THE EFFECTS OF ENDURANCE EXERCISE TRAINING ON ADIPOSE TISSUE METABOLISM

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ABSTRACT

Chronic exercise training is a common approach utilized to optimize health and prevent disease. While the beneficial physiological adaptations to long term exercise are well established in many systems such as skeletal muscle, the specific alterations that occur in adipose tissue under this condition are less clear. With conflicting results presented in the literature, this study set out to clarify the effects of chronic exercise on adipose tissue lipolysis and lipogenesis in both a visceral and subcutaneous fat depot. Six weeks of exercise training by treadmill running resulted in a significant 26% reduction in isoproterenol stimulated lipolysis in both epididymal and inguinal adipocytes of male Wistar rats. Attempts to elucidate the mechanism underlying this adaptation were unsuccessful but determined that ATGL, CGI-58, perilipin and HSL are likely not responsible. Exercise training also led to a reduction in fat mass that was accompanied by lower triglyceride synthesis.

DEDICATION

For my mom, **Flavia Del Bel Belluz**, who always approached life's challenges with patience, persistence and courage. Your example has been my inspiration throughout this thesis.

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LIST OF ACRONYMS

AC	Adenylate cyclase
ACC1	Acetyl CoA carboxylase 1
ACS-1	Acyl-CoA Synthetase isoform 1
AGPAT	1-acyl-sn-glycerol-3-phosphate acyltransferases
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
AR	Adrenergic receptor
ATGL	Adipose triglyceride lipase
ATP	Adenosine triphosphate
CGI-58	Comparative gene identification-58
CM	Chylomicron
DAG	Diacylglycerol
DGAT	Diacylglycerol acyltransferate
DHAP	Dihydroxyacetone phosphate
DNL	*De novo* lipogenesis
FA	Fatty acid
FA-CoA	Fatty acyl-CoA molecules
FAS	Fatty acid synthase
FAT/CD36	Fatty acid translocase/CD36
FATP	Fatty acid transport protein
FATP1	Fatty acid transport protein-1
G3P	Glycerol-3-phosphate
G3PD	Glycerol-3-phosphate dehydrogenase
G6P	Glucose-6-phosphate
Gi	G-inhibitory protein
GLUT	Glucose transporter
GPAT	Glycerol-3-phosphate acyltransferases
Gs	G-stimulatory protein

H&E	Hemotoxylin and Eosin
HSL	Hormone sensitive lipase
LCFA	Long chain fatty acid
LD	Lipid droplet
LPL	Lipoprotein lipase
LPA	Lysophosphatidic acid
MAG	Monoacylglycerol
NEFA	Non-esterified fatty acid
OA	Oxaloacetate
PA	Phosphatidic acid
PDE3B	Phosphodiesterase 3B
PEP	Phosphoenolpyruvate
PEPCK	Phosphoenolphruvate carboxykinase
PI3K	Phosphoinsoitide 3 kinase
PKA	Protein kinase A
PLIN	Perilipin
PM	Plasma membrane
PPH-1	Phosphatidate phosphohydrolase
SC	Subcutaneous
TAG	Triacylglyerol
TG	Triglyceride
VC	Visceral
VLDL	Very low density lipoprotein
qPCR	Quantitative polymerase chain reaction

1. INTRODUCTION

The primary role of white adipose tissue (WAT) is to act as an energy reservoir, storing and releasing substrate under conditions of positive and negative energy balance, respectively. This function is of particular importance for maintaining energy homeostasis when the system is challenged, such as when increased substrate is required during an exercise bout. Under this condition, it has been well documented that adipocytes are capable of adapting their metabolism to meet exercise imposed increases in energy demand (1). In fact, the release of fatty acid (FA) by the adipose tissue during acute exercise has been shown to far exceed what is required by peripheral tissues for oxidation (1). Over the long term, repeated bouts of exercise leads to adaptations in peripheral substrate requirements. More specifically, chronic training induces a shift towards greater FA utilization by skeletal muscle (2, 3). Therefore, with chronic exercise additional adaptations in the organism are required in order to better match the supply of substrate with changing metabolic demands. Many studies report that in the trained state, adipocyte mobilization of triglyceride (TG) stores is enhanced to supply the increased proportion of FA oxidized by working muscles (4-6). In this circumstance enhanced lipolysis seems reasonable; however, another well established training adaptation in skeletal muscle is that it increases oxidation of its own intrinsic fat stores with a proportionate reduction in reliance on adipose tissue FA (4, 5, 7). In this context, enhanced lipolysis by

adipose tissue seems counterintuitive given that training would reduce requirements for plasma fatty acids that are already liberated in excess of what is required by the periphery. In line with this evidence, other research has reported adipose tissue lipolysis to be reduced in the trained state (8). The mechanisms by which these various reported changes in lipolysis occur remains unclear.

