The Role of Lipocalin-2 in Exercise Induced Mitochondrial Metabolism in Thermogenic Adipose Tissue.

A THESIS
SUBMITTED TO THE FACULTY OF THE
GRADUATE SCHOOL OF
THE UNIVERSITY OF MINNESOTA
BY

SHAILA HUSSAIN CHOHAN

IN PARTIAL FULFILLMENT OF REQUIREMENT
FOR THE DEGREE OF
MASTERS OF SCIENCE

Advisor: Xiaoli Chen, M.D. PhD

August, 2019

Acknowledgements:

I express my deepest and sincere gratitude to my mentor Dr. Xiaoli Chen who supervised, supported and encouraged me all through this work. Her guidance helped me throughout the research process and writing of this thesis. I could not have imagined having a better advisor and mentor. Her current understanding of the molecular mechanisms underlying obesity and metabolism is steadfast. She has helped me grow on both personal and academic levels.

I would also like to thank my lab members Hong Guo and Derek Lin for creating a friendly and welcoming environment which made learning easy and fun. They provided valuable insight on different complicated techniques. They were always there to lend me a helping hand when I needed it.

I am forever grateful for the love and support of my family especially my kids who were patient enough to see me fulfil my dream. I am appreciative of the support of my husband Ahmed Pasha who always believed in me.

Abstract

Obesity is a major health issue faced by the present era. According to the CDC(Center for disease control and prevention), obesity is not just a weight-gain problem; it can have serious deleterious effects on an individual's physical, metabolic and psychological health. Dysfunctional adipose tissue is the major contributor to obesity and its associated metabolic syndrome. Brown adipose tissue (BAT) is a major thermogenic organ that regulates energy expenditure and is a potential target of drugs for combating obesity and type 2 diabetes. BAT is involved in non-shivering thermogenesis and is a site of glucose uptake and lipid oxidation. The mitochondrion plays a critical role in energy metabolism and it is a dynamic organelle that needs quality control while activated during thermogenesis. Exercise is a useful tool to activate brown adipose tissue. It helps stimulate the sympathetic nervous system to activate the mitochondrial oxidation of brown adipose tissue. When exercise activates adipose tissue, mitochondrial turnover is increased in BAT but decreased in beige adipose tissue. Lipocalin-2 is an adipokine that has a role in energy metabolism via activating BAT and white adipose tissue beiging. Our previous studies have demonstrated that Lcn-2 plays a key role in cold-induced thermogenesis. Herein, we sought to discover if Lcn-2 plays a role in exercise-induced thermogenesis and mitochondrial metabolism. We used a Lcn-2 KO mouse model to investigate the expression of thermogenic and mitochondrial genes. We showed that the expression

levels of Ucp-1 were not significantly changed in BAT of Lcn-2 KO mice, i.e. Ucp-1 expression can be induced by exercise when Lcn-2 is not present. We also determined if Lcn-2 had an effect on mitophagy involving PINK-1/Parkin/P-62 system in BAT and iWAT in response to exercise. Interestingly, we found that Lcn-2 deficiency does seem to affect mitochondrial quality control as Lcn-2 KO does not show exercise-induced mitochondrial biogenesis but shows increased mitophagy in white adipose tissue, indicating that the beiging process is defective. We conclude that Lcn2 plays an important role in exercise-induced mitochondrial turnover and metabolism in brown and beige adipose tissue.

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BAT: Brown Adipose Tissue

WAT: White Adipose Tissue

iWAT: Inguinal White Adipose Tissue

PGC1-α: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

Tfam: transcription factor A, mitochondrial

Prdm16: PR domain containing 16

Lcn-2: Lipocalin-2

Ucp-1: Uncoupling Protein 1

TNF-α: Tumor Necrosis Factor alpha

PKA: Protein Kinase A

CREBP: cAMP-response element-binding protein

PINK-1: PTEN(Phosphatase and tensin homolog) induced kinase 1

CVD: Cardiovascular Disease

BMI: Basal Metabolic Rate

TAG: Triacylgleceride

Literature Review

Overview

Adipose tissue is a highly metabolic, energy storing and an endocrine organ. The adipocytes are supported by the connective tissue matrix, nerve tissue, stromal vascular cells and immune cells. Adipose tissue responds to the various hormonal and the central nervous system signals by secreting hormones like leptin, adiponectin, complement components, and proteins from the renin angiotensin system(Kershaw & Flier, 2004).

It is traditionally thought that adipose tissue especially white adipose tissue is a major energy house of the body releasing fatty acids as fuel when needed. Thus, the size of the adipose tissue increases when there is positive energy balance and a decrease in utilization of the stored energy(Trayhurn & Beattie, 2019). It is also thought to act as an immune organ through interacting with immune cells in adipose tissue (Trayhurn & Beattie, 2019). Although white adipose tissue is the major site of releasing fatty acids, it also helps in the metabolism of steroid hormones. It does not synthesize the hormones de novo but helps to release the enzymes which regulate the metabolism of glucocorticoids and sex hormones(Mohamed-Ali, Pinkney, & Coppack, n.d.).

Leptin and adiponectin are the two major hormones secreted by white adipose tissue, playing an important role in energy metabolism and obesity. Physiologically leptin produced from adipose tissue suppresses hunger and improves insulin sensitivity. The rate of leptin production is directly related to adiposity. However, there are some inter individual

differences as well as gender differences in the levels of leptin and adiponectin secretion. In obese individuals, the leptin levels are increased, but this increased leptin does not produce food intake suppression or an increase in energy expenditure. This maybe due to a limitation of the blood brain barrier or leptin resistance (Jéquier, 2002) This is why obese individuals do not respond to leptin treatment for weight loss. Leptin resistance is commonly accompanied by the impairment of insulin action or insulin resistance in obesity. Insulin resistance causes a decrease in glucose transport, an upregulation of glycogen synthase, and an increase in lipogenesis. These are some common metabolic defects observed in obesity, insulin resistance and type 2 diabetes mellitus (Müller, Ertl, Gerl, & Preibisch, 1997). Adiponectin is another adipokine released from adipocytes and it has major effects on activating AMPK, regulating glucose homeostasis and increasing insulin sensitivity. It stimulates the phosphorylation of acetyl Co-A carboxylase enzyme leading to fatty acid oxidation(Yamauchi et al., 2002). From the above evidence it can be stated that adipose tissue is a major organ controlling metabolism of both glucose and lipids through secreting various hormones.

Obesity and its relation to Metabolic Syndrome:

Monitoring body weight and expansion of adipose tissue is now based on BMI and waist circumference set by the WHO. BMI between 18.00 - 24.99kg/m^2 is considered normal weight, between 25.00 - 29.99 kg/m^2 over-weight, and between 30.00 - 40 kg/m^2 is termed as obesity which is

further sub-classified into three types. One is morbidly obese when BMI is more than 40 kg/m^2.

Obesity is associated with a number of metabolic diseases such as cardiovascular diseases, insulin resistance, and type 2 diabetes. Individuals with cardiovascular diseases also have a cluster of other cardiometabolic risk factors that are commonly accompanied by obesity such as dyslipidemia, high blood pressure, high blood glucose and insulin resistance, chronic inflammation and a prothrombotic state(Ritchie & Connell, 2007). Reaven in 1988 was the first person to establish a relationship between insulin resistance, plasma insulin levels and glucose intolerance with a significant change in plasma fatty acid concentration. He found that patients with treated or untreated hypertension usually have insulin resistance and are hyperglycemic(Reaven, 1988). Now this aggregation of risk factors with obesity is termed metabolic syndrome(Lean, Han, & Morrison, 1995). The development of metabolic syndrome has been associated with an increase in the expansion of adipose tissue especially abdominal fat. Metabolic syndrome doubles the risk of a cardiovascular event in obese individuals. Therefore, the primary purpose of studying the fat tissue composition and function is important in the effective treatment of CVD(Han & Lean, 2016).

Anatomical Differences of Adipose Tissue:

Obesity with a BMI of $\geq$ 30 kg/m^2 is a major risk factor for CVD including hypertension, dyslipidemia, diabetes and metabolic syndrome. Fat accumulation in different fat compartments can have differential metabolic consequences in terms of risk for metabolic syndrome (Fox et al., 2007). Abdominal fat mass can be divided into two parts: 1) Subcutaneous abdominal fat mass. 2) Intra-abdominal fat mass. The intra-abdominal fat mass can be further divided into visceral fat mass and retroperitoneal fat mass(Mårin et al., 1992). Intra-abdominal visceral fat accumulation is considered to be a risk factor for metabolic diseases. This can be related to the depot difference in lipid/glucose metabolism, adipocytokine secretion, and inflammatory response. For example, plasma concentrations of adiponectin (one of the important adipocytokines) and collagen like protein which are protective against metabolic syndrome are reduced in individuals with increased visceral fat(Matsuzawa, 2006). The visceral adipose tissue has a high lipolytic activity due to presence of lipoprotein lipase enzyme. This causes mobilization of free fatty acids in both obese and non- obese individuals, particularly more in obesity (Björntorp, 1991). Due to this reason more free fatty acids enter plasma leading to increased fatty acid levels in the circulation in obese and type 2 diabetes individuals (Wajchenburg, 2014).

