15 and/or stimuli leading to IL-15 secretion in humans remain unknown (10, 93). Acute exercise dramatically increases lipolysis (8, 56, 114), and is also thought to be a potential stimulus leading to IL-15 secretion; yet both increases (21, 98, 115) and no changes (70, 99) in IL-15 following exercise have been observed. If exercise leads to increased IL-15, exercise-induced IL-15 could be partially responsible for exercise-induced lipolysis, further supporting an important *in vivo* role of IL-15 in AT metabolism. Therefore, the purpose of this study was to determine if interstitial IL-15 in SkM and SCAT are related to SCAT lipolysis in humans at rest as well as during and after exercise. A secondary aim was to determine if resting and/or exercise local IL-15 and SCAT lipolysis relationships were different between LN and OB individuals.

Materials and Methods

Screening visit

Participants. Potential subjects visited the Fitness Instruction, Training, & Testing (FITT) building at ECU where they were briefed on all study procedures, and signed an informed consent prior to any participation. Consented volunteers underwent a general health screening (height/weight/BMI, resting blood pressure and heart rate, etc.), and completed a brief medical history questionnaire to confirm qualification. Subjects aged 18-45 y were classified by "LN" or "OB" BMI, represented as 18.5-25 and 30-39.9 $kg \cdot m^{-2}$, respectively. Twenty LN (n=10, BMI: 23.1 ± 1.9 $kg \cdot m^{-2}$, age: 24.0 ± 3.7 yr) and OB (n=10, BMI: 34.7 ± 3.5 $kg \cdot m^{-2}$, age: 27.3 ± 9.1) men (n=11) and women (n=9) were used in the final groups. Subject demographic and physical characteristics are provided in Table 2.1. Potential subjects were excluded if they did not fit within the desired BMI categories, exercised ≥ 3 $d \cdot wk^{-1}$, were a smoker, had any known cardiovascular or neuromuscular disease, were taking any medication effecting metabolism, or were pregnant or lactating. We did not control for the timing of the experimental visit within the menstrual cycle phase in female subjects. However, we did measure follicle stimulating hormone (FSH), and luteinizing hormone (LH), and determined there were no differences between the LN and OB females in either measure (p=0.59 and p=0.42, respectively). All study procedures were approved by the ECU University and Medical Center Institutional Review Board (UMCIRB).

Body composition assessment. Participants had height measured to the nearest 0.5 cm using a wall-mounted stadiometer and weight assessed on an electronic scale to the nearest 0.1 kg, and had BMI ($kg \cdot m^{-2}$) calculated. To more accurately describe body composition, we performed a dual-energy X-ray absorptiometry (DXA) scan (Prodigy, GE Lunar Corp., Madison, WI), where subjects lied on the DXA table while a fan beam scanner obtained detailed lean and

fat mass densitometries. Provided software calculated specific parameters, which included bone mineral density and content, fat mass and percent body fat (total and regional estimates), lean mass and percent lean mass. DXA-assessed parameters are presented in Table 2.2.

Resting cardiovascular measures. During the screening visit, we measured resting blood pressure using a standard sphygmomanometer cuff while subjects sat upright in a chair. Participants were given a polar heart rate (HR) monitor (Polar Electro Inc, Lake Success, NY) and instructed how to obtain morning resting HR (HR_{rest}). Briefly, they were asked to secure the chest strap electrode before rising out of bed, and to read HR on the paired watch for ~ 60 sec. Three consecutive morning measurements were provided to the investigators and averaged for an accurate HR_{rest} value.

Experimental visit

Within 48h of the experimental visit, subjects were instructed not to perform vigorous exercise or ingest alcohol. The day prior, they were asked to eat meals close to their typical diet and only drink water from ~ 2200h the night before. Subjects were asked to eat a provided breakfast 2h before their experimental visit began (~0600h), which consisted of 2 crunchy granola bars (190 kcal, 29 g carbohydrate (CHO), 6 g fat (FAT), 4 g protein (PRO)) and 4 oz. ensure drink mix (125 kcal, 17 g CHO, 4.5 g FAT, 4.5 g PRO) for a total of 315 kcal (~ 60 % CHO / 30 % FAT / 10 % PRO). Subjects reported to the East Carolina Heart Institute (ECHI) for their experimental visit at approximately 0800h. After confirming that the meal was eaten at the time specified to standardize feeding, subjects were asked to rest in a semi-recumbent prone position in a hospital bed to begin the microdialysis (MD) probe insertion for interstitial fluid/dialysate sampling and intravenous catheter insertion for blood draws. Other than the exercise portion, subjects were allowed to work quietly, read, or watch television throughout the

visit. Subjects were allowed to drink water ad libitum during the day, but no food was allowed until the end of the experimental visit.

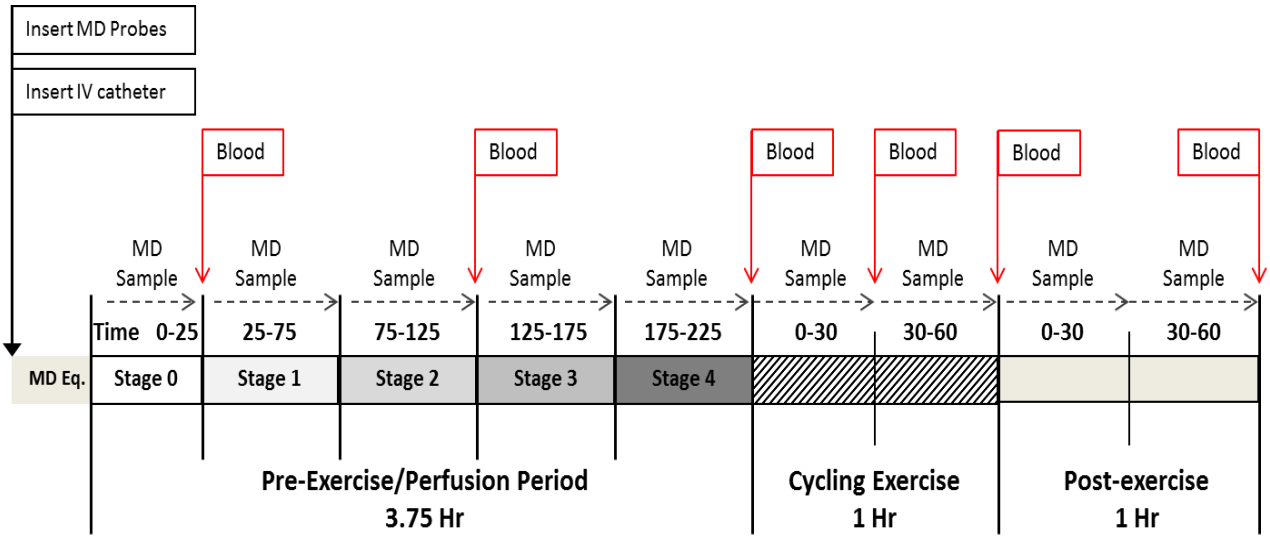

Figure 2.1. Study schematic of the Experimental Visit. Portions of this visit (Pre-exercise/Perfusion Period Stages 1-4) also served as the experimental protocol in Chapter 3. 'MD sample' indicates period of time that dialysate was sampled for IL-15 and glycerol. 'Blood' indicates where blood was sampled through IV catheter. MD = microdialysis; IV = intravenous.

MD Probes and Perfusates. Custom MD probes (CMA/20: 15 or 30 mm membrane length, 100 kDa cut off) were ordered from CMA Microdialysis (CMA/Harvard Apparatus, Hollison, MA), soaked for 30 min in 70% isopropanol the day before use and kept antiseptic in sterile water overnight. Perfusate solutions were prepared in a base solution of sterile 0.9 % NaCl (saline), where we added 10mM EtOH to estimate blood flow and calculate interstitial IL-15 and glycerol concentrations, and [30 g/L] Dextran (40 kDa molecular weight; Sigma Aldrich, St. Louis, MO) to prevent ultrafiltration across the large pore size membrane. Once agents were added, perfusates were sterile filtered with 0.22 micron filters into CMA 106 MD syringes (MDialysis, N. Chelmsford, MA), and kept overnight at 4°C until use the next day.

MD Probe Insertion. For SkM and SCAT MD Probe insertion, the skin around the insertion site was first cleaned using iodine swab sticks. To ease any discomfort from the probe placement, a small amount (~1-2 mL / insertion site) of 1% lidocaine was then injected with the use of ethyl chloride spray (cold numbing spray) under the skin surface and down into the vastus lateralis (VL) thigh muscle ~5-6 cm proximal to the patella or in the abdominal SCAT space ~2-3 cm lateral to the umbilicus. Next, a removable plastic sheath was inserted into the numbed area with the use of an 18-guage catheter needle. Once the needle was removed, the sterile MD probe was placed inside the plastic sheath. The plastic guide was then removed, leaving the dialysis membrane portion of the MD probe embedded below the skin and into the VL muscle or SCAT. See Figure 1.2 Panels A and B for an illustration of SkM and SCAT MD.

Inserted MD probes were secured to the subject and connected to perfusate syringes and calibrated syringe pumps (CMA 107 Pump, Mdialysis, North Chelmsford, MA). Once the pump was started, and flow was established (able to observe dialysate coming from exit tubing), we allowed a 1-hr equilibration before dialysate was collected for analysis. Dialysates were collected in 300 µL plastic microvials (Milian USA, Columbus, OH), and were weighed both prior to and following collection to monitor flow rate, set to 2 $\mu L \cdot min^{-1}$. Collected dialysates were kept at either 4°C or -20°C until desired analysis occurred (see below).

Blood collection. After the MD probe insertion and setup was completed, subjects had an intravenous (IV) catheter with a saline lock inserted into an antecubital vein in the forearm near the elbow. The line was kept patent by periodically flushing with 2-3 mL sterile saline. At the time indicated in Figure 2.1 (resting, exercise, recovery) blood was drawn into vacutainers (BD Diagnostics, Franklin Lakes, NJ) containing EDTA for plasma, or into serum separator tubes for serum. After 7-10 inversions (and allowing serum to clot for 30 min at RT), vacutainers were

spun for 15 min (plasma at 4°C; serum at RT), had plasma or serum aliquotted to micro-centrifuge tubes and frozen at -80°C until specific analysis.

Acute Exercise protocol. To determine if IL-15 was induced by exercise and/or related to exercise-induced lipolysis, we had subjects aerobically exercise on a Monark Ergomedic 828E cycle ergometer (Monark Exercise AB, Sweden). The difference between measured HR_{rest} and calculated HR_{max} (220 - age) was the HR reserve (HRR). On the day of the visit, subjects were fitted with a Polar HR monitor and asked to pedal for 60 min at the training HR, set to 60% HRR, using the Karvonen equation: $[(HR_{max} - HR_{rest})*0.6] + HR_{rest}$ (52). This 60% HRR should have roughly approximated their individual 60% VO_{2peak} (5), an intensity previously shown to robustly increase lipolysis (8, 30). Exercise intensity between a 55-60 rpm pedal rate was adjusted as necessary to keep participants as close to their training HR as possible.

Basic blood biochemistry. Resting serum (8-hr fasted from provided breakfast, end of 'Stage 4' in Figure 2.1) was analyzed for basic blood chemistries using the automated UniCel DxC 600i Synchron Access Clinical System (Beckman Coulter, Inc., Brea, CA) and are presented in Table 2.3. We measured glucose, insulin, triglycerides (TG), total cholesterol (TC), high density lipoprotein (HDL)-C, low density lipoprotein (LDL)-C. Using blood glucose and insulin, we calculated Homeostatic Model Assessment (HOMA) to quantify insulin resistance using the equation: HOMA = fasting glucose [mg / dL] x fasting insulin [µIU / mL] / 405 (63).

Glycerol. During each MD collection period (~30-45 min), indicated by 'MD Sample' in Figure 2.1, collected dialysate was assessed for glycerol on a specialized microdialysis analyzer (CMA 600, MDialysis, North Chelmsford, MA) according to manufacturer's directions. The analyzer measures glycerol concentration from < 2 µL samples using a colorimetric reaction.

Dialysate samples were kept at -20°C until analysis. All samples were run in duplicate, measured against provided calibrators, and averaged for data analysis.

EtOH Assay. During MD collection periods, perfusate and dialysate samples were taken and stored at 4°C until analyzed for EtOH (≤ 48 h from collection) as previously described (44). This fluorometric assay is based on the conversion of EtOH to acetylaldehyde *via* alcohol dehydrogenase (ADH) and co-factor NAD. The fluorescent NADH product (excitation/emission at 360/415) is directly proportional to EtOH in the original sample, and was detected using the Victor^3 Multilabel counter (PerkinElmer, Wellesley, MA). All samples were run in duplicate, measured against a standard curve, and averaged for data analysis. We calculated the *in vivo* EtOH O:I ratio as $[EtOH_{dialysate}]$ / $[EtOH_{perfusate}]$ to qualitatively estimate blood flow and calculate interstitial IL-15 and glycerol concentrations.

IL-15 Assays. IL-15 concentrations were measured in both SkM and SCAT dialysate and blood using a commercially-available electrochemiluminescent Ultra-sensitive IL-15 kit from Meso Scale Discovery (MSD; Rockville, MD). Briefly, sample medium was added to wells containing carbon electrodes pre-coated with IL-15 capture antibodies. After a labeled detection antibody and read buffer are added, the electrode is excited and the emitted light is proportional to the amount of IL-15 in the sample. The chemiluminescent signal was read against a standard curve using the MSD Sector 2400 instrument and quantified using MSD Discovery Workbench software v.4. Plasma samples were run according to the manufacturer's specifications without modifications, while dialysate samples were measured in a modified protocol developed along with the manufacturer. For dialysate samples, we prepared the calibration curve in a 0.9% saline and 1 % BSA background. A small amount of BSA (1 % final BSA) was added to the dialysate

samples to standardize matrices, and we applied appropriate dilution factors to all unknown samples. Samples were run in duplicate where volume allowed, and averaged for data analysis.