Another important adaptation to exercise training is a reduction in adiposity. Whether this results from an enhanced mobilization of adipose tissue TG stores that are subsequently utilized by peripheral tissues or a reduced ability of adipocytes to synthesize and store TG is not clear. In either case, understanding how training modifies adipose tissue metabolism to facilitate changes in adiposity is relevant for the treatment of disease because accumulation of adipose tissue has been correlated with the development of chronic illness (9, 10).

With inconsistencies pertaining to training induced alterations in adipose tissue metabolism, this study was designed to identify how a chronic exercise program effects adipocyte lipolysis and lipogenesis. Since physiological changes arising from chronic exercise have been shown to confer health benefits, molecular mechanisms governing training induced adaptations in adipose tissue will be explored. Identification of regulatory mechanisms improving adipose tissue metabolism may be of therapeutic relevance for the maintenance of health or correction of disease.

2. LITERATURE REVIEW

2.1 White adipose tissue (WAT)

The WAT plays an important role in the maintenance of whole-body energy homeostasis by functioning as a major energy reservoir capable of storing and releasing energy substrate (11). WAT is also an endocrine organ with the ability to secrete bioactive molecules (adipokines) that regulate many physiological processes throughout the body (11, 12). The WAT is primarily composed of adipocytes, spherical cells with a large central lipid droplet containing a TG core surrounded by a phospholipid monolayer. Adipocytes can range from 20 to 160 μm in diameter depending on the size of the lipid droplet and thus the amount of TG stored in it (13). TG pools within the adipocytes are continuously undergoing TG hydrolysis (lipolysis) and fatty acid (FA) esterification (TG synthesis) simultaneously, with the net flux of substrate regulated according to the nutritional status of the system (14). Overall, adipocytes are equipped with machinery to perform the primary functions of storing and releasing substrate under conditions of energy surplus and increased demand, respectively (15).

2.2 Lipogenesis

When energy intake exceeds expenditure, as is seen in a post-prandial state, excess substrates are taken up by adipocytes and stored as TG through a process known as lipogenesis (Figure 1). Briefly, non-esterified fatty acid (NEFA)