Developmental Differences of White, Brown and Beige Adipose tissue:

Humans histologically have two main types of adipose tissue; white adipose tissue that is widely distributed in the body and brown adipose tissue that is restricted to specific areas in the adult body. White adipose tissue is the major energy store house while brown adipose tissue functions in dissipating heat during the process of thermogenesis. Adipocytes in these different tissues are both molecularly and functionally different(Cypess et al., 2013). Peroxisome proliferator-activated receptor gamma (PPAR gamma) mRNA, a transcription factor is highly abundant in adipose tissue and plays a key role in adipogenesis or adipocyte differentiation. Mouse 3T3-L cell line has been used to study adipocyte differentiation. They are fibroblast like cells which can be induced to differentiate into adipocytes under appropriate stimulation e.g., by activating PPAR-gamma. The PPAR-gamma itself in the tissues and its activators are strongly associated with pre-adipocyte differentiation into mature adipocytes(Chawla, Schwarz, Dimaculangan, & Lazar, et al. 1994). It is an interesting fact that in humans the brown adipose tissue develops before the white adipose tissue during embryo genesis. This brown adipose tissue then starts to regress after infancy until it is restricted to only certain areas in the body(Nedergaard & Cannon, 2010). In one study, Crisan et al.(2008) have suggested that myf5^{+} cells give rise to both skeletal muscle and brown adipose tissue. On the other hand,

white adipose tissue is developed from a different origin (Crisan et al., 2008). This is why skeletal muscles and brown adipose tissue also have similar physiological functions of glucose clearance, lipid catabolism and oxidation. They are both innervated by sympathetic nervous system and have abundant mitochondria(Farmer, 2008). To further strengthen the notion that white and brown adipose tissue arise from different developmental origins, the differentiation of adipocytes in response to BMP (Bone morphogenetic protein) in vitro was studied. The results showed that BMP2 and BMP4 favor white adipose tissue development (Bowers & Lane, 2007) while BMP7 favors brown adipose tissue development providing further support to the notion that the stimulators of adipocyte differentiation are also differentially expressed in different tissues.(Tseng et al., 2008).

Classical BAT is located in the intrascapular, perirenal and axillary regions in mammals. This helps to keep the mammals warm by uncoupling mitochondrial oxidation from ATP production and releasing it as heat. This is maintained by the uncoupling protein 1 (Ucp-1) which is the major component driving this process of thermogenesis(Enerbäck et al., 1997). The thermogenic fat tissue can also develop within white adipose tissue when appropriately stimulated. These cells that undergo brown-like adaptation are called “Beige cells” or “Brite Cells”. Beige cells are basically mitochondrial rich and Ucp-1 expressing cells seeded within the white adipose tissue. Brown and beige adipocytes are very similar in

morphology and function. They both express key thermogenic genes like Ucp-1, Cidea, Ppara, Pgc1-alpha and both can perform functions of thermogenesis. The main difference between the two is that brown adipose tissue constitutively express Ucp-1 and mitochondria whereas, beige cells express thermogenic components only when stimulated(Harms & Seale, 2013) and also arise from a different cell lineage.

Brown Adipose Tissue in Energy Metabolism:

Brown adipose tissue contains beta, alpha 1 and alpha 2 adrenergic receptors. These receptors are responsive to both epinephrine and nor-epinephrine. Of the three receptors, beta 3 adrenergic receptor is most important as it is coupled to a signaling cascade that controls Ucp1 expression and thermogenesis in brown adipocytes. Beta 1 receptors are also expressed in brown adipose tissue, but they are not coupled to thermogenic signaling pathway (Bronnikov et al., 1999). The beta 2 receptors are not present on the brown adipocytes but in the surrounding vascular system (Bengtsson, Cannon, & Nedergaard, 2000). Whenever there is sympathetic stimulation by cold or exercise it causes the release of nor-epinephrine that stimulates the beta receptors in the adipose tissue. These receptors are coupled to the G_s stimulatory proteins and cause an increase in the sensitization of the adenylate system. The results in one particular study showed that surgical denervation of the BAT prevents cold-induced thermogenesis(Granneman, 1988), suggesting the important

role of beta adrenergic signaling in the control of thermogenesis. The nor-epinephrine activates beta adrenergic signaling and induces cAMP formation leading to activation of protein kinase A (PKA) (H Thonberg, Lindgren, Nedergaard, & Cannon, 2001). After the activation of the adenylyl cyclase system, the G_s proteins and protein kinase A activation, the protein kinase A phosphorylates and activates the transcription factor CREBP. This CREBP then activates the transcription of genes involved in the expression of Ucp-1(Håkan Thonberg, Fredriksson, Nedergaard, & Cannon, 2002).

There are three core regulators of beiging. 1) PPAR gamma 2) PRDM16 3) PGC-1 alpha. PPAR- gamma is the most important transcription factor responsible for the differentiation of both white and brown adipocytes. Activation of PPAR-gamma is also the principle activator of expression of Ucp-1. Its activation induces the expression of Ucp1 expression in brown-like adipocytes from within the white adipose depot. Upon PPAR- gamma activation, there is not only an increase in the Ucp-1 expression but also other genes responsible for mitochondrial biogenesis such as PGC-1 alpha, CPT 1M, Elov13 and Cidea(Petrovic et al., 2010b). On the other hand, PRDM 16 which is essential for the beiging of WAT is abundant in the subcutaneous tissue. In one study, depletion of PRDM16 by shRNA caused a decrease in the expression of thermogenic genes and uncoupled respiration(Seale et al., 2011). Interestingly, PRDM16 induces beiging but meantime represses the WAT genes. For

instance, it represses the expression of CtBP1 and CtBP2 at the promoters of WAT genes (Kajimura et al., 2008) hence, repressing WAT differentiation. PGC1-alpha is a co-activator of PPAR-gamma and helps in the up regulation of genes promoting Ucp-1 expression, respiratory protein levels, and fatty acid oxidation enzymes(Tiraby et al., 2003). It is only required for thermogenesis function of beige cells and not for beige cell differentiation.

Mitochondria, a Dynamic Organelle in thermogenesis:

Mitochondrion is a highly dynamic cytoplasmic organelle. Mitochondria are present in all cells of the body except erythrocytes. They have their own mtDNA and RNA, but most of their proteins are encoded by the nuclear DNA, formed in the cytoplasm, and then translocated to the mitochondria post transcriptionally(Logan, 2006). Mitochondria are important in driving many metabolic pathways like the TCA cycle, pyruvate decarboxylation, beta- oxidation, and catabolism of branched amino acids. It is also the site which provides intermediates for biosynthetic processes such as synthesis of urea, fatty acids and heme. However, the major function of the mitochondria is to serve as the energy house of the cell. The cell derives about 95% of its energy from mitochondrial metabolism of high energy NADH and $FADH_2$ and also by phosphorylation of ADP to ultimately produce ATP(Kim, Wei, & Sowers, 2008). Physiologically, the inner membrane of mitochondria contains a chain of complexes and the

main function of these complexes is to metabolize NADH, $FADH_2$ and ADP by creating an electrochemical gradient of protons that develops a membrane potential of 180V. This gradient prevents the leaking of protons and helps to release ATP(Benard et al., 2006).

Food combustion releases energy and the final products of this energy production is water and carbon dioxide after releasing ATP. This control of energy production is very important so that the substrates are properly channeled towards energy production instead of being wasted. The main task of the cell is to balance energy production and utilization. During metabolism of various food contents like carbohydrates, lipids and proteins along with micronutrients leads to the production of NADH and $FADH_2$ which in turn donate electrons to the electron transport chain located on the inner mitochondrial membrane. During this process the protons inside the mitochondria are pumped out. Thus, creating an electrochemical gradient. Proton from the exterior of the mitochondria re-enter the inner membrane through the actions of the F_0/F_1 units of ATP synthase. This re-entering of protons is coupled to the conversion of ADP to ATP. In this way, the cell produces its energy. On the contrary, beta-adrenergic stimulation due to various processes like cold exposure, exercise, pharmacological drugs can have both acute and chronic effects on Ucp-1 induction (which is located on the inner mitochondrial membrane). The acute stimulation increases Ucp-1 activity whereas, the chronic stimulation can increase both Ucp-1 protein levels and

mitochondrial biogenesis. Adrenergic stimulation causes an increase in cyclic AMP which in turn activates lipolysis. Fatty acids act as substrates for Ucp-1. The Ucp-1 which has been upregulated by the sympathetic stimulation transports the fatty acid anions and not protons to the outside of mitochondrial matrix. These fatty acids that have been transported outside become re-protonated and flip back into the mitochondrial matrix, in this way a protonophore cycle with a net transfer of fatty acid-protons into mitochondria is produced. This has dual functions of both thermogenesis as well as energy production. In this way, Ucp-1 present on the inner mitochondrial membrane uncouples proton entry from ATP synthesis. As a result, electrochemical gradient is lost and heat is generated(Lowell & Spiegelman, 2000).

Mitochondrial Dynamics and its role in Beiging

Mitophagy and its significance:

Mitochondria is a dynamic organelle with high oxidative capacity. It is constantly working to maintain cellular homeostasis. The removal of damaged mitochondria is critical for the normal functioning of the cell. It is important that mitophagy is selective so that only damaged mitochondria are removed. Mitophagy occurs in two steps: one is induction of mitophagic components, and second is priming of damaged mitochondria for selective degradation. It is thought that the induction of the

components include the activation of the PINK-1/Parkin system(Ding & Yin, 2012).

PINK-1(PTEN induced kinase-1) is a mitochondrial serine/threonine-protein kinase encoded by the PINK-1 gene. Whereas, Parkin is a ubiquitin ligase that plays an important role in ubiquitination process. Ubiquitination allows the ubiquitin to bond with the degradative matter and transport it to the proteasomes or lysosomes for digestion and removal. PINK-1 resides in the mitochondria and the Parkin is located in the cytosol. But through an unknown pathway PINK-1 can recruit Parkin on the damaged outer mitochondrial membrane. This Parkin then tags the damaged mitochondria and gets it ready for selective degradation(Geisler et al., 2010a). Under normal conditions, PINK-1 enters the mitochondria where it is cleaved at its hydrophobic domain by a protease called presenillin-associated rhomboid-like protein(PARL)(Jin et al., 2010). This import and degradation cycle keeps the amount of PINK-1 undetectable. However, when the mitochondrial membrane is disrupted due to membrane depolarization, this PINK-1 is not transported into the mitochondria and it accumulates non-cleaved on the outer membrane of mitochondria(Lazarou, Jin, Kane, & Youle, 2012). This aggregated PINK-1 then directly phosphorylates Parkin and causes recruitment of Parkin to the damaged mitochondria. This tags the mitochondria and the PINK-1/ Parkin system acts as a chaperone to further help in the removal of these mitochondria. (Lazarou et al., 2012).