Interstitial estimates. In order to estimate interstitial IL-15 or glycerol concentrations, we first conducted *in vitro* experiments using the same probes and flow rates as in the *in vivo*/*in situ* conditions. The probes were placed in a beaker solution (Dulbecco's phosphate-buffered saline (D-PBS), 0.1 % bovine serum albumin (BSA), 5 mM glucose, 0.2 mM glycerol, 5 mM ethanol (EtOH), 2 mM lactate, and varying concentrations of IL-15), similar to conditions previously described (72). The relationship between the *in vitro* recoveries of ethanol and a given substance of interest (e.g. IL-15) was used to calculate the *in vivo* interstitial concentration of the substance of interest, given the assumption that the relationship between the relative recoveries of two substances *in vitro* is the same as the relationship between the relative recoveries of those two substances *in vivo*. We used the following equation to calculate interstitial IL-15 (*i*IL-15):

$$i\text{IL15} = \left[\left(\frac{in\ vitro\ \text{EtOH relative recovery}}{in\ vitro\ \text{IL15 relative recovery}}\right) \times \frac{[d\text{IL15}]}{\underset{in\ vivo}{\text{1-EtOH O:I}}}\right]$$

where: *d*IL15 = measured *in vivo* dialysate IL-15; 1-EtOH O:I = *in vivo* EtOH lost to tissue from the perfusate; *in vitro* EtOH relative recovery = ([*in vitro* dialysate EtOH] / [*in vitro* beaker EtOH]); *in vitro* IL-15 relative recovery = ([*in vitro* dialysate IL-15]/[*in vitro* beaker IL-15]). Interstitial glycerol (*i*Glyc) was calculated similarly.

Statistical analysis

Most data analysis between LN and OB (demographics, blood measures such as basic chemistries and resting IL-15, etc.) was assessed using a Student's t-test. For differences examined across multiple time points (resting, exercise, recovery), we used a repeated measures

ANOVA. If a significant F-ratio was detected in the ANOVA models, we used a Fisher's LSD *post-hoc* analysis. To examine relationships between certain variables, we used Pearson Product Moment Correlation analysis. All statistical analyses were performed using SPSS Statistics for Windows v.20.0 (IBM, Artmonk, NY). Data is reported as mean ± SD, unless otherwise noted, and α-level was set at $p \leq 0.05$.

Results

Physical characteristics. In our final sample population, we had two distinct LN and OB BMI groups (LN: 23.1 ± 1.9 vs. OB: 34.7 ± 3.5 $kg \cdot m^{-2}$, $p < 0.01$). They were not significantly different in age (p=0.30), and there was a good distribution of men and women in LN (M/F: 5/5) and OB (M/F: 6/4) groups. This BMI stratification also yielded expected differences in DXA-assessed body composition parameters. In almost every DXA measure, the OB were significantly different than their LN counterparts as seen in Table 2.2. With regards to body fat distribution, OB individuals had a significantly higher % BF (p=0.02), but also carried more fat in their upper body, as suggested by a higher Android % fat (p<0.01) and Android:Gynoid ratio (p=0.02). However, there was no difference in lower body fat between LN and OB as indicated by a similar Gynoid % fat (p=0.26). The OB group, as expected, could be classified as "slightly less fit" vs. LN individuals, as evidenced by a lower HRR (122.4 ± 9.5 vs. 132.3 ± 10.1 bpm, p=0.04), driven mostly by a relatively higher resting HR.

Blood chemistry. Table 2.3 shows that despite some large magnitude differences in mean concentrations, most of the blood chemistry measures were not found to be statistically different between LN and OB due to considerable variation. However, the OB group demonstrated 2-fold

higher insulin concentrations and HOMA scores ($p<0.01$) than their LN counterparts. There were no differences in 8-hr fasted glucose between LN and OB subjects ($p=0.19$).

Resting IL-15 Concentrations. We did not find a significant difference in resting circulating IL-15 between LN and OB ($p=0.47$). Further, despite speculation that OB SkM would secrete less IL-15, we found that SkM IL-15 was also not significantly different between LN and OB, either for dialysate IL-15 (*d*IL15) ($p=0.45$) or calculated interstitial IL-15 (*i*IL15) ($p=0.35$) concentrations. Alternatively, we did observe an increased resting SCAT IL-15 in OB, both for *d*IL15 ($p=0.02$) and *i*IL15 ($p=0.02$) concentrations, as seen in Figure 2.2. With no evident differences in dialysate or interstitial relationships, we will only report findings for interstitial concentrations (*i*IL15 or *i*Glyc) throughout the remainder of the results section. Despite a significantly higher SCAT *i*IL15 in OB, plasma IL-15 was not significantly related to either SkM or SCAT *i*IL15 (Table 2.4). This was true for analysis of collapsed groups (Plasma IL-15 vs. SkM *i*IL15: $p=0.50$; Plasma IL-15 vs. SCAT *i*IL15: $p=0.92$), or within LN (Plasma IL-15 vs. SkM *i*IL15: $p=0.23$; Plasma IL-15 vs. SCAT *i*IL15: $p=0.85$) or OB (Plasma IL-15 vs. SkM *i*IL15: $p=0.55$; Plasma IL-15 vs. SCAT *i*IL15: $p=0.84$) separately.

Resting IL-15 and SCAT Glycerol relationships. Resting relationships between SkM/SCAT *i*IL15 and SCAT *i*Glyc can be seen in Figure 2.3, with LN and OB plotted by different symbols but with one regression line (± 95% CI) for the entire group. Resting SkM *i*IL15 was not significantly related with SCAT *i*Glyc (Panel A), either when analyzed by all subjects combined ($r = 0.01$, $p = 0.97$), or when analyzed in LN ($r = -0.13$, $p = 0.72$) and OB ($r = 0.21$, $p = 0.59$) separately. Interestingly, the amount of SCAT *i*IL15 was significantly related to the amount of SCAT *i*Glyc (Panel B) when the group was analyzed together ($r = 0.45$, $p = 0.05$), but not when LN ($r = 0.47$, $p = 0.17$) and OB ($r = 0.25$, $p = 0.49$) were analyzed separately.

Resting EtOH O:I Ratio. The resting EtOH O:I ratio was not different between LN and OB groups in SkM (LN: 0.36 ± 0.11 vs. OB: 0.32 ± 0.12, p = 0.46) or in SCAT (LN: 0.60 ± 0.14 vs. OB: 0.69 ± 0.09, p = 0.10), suggesting that there were no qualitative differences in blood flow within a given tissue between LN and OB. However, a significant tissue (SkM/SCAT) x BMI interaction ($p<0.01$) was observed. Post-hoc tests revealed that within LN or OB, the tissue EtOH O:I was different (LN SkM: 0.36 ± 0.11 vs. LN SCAT: 0.60 ± 0.14, $p<0.01$; OB SkM: 0.32 ± 0.12 vs. OB SCAT: 0.69 ± 0.09, $p<0.01$), suggesting higher blood flow in SkM than SCAT, as expected.

Exercise

Exercise Intensity. Throughout the 60 min exercise bout, work load was slightly adjusted to keep the subjects' training HR as close as possible to 60% HRR. Figure 2.4 shows the HR data collected every 15 min. The LN subjects maintained a mean HRR of 57.1 ± 5.4 %, while the OB maintained a mean HRR of 56.1 ± 5.4 % HRR. There were no significant main effects of time ($p=0.76$) or BMI ($p=0.72$), or a time x BMI interaction ($p=0.08$) for HRR.

Exercise-induced Plasma IL-15. Figure 2.5 Panel A shows Plasma IL-15 throughout the 60-min aerobic exercise bout and up to 1-hr into recovery. A significant main time effect ($p<0.01$) and *post hoc* tests indicated that in both LN and OB groups, exercise resulted in an increased circulating IL-15 during (30 min) and at the cessation of exercise (60 min), and that Plasma IL-15 returned to baseline within 1-hr of recovery. There was a BMI x time interaction ($p<0.01$), but *post hoc* testing did not reveal any differences between LN and OB in plasma IL-15 at any time. However, the OB group demonstrated a greater relative increase from their pre-exercise to their AVG exercise value (OB: 20.3 ± 10.8 vs. LN: 10.8 ± 7.5 %, $p=0.03$). Further, the OB also demonstrated a higher total amount of IL-15 over the 60-min exercise bout (OB: 9.5

± 4.1 vs. LN: 4.5 ± 2.4 pg/mL*60 min, respectively; $p<0.01$), indicated by the area under the curve (AUC) analysis in Figure 2.5 Panel B.

Exercise-induced SCAT IL-15. Although there was an increased circulating IL-15 in LN and OB during, as compared to before exercise, there were no increases in SCAT *i*IL15 during exercise. Further, there were no differences between the LN and OB groups in SCAT *i*IL15 when expressed as a raw concentration change (LN v. OB SCAT *i*IL15 change: $p=0.35$) or percent change (LN v. OB SCAT *i*IL15 % change: $p=0.97$).

Exercise-induced SCAT glycerol. As a result of exercise, we observed an expected increase in SCAT *i*Glyc in both LN and OB groups (Resting *i*Glyc: 200.4 ± 50.9 vs. Exercise AVG *i*Glyc: 404.5 ± 156.8 µM, $p<0.01$); however, there were no differences between the two groups (*i*Glyc BMI x time interaction: $p=0.85$). Additionally, there were no differences between LN and OB groups in exercise-induced SCAT *i*Glyc when expressed either as a raw concentration change (LN v. OB SCAT *i*Glyc change, $p=0.85$) or percent change (LN v. OB SCAT *i*Glyc % change, $p=0.45$) from resting values.

Exercise-induced IL-15 and SCAT glycerol relationships. Exercise-induced (% change) relationships between Plasma IL-15 and SCAT *i*Glyc, and between SCAT *i*IL15 and SCAT *i*Glyc can be seen in Figure 2.6. Although there were differences in the exercise-induced plasma IL-15 change between LN and OB, there were no significant relationships observed between the exercise-induced Plasma IL-15 % change and SCAT *i*Glyc % change (Panel A), analyzed by the whole group ($r = -0.15$, $p=0.59$) or within LN ($r = 0.16$, $p=0.71$) and OB ($r = -0.29$, $p=0.48$) separately. Likewise, we did not observe any significant relationships between exercise-induced SCAT *i*IL15 % change and SCAT *i*Glyc % change (Panel B), either analyzed by the whole group ($r = 0.21$, $p=0.45$) or within LN ($r = 0.09$, $p=0.85$) and OB ($r = 0.41$, $p=0.32$) separately.

Discussion

In this study we used a combination of blood and microdialysis sampling techniques, which allowed us to gain a unique perspective into local IL-15 contributions to the circulating IL-15 pool. These are the first data of IL-15 in SkM and SCAT in humans, and several novel findings emerged from this study. The primary findings were the following: 1) there was no difference in circulating IL-15 or SkM IL-15 between distinct LN and OB groups, 2) OB SCAT contained more IL-15 than LN SCAT, 3) the two local IL-15 sources (SkM and SCAT) examined did not appear related to systemic IL-15, 4) there was a significant positive correlation between SCAT IL-15 and SCAT lipolysis at rest, 5) there were expected rises in SCAT lipolysis during 60 min of aerobic exercise in both LN and OB, but the degree to which lipolysis increased was not related to the amount of IL-15 released during exercise. These results suggest that IL-15 in SCAT tissue may serve a more important autocrine/paracrine role in humans (AT IL-15 acting in AT) rather than an endocrine role (SkM IL-15-blood-AT).

In contrast to previous reports, we did not observe higher systemic IL-15 concentrations in LN individuals, despite similar BMI stratifications between our study and those previously reported. For instance, both Barra, et al., (10), and Nielsen, et al., (69), reported that LN individuals had a higher circulating IL-15 than OB. A third more recent study (21) also reported an apparently higher resting concentration in LN than OB, although it was not clear whether there was a significant difference between their LN and OB groups. While we do not report the same findings as those previously reported (higher circulating IL-15 in LN than OB), our mean concentrations are within reason of those previously reported, as well as within ranges given for non-diseased subjects (e.g. individuals not known to have a clinical condition where circulating IL-15 is expected to be abnormally high) (37, 47). Some possible explanations for the discrepant

results could be the use of different assays or distinct sample populations. With regards to the latter, the studies above in which age was reported, those subjects were slightly to substantially older than the subjects used in our study. The influence of age on circulating IL-15 is not known, but potentially of interest with IL-15's potential role in both body composition and inflammatory processes. More research will be required to clarify this potential interaction and its implications for human health and disease in aging populations.

We did not observe any significant relationships between resting SkM or SCAT IL-15 and plasma IL-15, suggesting that the circulating pool might not accurately reflect local tissue concentrations. In fact, our estimated interstitial SkM and SCAT IL-15 concentrations are several orders of magnitude higher than the circulation. Similar observations have been reported for other proteins sampled between blood and local tissues, showing disconnection between these distinct biocompartments and the importance of measuring tissue concentrations (71, 72). To our knowledge, our study is the first to report IL-15 concentrations in human fat tissue. Given that obesity is generally considered a low-grade inflammatory condition, this result is not completely surprising. Using microdialysis sampling similar to that used in the current study, Murdolo, et al., demonstrated several inflammatory peptides were elevated in the SCAT dialysate collected from OB subjects compared to their LN counterparts (67). The exact purpose for elevated IL-15 in SCAT tissue is unknown. Yet, with strong links between obesity and inflammatory processes (26), elevations in local AT IL-15 are likely to mediate the proliferation of IL-15 dependent immune cells (23) and related inflammatory cascades in this biocompartment. For example, compared to control mice, IL-15 OE mice demonstrated an accumulation of natural killer (NK) cells in AT, while IL-15 KO mice demonstrated decreased NK cell populations in AT (10). It does not seem likely that there exists a SkM-blood-AT axis in humans given the large gradient

difference that IL-15 would have to overcome traveling from the blood to SCAT. We contend that IL-15 in SCAT or SkM IL-15 likely remains in, and acts on the local tissue where it was produced.