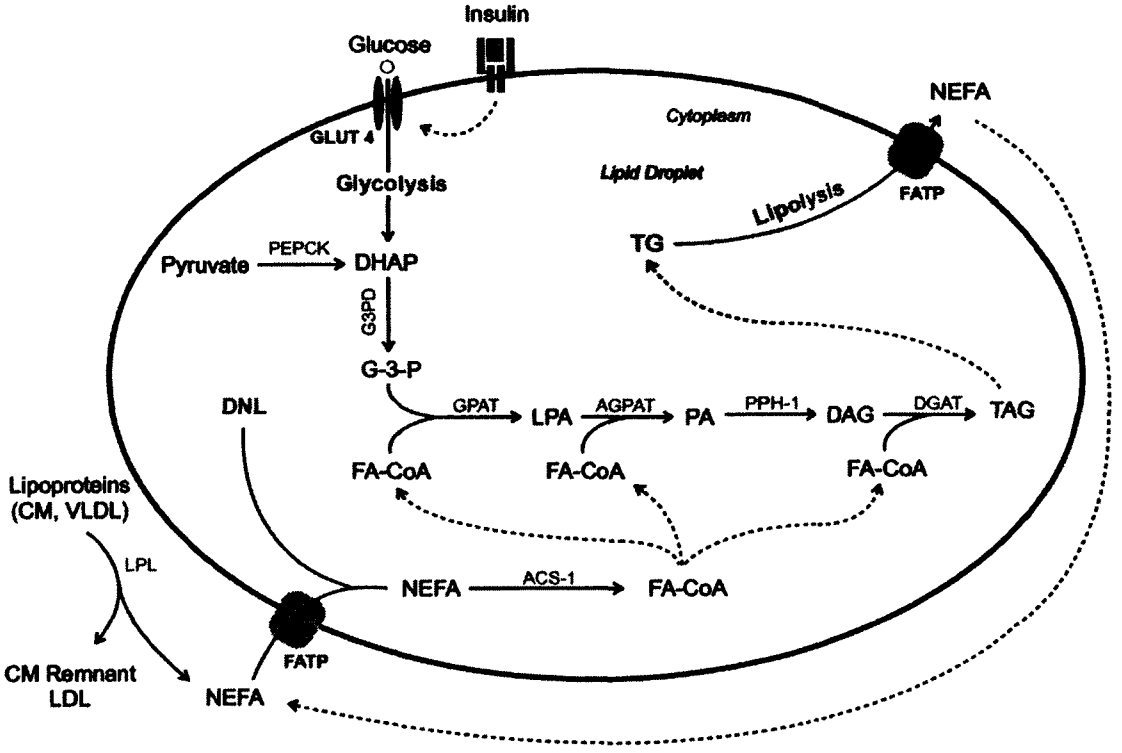

Figure 1. Overview of TG synthesis in the adipocyte as adapted from (15). Glucose uptake is stimulated by insulin dependent GLUT 4 translocation to the plasma membrane (PM). Glucose is converted to dihydroxyacetone phosphate (DHAP) by glycolysis and finally to glycerol-3-phosphate (G3P) by glycerol-3-phosphate dehydrogenase (G3PD) to form the backbone of the TG. G3P can also be generated from precursor molecules such as pyruvate through the process of glyceroneogenesis by phosphoenolphruvate carboxykinase (PEPCK). NEFA utilized for TG synthesis can be derived from 3 sources: 1) hydrolysis of lipoproteins by lipoprotein lipase (LPL) 2) conversion of glucose to long chain fatty acids (LCFA) by *de novo* lipogenesis (DNL) and 3) liberation of FA from intracellular TG stores through lipolysis. NEFAs generated from these sources are activated via acylation by acyl-CoA synthetase isoform 1 (ACS-1) to produce molecules of fatty acyl-CoA (FA-CoA) and esterified to G3P to form TG. The initial FA-CoA is esterified to G3P by glycerol-3-phosphate acyltransferases (GPAT) producing lysophosphatidic acid (LPA). In turn, LPA is acylated by 1-acyl-sn-glycerol-3-phosphate acyltransferases (AGPAT) producing phosphatidic acid (PA). Hydrolysis of PA by phosphatidate phosphohydrolase (PPH-1) leads to the production of diacylglycerol (DAG), which is subsequently acted upon by diacylglycerol acyltransferate (DGAT) to esterify the final FA-CoA. The newly formed TG merges with the central lipid droplet (LD).

originating from a number of sources are acylated in the cytoplasm by ACS-1 (16). Three FA-CoA molecules are then esterified sequentially to a G3P molecule derived via glyceroneogenesis or glycolysis (17). The initial FA-CoA is esterified to G3P by GPAT producing LPA (16, 18). LPA in turn undergoes acylation by AGPAT producing PA (18). Hydrolysis of PA by PPH-1 then generates the TG precursor DAG, which is subsequently acted upon by DGAT to esterify the third and final NEFA (16). The newly formed TG merges with the central LD. TG storage by the WAT is very important for NEFA and glucose clearance from the blood, as high plasma concentrations of these substrates may disrupt glucose and lipid homeostasis (11). Under a state of chronic positive energy balance, the increased flux of substrate through the lipogenesis pathway can lead to excessive expansion of WAT mass also increasing the risk of developing dysfunctional metabolic alterations as seen in obesity (19).

2.2.1. Glyceride-glycerol precursors

Due to a lack of significant glycerol kinase activity (20,21), adipocytes cannot phosphorylate free glycerol to produce G3P for direct use in FA esterification and TG synthesis (22). In this context, esterification of NEFAs in adipocytes requires the formation of G3P from other precursors to produce the glycerol backbone of the TG molecule. The inability of the adipocyte to utilize free glycerol for TG synthesis is an important metabolic feature as it prevents the

immediate re-synthesis of TGs from substrates intended for peripheral tissues released by the cell through lipolysis (23). A large amount of G3P is produced from the conversion of glucose via the glycolytic pathway. In this process, insulin acts as a key regulatory hormone that stimulates GLUT 4 translocation to the PM subsequently increasing glucose uptake into the adipocyte by approximately 30-fold as compared to non-insulin stimulated conditions (24). Once inside the cell, glucose is phosphorylated by hexokinase producing glucose 6-phosphate (G6P), preventing it from exiting the cell (24). G6P is then sequentially converted into intermediary molecules that ultimately undergo a reduction reaction by G3PD producing G3P from DHAP (24). G3P can then be utilized to esterify incoming fatty acids and be stored as a TG. Other intermediary molecules such as pyruvate and lactate can also be used to generate G3P in the adipocyte through glyceroneogenesis (25). Briefly, oxaloacetate (OA) is produced from glyceroneogenic precursors (pyruvate and lactate) and then converted into phosphoenolpyruvate (PEP) by the enzyme PEPCK (26). Through additional reactions, PEP is converted to DHAP and finally to G3P (26). Glyceroneogenesis is particularly important in glyceride-glycerol production during conditions where glucose is limited or insulin levels are low (fasting/exercise) because uptake of glucose for G3P production is reduced (27). In fact, in the epididymal fat pads of carbohydrate restricted rats more than 80% of the glyceride-glycerol utilized for TG synthesis has been reported to be produced via glyceroneogenesis (28).

2.2.2. Sources of FA for TG synthesis

The majority of FA utilized by adipose tissue for TG synthesis is derived from the plasma by the hydrolysis of circulating lipoproteins or uptake of un-utilized albumin bound NEFA, endogenously generated by adipocyte lipolysis (29). FAs can also be synthesized by the adipocyte intracellularly from glucose through a process termed *de novo* lipogenesis (29).