Parkin-Mediated Mitophagy in Beige Cells:

Ucp-1 present on the inner mitochondrial membrane utilizes fatty acids as substrates to increase proton conductance to dissipate membrane potential for heat generation. The membrane potential of brown adipose tissue mitochondria is between -30 to -50mV. In contrast the membrane potential of Ucp-1 ablated mice is -200mV. The less negative membrane potential is very important for browning to occur(A Matthias, Jacobsson, Cannon, & Nedergaard, 1999). This drop-in membrane potential is beneficial for the brown adipose tissue. Treating 3T3 L-1 adipocytes with rosiglitazone provided evidence that activation of beiging in white adipocytes requires the membrane potential to fall. During the transition of WAT to Beige cells, Parkin levels are decreased and subsequent Parkin mediated mitophagy is inhibited. This can be explained partly by the fact that WAT browning permits an increase in the mitochondrial mass but this amount is not as sufficient as it would be in BAT. So the mitochondria need to persist longer so that browning can occur(Taylor & Gottlieb, 2017a). Once the stimulus is removed, the Ucp-1 expression decreases and mitochondrial electron chain complex activity is reduced, leading to a stabilization of PINK-1/Parkin system and an increase in mitophagy. This increase in mitophagy leads to a conversion of beige adipocytes back to white(Altshuler-Keylin & Kajimura, 2017a).

Parkin-Mediated Mitophagy in Brown Adipose Tissue:

Brown adipose tissue have abundant mitochondria and Ucp-1 is constitutively expressed on the inner mitochondrial membrane. As the brown adipose tissue is activated during cold or exercise it is important that the turnover of mitochondria is increased to maintain mitochondrial quality. As the mitochondria are constantly active during thermogenesis they are under oxidative stress so they are constantly being removed through selective mitophagy yet, it is crucial that mitochondrial biogenesis is also increased. Mitochondrial biogenesis can be regulated through mitochondrial biogenic genes like PPAR-γ, PGC-1-α, Prdm-16 and Tfam(Kelly & Scarpulla, 2004). As far as mitophagy in brown adipose tissue is concerned, there is an evidence-based study that describes the increase in PINK-1 /Parkin system whenever the mitochondrial membrane is depolarized. Whenever there is a stimulation in the form of cold, exercise, diet induced thermogenesis and pharmacological drugs, BAT is activated. This adaptive non-shivering thermogenesis increases Ucp-1 activity as a result the membrane potential decreases. This decrease in membrane potential is perceived as a stress to the mitochondria and they need to be replaced. The PINK-1 gets stabilized on the outer membrane of mitochondria and cause Parkin activation and thus, tag the damaged mitochondria for degradation by the lysosomes. This mitophagy is coupled to an increase in the mitochondrial biogenic genes which simultaneously

increase the number of mitochondria ,hence increase the turnover(Altshuler-Keylin & Kajimura, 2017a).

In summary, PINK-1/Parkin system needs to decrease when there is a transition from white to beige cells, while the mitophagy machinery needs to be activated in brown adipose tissue in order to maintain mitochondrial quality.

The role of Downstream p-62 in Parkin-Induced mitophagy:

Parkin-mediated mitophagy is carried out in three important steps. The first step requires the activation of functional Parkin. The Second involves, the mitochondrial clustering with resultant poly-ubiquitin chains. Lastly, the third step requires the activation p62/SQSTM1/Sequestosome-1 recruitment(Geisler et al., 2010b).

As mentioned previously, the PINK-1 clusters on the outer membrane of mitochondria when the mitochondrial inner membrane is depolarized. Once Parkin is recruited to mitochondria, the p-62 is ubiquitinated by the phosphorylated Parkin. This helps to recruit the damaged mitochondria to the phagophore (Liu et al., 2017). The adapter protein p-62 has two domains: one is the ubiquitin binding domain for the recognition of the ubiquitin PINK-1/Parkin system and the other is the LIR domain (LC3 interacting domain) that binds to the LC3 of the phagosome(Altshuler-Keylin & Kajimura, 2017b). Thus, p-62 appears to act as a bridge between the depolarized mitochondria ubiquitinated by the PINK-1/Parkin

system and its removal by phagosome/lysosome through mitophagy. However, recently there has been discovery of proteins that provide evidence that p-62 is a dispensable factor in the mitophagic process. Therefore, p-62 is not always required for PINK-1/Parkin-induced mitophagy(Hollville, Carroll, Cullen, & Martin, 2014).

Exercise in Adipose Tissue Remodeling

Effects of Exercise on Energy Balance, Metabolism, and Beiging:

It is estimated that humans contain approximately 50g of brown adipose tissue. This can give rise to resting energy expenditure of almost 20%. (van Marken Lichtenbelt & Schrauwen, 2011). Optimistically speaking a chronic 5% increase in the resting energy expenditure can dissipate 75-100kcal/day over the course of one year which in turn can lead to a loss of 4-4.5kg of fat mass yearly(Hill, 2009). This fact is important when we try to study the beneficial effects of activating brown adipose tissue. Recent studies done on animal models have provided the evidence supporting the fact that activating BAT reduces plasma TAG levels. The study showed that beta 3 adrenergic stimulation of APOE-3 Leiden CETP mice not only increased resting energy expenditure but also improved plasma TAG levels. The proposed pathway of this beneficial role is through selective uptake of fatty acids into BAT and clearing of plasma from the cholesterol rich remnants(Hill, 2009). BAT activation is also thought to increase

glucose oxidation and improve insulin sensitivity of cells. Therefore, it can be directly used as a method to prevent type-2 diabetes mellitus(Peirce & Vidal-Puig, 2013). Apparently in one study, transplantation of 100 mg of BAT into the visceral cavity of age matched lean mice increased energy expenditure and improved glucose tolerance. Although the effect of transplanted BAT was relatively decreased as compared to the endogenous BAT but it proved that BAT helps in glucose clearance(Stanford et al., 2013a).

Exercise itself is a heat generating process; it was reported that endurance exercise that lasted just a few hours a day had no effects on the Ucp-1 expression in BAT. But when endurance training was coupled with cold climate, an increase in Ucp-1 mRNA was observed(Segawa et al., 1998). However, although exercise did not increase Ucp-1 expression in BAT, it did have effect on beiging, i.e. increased Ucp1 expression in WAT. It is known that Ucp-1 is induced by exercise in scWAT through a sympathetic tone route. In this regard, several hypotheses have been put forward. One hypothesis is that during sustained exercise when the TAG get mobilized and the fat cells decrease in size due to a decrease in lipid content by increase in lipolysis and fatty acid oxidation, it decreases the insulation of the body which acts as a compensatory mechanism and increases heat production through beiging of subcutaneous WAT(Nedergaard & Cannon, 2014). Another hypothesis of exercise-induced beiging stated that myokines e.g., irisin, meteorin like -1,myostatin

and beta-aminobutyric acid are released after exercise that cause beiging in scWAT (Pedersen & Febbraio, 2012). In rodent studies of 3-4 weeks of exercise, the marker genes for the process of beiging such as Ucp-1 and Prdm 16 were observed to be increased. Several other genes which were indicative of the process of beiging including Cidea, Elovl-3, Pgc1-alpha and Cox-8 were also found to be increased (Stanford, Middelbeek, & Goodyear, 2015).

Effects of Exercise on Adipose Tissue Mitochondrial Function:

Previously it has been proposed that exercise increases mitochondrial activity. In one study, 11 days of endurance exercise increased the oxygen consumption rate which was measured through the respirometry in the scWAT. This study showed that the mitochondrial function is up-regulated mostly in the white adipose tissue leading to activation of beige cells(Stanford et al., 2015).

In another study, rats were placed on 10 weeks of training exercise and the effects of exercise on mitochondria and mitochondrial enzymes were examined. After 10 weeks of exercise, the activity of cytochrome-c oxidase from the respiratory chain and malate dehydrogenase from the TCA cycle were significantly increased in white adipose tissue (Stallknecht, Vinten, Ploug, & Galbo, 1991). Hence, it can be concluded that exercise increases the mitochondrial function by increasing the

activity of enzymes involved in the processes taking place in the mitochondria.

Change in Mitochondrial Dynamics after exercise in adipose tissue

I have previously mentioned that increase in the white adipose tissue size leads to WAT dysfunction. There is subsequent down regulation of anti inflammatory adipokine secretion and an increase in the inflammatory cytokines which worsen the imbalance in systemic metabolic homeostasis. In contrast the BAT has high number of mitochondria enriched in Ucp-1 thermogenin protein which increase energy expenditure by increasing the redox potential of the mitochondria. Cold exposure increases glucose uptake and helps decrease insulin resistance. One way to combat obesity is to increase the negative energy balance. This can be achieved by recruiting more brown-like cells with in WAT to increase energy expenditure. But this concept has been met with challenges because it is thought that the beige cells are quantitatively less likely to have a significant impact on the metabolic system of the body due to their low thermogenic capacity(Shimizu & Walsh, 2015).

Lipocalin-2 and its significance in Adipose tissue Modulation

Lipocalin-2 Expression and Regulation

Lipocalin-2 is also called neutrophil gelatinase- associated lipocalin. It is coded by the Lcn-2 gene. It has been known to regulate innate immunity via binding siderophores and sequestering iron to limit bacterial growth. Our lab has done extensive work on Lipocalin-2 and its role in adipose tissue and energy metabolism. Adipose tissue releases several adipokines that are associated with obesity and inflammation. Through proteomics and microarray screening our lab has identified lipocain-2(Lcn-2) as an adipokine connecting obesity and chronic inflammation. We have evidence showing that Lcn-2 is released from adipose tissue including brown and white adipose tissue and functions to mitigate inflammation in adipose tissue as well as improve glucose uptake and insulin resistance. Obesity can lead to low-grade chronic inflammatory states; this can cause release of inflammatory cytokines. Results from our lab also clearly showed that Lcn-2 deficiency increased inflammation in adipose tissue and worsened insulin resistance and dyslipidemia in high fat diet-induced obesity. Moreover, our results also demonstrated that Lcn-2 recombinant protein is able to reverse the TNFα inhibition of leptin and adiponectin secretion from adipocytes(J. Zhang et al., 2008). The Lipocalin structure consists of a highly conserved anti-parallel beta barrel shape. It has an internal binding ligand sight. This provides the lipocalin the ability to

recognize and bind small hydrophobic molecules, bind specific-cell surface receptors, and form complexes with soluble macromolecules(FLOWER, 1996). It is expressed in the neutrophils, bone marrow, trachea, lungs, stomach, salivary gland, and colon. This diverse tissue expression shows that Lcn-2 is expressed in those tissues that are continuously exposed to micro-organisms(Cowland & Borregaard, 1997). In the neutrophils, the Lcn-2 is released whenever the cells encounter inflammation. Lipopolysaccharide and TNFα are the two main inducers of Lipocalin-2. Lcn-2 deficiency leads to an increased susceptibility of infection in mice(Berger et al., 2006).