In the current study, we sought to answer what role local IL-15 may play in AT biology (lipolysis) with regard to human obesity. It has been speculated that SkM IL-15 plays a role in reducing fat mass in humans through an "endocrine-like" axis (SkM-blood-AT) (92, 94). In the current study, we aimed to assess IL-15's potential role in increased lipolysis, a critical first step in the overall reduction of fat mass. Our data indicate however that SkM IL-15 is not related to SCAT lipolysis. Therefore, it seems unlikely that IL-15 from SkM would play a substantial role in reducing fat mass in humans through lipolysis. To the contrary, we observed that SCAT IL-15 was related to SCAT lipolysis when analyzed across all our subjects combined. This finding potentially represents a previously uninvestigated IL-15 autocrine/paracrine axis in human AT, which has been raised by Ajuwon and colleagues in porcine cell models. Under certain conditions such as an immune challenge, isolated porcine adipocytes can upregulate IL-15 production and secretion to the surrounding area (1). IL-15 has also been shown to robustly induce lipolysis in the porcine adipocytes after a 2-hr exposure to the pro-inflammatory peptide (2). These authors suggested that lipolytic induction *via* IL-15 may repartition energy away from the adipocyte towards other bioenergetic needs related to mounting a successful immune response. Together with our results that IL-15 in the SCAT is related to the amount of SCAT lipolysis, a similar autocrine/paracrine IL-15 axis may exist in human AT. It would be important to perfuse IL-15 into LN and OB SCAT to assess the direct lipolytic effects of IL-15 at more physiologically relevant doses, as most studies treating animals or cells with IL-15, including the one mentioned above (2), have used concentrations rarely seen in AT. This could clarify IL-15's

in vivo lipolytic effects. It is also possible that LN and OB respond differently to IL-15 perfusion in terms of their lipolytic response, as seen with other pharmacologic agents stimulating lipolysis (48, 49). Until these experiments are performed however, we can only speculate about this latter point.

With the potential that exercise/muscle contraction leads to IL-15 secretion (21, 98, 115), and the previous findings that IL-15 increases lipolysis (2), the other sub-aim of this study was to examine if acute aerobic exercise leads to increases in SCAT IL-15 and plasma IL-15 and if these changes were related to exercise-induced SCAT lipolysis. First, we observed that plasma IL-15 increased in both LN and OB in a transient manner, returning to baseline values within 60 min of exercise cessation. Absolute differences in plasma IL-15 between LN and OB were not evident during exercise and recovery; however, relative increases from baseline and an increased total IL-15 in the exercise-induced AUC analysis suggests that OB have an increased IL-15 secretion from some tissue/organ draining to the circulation. It is also possible that these changes could be driven by differences in IL-15 clearance or changes in bioavailable IL-15 (e.g. not bound to IL-15Rα). Christiansen, et al., (21) also reported increased plasma IL-15 with 2-hr of cycling exercise with LN and OB subjects. While they observed an apparently higher fold change in their OB vs. LN subjects at 2-hr, there was no difference in the magnitude change of IL-15 between LN and OB subjects in their study. It has been speculated that SkM may secrete IL-15 during exercise. We did not keep the microdialysis probes in the muscle during exercise in the majority of our subjects due to the relatively fragile large pore-size membrane we used for IL-15 sampling. In fact, we only kept probes in muscle in 2 LN and 3 OB subjects in our first few trials. In those subjects where we assessed SkM IL-15 with exercise, there appeared to be a slight increase in SkM interstitial IL-15 (data not shown), but the variability was quite spread

among these subjects. As such, we cannot exclude SkM as a contributing source to the elevated circulating IL-15 pool during exercise without the use of a different methodology, and in more subjects. We were able to measure SCAT IL-15 during exercise *via* microdialysis. We found no clear changes in exercise-induced SCAT IL-15, and no significant correlations between exercise-induced SCAT and plasma IL-15; therefore, SCAT IL-15 is not likely a significant contributor to increased plasma IL-15 during exercise.

As for metabolic significance of the plasma and SCAT IL-15 during exercise, exercise-induced changes in plasma or SCAT IL-15 were not significantly related to the amount of exercise-induced lipolysis. More specifically, despite a larger plasma IL-15 increase in OB than LN, and no clear directional change in SCAT IL-15 with exercise, both LN and OB subjects underwent similar increases in interstitial glycerol and glycerol AUC (AUC data not shown) during exercise. Thus, it is likely that IL-15 is not a main contributor to exercise-induced lipolysis. Reasons for augmented increases in plasma IL-15 in OB during exercise remain to be answered. Regardless, this should not discount the potential for IL-15 to contribute to lipolysis during exercise, as the effect of IL-15 may be masked by other well-known factors such as catecholamines and ANP that are likely responsible for the majority of exercise-induced lipolysis in humans (8, 64).

In summary, we did not observe differences in resting SkM or plasma IL-15 between LN and OB subjects. There was a higher SCAT IL-15 in OB than LN subjects, but there were no associations between the local sources and circulating IL-15. Additionally, we found that SCAT IL-15 was related to SCAT lipolysis at rest, suggesting an IL-15 autocrine/paracrine axis may exist in humans, and may be more important in human obesity than previous postulations of an endocrine (SkM-blood-AT) axis. While we also report that plasma IL-15 increased more in OB

human subjects during exercise, the biological significance for such increases remain to be clarified, as exercise-induced changes in IL-15 were not related to exercise-induced changes in lipolysis.

Table 2.1 Subject demographic and anthropometric characteristics

	Lean (n=10)	Obese (n=10)	p-value
Male/Female	5 / 5	6 / 4	n/a
Age (yr)	24 ± 4	27 ± 9	0.30
Height (m)	1.7 ± 0.1	1.8 ± 0.1	0.23
Weight (kg)	66.4 ± 11.0	104.2 ± 11.7 *	< 0.01
BMI (kg/m^2)	23.1 ± 1.9	34.7 ± 3.5 *	< 0.01
Resting heart rate; HR (bpm)	63.7 ± 7.9	70.3 ± 6.5	0.06
Heart rate reserve; HRR (bpm)	132.3 ± 10.1	122.4 ± 9.5 *	0.04
Target HR (bpm)	143.1 ± 2.9	143.6 ± 6.7	0.81

Values are mean ± SD; *P<0.05 vs. Lean group.

Table 2.1. Basic demographic information for study volunteers obtained during the Screening visit. Details for calculated HRR and Target HR are provided in the Methods section; bpm = beats per minute.

Table 2.2. DXA-assessed body composition parameters for participants

	Lean (n=10)	Obese (n=10)	p-value
Bone mineral density; BMD (g/cm^2)	1.20 ± 0.13	1.32 ± 0.11 *	0.03
Bone mineral content; BMC (g)	2855.96 ± 729.60	3285.96 ± 497.74	0.14
Total mass (kg)	66.5 ± 11.2	103.3 ± 11.0 *	< 0.01
Fat mass (kg)	19.7 ± 5.9	43.7 ± 10.3 *	< 0.01
Body fat (%)	30.6 ± 10.8	42.2 ± 8.4 *	0.02
Lean mass (kg)	43.9 ± 13.0	56.4 ± 9.9 *	0.03
Lean body mass (%)	65.2 ± 10.3	54.7 ± 8.1 *	0.02
Fat free mass (%)	69.4 ± 10.7	57.8 ± 8.4 *	0.02
Android fat (%)	37.2 ± 12.0	53.7 ± 4.6 *	< 0.01
Gynoid fat (%)	39.0 ± 14.0	45.8 ± 12.1	0.26
Android: Gynoid ratio	0.98 ± 0.22	1.23 ± 0.24 *	0.02

Values are mean ± SD; *P<0.05 vs. Lean group.

Table 2.2. Data are anthropometric data obtained from the DXA scan during the Screening visit. All variables are provided using the standard software provided with DXA instrument.

Table 2.3. Basic blood chemistry for participants

	Lean (n=10)	Obese (n=10)	p-value
Glucose [mg / dL]	81.9 ± 6.5	82.0 ± 6.8	0.19
Insulin [µIU / mL]	5.4 ± 2.4	11.0 ± 3.1 *	< 0.01
HOMA	1.10 ± 0.51	2.23 ± 0.69 *	< 0.01
Triglycerides [mg/dL]	56.7 ± 24.2	94.2 ± 67.2	0.11
Total Cholesterol [mg/dL]	155.8 ± 16.0	190.4 ± 66.5	0.13
HDL-C [mg/dL]	48.9 ± 7.7	48.6 ± 9.7	0.95
LDL-C [mg/dL]	99.9 ± 14.5	126.3 ± 57.4	0.17

Values are from resting blood drawn 8 hr fasted and are presented as mean ± SD; *P<0.05 vs. Lean group.

Table 2.3. Blood chemistry values obtained from UniCel DxC 600i Synchron® Access® Clinical System (Beckman Coulter, Inc., Brea CA). Details for calculated HOMA (Homeostatic Model Assessment) values are provided in the Methods section.

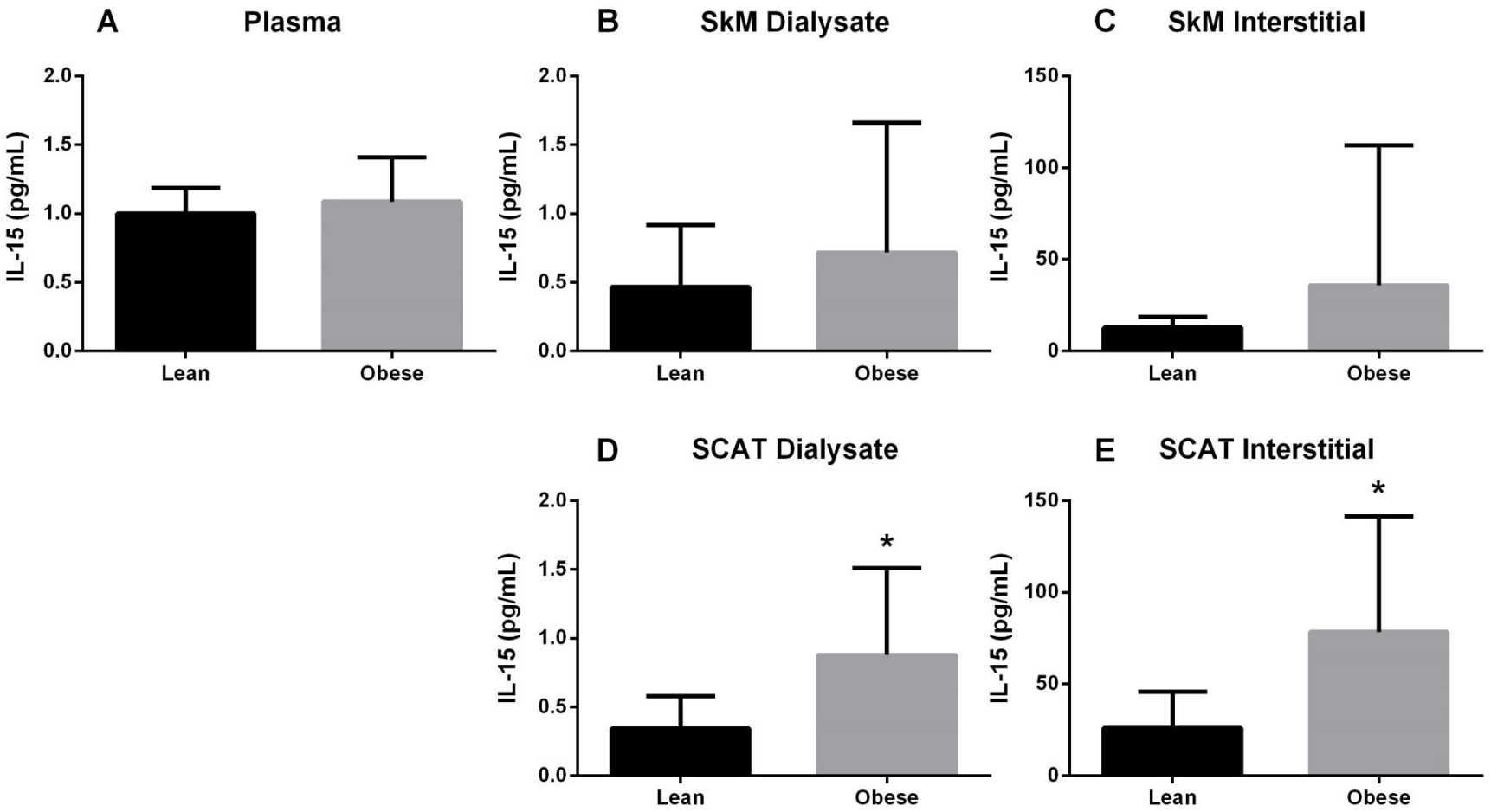

Figure 2.2. Resting IL-15 concentrations (pg/mL) compared among Plasma (A), skeletal muscle (SkM) Dialysate and Interstitial IL-15 (B, C) and subcutaneous adipose tissue (SCAT) Dialysate and Interstitial IL-15 (D, E) in Lean (n=10) and Obese subjects (n=10). Plasma was obtained from I.V. catheter, and SkM and SCAT Dialysate was obtained from microdialysis probes inserted into SkM or SCAT. Details on Interstitial IL-15 calculations from Dialysate Il-15 concentrations are provided in the Methods section. Data are mean ± SD. *****P<0.05 vs. Lean.