2.2.2.1. Lipoprotein derived FA

Lipoproteins are lipid-rich particles that travel through the circulation to deliver FAs to peripheral tissues (30). A proportion of the FA stored by the adipose tissue comes from the hydrolysis of TG in lipoproteins such as chylomicrons (CM) and very low density lipoproteins (VLDL) (29). CM are composed mainly of exogenous TG and are packaged by enterocytes after intestinal absorption of dietary fats (30). Post-prandially, CM TG is the dominant source of FA (29,31) but the presence of this lipoprotein is short lived as it is only available after a meal (32). VLDL is an endogenous TG containing lipoprotein that is produced by the liver (30). The provision of FA by VLDL predominates during the post-absorptive state, when CM are typically not available (29). The hydrolysis of lipoprotein TG for FA storage in adipocytes is dependent on adipose tissue LPL. LPL is a glycoprotein enzyme secreted by the adipocytes and transported to the luminal surface of capillaries where it anchors to epithelial cells

(33). In the capillary, LPL cleaves lipoprotein TG releasing FAs that can be taken up into adipocytes via diffusion across the plasma membrane or by fatty acid transport proteins (FATP) such as fatty acid translocase/CD36 (FAT/CD36) and fatty acid transport protein-1 (FATP1) (32, 34, 35). LPL is an important enzyme for lipogenesis as *in vitro* models have demonstrated an 80% reduction in fat storage when LPL expression is reduced by 50% (32). Similarly, defective FATP impair the ability of adipocytes to take up FA for storage. This can result in accumulation of fat in the plasma as is evident in spontaneously hypertensive rats, which have CD36 deficiency that leads to hypertriglyceridemia (36).

2.2.2.2. Fatty acid re-esterification

NEFAs can also become available by the hydrolysis of intracellular adipocyte TG pools through a well-regulated process known as lipolysis. Lipolysis results in the release of FAs into the circulation for utilization by peripheral tissues. Those unutilized FAs (~40%) of the total released by the adipocytes, return to the WAT to be re-esterified back into TG (37). The "futile cycle" (38) of TG release and re-esterification plays an important role in allowing the adipose tissue to respond to rapid changes in whole body energy metabolism (1, 39). For example, Wolfe and colleagues showed that a reduction in the rate of re-esterification (~45%) that accompanied the first 30 minutes of an exercise session in men effectively doubled the NEFA availability to working muscles (1).

In the same study, the cessation of exercise elicited a sharp increase in the rate of re-esterification, preventing an elevation in plasma NEFAs beyond the carrying capacity of albumin (1). This is important because immediately post-exercise, lipolysis remains elevated even though skeletal muscles require less FA for oxidation (1), increasing the potential for hazardous accumulation of FA in the plasma. While the futile cycle plays an essential role in regulating FA homeostasis, the energy cost of re-esterification is considerably large for a single biochemical pathway (37, 40) requiring approximately 7-9 ATP for the production of every TG molecule (41).

2.2.2.3. *De novo* lipogenesis

NEFAs are also constructed by the adipocyte through *de novo* lipogenesis (DNL), a process which utilizes glucose to produce LCFA in the cell (42). In this metabolic pathway, glucose is ultimately converted to citrate through glycolysis and the Kreb cycle, which then exits the mitochondria to be cleaved into OA and acetyl CoA by ATP citrate lyase (27). Acetyl CoA carboxylase 1 (ACC1) then converts acetyl CoA to malonyl CoA (24). Fatty Acid Synthase (FAS), a multienzyme complex, subsequently joins an acetyl CoA molecule to malonyl CoA to produce a 4 carbon structure (24, 43). FAS then sequentially catalyzes the addition of multiple malonyl CoA molecules, increasing the length of the fatty acid chain by the successive addition of 2 carbons until the desired length is

reached (43). The absolute contribution of this system to FA synthesis under general conditions is unclear and thought to be minimal in human adipose tissue as the energy requirement for FA production from carbohydrate is high (44, 45).

2.3. Lipolysis

Under conditions of increased energy demand (i.e. exercise) or energy restriction (i.e. fasting), the WAT mobilizes its intracellular TG stores to provide substrate to peripheral tissues for energy production. The breakdown of TG occurs in a stepwise manner, through a process known as lipolysis that culminates with the release of three FAs and one glycerol molecule (Figure 2). FAs serve as an important fuel used by tissues such as skeletal muscle, liver, and heart for energy production, while glycerol can be used by the liver to produce glucose through gluconeogenesis (24).

2.3.1. Hormonal regulation of lipolysis

The hydrolysis of adipose tissue TG is primarily regulated by catecholamines (adrenaline and noradrenaline) and the hormone insulin. The relative balance between stimulatory and inhibitory signals transmitted by these opposing agents in combination with the density of their respective receptors mediates the net breakdown of TG stores in WAT (46).