Lipocalin-2 In Adipose Tissue Metabolism:

Obesity leads to a change in the adipose tissue composition in the form of both adipocyte hyperplasia and hypertrophy. The adipocytes become fat filled and increase in size, endothelial cells become laden with inflammatory cells and the immune cells migrate into the connective tissue surrounding adipocytes in adipose tissue. These events lead to an endocrine dysfunction causing an imbalance in the regulation of energy, body weight homeostasis, inflammation, and insulin resistance. Insulin resistance leads to dyslipidemia and hence a full blown picture of metabolic disease is created(Sun, Kusminski, & Scherer, 2011). Our lab has found that Lcn-2 KO mice when fed a high fat diet displayed worsening of obesity, insulin resistance and fatty liver. The insulin

responsiveness is decreased in adipose tissue, muscle and liver. Further we also showed that the expression of enzymes PEPCK and G6Pase in the liver of Lcn-2 KO mice was upregulated, suggesting an increased hepatic glucose production in Lcn-2 KO mice. Dyslipidemia and hypertriglyceridemia were prominent in Lcn-2 KO mice. This was shown to be due to impaired lipid metabolism. Lcn-2 KO mice fed on RCD showed an increase in serum triglyceride levels, while on a HFD a decrease in the triglyceride levels was observed. This decrease can be due to a decrease in the production of VLDL and led to a development of fatty liver. (Hong Guo et al., 2013). However, another group of researchers found that Lcn-2 KO mice were insulin sensitive. Lcn-2 KO, in their study significantly decreased fasting glucose and improved insulin sensitivity compared to their WT group. They also found that although the fat mass was increased, the inflammatory marker Tnf-α was decreased. The enzyme 12-lipoxygenase, which under normal conditions helps breakdown the arachidonic acid usually elevated in aging and obesity is inhibited in Lcn-2 KO. The results in this study showed that Lcn-2 KO provided protection against aging and obesity related insulin resistance(Law et al., 2010).

Lcn-2 in Beiging and Mitochondrial Biogenesis:

Another interesting study conducted by our lab on Lcn-2 described that Lcn-2 plays a role in regulating brown adipose tissue activation by modulating the adrenergic independent PGC1-α-Ucp-1 pathway. This

study was based on a global Lcn-2 knockdown mouse model under the condition of cold stimulation. Under normal physiological conditions, cold stimulation increases the mobilization of fatty acids, the expression of thermogenic gene Ucp-1, and the expression of mitochondrial biogenic genes. The results showed that in Lcn-2 KO the thermogenic activation of BAT induced by cold stimulation (the activation of the sympathetic system due to catecholamine release) remained intact, But the mitochondrial and peroxisomal lipid oxidation was decreased. For instance, Lcn-2 KO mice had a normal cold-induced HSL phosphorylation that signals the release of free fatty acids into the blood stream which act as a substrate for thermogenesis. However, cold-induced expression of Ucp-1 and mitochondrial genes was decreased. The mitochondrial biogenic genes PGC-1α, prdm 16 were also markedly reduced in Lcn-2 KO mice (PGC-1 α and prdm16 are the brown adipocyte markers that get upregulated when the mitochondrial activity is increased during the beiging process to increase the number of mitochondria). This shows that brown adipose tissue activation does not occur in Lcn-2 KO mice due to decreased thermogenesis and oxidative capacity of the mitochondria. Additionally, the Ucp-1 expression was not significantly affected under normal physiological conditions but there was a decreased response to cold-induced Ucp-1 expression in Lcn-2 KO mice. To further analyze the functional regulation of mitochondrial activity by Lcn-2, the redox state (ratio of NAD^+/NADH) was calculated. This ratio was decreased in

differentiated brown adipocytes from Lcn-2 KO mice. This redox reaction takes place in the mitochondria by the electron transport chain, thereby serving as a good indicator of mitochondrial function(Y. Zhang et al., 2014). In summary Lcn-2 affects cold-induced thermogenesis, mitochondrial biogenesis and mitochondrial oxidative capacity.

Current Objectives:

Lipocanlin-2 is released from adipose tissue and is an important factor regulating metabolism of brown and beige adipose tissue. Our previous studies have demonstrated that Lcn-2 plays a key role in cold-induced thermogenesis. In this thesis project, we sought to discover if Lcn-2 plays a role in exercise-induced thermogenesis and mitochondrial metabolism. We used a Lcn-2 KO model to investigate the Ucp-1 expression, mitochondrial biogenesis, and mitophagy involving PINK-1/Parkin/P-62 system in BAT and iWAT in respect to exercise.

Introduction

Obesity and its associated metabolic syndrome are the major health issues the modern world is dealing with today. There have been many pharmaceutical therapies that have been devised to combat these medical issues, but the side effects are concerning. There is a need of using the physiological model of the body system in our favor to fight against and resist an adverse effect to the body. Obesity is a result of an increase in energy consumption and/or a decrease in energy expenditure. The body is constantly fighting to stay in a state of energy equilibrium to maintain body weight but whenever this balance is disturbed we see an increase in the accumulation of body fat(Cui & Chen, 2016).

Brown fat that was once thought to be present or activated only in infants has now been discovered to be present in the adult life as well. The browning of white adipose tissue also has the same function as the brown adipose tissue with an overexpression of Ucp-1. There are two main differences in terms of metabolic function and developmental origin between the brown adipose tissue and the beige adipocytes. Firstly, the BAT constitutively expresses Ucp-1 while the beige adipocytes express Ucp-1 upon stimulation. Secondly, they originate from different cell lineages(Seale et al., 2008).

Beiging is a complex process involving an interplay of many hormonal and environmental factors. The appropriate signal in the form of beta sympathetic stimulation or cold can induce the activation of signaling pathway involving PPAR-γ and its co-activator PGC-1α. This can cause

upregulation of the expression of Ucp-1 and cause the white adipocytes to convert to beige adipocytes (Puigserver et al., 1998). These beige adipocytes then cause an uncoupling of the respiratory chain, leakage of protons, and loss of membrane potential as a result of which there is loss of energy in the form of heat.

Lipocalin-2 belongs to the lipocalin subfamily of low molecular weight secreted proteins. We have characterized Lcn-2 as an adipose-derived cytokine that is increased in obesity. It is also secreted by the neutrophils which indicate that obesity is sensed by the body as an insult or stress to the body's defenses(Abella et al., 2015). In the absence of lipocalin-2, mice are cold intolerant and cannot maintain their body temperature appropriately(Roudkenar et al., 2009), suggesting Lcn-2 plays a critical role in thermogenesis. Our previous studies also show that Lcn-2 KO impairs mitochondrial function in brown and inguinal white adipocytes(H. Guo et al., 2010). Since exercise is known to induce browning of white adipose tissue and mitochondrial oxidation, we hypothesize that modulating Lipocalin-2 activity can have effects on exercise-induced browning and mitochondrial metabolism of adipose tissue. As Lcn-2 is associated with thermogenesis, there would be a decrease in the expression of Ucp-1 in the Lcn-2 KO models after exercise as compared to their wild type controls. Additionally, we determined if Lcn-2 KO has an effect on mitophagy after exercise in both brown and white adipose tissue.

Results

1. Effects of Lcn-2 Deficiency on Exercise Performance

To determine the impact of Lcn-2 deficiency on exercise performance, Lcn-2 WT and KO mice were kept individually in a cage with or without a running wheel. The running distance of mice was monitored and recorded and then a cumulative distance of every 6 hours was calculated. Body weight was measured at the beginning of the experiment and at the end of exercise period. At day 14, mice were sacrificed and the blood samples and tissue samples were collected.

We found that WT mice ran for a 24h average distance of 6.59km/day. When compared to WT mice, Lcn-2 KO mice ran a lower 24 h Average distance of 4.47 km/day. However, the difference in average running distance between WT and Lcn-2 KO mice did not reach a statistical significance ($p = 0.33$) as shown in Figure 1A.

As shown in our previous publications, Lcn-2 KO mice have decreased activation of brown adipose tissue by cold stimulation (Y. Zhang et al., 2014) and exacerbated obesity induced by high fat diet feeding (Hong Guo et al., 2013). We then determined if voluntary wheel running exercise affect body weight differently in WT and Lcn-2 KO mice. Figure 1B showed that both the groups started with almost the same body weight. Over 13 days of voluntary wheel running exercise, WT mice had a trend towards a decrease in body weight while Lcn-2 KO mice had no significant change in body weight. Moreover, the tissue weight of fat depots including brown adipose tissue (BAT), inguinal adipose tissue

(iWAT) and epididymal adipose tissue (Epi-WAT) as well as liver and muscle was not significantly different between WT and Lcn-2 KO (Figure 1C).

2. Effect of Lcn-2 Deficiency on Exercise-Induced Changes in Ucp-1 Protein Levels in Brown and White Adipose Tissue

Brown adipose tissue, as the main thermogenic organ in the body, constitutively expresses Ucp-1 protein. But recent research has found that Ucp-1 is also expressed in white adipose tissue upon proper stimulation(De Matteis et al., 2013), a process called browning of white adipose tissue. It has been proposed that exercise can induce the browning process through a series of mechanisms that cause the phenotypic switch from white to brown-like adipocytes (Aldiss et al., 2018). BAT is known to provide thermogenesis by uncoupling oxidative metabolism from ATP production to heat production during adaptive thermogenesis. Recent investigations have proved that after exercise, the browning of white adipose tissue or brown-like adipocytes (beige adipocytes) appear within white adipose tissue(Sidossis et al., 2015). In our previous studies, we have shown that Lcn-2 deficiency reduces BAT thermogenic activation and Ucp1 expression in BAT (Y. Zhang et al., 2014). We also demonstrated that Ucp-1 expression in white adipose tissue or the browning of WAT was impaired in Lcn-2 KO mice. Therefore,

herein we examined if Lcn-2 deficiency affects exercise-induced thermogenic activation of BAT and the browning of WAT. We assessed the Ucp1 protein level in BAT and iWAT of WT and Lcn-2 KO mice after voluntary wheel running exercise.