Table 2.4. Resting Local and Systemic IL-15 relationships		
	SkM Interstitial vs. Plasma IL-15	**SCAT Interstitial vs. Plasma IL-15**
All (n=20)	r = – 0.16 P = 0.50	r = 0.03 P = 0.92
LN (n=10)	r = – 0.42 P = 0.23	r = – 0.07 P = 0.85
OB (n=10)	r = – 0.22 P = 0.55	r = –0.08 P = 0.84

Table 2.4. Relationships between local (SkM and SCAT) interstitial sources of IL-15 and circulating IL-15. As indicated in the table, there are no significant relationships ($p \geq 0.23$ for all analyses) between local and systemic IL-15.

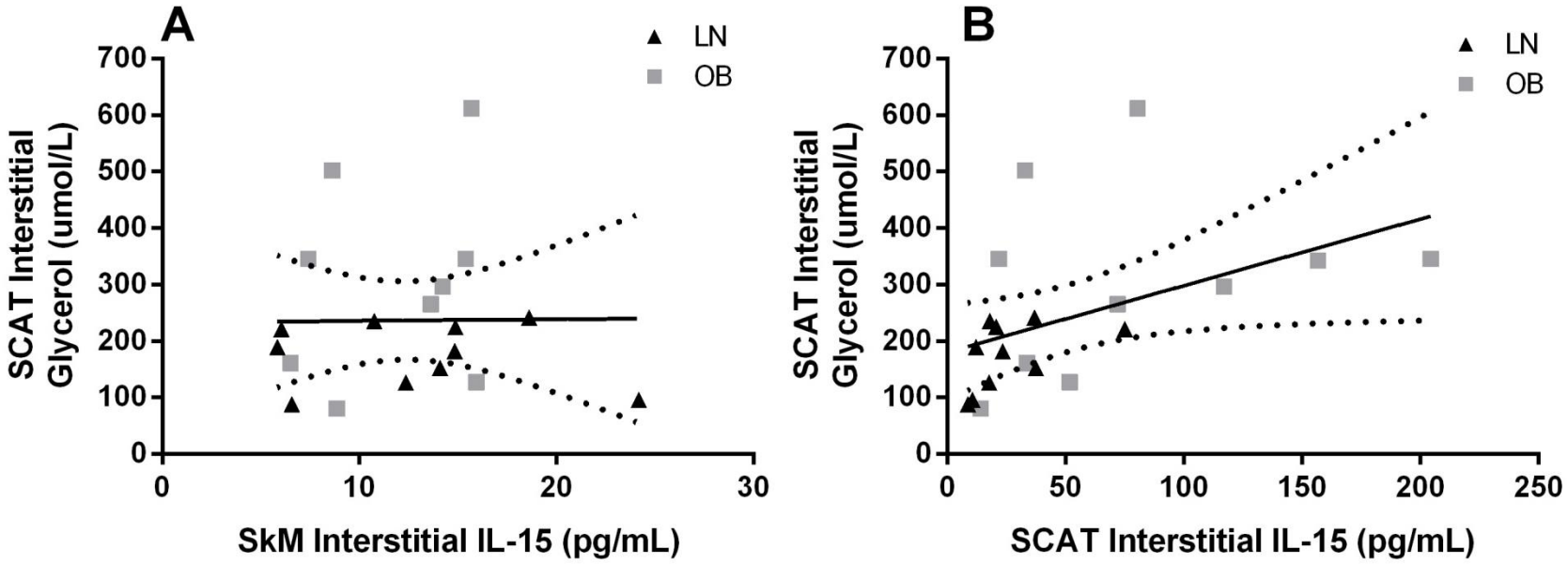

Figure 2.3. Relationships between resting SkM/SCAT Interstitial IL-15 and SCAT Interstitial glycerol. SkM and SCAT Dialysate IL-15 and glycerol were obtained from inserted microdialysis probes in SkM and SCAT. Details for calculations of SkM and SCAT Interstitial IL-15 and glycerol from Dialysate IL-15 and glycerol are provided in the Methods section. LN = lean subjects, black triangles; OB = obese subjects, gray squares. Solid lines are the trendlines for entire group, and dotted curved lines are ±95% CI for entire group (n=19 for SkM IL-15/ SCAT glycerol relationship; n=20 for SCAT IL-15/ SCAT glycerol relationship).

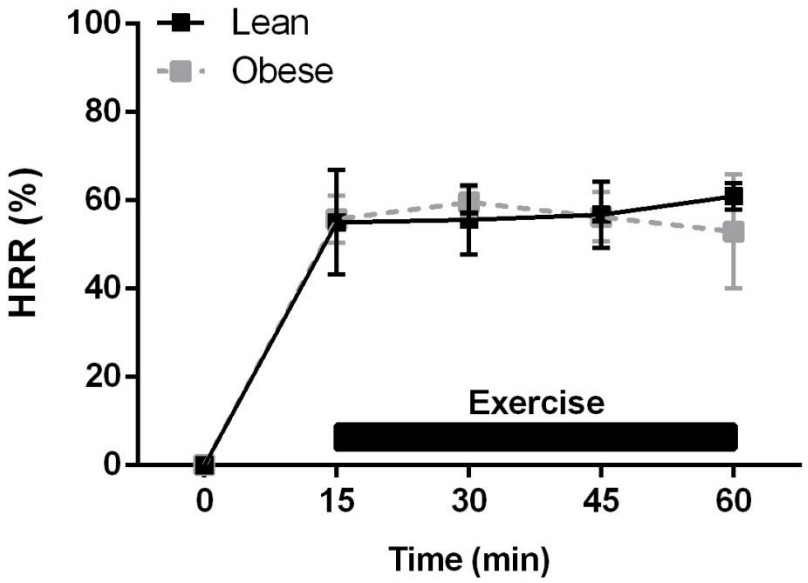

Figure 2.4. Exercise-induced heart rate (HR) responses. Data are HR measured during the exercise bout, displayed as a percentage of the Heart Rate Reserve (HRR) in Lean (n=9) and Obese (n=9) subjects. Details on HRR calculation using the Karvonen equation are found in the Methods section. Data are presented as mean ± SD.

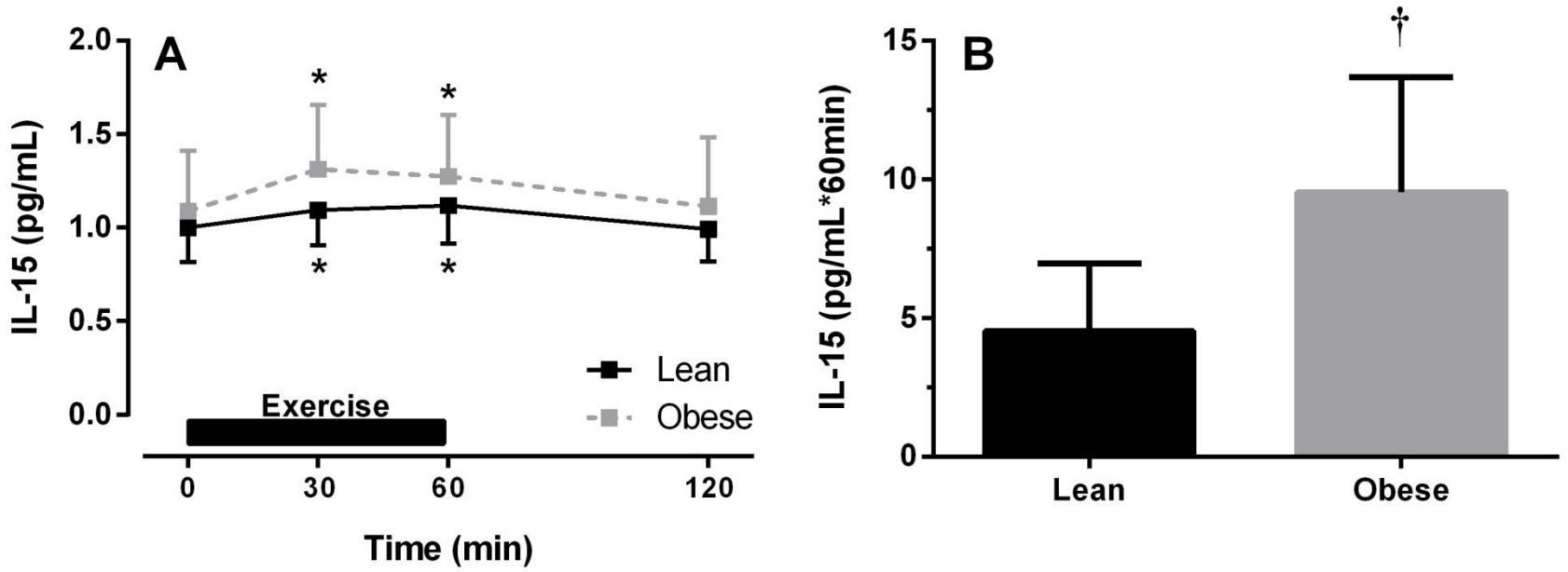

Figure 2.5. Exercise-induced plasma IL-15. Panel A shows mean plasma IL-15 concentration at time point sampled during exercise. A significant and transient rise in IL-15 occurred in both Lean (n=10) and Obese (n=10) as a result of acute aerobic exercise. *P<0.05 vs. Pre-exercise ('0' timepoint). Panel B shows the calculated area under the curve for plasma IL-15 during the 60 min of exercise. †P<0.01 vs. Lean.

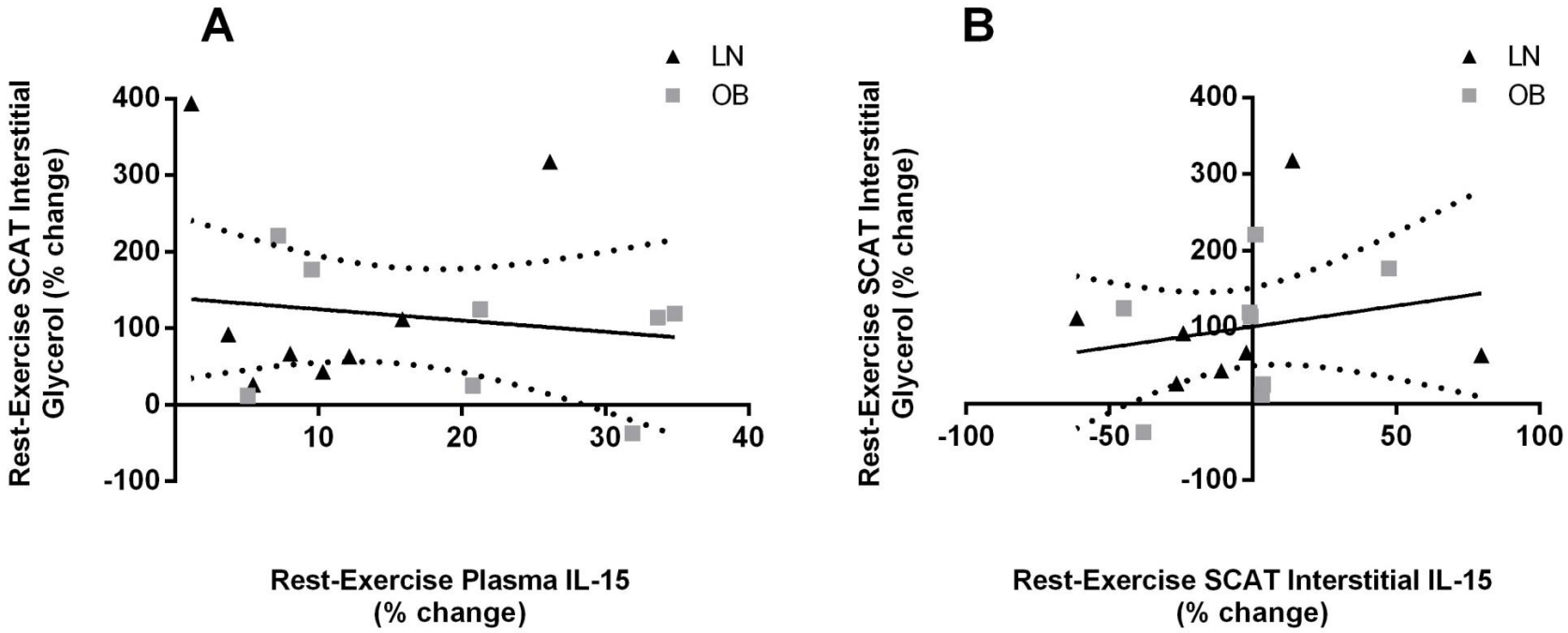

Figure 2.6. Exercise-induced relationships between IL-15 and SCAT interstitial glycerol. Relationships are between the exercise-induced percent change in plasma IL-15 and the exercise-induced percent change in SCAT interstitial glycerol (A), and between exercise-induced percent change in SCAT interstitial IL-15 and exercise-induced percent change in SCAT interstitial glycerol (B). SCAT Dialysate IL-15 and glycerol was obtained from inserted microdialysis probes in SCAT and used to calculate Interstitial IL-15 and glycerol. Details on the Interstitial calculations from Dialysate concentrations are provided in the Methods section. LN = lean subjects (n=8), black triangles; OB = obese subjects (n=8), gray squares. Solid lines are trendlines for the entire group, and dotted curved lines are ±95% CI for entire group.