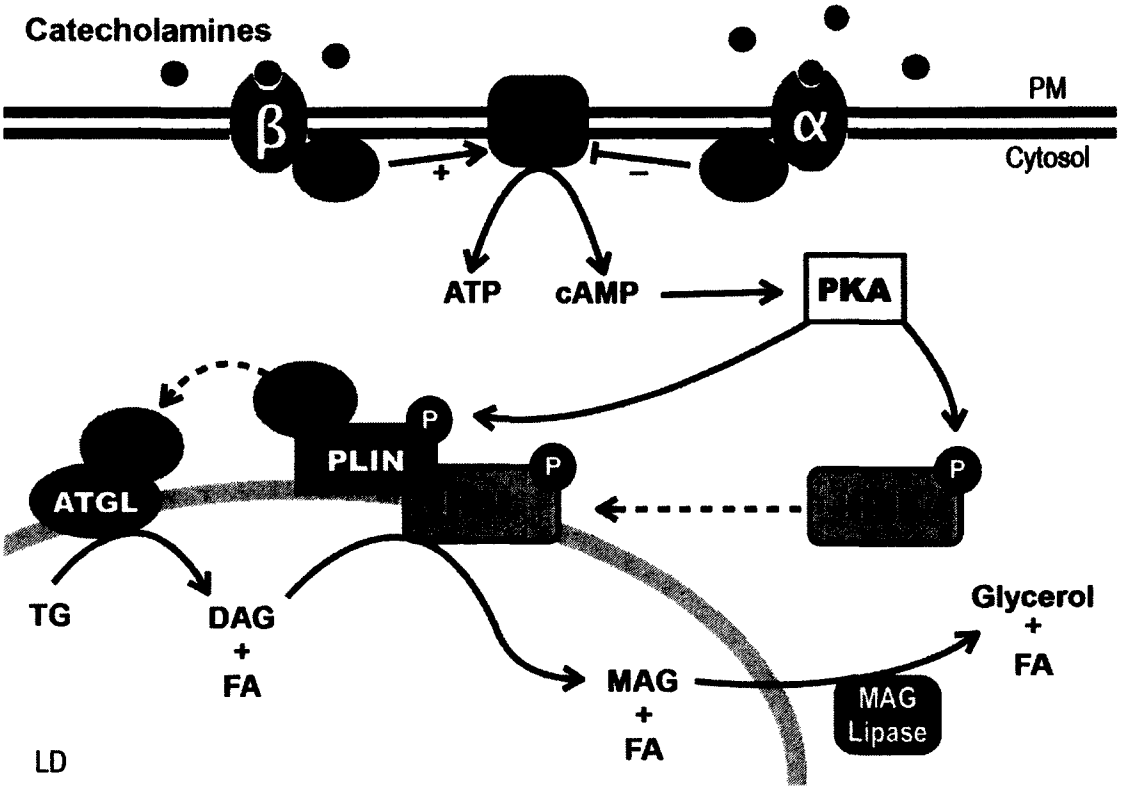

Figure 2. The lipolytic cascade. Adapted from Lafontan & Langin (47). Catecholamines bind to beta adrenergic receptors (β-AR) activating adenylate cyclase (AC) via stimulatory G-protein coupled receptors (Gs). This leads to a rise in the concentration of cyclic AMP (cAMP), which subsequently activates protein kinase A (PKA). PKA phosphorylates perilipin (PLIN) and hormone sensitive lipase (HSL) at multiple Serine (Ser) residues. Phosphorylation induces a conformational change in PLIN that releases comparative gene identification-58 (CGI-58) into the cytosol where it is free to co-localize with adipose triglyceride lipase (ATGL), activating it. Activation of ATGL enhances hydrolase activity of this enzyme leading to the initial breakdown of TG producing DAG and a FA. Simultaneous phosphorylation of HSL at $Ser_{569/660}$ and PLIN at Ser_{517} allow for the activation and translocation of HSL, with phosphorylated PLIN providing a docking site for increased access of HSL to the LD. HSL then catalyzes the catabolism of DAG producing monoacylglycerol (MAG) and an additional FA. The final step in the lipolytic cascade is the breakdown of MAG by MAG lipase liberating glycerol and the final FA. Conversely, catecholamines binding to alpha-2 adrenergic receptors (α_2-AR) exert an inhibitory effect on lipolysis by preventing activation of AC through stimulation of inhibitory G-protein coupled receptors (Gi).