From the data of Western Blot analysis presented in Figure 2A, brown adipose tissue from WT mice showed a significant increase in the Ucp-1 expression in the voluntary wheel running group compared to the sedentary controls in WT mice. However, in Lcn-2 KO mice, Ucp1 expression levels were not increased after voluntary wheel running exercise.

Figure 2B showed that in white adipose tissue, there was no significant change in the Ucp-1 expression after 14 days of voluntary wheel running in the running group as compared to the sedentary group in both WT and Lcn2 KO mice. This result suggests that Lcn-2 deficiency may not be involved in exercise-induced Ucp-1 expression in white adipose tissue or browning of white adipose tissue.

The main control of Ucp-1 expression lies at the transcriptional level. Within minutes of exercise the increase in Ucp-1 gene transcription and Ucp-1 mRNA is observed. This is primarily due to the action of catecholamines released by the beta adrenergic system as a response to exercise (del Mar Gonzalez-Barroso et al., 2000). We then confirmed the effect of Lcn2 deficiency on Ucp-1 expression by exercise in iWAT at the mRNA level. The qPCR data of Figure 2C indicate similarly there was no

statistically significant increase in the Ucp-1 gene expression in WT mice after voluntary wheel running exercise. Consistent with our previous published results, Ucp-1 gene expression was significantly decreased in Lcn-2 KO mice compared to WT mice under sedentary condition. Voluntary wheel running exercise led to a trend increase in the Ucp-1 gene expression in Lcn-2 KO mice.

3. Effect of Lcn-2 Deficiency on Changes in Mitochondrial Biogenesis Gene Expression in Inguinal White Adipose Tissue by Exercise

Mitochondrial oxidation plays a critical role in providing energy for thermogenesis. PGC-1α (Peroxisome proliferator-activated receptor gamma coactivator 1-alpha), Tfam (transcription factor A, mitochondrial) and Prdm-16 (PR domain containing 16) are the important regulators of genes involved in mitochondrial biogenesis. PGC-1α, a PPAR-γ co-regulator is thought to be a cold inducible protein. The activation of PPAR-γ causes the differentiation of pre-adipocytes to mature brown adipocytes together with Prdm 16 in BAT as well as into mature brown-like-adipocytes (beige adipocytes) in white adipose tissue (Fernandez-Marcos & Auwerx, 2011). Tfam is another transcriptional factor that plays an important role in this browning process. It binds to the DNA and up-regulates mitochondrial gene transcription. This occurs when mitochondrial biogenesis is needed to increase the number of mitochondria for browning and mitochondrial turnover. As shown in Figure

3A, B, and C, the expression of PGC-1α, Tfam and Prdm-16 gene was significantly increased in the inguinal white adipose tissue of WT mice after voluntary wheel running exercise compared to sedentary WT mice. However, the increase in the expression of these mitochondrial biogenesis genes by exercise was not significant in Lcn--2 KO mice. These results suggest that exercise-induced mitochondrial biogenesis and browning of white adipose tissue may be defective in Lcn2 KO mice.

4. Effect of Lcn-2 Deficiency on Changes in Mitochondrial Dynamics in Brown and Inguinal White Adipose Tissue by Exercise

Lipocalin-2 is expressed in many tissues and organs such as adipose tissue, liver, lung, spleen, bone marrow, uterus, and gastrointestinal tract. Liver and adipose tissue are the two most important organs where Lcn-2 performs anti-inflammatory functions combating cellular and oxidative stress. The elevation of Lcn-2 as a result of tissue inflammation is closely associated with obesity, insulin resistance, mitochondrial dysfunction and adipocyte metabolism. It has also been reported that Lcn-2 deficiency alters the mitochondrial membrane potential rendering the mitochondria dysfunctional and ready for removal through mitophagy(Asimakopoulou et al., 2017).

a. Lcn-2 deficiency and Parkin levels:

While an increase in mitophagy is required in brown adipose tissue after exercise to increase the turnover of mitochondria, a decrease in mitophagy is expected to allow the conversion of white adipocytes to beige cells.

From Fig 4A, we showed that the Parkin protein levels had a trend towards an increase in brown adipose tissue of WT mice after exercise. But this increase was not observed in Lcn-2 KO mice.

In Fig 4B, in inguinal white adipose tissue, there was a trend towards a decrease in the Parkin levels in the running group of WT mice after exercise. This is in accordance with the known fact that mitophagy should be decreased so that beiging can occur. However, in Lcn-2 KO mice, we saw a trend towards an increase in the Parkin expression in the running group as compared to its sedentary control group. Parkin levels in iWAT were significantly higher in Lcn2 KO mice compared to WT mice after exercise. This result indicates that there is an increase in mitophagy in iWAT of Lcn-2 KO mice, suggesting that beiging is impaired. As the results are statistically significant in white adipose tissue, we can conclude that Lcn-2 might only play an important role in regulating Parkin-mediated mitophagy during exercise in white adipose tissue only and have no effects on brown adipose tissue..

b. Lcn-2 deficiency and P-62 Expression:

In addition to Parkin, p-62 plays an important role in mitophagy. It causes the clustering of parkin targeted depolarized mitochondria and this helps to decrease the surface area for the lysosomal degradation.

Fig 4C shows that the p-62 levels in brown adipose tissue was significantly higher in Lcn-2 KO mice compared to WT mice under the sedentary condition. This can be explained by the fact that Lcn-2 KO may cause mitochondrial damage even in the absence of exercise. Additionally, there was a significant increase in the p-62 expression in Lcn-2 KO when compared to WT mice under the wheel running condition. This gives clear evidence that Lcn-2 KO causes an increase in mitophagy through increasing the expression of p-62, thereby leading to an increase in the turnover of mitochondria.

In the white adipose tissue, there was a significant increase in the p-62 levels in the running group of Lcn-2KO mice. This shows that the p-62 levels are increased after exercise when Lcn-2 is not present, leading to an increase in mitophagy and a defective browning process. (Fig 4 D).

Discussion

Lipocalin-2, a Neutrophil gelatinase-associated lipocalin plays an important role in innate immunity. Extensive work has been done on the role of Lipocalin-2 in innate immune responses as well as its effects on lipid metabolism in adipose tissue and liver(Flo et al., 2004). Lcn-2 promotor region contains the CCAAT enhancer binding protein and nuclear factor κB binding sites(Shen, Hu, Goswami, & Gaffen, 2006), and a glucocorticoid response element(Garay-Rojas, Harper, Hraba-Renevey, & Kress, 1996) which suggests that Lcn-2 has some role in inflammation and adipose tissue remodeling. Studies from our group and others have demonstrated that Lcn-2 plays an important role in lipid metabolism, diet-induced obesity, and insulin resistance. Our lab has done a great deal of work on the effects of Lcn-2 in thermogenesis and consequent lipid metabolism and insulin resistance in adipose tissue. Our results prove that when fed a high fat diet Lcn-2KO mice develops increased fat mass and adipose tissue expansion leading to dyslipidemia and insulin resistance. The highlighting feature of the study was that Lcn-2KO mice were cold intolerant and had a defective thermogenesis when exposed to cold stimulation(Hong Guo et al., 2010).

Brown tissue activation requires the recruitment and biogenesis of mitochondria. In brown adipocytes mitochondria, the energy from the oxidation of nutrients is stored as membrane potential energy and then liberated as heat through the uncoupling protein 1(Ucp-1) which causes proton leak and membrane depolarization of the mitochondria. Since

mitochondrial function is the central point of brown tissue activation, the regulation of its biogenesis and increase in the turnover is of utmost importance (Lu et al., 2018a). In our previous studies, we found Lcn-2 KO mice have impaired mitochondrial function in both brown and beige adipose tissue under the cold stimulation. Therefore, in this study we investigated if Lcn-2 deficiency had any effects on the mitochondrial biogenesis and turnover in brown and beige adipose tissue after exercise.

1. Lcn-2 Deficiency Does Not Affect Exercise Performance

Exercise causes profound changes in the morphology and biochemical properties of white adipose tissue. It causes a decrease in adipose cell size and the lipid content of adipocytes resulting in a decrease in adiposity(Gollisch et al., 2009). Brown fat like adipocytes are seeded in the white adipose tissue and cold, exercise or pharmacological activation can cause the beta adrenergic stimulation that increases the browning of the white adipose tissue(Petrovic et al., 2010a). Our results show that Lcn-2KO has no effects on the exercise performance. Although the Lcn-2KO mice ran for a shorter distance than the WT mice the result was non-significant. The body weight changes are apparent which show that the Lcn-2 WT mice did lose body weight at the end of the 13-day exercise period but the results were not statistically significant. This can be explained by the fact that Lcn-2 does not directly cause any weight change by increasing the browning of white adipose tissue.

2. Lcn2 Deficiency Does Not Alter exercise-induced Ucp1 Expression in Brown and White Adipose Tissue

The process of thermogenesis in BAT cannot take place without the presence of the thermogenin protein Ucp-1. It uncouples the oxidative phosphorylation in the mitochondria and dissipates energy in the form of heat. In the absence of Ucp-1 thermogenesis cannot be induced by nor-epinephrine. Lipolysis is an important step for the process of thermogenesis which releases fatty acids for being utilized as fuel substrates during heat production(Anita Matthias et al., 2000). It is generally accepted that Ucp-1 is exposed to the cytosolic nucleotides during resting phase. On stimulation fatty acids that are released from the white and brown adipose tissue by the action of hormone sensitive lipase act as an activator of Ucp-1. We also know from previous studies that beta adrenergic activation leads to protein kinase A activation and this in turn induces p 38 MAP kinase pathway. This leads to fatty acid release which in turn causes Ucp-1 activation(CANNON & NEDERGAARD, 2004). Therefore, we expect that exercise causes Ucp-1 upregulation. In this study, we determined if Lcn-2 deficiency had any effects on Ucp-1 activation and thermogenesis by exercise respectively.