CHAPTER 3: DOES DIRECT PERFUSION OF IL-15 INTO SUBCUTANEOUS ABDOMINAL ADIPOSE TISSUE STIMULATE LIPOLYSIS IN LEAN AND OBESE HUMANS?

Abstract

Investigations into the ability of the cytokine IL-15 to drastically reduce adipose tissue (AT) fat mass across several animal models have led researchers to speculate that IL-15 can directly decrease AT fat mass in humans as well. While one mechanism responsible for IL-15 mediated decreases in fat mass includes increased mobilization (lipolysis), studies investigating IL-15 actions have not been physiologically relevant and have not been performed directly in human AT. **PURPOSE**: To investigate if IL-15 can induce lipolysis in human AT over a range of physiologically relevant to pharmacological concentrations. **METHODS**: We inserted microdialysis probes (MD; CMA/20: 100 kDa pore size membrane) into subcutaneous (SC) abdominal AT of 20 lean (LN; n=10, BMI: 23.1 ± 1.9 $kg \cdot m^{-2}$, age: 24.0 ± 3.7 yr) and obese (OB; n=10, BMI: 34.7 ± 3.5 $kg \cdot m^{-2}$, age: 27.3 ± 9.1) men and women. Probes were perfused with recombinant human (rh) IL-15 at concentrations of 0, 20, 650, 6500, and 65000 pg/mL and 10 mM ethanol (EtOH). The lipolytic response to IL-15 perfused through the probe was monitored by measuring dialysate glycerol: used to calculate interstitial glycerol (*i*Glyc). The EtOH outflow/inflow ratio (EtOH O:I) was used as an index of local blood flow, and in the calculation of *i*Glyc. **RESULTS**: The OB had a higher resting *i*Glyc than LN (OB: 307.7 ± 164.2 vs. LN: 175.5 ± 57.5 µmol/L; p=0.03), but no difference in resting blood flow (p=0.10), as indicated by resting EtOH O:I. IL-15 perfusion in LN resulted in an increase in lipolysis at the second lowest IL-15 perfusion concentration [650 pg/mL] (p=0.04). In contrast, lipolysis was suppressed in OB

at the lowest IL-15 concentration [20 pg/mL] ($p<0.01$). Importantly, both of these perfusate concentrations result in delivery of IL-15 to the interstitium in concentrations likely present *in vivo* in AT, and occurred without any marked changes in blood flow. **CONCLUSIONS**: Human AT can respond to some physiological doses of IL-15 with alterations in lipolysis; however, there are differences in the lipolytic responses between LN and OB SCAT and there was no response at doses above likely physiological concentrations. These results could suggest an important autocrine/paracrine role for IL-15 in human SCAT, with stimulation of lipolysis in lean individuals and suppression of lipolysis in obesity.

Introduction

Several paradigms in which IL-15 led to decreases in fat mass have identified this traditional cytokine as a regulator of fat mass (4, 10, 18, 94) in addition to the well-known proliferation and differentiation effects of IL-15 on natural killer (NK) immune cells (23). For instance, IL-15 overexpression (OE) animals have expanded NK cell populations, as well as decreased fat cell size and lower fat mass vs. control or IL-15 knock out (KO) counterparts (10). Strategies increasing endogenous circulating IL-15 concentrations have been shown to decrease fat mass in animals on a standard diet (94) and to mitigate fat gains in animals from diet induced obesity (10). Finally, administration of exogenous IL-15 has led to substantial decreases (up to 44% in some cases) in fat mass across several models of animal obesity (4, 10, 18). Thus, both endogenously produced and exogenously delivered IL-15 appears to play a substantial role in the regulation of AT. Potential mechanisms for these fat losses via IL-15 include both a decreased lipid uptake and deposition (2, 4, 10, 96) and increased mobilization (2). Importantly, these

models still represent conditions (e.g. supraphysiologic concentrations) rarely seen *in vivo*, and are not well studied in humans.

There is a relatively high expression of IL-15 in skeletal muscle (SkM), although it has also been demonstrated that the relatively low adipose tissue (AT) IL-15 expression can be increased under certain conditions in both animal (1) and human models (Ch. 2 Results). Ajuwon, et al., first showed that the adipocyte's relatively low expression of IL-15 was increased upon exposure to IFN-γ, a pro-inflammatory mediator (1). In addition, we recently observed that IL-15 concentrations were higher in the subcutaneous (SC) AT of obese (OB) than lean (LN) humans. Thus, there are situations in which adipocytes are likely to be exposed to elevated IL-15 concentrations *in vivo*. The adipocyte expresses all 3 IL-15R subunits (4), suggesting the adipocyte also has the capability to respond to this locally elevated IL-15. Indeed, IL-15 has been shown to directly induce lipolysis in isolated porcine adipocytes (2). We also recently observed that in a cohort of LN and OB subjects, the amount of SCAT IL-15 was significantly related to the amount of resting SCAT lipolysis (Ch. 2 Results). It has been suggested that IL-15 released from, and acting within, AT may represent an important autocrine/paracrine axis in which fatty acids are directed from the adipocyte and toward other innate immunity needs (2). It remains to be clarified if IL-15 exposure increases lipolysis in human AT, or if this response is different between LN and OB, as previously demonstrated with other lipolytic stimulatory agents (48).

In the current study, we aimed to determine if the direct perfusion of IL-15, at physiologically-relevant up through pharmacological concentrations, could induce lipolysis *in vivo* in human SCAT. A secondary aim was to determine if the SCAT lipolytic response to IL-15 perfusion was different between lean and obese humans. These perfusion studies were performed to clarify the *in vivo* role of IL-15 in mediating AT lipolysis (at the lower physiological doses),

while also to indicate potential therapeutic applications (at higher pharmacological doses) of IL-15 in decreasing fat mass in obese individuals. We hypothesized that IL-15 would increase lipolysis in both LN and OB individuals, but that the lipolytic induction would be blunted in OB subjects.

Materials and Methods

Screening visit

Participants. We used the same subjects as in Chapter 2 (Study 1). Therefore, the same screening procedures and inclusion/exclusion criteria applied. In short, after being briefed on all study procedures, and having signed an informed consent, volunteers underwent a general health screening (height, weight, BMI, etc.), and completed a brief medical history questionnaire to confirm qualification. Subjects aged 18-45 y were classified by "LN" or "OB" BMI, represented as 18.5-25 and 30-39.9 $kg \cdot m^{-2}$, respectively. Twenty LN (n=10, BMI: 23.1 ± 1.9 $kg \cdot m^{-2}$, age: 24.0 ± 3.7 yr) and OB (n=10, BMI: 34.7 ± 3.5 $kg \cdot m^{-2}$, age: 27.3 ± 9.1) men (n=11) and women (n=9) were used in the final groups. Subject demographic and anthropometric characteristics are provided in Table 3.1. Subjects were excluded if they did not fit within the desired BMI ranges, exercised ≥ 3 $d \cdot wk^{-1}$, were a smoker, had any known cardiovascular or neuromuscular disease, were taking any medication known to effect metabolism, or were pregnant or lactating. All study procedures were approved by the ECU University and Medical Center Institutional Review Board (UMCIRB).

Body composition assessment. Participants had height measured to the nearest 0.5 cm using a wall-mounted stadiometer and weight assessed on an electronic scale to the nearest 0.1 kg, and had BMI ($kg \cdot m^{-2}$) calculated. We also performed a dual-energy X-ray absorptiometry

(DXA) scan (Prodigy, GE Lunar Corp., Madison, WI), where subjects lied on the DXA table while a fan beam scanner obtained detailed lean and fat mass densitometries. Provided software calculated specific parameters, which included bone mineral density and content, fat mass and percent body fat (total and regional estimates), lean mass and percent lean mass. Select DXA-assessed parameters are presented in Table 3.1.

IL-15 Perfusion visit

Within 48h of the perfusion visit, subjects were instructed not to perform vigorous exercise or ingest alcohol. The day prior, they were asked to eat meals close to their typical diet and only drink water from ~ 2200h the night before. Subjects were asked to eat a provided breakfast 2h before their experimental visit began (~0600h), which consisted of 2 crunchy granola bars (190 kcal, 29 g carbohydrate (CHO), 6 g fat (FAT), 4 g protein (PRO)) and 4 oz. ensure drink mix (125 kcal, 17 g CHO, 4.5 g FAT, 4.5 g PRO) for a total of 315 kcal (~ 60 % CHO / 30 % FAT / 10 % PRO). Subjects reported to the East Carolina Heart Institute (ECHI) for their experimental visit at approximately 0800h. After confirming that the meal was eaten at the time specified to standardize feeding, subjects were asked to rest in a semi-recumbent prone position in a hospital bed to begin the microdialysis (MD) probe insertion for SCAT dialysate sampling. During the IL-15 perfusion period, subjects were allowed to work quietly, read, or watch television, and only allowed to drink water ad libitum during the visit.

MD Probes and Perfusates. Custom MD probes (CMA/20: 100 kDa cut off) were ordered from CMA Microdialysis (CMA/Harvard Apparatus, Hollison, MA), soaked for 30 min in 70 % isopropanol the day before use, and kept antiseptic in sterile water overnight. Recombinant human (rh) IL-15 was purchased sterile filtered and lyophilized from PeproTech (Rocky Hill, NJ), and reconstituted prior to use. IL-15 perfusate solutions of varying

concentrations 0, 20, 650, 6500, and 65000 pg/mL were prepared in a base solution of sterile 0.9 % NaCl (saline), where we added 10mM EtOH to estimate blood flow and calculate interstitial glycerol concentrations, and [30 g/L] Dextran (40 kDa molecular weight; Sigma Aldrich, St. Louis, MO) to prevent ultrafiltration across the large pore size membrane. Once these agents were added, perfusates were sterile filtered with 0.22 micron filters into CMA 106 MD syringes (MDialysis, North Chelmsford, MA), and kept overnight at 4°C until use the next day.

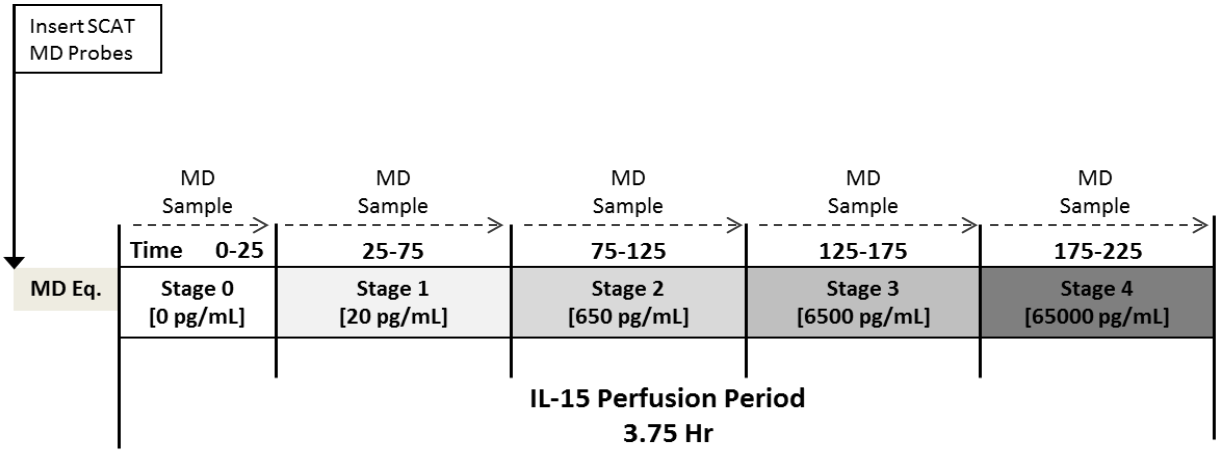

Figure 3.1. Study schematic of the IL-15 Perfusion Timeline. MD = microdialysis. 'MD Eq.' was 60 min of equilibration allowed after probe insertion before any sample was collected. 'MD sample' indicates period of time (25-50 min) that dialysate was sampled for glycerol. At the end of each stage (0-4), perfusates were also individually sampled for the IL-15 concentration being delivered to the SCAT.

MD Probe Insertion. For SCAT MD Probe insertion, the skin around the insertion site was cleaned using iodine swab sticks. To ease any discomfort from the probe placement, a small amount (~1-2 mL / insertion site) of 1% lidocaine was then injected with the use of ethyl chloride spray (cold numbing spray) under the skin surface into the abdominal SCAT space ~2-3 cm lateral to the umbilicus. Next, a removable plastic sheath was inserted into the numbed area with the use of an 18-guage catheter needle. Once the needle was removed, the sterile MD probe

was placed inside the plastic sheath. The plastic guide was then removed, leaving the dialysis membrane portion of the MD probe embedded below the skin and in SCAT (Figure 1.2 Panel B).

Inserted MD probes were secured to the subject and connected to perfusate syringes and calibrated syringe pumps (CMA 107 Pump, Mdialysis, North Chelmsford, MA). Once the pump was started, and flow was established (able to observe dialysate coming from exit tubing), we allowed a 1-hr equilibration before dialysate was collected for analysis. Dialysates were collected in 300 µL plastic microvials (Milian USA, Columbus, OH), and were weighed both prior to and following collection to monitor flow rate, set to 2 $\mu L \cdot min^{-1}$. Collected dialysates were kept at either 4°C or -20°C until desired analysis occurred (see below).

Dialysate Glycerol Measurement. During each MD collection period (~25-50 min), indicated by 'MD Sample' in Figure 3.1, collected dialysate was assessed for glycerol on the CMA 600 Analyzer (MDialysis) according to manufacturer's directions. The analyzer measures glycerol from < 2 µL samples using a colorimetric reaction. Dialysate samples were kept at -20°C until analysis. All samples were run in duplicate, measured against provided calibrators, and averaged for data analysis.