2.3.1.1. Catecholamines

Under fasting and exercise conditions, adrenaline and noradrenaline are released due to increased activity of the sympathetic nervous system. Adrenaline is a hormone secreted by the adrenal medulla and reaches the adipose tissue by way of the circulation (48). Noradrenaline is a neurotransmitter released by sympathetic neurons innervating the WAT (48). These catecholamines bind to multiple α/β adrenergic receptor (AR) subtypes located in the adipocyte PM (49). There are three β-ARs currently described. β_1 and β_2 are the predominant isoforms regulating lipolysis in human WAT while β_3-AR is the main form responsible for lipolysis in rodents (49, 50). The binding of catecholamines to β-adrenergic receptors leads to the activation of AC via Gs (47, 51). This increases formation of cAMP by AC, which activates PKA initiating the phosphorylation of key regulatory proteins involved in the hydrolysis of TG including HSL and PLIN (46-48, 51). In addition, WAT contains α-AR, specifically the α_2 isoform (α_2-AR), which also bind catecholamines (49). Binding of catecholamines to the α_2-AR elicits an antilipolytic response by inhibiting AC via activation of Gi, thus preventing the production of cAMP (51). Catecholamines preferentially bind to α_2 -AR over β-AR, this dual adrenoceptor function in WAT may serve to prevent unrestrained lipolysis from occurring under resting conditions (52). The dual receptor control of lipolysis in adipocytes is a species-specific phenomenon, since α_2-ARs are present in human but not to a great extent in rat WAT (49).

2.3.1.2. Insulin

While β-AR stimulation is responsible for inducing lipolysis, counter regulation occurs primarily through the hormone insulin. Insulin is released by the pancreas in response to increased plasma glucose levels (i.e. post-prandially). Upon insulin binding to its receptor, a signaling cascade ensues leading to phosphorylation of Akt mediated by the activation of phosphoinsoitide 3 kinase (PI3K) (24). In turn, Akt phosphorylates phosphodiesterase 3B (PDE3B), which inactivates cAMP by converting it to 5'AMP (53). This inhibits lipolysis by preventing cAMP dependent activation of AC. Insulin has also been shown to inhibit lipolysis through an Akt-independent process. Researchers found that activating PI3K by insulin while blocking Akt lead to reduced phosphorylation of the lipid droplet protein PLIN, which lead to decreased stimulated lipolysis (54).

2.3.2. Molecular basis of lipolysis

Hydrolysis of adipocyte intracellular TG stores requires the orchestration of a number of lipases and lipid droplet associated proteins initiated by the β-AR signaling cascade. Ultimately, each ester bond attaching the 3 FAs to the glycerol backbone in a TG is cleaved sequentially by 3 major lipases: ATGL, HSL, and MAG lipase. TG catabolism is also regulated by the interaction of the rate limiting lipases ATGL and HSL with proteins present on the surface of the lipid droplet such as PLIN and CGI-58 (55).

2.3.2.1. ATGL

Originally, HSL was thought to be the primary lipase involved in neutral TG hydrolysis but research with HSL knockout models has shown that HSL is primarily responsible for the hydrolysis of DAG (56). More recently, ATGL has been credited for the initial FA release and formation of DAG as it was shown to have much higher specificity for TG compared to HSL (57). In addition, the enzyme has been identified specifically as a TG hydrolase, having 10-fold higher activity against TG versus DAG and no activity against other substrates (58). The role of ATGL in the initial step of TG catabolism has been confirmed by ATGL knock out models. Animals lacking this enzyme were shown to accumulate TG in multiple tissues while displaying severely blunted basal and stimulated lipolysis (59). Importantly, ATGL is regulated independently of HSL and is not a direct downstream target of PKA (58). ATGL is however under hormonal regulation through its activation by another protein, CGI-58 (60). It is postulated that under basal conditions, CGI-58 is co-localized with PLIN (58). Upon phosphorylation of PLIN through hormonal activation of the β-AR cascade, CGI-58 is able to dissociate from this PLIN and be released into the cytosol to activate ATGL (58). ATGL hydrolase activity has been shown to increase substantially (20-fold) when it is co-localized with CGI-58 (61). In addition, people with Chanarin-Dorfman Syndrome possessing mutated forms of CGI-58 display TG accumulation in multiple tissues also suggesting that CGI-58 is essential for ATGL activation (61).

2.3.2.2. HSL

HSL displays activity against a number of substrates including TG, DAG, MAG and cholesterol esters (62) and for a long time researchers believed it played the central role in TG breakdown. However, with the discovery of ATGL, it was found that HSL actually acts primarily as a DAG lipase with 11 and 5-10 fold higher activity against DAG versus TG and MAG, respectively (62, 63). Furthermore, animals lacking HSL show accumulation of DAG with normal T G levels, confirming that HSL is mainly responsible for DAG catabolism (56). Structural analysis of HSL revealed that the C-terminal catalytic domain houses the regulatory module containing all known phosphorylation sites of this lipase (62, 64). Of particular importance for hydrolytic activity of HSL is the phosphorylation of Serine residues 569 and 660 in the regulatory module, which have been shown through mutagenesis studies to be the major sites controlling enzymatic activity during lipolysis (65). The activation of these residues is under direct hormonal control via active PKA, but phosphorylation alone only increases catabolic activity moderately by approximately 2-fold (64). For full activity, the enzyme must translocate to the LD where it can better access lipid stores, a process that is mediated by the phosphorylation of the lipid droplet associated protein, PLIN (66). The phosphorylation and translocation of HSL in combination with activation of ATGL by CGI-58, results in a 100-fold increase in the hydrolysis of triglycerides (64). Another HSL phosphorylation site is the Serine 565 residue,