Our results showed that the exercise-induced expression of Ucp-1 protein was not significantly attenuated in BAT of Lcn-2 KO mice, suggesting that Lcn-2 deficiency does not affect exercise-induced Ucp1 expression in brown adipose tissue. In the inguinal white adipose tissue, we also

couldn’t see a significant rise in Ucp-1 expression after exercise in both WT and Lcn-2 KO mice. This suggests that 14 days of voluntary wheel running may not be able to induce beiging of iWAT. Therefore, we cannot conclude if Lcn-2 has any effect on the Ucp-1 expression in iWAT or beiging induced by exercise under this particular experimental condition. Next, we wanted to see if the Ucp-1 is affected at the gene expression level. We found that the Ucp-1 gene expression was increased in the running group of both the Lcn-2 WT and Lcn-2 KO. The statistically non-significant results confirm the conclusion that despite the fact that Lcn-2 is released after exercise and helps in the beiging process it directly does not have any effects on the Ucp-1 protein and gene expression and that exercise-induced thermogenesis in beige adipose tissue may not be impaired.

3. Lcn-2 Deficiency Changes Mitochondrial Biogenesis Gene Expression in Inguinal White Adipose Tissue by Exercise

It has been known from previous research that exercise increases the beiging of white adipose tissue and that the expression of mitochondrial biogenic genes are increased during the process of beiging. However, it is unclear if Lcn-2 has any effects on the mitochondrial biogenic genes induced by exercise. Increase in mitochondrial activity is required for the beiging of white adipose tissue; for this reason, an increase in the

expression of mitochondrial genes is required. Exercise increases the energy demand of the cells,which increases cAMP and activates the calcium calmodulin complex. This in turn activates AMP activated protein kinase which eventually activates the P-38 MAPK pathway(Håkan Thonberg et al., 2002). This pathway is important in the process of thermogenesis as it helps in the upregulation of the thermogenic genes. For example, the activation of P-38 MAPK pathway in turn helps to trigger the activation of PGC-1α and Tfam(Kang & Li Ji, 2012) which increase the number of mitochondria (mitochondrial biogenesis). Comparing Lcn-2 KO with WT mice, we showed that the mitochondrial biogenic genes (PGC1-α, Tfam) were significantly increased after exercise in Lcn-2 WT mice. However, this exercise-induced increase in mitochondrial biogenesis was not observed in Lcn-2 KO mice. These results led us to interpret that Lcn-2 deficiency reduces the effect of exercise on the expression of mitochondrial biogenic genes. Prdm16 performs thermogenic functions in both the developmental and induced brown adipose tissue. It has a central role in almost all steps of thermogenesis starting from the differentiation of brown adipocyte precursors to the co-activation of thermogenic gene expression. Studies in mice have also confirmed the findings that thermogenic adipose tissue activation depends on Prdm16 expression and the metabolic function of Prdm16 in protecting against obesity and insulin resistance (Ishibashi & Seale, 2015). In our studies, Prdm16 was significantly increased in the running group of Lcn-2 WT mice compared to

the sedentary group of mice but showed no significant changes in Lcn-2 KO mice. This suggests that Lcn-2 is required for exercise-induced expression of Prdm16 expression.

4. Lcn-2 Deficiency Alters Mitochondrial Dynamics in Brown and Inguinal White Adipose Tissue by Exercise

Mitochondria performs many important functions in adipose tissue. Some of these involve lipogenesis, lipolysis, adipocyte differentiation, production of radical oxygen species, ATP production, heat generation through the uncoupling of oxidative phosphorylation(De Pauw, Tejerina, Raes, Keijer, & Arnould, 2009). Thus, an intervention in any one of the functions can help in reducing adiposity, decreasing fat mass and preventing the development of metabolic syndrome. A correlation has been established between the activation of brown adipose tissue and the decrease in insulin resistance and fat mass. In an experiment performed by a group of researchers, transplanting brown adipose tissue into the subcutaneous white adipose tissue showed promising results with a decrease in blood glucose and insulin resistance (Stanford et al., 2013b). The utilization of exercise to promote the beiging of white adipose tissue can have beneficial effects on overall metabolic health. For this to occur, the role of mitochondria is of central importance. It is important to maintain good quality of mitochondria so that the beiging can efficiently take place.

When Ucp-1 is upregulated upon stimulation the membrane of the mitochondria become depolarized and the quality of these mitochondria is decreased. It is necessary that these mitochondria get removed and is coupled to mitochondrial biogenesis so that the turnover of mitochondria is increased (Lu et al., 2018a). In this study, we wanted to explore the effect of Lcn-2 deficiency on the process of mitophagy during exercise. Our results showed that both Parkin and p-62 were increased in brown and inguinal white adipose tissue of Lcn-2 KO mice as compared to WT mice. This suggests that Lcn-2 is important for mitochondrial turnover in brown adipose tissue. It is likely that Lcn-2 deficiency causes membrane depolarization of mitochondria leading to activation of PINK-1 and Parkin system along with P-62 so that the depolarized mitochondria can be removed. It is also known that increased mitophagy is coupled with increased mitochondrial biogenesis in brown adipose tissue in order to keep appropriate quantity and quality of mitochondria. For instance, the PGC-1α is also activated when the PINK-1 and Parkin system is activated after exercise(Lu et al., 2018b). In Lcn-2 KO mice, we saw an increase in mitophagy but a decrease in mitochondrial biogenesis in brown adipose tissue, suggesting an impairment of mitochondrial turnover in the absence of Lcn-2.

However, a decrease in mitophagy is required for the beiging of white adipose tissue. The number of mitochondria and the amount of Ucp-1 is very scant in the beige cells. A decrease in mitophagy is beneficial in this

case so that the mitochondria that have over expressed Ucp-1 can stay for a longer period of time (Taylor & Gottlieb, 2017b). Our results showed that Lcn-2 deficiency causes a trend increase in Parkin in inguinal white adipose tissue in the running group compared to WT mice. However, p-62 levels were significantly increased in the running group of Lcn-2 KO mice as compared to Lcn-2 WT mice. This suggests that in the absence of Lcn-2 mitophagy is increased leading to defective beiging after exercise. Together with the results from figure 3A, we have demonstrated that Lcn-2 deficiency increases mitophagy which is uncoupled with an increase in mitochondrial biogenesis leading to a reduction in beiging during exercise.

Conclusion

Lipocalin-2 is important for adipose tissue metabolism as well as mitochondrial quality control. Based on our results, Lcn-2 deficiency does not seem to affect the running performance. There is no change in body weight of mice after 13 days of exercise in Lcn-2 KO mice as compared to WT controls. Ucp-1 levels also show no significant change in Lcn-2 KO mice, i.e. Ucp-1 expression can be induced by exercise when Lcn-2 is not present, but the process of thermogenesis may be decreased. Lcn-2 deficiency does seem to effect mitochondrial quality control as Lcn-2 KO does not show exercise-induced mitochondrial biogenesis but shows increased mitophagy in white adipose tissue, indicating that the beiging process is defective.

Materials and Methods

1. Animal Handling

Animals were housed at thermoneutral environment in a specific pathogen-free facility at the University of Minnesota. Animal studies were conducted with the approval of the University of Minnesota Animal Care and Use Committee and conformed to the National Institute of Health guidelines for laboratory animal care. WT and Lcn2 KO mice from the same litter were housed at thermoneutrality in 12:12-h light-dark cycle with free access to water. Male mice were fed a regular chow diet (RCD) after weaning.

2. Wheel Running:

At 12 weeks of age, mice were individually housed and placed in cages with the wheel running. Mice were housed without a running wheel (sedentary; SED) or were given voluntary access to an active running wheel (VWR; 24 cm diameter; Nalgene) for 2 weeks. Wheel revolutions were measured daily using odometers. In short, after acclimatization, 12-week old mice were housed without (sedentary) or with free (VWR) access to a running wheel (24 cm diameter, 8 cm wide; Nalgene, Rochester, NY) for the indicated experimental time.

3. Generation of Lcn-2 KO mice

Lcn-2 KO mice were kindly provided by Dr. Alan Aderem (Institute for Systems Biology, Seattle, Washington), which were originally generated by Dr.Shizuo Akira (Research Institute for Microbial Diseases, Osaka University,Osaka, Japan). Lcn-2 KO mice were generated by gene targeting in embryonic stem (ES) cells from mouse strain 129, and targeted ES cells were injected into C57BL/6 blastocysts. C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Lcn-2-null mice were backcrossed onto the B6 background for 10 generations before mice were used for the experiments. Heterozygous mating scheme was used to generate wild-type and Lcn-2 KO mice for the experiments.

4. Western blot analysis

Brown adipose tissue and Inguinal WAT were homogenized in RIPA buffer (Sigma, St. Louis, MO). Protein was quantified using the bicinchoninic acid method (Pierce, Rockford, IL). Equivalent amounts of protein were run on an SDSPAGE gel and transferred to a nitrocellulose membrane prior to incubation with primary and secondary antibodies. All primary antibodies were obtained from Cell Signaling Technology (Danvers, MA) except for mouse monoclonal UCP-1 antibody from R&DSystems (Minneapolis, MN). Antibodies included β-Actin, Ucp-1, Parkin, P-62, Lcn-2. Anti-rabbit secondary antibody and antimouse secondary antibody are from R&D

Systems (Minneapolis, MN) and used at a dilution of 1:5000. ECL western blotting substrate (Pierce, Rockford, IL) was used to detect reactivity.