EtOH Assay. During MD collection periods, perfusate and dialysate samples were taken and stored at 4°C until analyzed for EtOH (≤ 48 h from collection) as previously described (44). This fluorometric assay is based on the conversion of EtOH to acetylaldehyde *via* alcohol dehydrogenase (ADH) and co-factor NAD. The fluorescent NADH product is directly proportional to EtOH in the original sample, was detected using the Victor^3 Multilabel counter (PerkinElmer, Wellesley, MA). All samples were run in duplicate, measured against a standard curve, and averaged for data analysis. We calculated the *in vivo* EtOH O:I ratio as $[EtOH_{dialysate}]$ / $[EtOH_{perfusate}]$ to qualitatively estimate blood flow and calculate interstitial concentrations.

IL-15 Assays. IL-15 concentrations were measured in collected SCAT dialysate and IL-15 perfusates using a commercially-available electrochemiluminescent Ultra-sensitive IL-15 kit from Meso Scale Discovery (MSD; Rockville, MD). Briefly, sample medium was added to wells containing carbon electrodes pre-coated with IL-15 capture antibodies. After a labeled detection antibody and read buffer are added, the electrode is excited and the emitted light is proportional to the amount of IL-15 in the sample. The chemiluminescent signal was read against a standard curve using the MSD Sector 2400 instrument and quantified using MSD Discovery Workbench software v.4. Dialysate and perfusate samples were measured in a modified protocol developed along with the manufacturer, where we prepared the calibration curve in a 0.9% saline and 1 % BSA background. A small amount of BSA (1 % final BSA) was added to the dialysate samples to standardize matrices, and we applied appropriate dilution factors to all unknown samples. Samples were run in duplicate where volume allowed, and averaged for data analysis.

Interstitial glycerol estimates. In the current study, we calculated the interstitial glycerol (*i*Glyc) in response to IL-15 perfusion in the same manner as in Chapter 2 using relative recoveries of glycerol to EtOH. Briefly, *in vitro* experiments were conducted where the same probes and flow rates as used in the *in vivo*/*in situ* conditions were placed in a beaker solution (Dulbecco's phosphate-buffered saline (D-PBS), 0.1 % bovine serum albumin (BSA), 5 mM glucose, 0.2 mM glycerol, 5 mM ethanol (EtOH), and 2 mM lactate). By adding 10 mM EtOH to the *in vivo* perfusate and given the assumption that the relationship between the recoveries of two substances *in vitro* is the same as the relationship between the relative recoveries of those two substances *in vivo,* we used the following equation to calculate Interstitial glycerol (*i*Glyc):

$$i\text{Glyc} = \left[\left(\frac{in\ vitro\ \text{EtOH relative recovery}}{in\ vitro\ \text{glycerol relative recovery}}\right) \times \frac{[d\text{Glyc}]}{\underset{in\ vivo}{\text{1-EtOH 0: I}}}\right]$$

where: *d*Glyc = measured *in vivo* dialysate glycerol; 1-EtOH O:I = *in vivo* EtOH lost to tissue from the perfusate; *in vitro* EtOH relative recovery = ([*in vitro* dialysate EtOH] / [*in vitro* beaker EtOH]); *in vitro* glycerol relative recovery = ([*in vitro* dialysate glycerol] / [*in vitro* beaker glycerol]).

Statistical analysis

Subject characteristics and resting baseline measures (EtOH O:I, *i*Glyc) between LN and OB was assessed using a Student's t-test. For differences examined across multiple time points during the IL-15 perfusion stages, we used repeated measures ANOVAs. If a significant F-ratio was detected in the ANOVA, we used a Fisher's LSD *post-hoc* analysis. All statistical analyses were performed using SPSS Statistics for Windows v.20.0 (IBM, Artmonk, NY). Data are reported as mean ± SD, unless otherwise noted, and α-level was set at $p \leq 0.05$.

Results

Physical characteristics. The same subjects as were used in Chapter 2 (Study 1), were used for this study; therefore, the subject characteristics remained the same. Briefly, and as shown in Table 3.1, between the two distinct LN and OB BMI groups (LN: 23.1 ± 1.9 vs. OB: 34.7 ± 3.5 $kg \cdot m^{-2}$, $p < 0.01$), LN and OB were not different in age (p=0.30), but the OB had a higher percent fat mass (p=0.02) and a lower percent fat free mass (p=0.02) vs. their LN counterparts. The OB also had a higher Android % fat (p<0.01) and Android:Gynoid ratio (p=0.02), but were not different in their lower body fat distribution (Gynoid % fat) (p=0.26).

IL-15 Perfusates. In this study we aimed to perfuse IL-15 directly into SCAT tissue at physiologically-relevant up through pharmacological concentrations. The investigations from which we based our IL-15 perfusate concentrations were from measured IL-15 concentrations

(10, 37, 69), and those studies which delivered exogenous IL-15 to animals (4, 10, 18) or incubated cells with IL-15 (2, 10). As Presented in Figure 3.2, IL-15 perfusates were found to be at mean concentrations of: 20, 650, 6500, and 65000 pg/mL for Stages 1-4, respectively. Importantly, there were no BMI ($p=0.46$) or BMI x concentration interactions ($p=0.68$) for the IL-15 perfusate concentrations used.

Ethanol (EtOH) O:I Ratio. Resting (Stage 0) EtOH O:I was not different between LN and OB individuals (LN: 0.60 ± 0.14 vs. OB: 0.69 ± 0.09, $p=0.10$). As a result of the IL-15 perfusion, there were no main (time effect, $p=0.12$; BMI effect, $p=0.12$) or interaction effects (time x BMI interaction effect, $p=0.87$) observed, suggesting IL-15 perfusion did not alter blood flow, as indicated by the EtOH O:I ratio (Figure 3.3).

Interstitial Glycerol. Resting (Stage 0) calculated *i*Glyc was higher in OB than LN subjects (OB: 307.7 ± 164.2 vs. LN: 175.5 ± 57.5 µmol/L, $p=0.03$). SCAT IL-15 perfusion (Figure 3.4) resulted in a significant BMI x time interaction ($p=0.02$) with regard to the SCAT *i*Glyc (lipolytic) response. In the LN subjects, there was significant 13.7 % increase ($p=0.04$) in *i*Glyc between the two lowest IL-15 perfusion concentrations [20 and 650 pg/mL], suggesting that IL-15 increased lipolysis in AT of LN subjects. By the next highest concentration [6500 pg/mL], *i*Glyc concentrations were no longer elevated in the LN subjects and were similar to baseline for the remainder of the IL-15 perfusion period ($p>0.05$). To the contrary, SCAT IL-15 perfusion significantly decreased *i*Glyc by -18.8 % between the Resting (Stage 0) and lowest IL-15 perfusion concentration [20 pg/mL] in OB subjects ($p<0.01$), suggesting that IL-15 suppressed SCAT lipolysis. The *i*Glyc concentrations remained suppressed, and were still below baseline values ($p=0.01$) at the end of the IL-15 perfusion period [65000 pg/mL]. When expressed as a percent change from to baseline (Stage 0, perfused with 0 pg/mL), the *i*Glyc

change from 0 pg/mL to 20 pg/mL perfusion was nearly different between LN and OB (p=0.07), but the *i*Glyc change from 0 pg/mL to 650 pg/mL perfusion was statistically different between LN and OB (p=0.03).

Discussion

Previous investigations of IL-15 effects on AT metabolism have only been performed in animal or cell culture models, and have used concentrations rarely seen *in vivo*. We therefore perfused LN and OB human SCAT with a range of physiologically relevant to pharmacological concentrations. To our knowledge, we are the first to show that human AT does respond to IL-15, even at physiologically-relevant concentrations. We report in the current study that the SCAT lipolytic response to IL-15 was different in LN and OB human AT without significant alterations in blood flow. These findings demonstrate that human IL-15 in AT can effect lipolysis in AT, highlighting an autocrine/paracrine axis that could have implications in human obesity.

Studies that have increased circulating IL-15 through genetic manipulations or exogenous IL-15 administration have observed robust anti-obesogenic responses. Decreased fat mass resulting from IL-15 have mostly been attributed to decreased lipid uptake and deposition (4, 10, 96), but could also be related to an increased IL-15 lipolytic response (2). Based on the observation in our lab (Ch 2 results) that the amount of SCAT interstitial IL-15 was related to the amount of SCAT lipolysis, and a study showing that IL-15 could induce lipolysis in porcine adipocytes (2), we hypothesized that IL-15 would induce lipolysis in human AT. In line with our hypothesis, we observed that LN demonstrated a modest (13.7 %), but significant increase in lipolysis by the second lowest IL-15 perfusion concentration [650 pg/mL]. Since this increase only occurred after being exposed to a previous (lower) IL-15 perfusion dose and after

approximately 1 hr 40 min from the initial exogenous exposure, it could be argued that this increase in human SCAT glycerol not only has a concentration dependence, but a time-dependence as well, as previously highlighted in porcine adipocytes.(2). In that investigation, the authors showed a significant two-fold induction with 50 ng/mL IL-15 and 3-fold induction with 100 ng/mL IL-15 over control media conditions. They further demonstrated that with 50 ng/mL IL-15 exposure, there was a transient lipolytic response, where the largest increase was observed at 2-hr from the initial IL-15 exposure and was followed by a decline thereafter (2). In accordance with that study, the end of the 650 pg/mL perfusion in the present study was nearly 2-hr from the start of IL-15 perfusion, and was followed by a decline towards basal levels after. This transient lipolytic response could involve an IL-15 time or concentration threshold being met in the lean subjects, in which IL-15-induced lipolysis is suppressed after a given IL-15 exposure in lean human adipose tissue. Further experiments are needed to determine both the nature and significance of this finding.

In contrast to the lipolytic stimulation observed in the LN subjects, OB demonstrated a suppressed lipolytic response (-18.8 % change) between the basal perfusion and the lowest IL-15 exogenous perfusion concentration of 20 pg/mL. Attenuated lipolysis in OB compared to LN humans has been shown previously upon exposure to the β-AR stimulant, isoproterenol (49). It has been postulated that the reason for this blunted response is to minimize tissue exposure to FFA released from such a large amount of AT present in OB (48), and is likely due to alterations in receptor responsiveness and defects in lipolytic cascade proteins, such as PKA, HSL, ATGL, and other associated proteins (56). If one of IL-15's roles is to mobilize lipids, redirecting energy from adipocytes toward other needs (2), the suppressed IL-15 lipolytic response in OB could be a similar protective mechanism as mentioned above (e.g. to reduce tissue exposure to FFA). Given

that OB SCAT is a larger fat depot and contains higher IL-15 concentrations than LN SCAT (Ch 2 Results), this protective mechanism seems plausible. It is possible that an increased inflammatory profile (elevated SCAT IL-15 in this particular case) could also suppress lipolysis in OB SCAT, further supporting the link between a decreased lipid mobilization and an increased fat mass.

Obese individuals have also been shown to have altered NK cell populations (19, 59, 80) and decreased NK function (61, 80). If the lipolytic response to IL-15 in OB is suppressed and not elevated as expected (to redirect lipids toward other innate immunity needs), this could also point to a defect in obesity where obese individuals might not be able to meet immune challenges *via* IL-15 mediated mechanisms. It would be of particular interest to determine if the lower cytotoxicity activity and tumor fighting abilities in NK cells of OB than LN (80) might be linked to IL-15 signaling dysfunction.

Previous studies including the one mentioned above (2, 4, 10, 18) have exposed adipose tissue to substantially higher IL-15 concentrations than would be expected to occur in *in vivo* conditions (e.g. ng/mL range). This became the impetus for the current study: to determine of IL-15 could induce lipolysis both in human tissue and at physiological concentrations. Indeed, we observed an increased lipolytic response in LN SCAT with concentrations closer to the physiological range. Using our *i*IL15 data from Chapter 2, we recovered an estimated 1.4 ± 0.9 % IL-15 across the membrane (*in vivo* IL-15 recovery). It is assumed that the same amount of IL-15 was able to cross the membrane from the perfusate into the tissue during this perfusion study. Thus, the lowest IL-15 perfusion concentrations [20, 650, and 6500 pg/mL, respectively] would represent SCAT IL-15 exposure between 0.3 and 91 pg/mL (assuming 1.4% crossed the 100 kDa membrane used). Based on our estimated *in vivo* SCAT interstitial IL-15 concentrations

in Chapter 2 (LN: 11.8-40.3 pg/mL, OB: 33.5-123.5 pg/mL, 95 % CI range), the IL-15 perfusate concentrations used in the current investigation that stimulated/suppressed lipolysis are concentrations that SCAT could be exposed to when considering the range of human obesity. These calculations should be interpreted with caution due to the assumptions involved in probe recovery and diffusion through tissue in lean and obese individuals, which may vary based on adiposity, tissue characteristics, and possible binding of the IL-15.

Limitations and future directions

Some limitations of the current study must also be addressed. We chose to measure IL-15 lipolytic induction in this study to glean information about IL-15's potential to decrease fat mass *in vivo*. Choosing only lipolysis has limited our ability to draw complete conclusions on IL-15's role in human obesity. Specifically, we cannot comment on the ability of IL-15 to decrease fat mass in humans *via* decreased lipid uptake and deposition, as highlighted in several animal and cell models (2, 4, 10, 96). Further, we can only comment on the lipolytic suppression in response to IL-15 in the OB and cannot determine where this suppression is regulated along the lipolytic cascade. However, IL-15 perfusion into LN and OB SCAT has offered insight into a previously unexplored autocrine/paracrine axis in human AT. It seems plausible that pro-inflammatory molecules, such as IL-15, may be related to obesogenic processes and not solely a consequence of obesity per se, although further experiments would be needed to clarify this. We also chose a wide range of IL-15 perfusate concentrations. Based on assumptions of the microdialysis technique and estimated *in vivo* relative recoveries, we estimate that the tissue exposure was within physiological range. Future experiments with isotopic tracers are needed to better assess the delivery of the IL-15 over the membrane.