a target for active AMP-activated protein kinase (AMPK). Upon phosphorylation, Ser_{565} exerts an antilipolytic effect by preventing subsequent phosphorylation of the lipolytic Ser_{563} residue (41, 62). AMPK becomes activated due to an increased intracellular AMP:ATP ratio accompanied by an elevated rate of re-esterification that is proportional to the increased liberation of FAs (41). This activation of AMPK as a consequence of lipolysis may be an important regulatory mechanism preventing unrestrained mobilization of FAs beyond what is required. However, there is skepticism surrounding the antilipolytic role of Ser_{565} phosphorylation because the phosphorylation of Ser_{563} is not essential to stimulate lipolysis (62). Following the HSL mediated breakdown of DAG, the final step in the lipolytic cascade is the catabolism of MAG by the constitutively active MAG lipase, releasing the final FA and the glycerol molecule (47).

2.3.2.3. Perilipin

The adipocyte LD was initially thought to be an inert storage reservoir for excess energy substrate but is now known to be a dynamic organelle that plays an important part in the regulation of fat metabolism (67). A LD enzyme with a central role in adipose tissue lipolysis is PLIN. In the adipocyte, PLIN exists mainly as the A isoform and contains multiple Serine residues for phosphorylation by activated PKA (68). Under basal conditions, PLIN is found on the surface of the lipid droplet co-localized with CGI-58. In this un-phosphorylated state, PLIN is

thought to provide a protective barrier to the LD, preventing degradation of TG stores by intracellular lipases (68, 69). Upon phosphorylation of the Ser_{517} residue by PKA, PLIN mediates the action of both primary lipases involved in lipolysis (70). Phospho-PLIN (P-PLIN) induces a conformational alteration in the structure of the enzyme such that the co-localized protein CGI-58 is released into the cytosol to activate ATGL, facilitating a higher rate of TG breakdown (70). P-PLIN is also required for the full activity of HSL. The simultaneous phosphorylation of PLIN and HSL by PKA results in the translocation of HSL to the lipid droplet where PLIN provides a docking site so that HSL can access the lipid core (68). In addition to lipase regulation, phosphorylation of PLIN at Ser_{492} after prolonged stimulation of the β-AR pathway causes dispersal and fragmentation of the lipid droplet (69, 70). This increases the surface area for lipase access to lipid, effectively increasing lipolysis.

2.4. Regional differences in adipose tissue metabolism

While the molecular mechanisms underlying the regulation of metabolic processes such as lipolysis are maintained throughout all white adipocytes, the rate and contribution to total body metabolism of these processes depends on the regional location of the cells. Adipocytes are widely dispersed throughout different tissues but the majority are contained within two compartments of adipose tissue: subcutaneous (SC) or visceral (VC). SC adipose tissue is found

just beneath the skin, while VC depots are located deeper, within the thorax and abdominal cavity closely associated with internal organs (14). Research has shown that it is the expansion of VC and not the SC adipose tissues that is related to the development of metabolic abnormalities, in this context the VC compartment has been described as a pathogenic fat depot (71). VC fat has been demonstrated to have a more lipolytic phenotype than SC fat, displaying an increased rate of lipolysis due to an enhanced catecholamine response and reduced insulin sensitivity (9). In humans, an enhanced catecholamine response can be attributed to a 10-20 fold increased sensitivity with a 2 fold increased density of β-AR in VC versus SC adipose tissue (72). In addition, α_2 -AR sensitivity in human omental (VC) adipose tissue was shown to be 40 times lower than in gluteal (SC) adipocytes, thus exhibiting a reduced antilipolytic response to stimulated lipolysis in VC tissue (72). Since the VC depot is drained by the portal system, increased release of FAs by this tissue can directly affect liver metabolism and therefore contribute to metabolic dysfunction in this and other tissues (9, 10). Additionally, VC adipose tissue is less sensitive to the effects of the antilipolytic hormone insulin as compared to SC adipose tissue due to a reduced insulin receptor affinity and signal transduction (9). In contrast, the SC adipose depot exhibits a more lipogenic phenotype in which there are lower rates of FA mobilization due to a lower number of β-AR versus α_2 -AR (73). Also, adipocytes in the SC depot are more responsive to the antilipolytic effects of

insulin (9). The regional differences in adipose tissue metabolism are relevant for understanding the regulatory mechanisms leading to increase metabolic risk specific to the presence of VC fat.