5. Real-time PCR

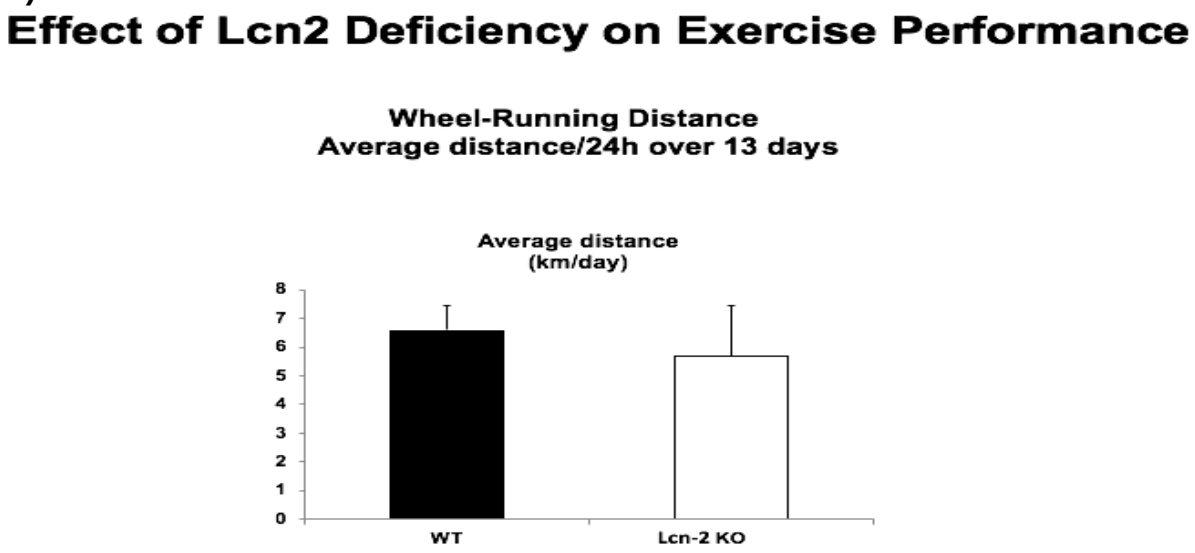
Total RNA was isolated from tissue using TRIZOL reagent (Invitro, Carlsbad, CA). RNA was DNase-treated prior to the synthesis of cDNA using Superscript II reverse transcription kit (Invitrogen, Carlsbad, CA). Real-time quantitative PCR was conducted using SYBR Green qPCR Master Mix (SABiosciences, Frederick, MD) with a StepOne Real-time PCR System (Applied Biosystem, Foster City, CA). The $\Delta\Delta$Ct method was used to calculate relative mRNA expression. Tbp or Cyclophillin served as an internal control.

6. Statistical Analysis

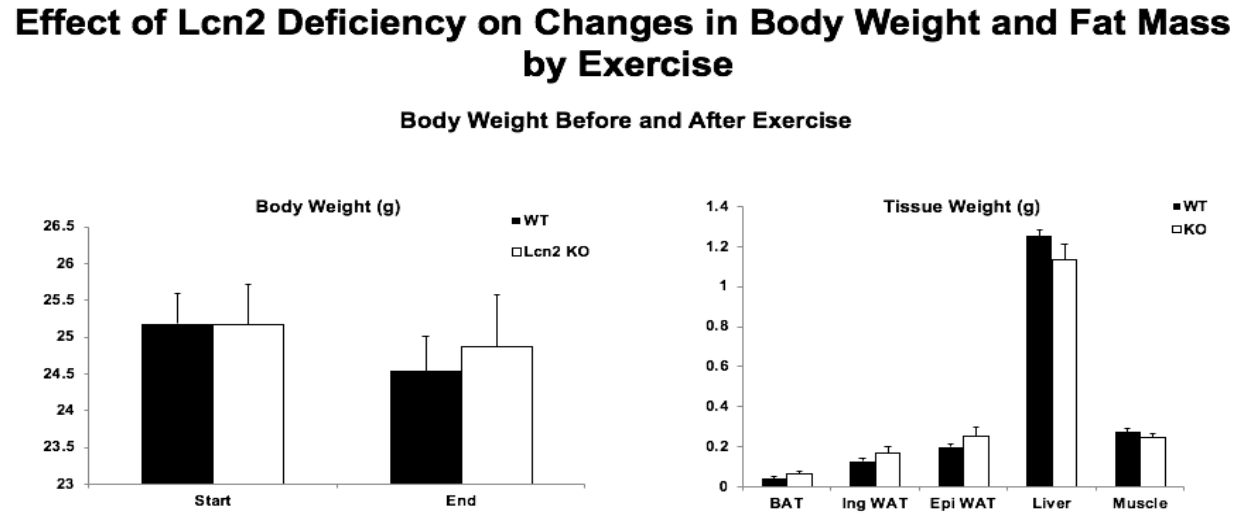
Values are reported as mean +/- standard error of the mean (SEM). Statistical significance was determined by two-tailed Student's t test, where a P value less than 0.05 was considered significant. For statistical analysis of the indirect calorimetry data, repeated-measure ANCOVA was used. The analysis included the absolute values with the associated body weight used as a covariate and the data presented as least-square means.

Figure 1:

A)



B)

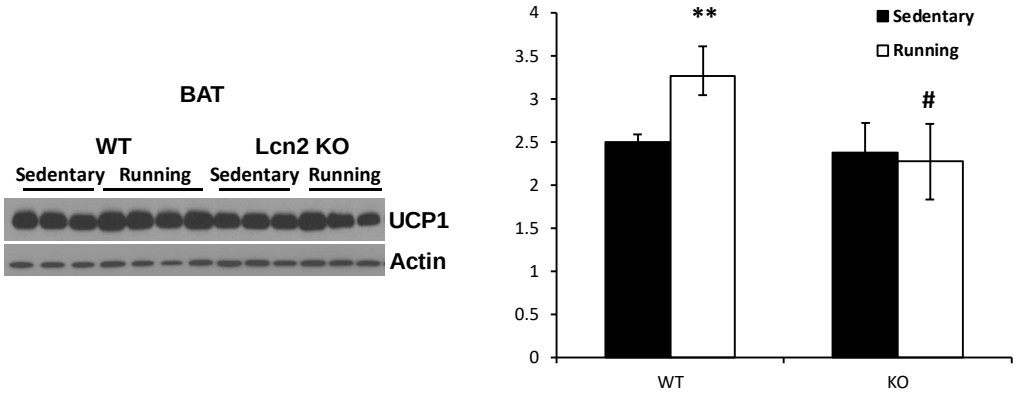


1. Effects of Lcn-2 Deficiency on Exercise Performance. A) Average distance covered by the mice after 13 days of wheel running in cages. B) Body Weight measured before and after Exercise in Brown adipose tissue, Inguinal adipose tissue, epi-gonadal adipose tissue, Liver and Muscle.

Figure 2

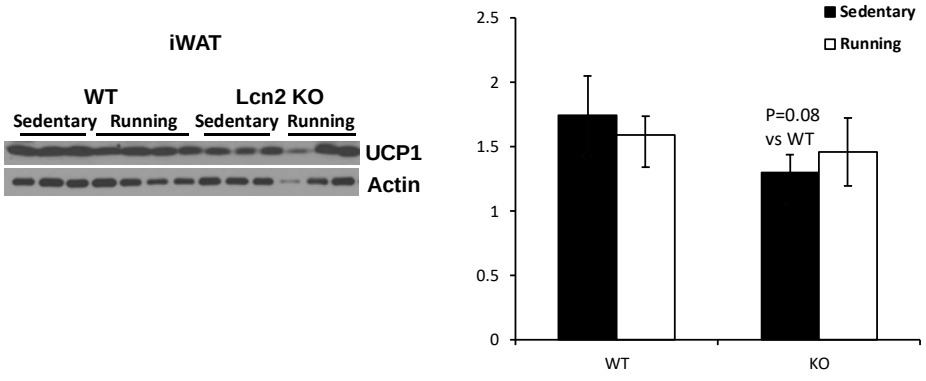
A)

Effect of Lcn2 Deficiency on Changes in UCP1 Protein Levels in Brown Adipose Tissue by Exercise

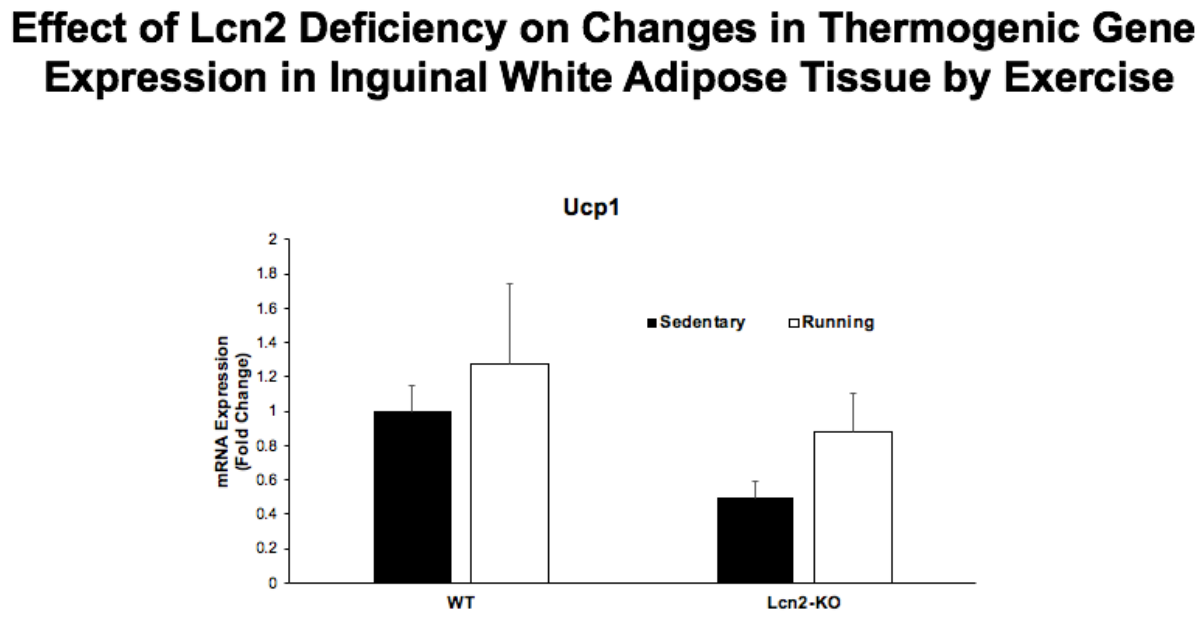


B)

Effect of Lcn2 Deficiency on Changes in UCP1 Protein Levels in Inguinal White Adipose Tissue by Exercise



C)