In conclusion, IL-15 can induce lipolysis in human SCAT at physiologically-relevant concentrations without significant alterations in blood flow. However, this response was only shown to occur in lean subjects, while obese subjects demonstrated lipolytic suppression to elevated IL-15 concentrations. These findings may help to understand interactions between a low-grade inflammatory state and the ability to mobilize lipids, and how this may contribute to obesogenic processes. It is also likely that supraphysiological concentrations of IL-15 would not appreciably contribute to losses in fat mass *via* lipid mobilization, limiting therapeutic applications of IL-15 in humans.

Table 3.1. Subject demographic and anthropometric characteristics

	Lean (n=10)	Obese (n=10)	p-value
Male/Female	5 / 5	6 / 4	n/a
Age (yr)	24 ± 4	27 ± 9	0.30
Height (m)	1.7 ± 0.1	1.8 ± 0.1	0.23
Weight (kg)	66.4 ± 11.0	104.2 ± 11.7 *	< 0.01
BMI (kg/m^2)	23.1 ± 1.9	34.7 ± 3.5 *	< 0.01
Body fat (%)	30.6 ± 10.8	42.2 ± 8.4 *	0.02
Fat free mass (%)	69.4 ± 10.7	57.8 ± 8.4 *	0.02
Android fat (%)	37.2 ± 12.0	53.7 ± 4.6 *	< 0.01
Gynoid fat (%)	39.0 ± 14.0	45.8 ± 12.1	0.26
Android: Gynoid ratio	0.98 ± 0.22	1.23 ± 0.24 *	0.02

Values are mean ± SD; *P<0.05 vs. Lean group.

Table 3.1. Table contains basic demographic information for study volunteers, including select DXA-assessed body composition measures.

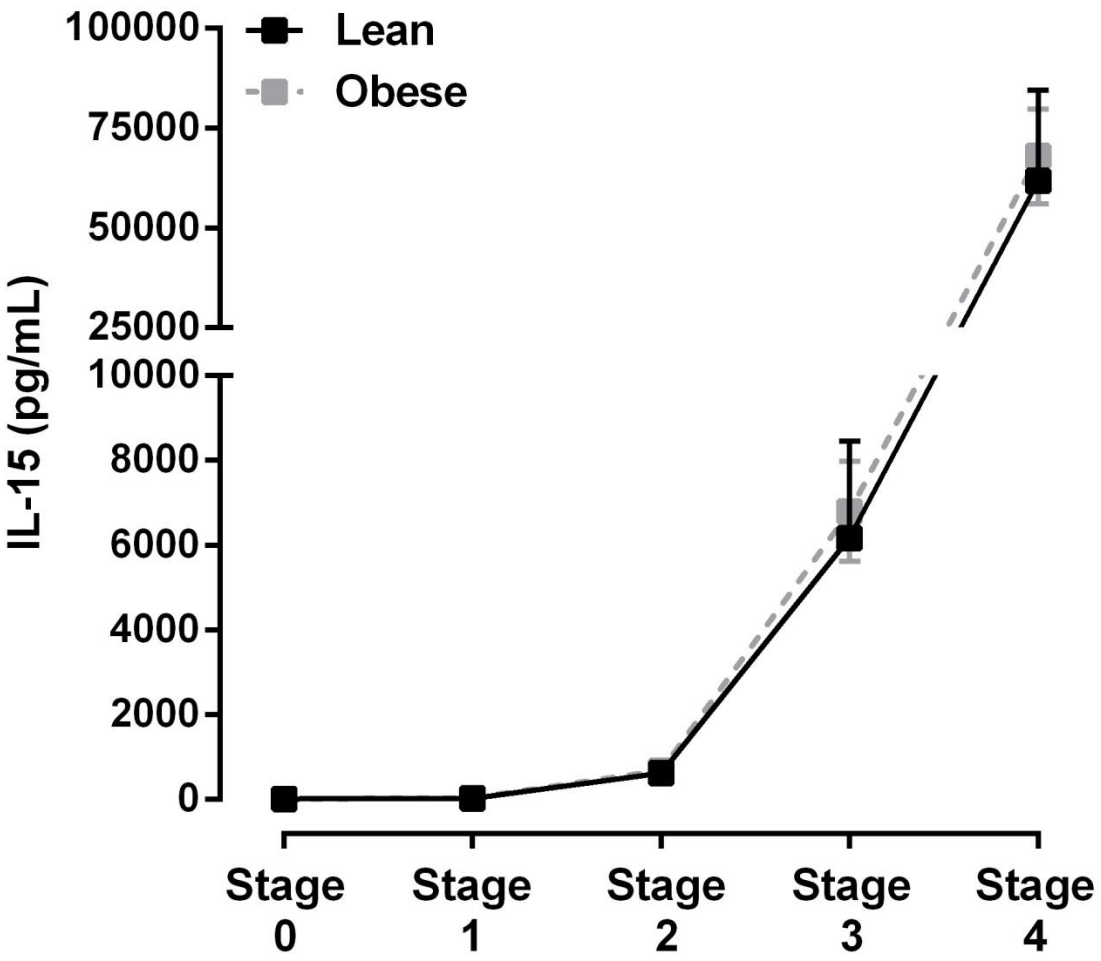

Figure 3.2. Measured IL-15 perfusate concentrations. Microdialysis probes were placed in the subcutaneous abdominal adipose tissue (SCAT) and perfused with various IL-15 concentrations to assess lipolytic responses in Lean (n=10) and Obese (n=10) subjects. Values are mean ± SD.

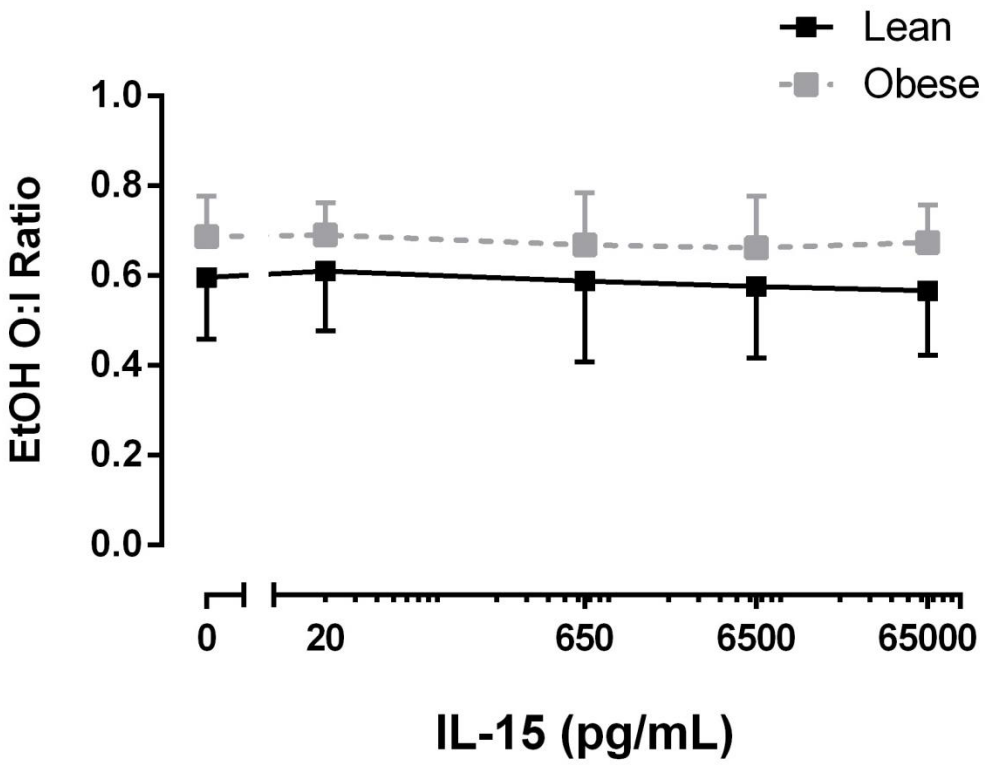

Figure 3.3. Ethanol outflow/inflow ratio (EtOH O:I) with IL-15 perfusion. Microdialysis probes were placed in the subcutaneous abdominal adipose tissue (SCAT) of Lean (n=10) and Obese (n=10) subjects, and perfused with various IL-15 concentrations (x-axis). Values on the y-axis are the EtOH O:I ratio (EtOH Dialysate / EtOH Perfusate) for each IL-15 perfusion concentration, and are presented as mean ± SD.

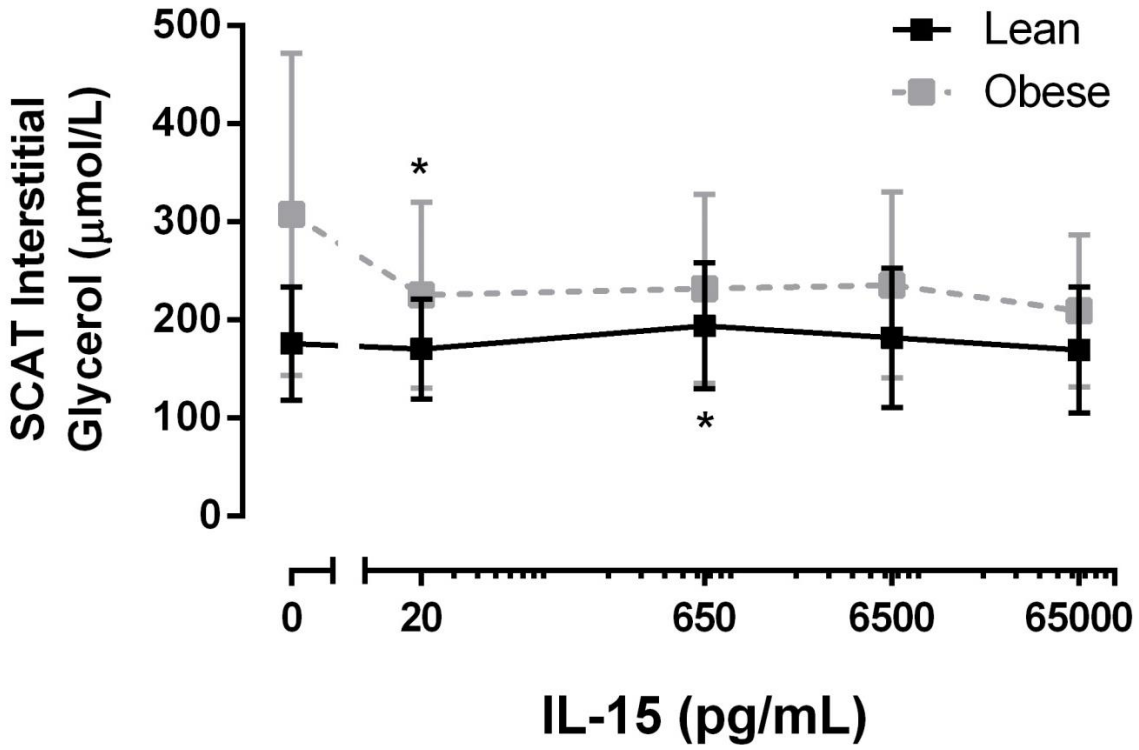

Figure 3.4. Subcutaneous adipose tissue (SCAT) interstitial glycerol with IL-15 perfusion. Microdialysis probes were placed in the SCAT and perfused with various IL-15 concentrations to assess lipolytic responses in Lean (n=10) and Obese (n=10) subjects. Values on the y-axis are the calculated SCAT Interstitial Glycerol for each IL-15 perfusion stage, and are presented as mean ± SD. *P<0.05 vs. preceding perfusion stage within BMI group.

CHAPTER 4: INTEGRATED DISCUSSION AND CONCLUDING REMARKS

Emerging models examining the secretion of factors between and within organ systems (4, 18, 26, 58, 79, 92, 94, 96), particularly involving adipose tissue (AT), have highlighted numerous viable targets in the fight against obesity. A review of the literature (Chapter 1) revealed that an endocrine-like axis may exist, in which skeletal muscle (SkM) is capable of secreting IL-15 to the blood that could ultimately reach and have effects in AT (e.g. reduce fat mass). However, as also pointed out throughout this dissertation, the most concrete evidence for this axis exists in animal and cell culture models (4, 10, 18, 92, 94, 96). Therefore, further data are needed to support this axis in human models if it is to aid in the understanding and treatment of human obesity. We designed the studies contained within this dissertation to: 1) provide evidence that this axis exists in humans, 2) demonstrate that IL-15 could induce lipolysis (a potential mechanism in a loss of fat mass), and 3) to determine if this axis and/or the responses within it were different between lean and obese humans. While our data do not support the proposed IL-15 endocrine axis in humans, we present data that support the possible existence of a previously uninvestigated IL-15 autocrine/paracrine axis in human adipose tissue. That is, IL-15 found in human subcutaneous AT (SCAT) appears able to exert actions in human SCAT, and may play a role in human obesity.

In Chapter 2 (Study 1), we aimed to determine the amount of IL-15 in lean and obese SkM and SCAT, and the degree to which interstitial IL-15 in SkM and SCAT was correlated with SCAT lipolysis. At least two studies had previously demonstrated that plasma IL-15 was higher in lean compared to obese subjects (10, 69). In these studies, it was speculated that the reason for a higher systemic IL-15 in lean individuals was a higher production and secretion of IL-15 from SkM compared to SkM in obese. Importantly, IL-15 secretion from SkM (e.g. SkM

interstitial IL-15) had not been assessed in humans until the current study. In fact, one of the investigations mentioned above reported that there was no association between SkM IL-15 mRNA and plasma RNA, suggesting that SkM may not be the main source of a lower plasma IL-15 in humans (69). Through a direct assessment of IL-15 using *in situ* microdialysis and blood samples, we found no difference in SkM IL-15 or plasma IL-15 between lean and obese subjects. In contrast to SkM IL-15, we observed a higher interstitial IL-15 in obese than in lean SCAT. This result was not completely surprising given the pro-inflammatory nature of IL-15 (33, 34), and the view that obesity is considered a low-grade inflammatory condition, particularly in the AT space (26, 58, 101). Furthermore, based on the magnitude of difference between SCAT and plasma IL-15 (higher in SCAT than in plasma), suppositions that IL-15 would reach AT from the circulation, operating as an endocrine mediator of body composition, do not seem likely. Therefore, we contend that the proposed SkM-blood-AT IL-15 axis is not likely to exist in humans. Regardless, we are the first to report data indicating the possible existence of an autocrine/paracrine IL-15 axis (IL-15 released in AT acting in AT) is more likely to exist in humans, which is in line with a similar model identified in porcine adipocytes (1, 2).

In the same study we demonstrated that exercise could increase plasma IL-15, as previously reported (21, 81, 115). We are the first to show, however, that plasma IL-15 increased to a greater extent in obese when compared to lean subjects. Because we were not able to leave the microdialysis probes in SkM during exercise, it is difficult to rule out the possibility that changes in SkM IL-15 secretion could account for changes observed in plasma IL-15, as previously suggested. Based on the concentration gradient between SkM and blood, this is a possible scenario. However, even if SkM IL-15 is released to the blood during exercise, our observation that plasma IL-15 changes during exercise were not positively correlated with

changes in lipolysis during exercise suggests that IL-15 produced in and secreted from SkM would probably not alter fat mass *via* lipolysis in SCAT of humans during exercise. Further, without any clear direction in SCAT IL-15 exercise-induced changes, we believe that SCAT IL-15 is not a major contributor to the elevations in plasma IL-15 during exercise, although the study of more subjects could clarify this point. As with exercise-induced changes in plasma IL-15, exercise-induced changes in SCAT IL-15 were not correlated with the changes in SCAT lipolysis during exercise. Thus, we believe that acute exercise-induced changes in lipolysis most likely occur through other well-established lipolytic agents such as catecholamines and ANP (8, 56), and not IL-15.

We further set out to address the direct effects of IL-15 perfusion on human AT using a wide range of concentrations, as the studies demonstrating IL-15 mediated metabolic actions used methods resulting in tissue exposure to IL-15 concentrations rarely seen *in vivo* (2, 10, 94). This design allowed us to gain both an *in vivo* perspective and insight into the potential use of IL-15 as a therapeutic tool in reducing human obesity through lipolysis. As reported in Chapter 3, we found that IL-15 was capable of increasing lipolysis in lean human AT, confirming the findings of the significant positive correlation between SCAT IL-15 and SCAT lipolysis reported in Chapter 2. Alternatively, we found that the obese showed a suppression of lipolysis, which was unexpected from the same analysis. It is possible that our earlier positive correlation between SCAT IL-15 and SCAT glycerol was driven by our obese subjects containing more IL-15 in SCAT as well as a higher local glycerol concentration, as opposed to this observation resulting from a potential causal relationship. Importantly, the direct IL-15 mediated changes in lipolysis were observed at perfusate concentrations likely to be seen by adipocytes *in vivo*. For instance, we observed that lean and obese SCAT interstitial IL-15 was 11.8-40.3 pg/mL (95 %

Confidence interval: ((CI)) in lean SCAT and 33.5-123.5 pg/mL (95 % CI) in obese SCAT. Our perfusate concentrations during Stages 1-3 ranged from 20-6500 pg/mL. It is likely that the amount of IL-15 perfused through the MD probe and able to cross the porous membrane could be between 0.3 - 91 pg/mL, if we assume the approximate 1.4% *in vivo* IL-15 recovery, as previously discussed. Compared to the calculated interstitial concentrations, these perfusate concentrations approximate possible *in vivo* conditions and thus represent practical *in vivo* responses. Using the same estimates of perfused and interstitial IL-15 given the observed *in vivo* recovery, it is also possible that the perfused IL-15 was not capable of elevating interstitial IL-15 concentrations. This becomes especially important among the stages where we observed lipolytic alterations (between Stage 0/basal perfusion [0 pg/mL] and Stage 3 perfusion [650 pg/mL]). For example, the estimated 0.3 pg/mL tissue exposure with 20 pg/mL perfusion is far below the estimated interstitial SCAT IL-15 concentration [~80 pg/mL] in obese humans where we observed a decreased lipolytic response. During Stage 3 perfusion [650 pg/mL], where we observed increased lipolysis in lean SCAT, a 1.4% recovery estimates an IL-15 tissue exposure of 9.1 pg/mL, also below the estimated interstitial SCAT IL-15 [~25 pg/mL] in lean individuals. To better understand the potential *in vivo* role that IL-15 could have in altering lipolysis, further experiments would be required to verify the true interstitial concentrations, and to calculate with confidence the amount of IL-15 to which SCAT tissue is exposed (e.g. diffusion of IL-15 into the tissue surrounding the probe) at a given IL-15 perfusate concentration.

Based on the positive correlation between SCAT IL-15 and SCAT glycerol presented in Chapter 2, and the lipolytic induction *via* IL-15 reported to occur in porcine adipocytes (2), we hypothesized that lean and obese SCAT would undergo an increased lipolysis with IL-15 exposure. We also expected that obese individuals would demonstrate a blunted response to IL-

15 perfusion compared to lean individuals, as previously shown with lipolytic inducing agents, such as catecholamines (48, 49). Given the large amount of fat tissue in obese individuals, it is thought that this blunted response is protective in nature and can be partially explained by defects along the lipolytic cascade (56). That is, to reduce overall tissue exposure to the liberated free fatty acids (FFA) (48). Once again, we observed an increased lipolysis at a relatively low IL-15 concentration in the lean subjects, which was later decreased with higher concentrations, and a suppressed lipolysis in the obese with the lowest IL-15 perfusion concentration. It is possible that a decreased lipolysis might have occurred once a certain IL-15 exposure threshold was met, and that this IL-15 threshold occurred earlier in the obese than in the lean subjects. This threshold response could be a protective mechanism by which IL-15-induced lipolysis becomes suppressed to reduce tissue exposure to FFA. Whether or not lipolytic suppression induced by IL-15 is a potential cause or consequence of obesity remains to be clarified.

The highest IL-15 perfusion concentration (Stage 4, 65000 pg/mL) could represent a potential dose of IL-15 used as a therapeutic application. Assuming the same 1.4% recovery rate as above, a 65000 pg/mL (65 ng/mL) dose would represent a tissue exposure of ~ 900 pg/mL (~ 0.9 ng/mL), clearly above the SCAT interstitial estimations reported in Chapter 2 (maximum value was ~ 125 pg/mL), as well as far above diseases with elevated IL-15 concentrations. For example, patients with the inflammatory Kawasaki disease have been shown to have levels ~ 10 pg/mL (47), while those with long term rheumatoid arthritis have circulating concentrations of ~ 150 pg/mL (37). Still, at this highest dose, lipolysis in lean subjects was not different than basal values, and the obese were still suppressed below their basal values. Thus, if IL-15 is to lead to decreased fat mass in obese individuals, these changes would most likely not occur through lipolytic induction due to IL-15, even at the highest doses investigated.

Limitations and future directions

While there are apparent differences in the metabolic (lipolytic) responses between lean and obese individuals attributed to IL-15, the biological significance of this IL-15 local axis with regard to human obesity remains to be clarified. Specifically, how these alterations in SCAT lipolysis *via* IL-15 could potentially alter specific cell (e.g. NK cell) function in obesity would be important to know for a more complete understanding of immune modulation in obesity. Further, if IL-15 is indeed important for human body composition changes, future studies may need to examine other metabolic mechanisms decreasing fat tissue with IL-15 exposure, such as reduced lipid uptake and deposition and increased fatty acid oxidation in human tissues. Finally, examining the true potential of IL-15 to mediate AT metabolism and body composition from a clinically-relevant and therapeutic application would require human pharmacokinetic studies on the effects of IL-15 to support these claims. Such studies may be of interest to clinicians given ongoing clinical trials using IL-15 as a chemotherapeutic agent (68).

Conclusions

There is a plethora of literature supporting an IL-15 endocrine axis (SkM-blood-AT) in the reduction of fat mass in cell culture and animal models; yet, the studies in this dissertation do not support the existence of a similar axis in humans. Our data suggest that an uninvestigated IL-15 autocrine/paracrine axis might exist in human AT (e.g. SCAT IL-15 produced in, and acting on SCAT). That is, it appears that IL-15 at certain doses can induce lipolysis in lean individuals and can suppress lipolysis in obese individuals. The collective findings in this dissertation further support links between obesity and increased pro-inflammatory molecules in SCAT (26, 67, 75, 79). How these specific increases in IL-15 and associated lipolytic responses in obese SCAT contribute to obesogenic and related co-morbidities remain to be determined.

Alternative Interpretations

While we presented evidence indicating the unlikely existence of an IL-15 endocrine axis in humans, and that IL-15 could directly impact lipolysis in human SCAT, our interpretations/ conclusions should also be guarded at the present time. As discussed, our conclusion regarding the existence of this endocrine axis (SkM-blood-AT) in humans was based on IL-15 concentrations in distinct biocompartments and correlation analyses. Based on the blood-tissue (e.g. plasma-AT) IL-15 gradient and correlations between local tissue and the systemic circulation, it would seem that IL-15 traveling from blood to AT would be extremely difficult, and that our conclusion is viable. However, it is also possible that a carrier protein could exist which would allow for IL-15 to traverse this barrier. The one IL-15 receptor subunit, IL-15 Rα, which has an extremely high affinity for IL-15 and can exist as a soluble receptor, has also been shown to enhance IL-15 biological activity, such as extending biological half-life and/or acting as a reservoir of IL-15 in tissue compartments. Because we did not assess the impact of IL-15 Rα in the current study, we cannot exclude the possibility that IL-15 Rα could serve this carrier protein role, or modify plasma concentrations substantially. We further concluded that IL-15 appears to directly modify lipolysis in human SCAT. As discussed above, these observations of IL-15 impacting lipolysis through microdialysis perfusion could be considered difficult to reconcile based on perfusate and interstitial concentrations, and given our estimated IL-15 *in vivo* recovery. At the same time, we cannot exclude the possibility that our 1.4% *in vivo* recovery estimates (based on the *in vitro* and *in vivo* relative recoveries of IL-15 and ethanol) might be underestimates of probe recovery. Once again, until further experiments are performed to better gauge the tissue exposure and responses to IL-15 in humans, this stresses the need for further research in this area if it is to be clarified that IL-15 alters lipolysis within human adipose tissue.

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APPENDIX: UNIVERSITY MEDICAL CENTER INSTITUTIONAL REVIEW BOARD (UMCIRB) AT EAST CAROLINA UNIVERSITY HUMAN SUBJECT APPROVAL

EAST CAROLINA UNIVERSITY
University & Medical Center Institutional Review Board Office
1L-09 Brody Medical Sciences Building· Mail Stop 682
600 Moye Boulevard · Greenville, NC 27834
Office **252-744-2914** · Fax **252-744-2284** · **www.ecu.edu/irb**

Notification of Initial Approval (Committee)

From: Biomedical IRB
To: Joseph Pierce
CC: Robert Hickner
Joseph Pierce
Date: 3/30/2012
Re: UMCIRB 11-001491
IL-6 and IL-15 cytokines on human lipolytic control

I am pleased to inform you that at the convened meeting of the Biomedical IRB on 3/14/2012, the committee voted to approve the above study. Approval of the study and the consent form(s) is for the period of 3/14/2012 to 3/13/2013.

The Biomedical IRB deemed this study Greater than Minimal Risk.

Changes to this approved research may not be initiated without UMCIRB review except when necessary to eliminate an apparent immediate hazard to the participant. All unanticipated problems involving risks to participants and others must be promptly reported to the UMCIRB. The investigator must submit a continuing review/closure application to the UMCIRB prior to the date of study expiration. The investigator must adhere to all reporting requirements for this study.

The approval includes the following items:

Name	Description	Modified	Version
2038 Pilot_Informed Consent_v3_032612 (clean copy) | History	Consent Forms	3/26/2012 12:19 PM	0.03
2038 Pilot_Medical History Questionnaire.docx | History	Surveys and Questionnaires	12/19/2011 4:57 PM	0.01
2038 Pilot_Protocol_v3	Study Protocol or Grant Application	3/26/2012 3:11 PM	0.03
HIPAA Authorization Form | History	HIPAA Authorization	12/16/2011 5:15 PM	0.01
HIPAA Authorization Form for Future Use of Samples | History	HIPAA Authorization	12/16/2011 5:16 PM	0.01
Pilot Study Recruitment Flyer_120111.pptx | History	Recruitment Documents/Scripts	12/16/2011 11:47 AM	0.01

The following UMCIRB members were recused for reasons of potential for Conflict of Interest on this research study:

R. Hickner

The following UMCIRB members with a potential Conflict of Interest did not attend this IRB meeting: None

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