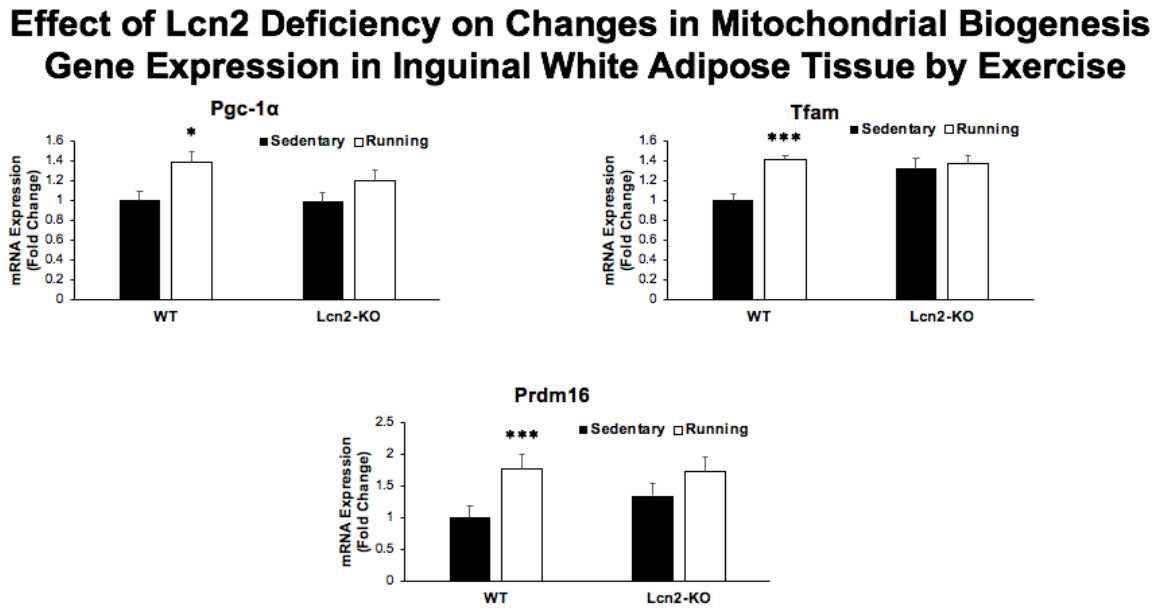


Effect of Lcn2 Deficiency on exercise-induced Changes in UCP1 Protein Levels in Brown and White Adipose Tissue. A) Immunoblotting for Ucp-1 in brown adipose tissue. Quantification Of Ucp-1 protein expression in Brown adipose tissue. B) Immunoblotting for Ucp-1 in White adipose tissue and quantification of protein expression in white adipose tissue. Significant increase in Ucp-1 expression after 13 days of exercise in Lcn-2 KO mice. C) Quantification of Ucp-1 mRNA expression through qPCR.

*p<0.05

Figure 3

A), B), C)



Effect of Lcn2 Deficiency on Changes in Mitochondrial Biogenesis Gene Expression in Inguinal White Adipose Tissue by Exercise. A) mRNA expression of Pgc-1α. Significant increase in Pgc-1α after 13 days of exercise in the Lcn-2 WT mice. B) mRNA expression of Tfam. Significant increase in Tfam after 13 days of exercise in the Lcn-2 WT mice C) mRNA expression of Prdm16. Significant increase in Prdm 16 after 13 days of exercise in the Lcn-2 WT mice Quantification of qPCR results showing expression of mitochondrial biogenic genes.

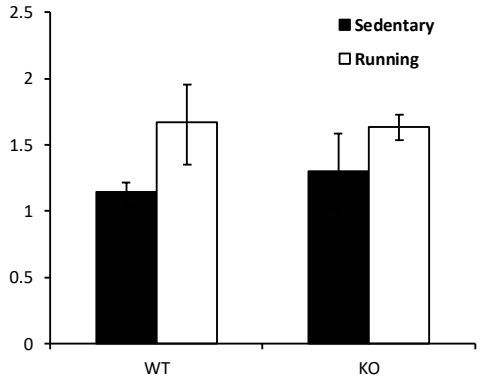
*p< 0.05 *** p<0.01

Figure 4

Parkin Protein Expression:

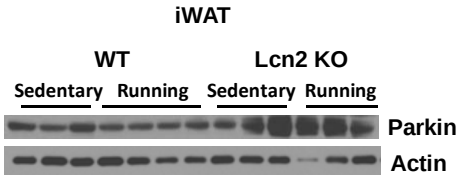
A)

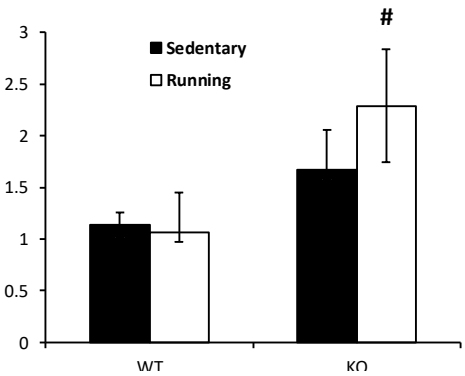
Effect of Lcn2 Deficiency on Changes in Mitochondrial Dynamics in Brown Adipose Tissue by Exercise



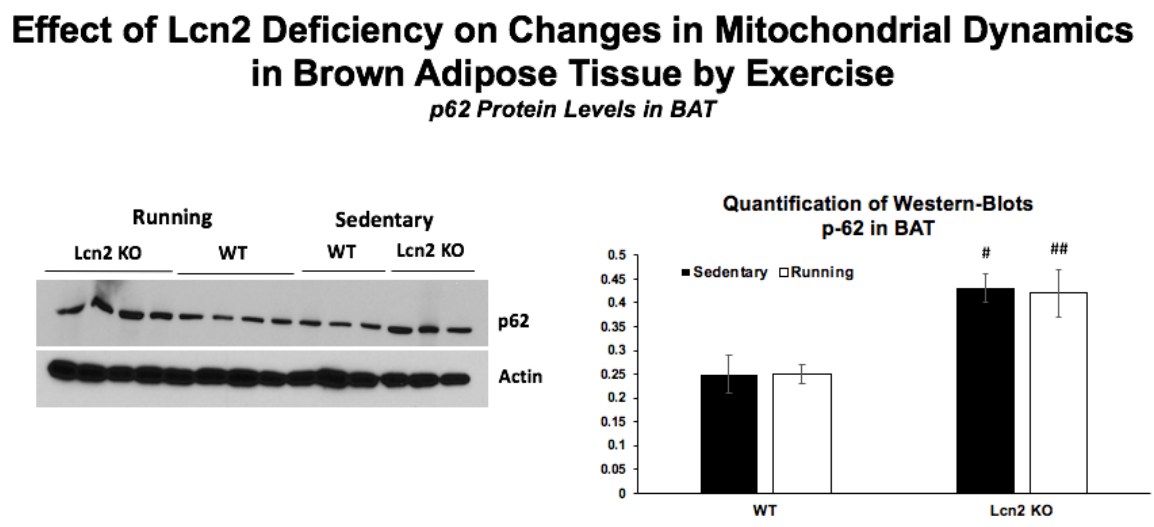
B)

Effect of Lcn2 Deficiency on Changes in Mitochondrial Dynamics in Inguinal White Adipose Tissue by Exercise

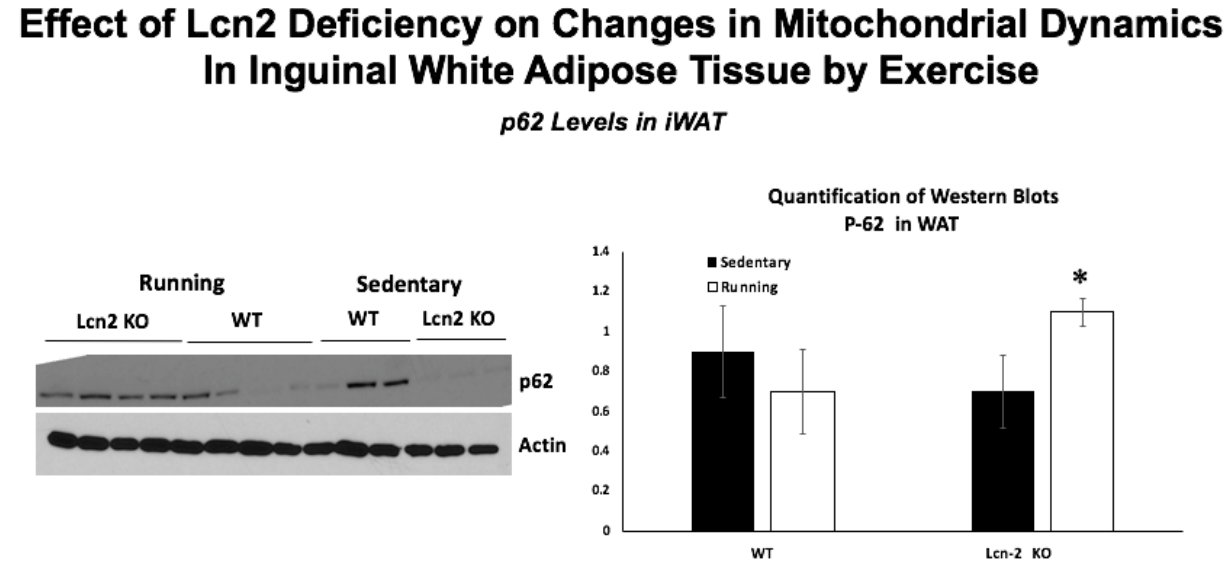




C)



D)



Effect of Lcn2 Deficiency on Changes in Mitochondrial Dynamics in brown and Inguinal White Adipose Tissue by Exercise. A) Immunoblot results of Parkin expression in brown adipose tissue. B) Immunoblot results of Parkin in Inguinal white adipose tissue. C) Immunoblot results of P-62 in brown adipose tissue. #p<0.05: significant change between

sedentary groups of Lcn-2 WT and Lcn-2KO mice. ##p<0.05: significant change between running groups of Lcn-2 WT and Lcn-2KO mice D) Immunoblot results of P-62 in Inguinal white adipose tissue. *p<0.05: Significant increase in p-62 expression after 13 days of exercise in Lcn-2 KO mice.

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