2.5. Exercise training and WAT metabolism

Given the evidence that VC fat deposition increases the risk for metabolic dysfunction (9, 71, 72), preventing or reversing visceral adiposity has been the logical route for disease prevention. In this context, a well accepted recommendation has been to utilize exercise as a means to control adiposity. Furthermore, endurance training has been shown to increase the use of fat as a substrate for energy generation by increasing the oxidative capacity of skeletal muscles (2, 3). This alteration in itself could be sufficient to reduce adipose stores; however, chronic endurance training has been shown to increase the utilization of fat sources located within the muscle with a proportionate reduction in utilization of adipose tissue-derived NEFAs (2-4, 74). While a shift towards greater FA oxidation of intramuscular TG (IMTG) is apparent with chronic exercise, the accompanying training induced adaptations in adipose tissue metabolism remain unclear. Many sources report enhanced stimulated lipolysis in adipocytes from endurance trained subjects that is attributed to alterations in the lipolytic cascade (4-6, 75, 76). For example, work by Nomura and colleagues demonstrated an increase in PKA and HSL activity accompanying enhanced

stimulated lipolysis in the adipocytes of 9 week trained rats (75). Similarly, another investigation found that elevated adipose tissue lipolysis in swim-trained rats resulted from greater HSL sensitivity to adrenaline stimulation as well as depot specific increases in HSL protein content (77). Lipolysis has also been reported to be elevated at rest in trained individuals (78). In this study, elevated resting lipolysis was involved in increased TG-FA cycling, which was suggested to allow for redistribution of adipose FA stores to muscle, enhancing the potential for FA oxidation (78). Conversely, *in vivo* studies report either unchanged (79) or decreased rates of lipolysis after chronic endurance exercise (7). Martin and colleagues showed a reduced rate of appearance of glycerol and FA with an increase in total FA oxidation during exercise after a period of chronic training, suggesting that less substrate is being liberated from adipose tissue stores in the trained state during submaximal exercise (7).

While alterations in β-AR signaling have not been fully explored in the context of an observed decrease in lipolysis, other research has demonstrated that it may be the result of training induced reductions in catecholamine release (80). Research has shown that a reduced hormonal response to submaximal exercise is an adaptation that occurs relatively early on in the endurance training process (80). In addition to changes in hormonal release, other work has looked at training induced adaptations to hormonal sensitivity. Suda and colleagues found that while isolated adipocytes had an enhanced lipolytic response to AR

agonists after training, there was also increased responsiveness to the antilipolytic hormone insulin mediated through increased activity of phosphodiesterase (76). This may be one explanation for why a number of studies utilizing isolated adipocytes in the absence of insulin report enhanced lipolysis while *in vivo* work, which cannot eliminate the presence and effect of insulin, has shown reduced lipolysis after training. The inconsistencies found in the literature may also be due to differences in methodology related to exercise protocol or adipocyte preparation.

With respect to lipogenesis, Askew and colleagues showed that chronic exercise training did not alter resting rates of TG synthesis but rather suppressed rates only during acute exhaustive exercise (81). It was postulated that this effect may contribute to carbohydrate sparing that allows for greater exercise capacity in the trained state (81). In addition, other work described reduced G6PD activity in adipose tissue affecting TG synthesis only immediately after an exercise bout (82). In contrast, other studies have reported alterations in the adipocytes' ability to synthesize TG from glucose attributed to an effect of long term exercise training (83). Aside from debatable changes in TG storage with chronic exercise, others have found that reductions in adiposity imposed by exercise are mediated by a training induced inhibition of adipogenesis (84).

Overall, the multifaceted role adipose tissue plays in physiology has systemic implications on maintaining optimal metabolic functioning. As such,

alterations in the regulation of this organ can lead to metabolic disease (13) that appears to be, at least in part, ameliorated through the use of exercise therapy. Understanding how WAT metabolism is affected by chronic endurance training may be of great therapeutic relevance for the maintenance of health or correction of disease.

3. OBJECTIVES

Given the lack of conclusive findings in this research field, the aim of the current study is to determine the effects of chronic endurance exercise training on adipose tissue metabolism. The specific objectives of this investigation are:

1. To determine the effects of long-term endurance exercise training on the rate of basal and insulin-stimulated lipid synthesis in VC and SC adipocytes.
2. To determine the effects of chronic endurance training on basal and catecholamine-stimulated lipolysis in VC and SC adipocytes.
3. To determine the molecular mechanisms underlying the training-induced alterations in TG metabolism in VC and SC adipose tissues.

HYPOTHESES

Based on the fact that chronic endurance training increases energy expenditure and improves the ability of skeletal muscle cells to utilize their intracellular TG stores for energy production, it is hypothesized that:

1. Lipogenesis will be reduced in VC and SC adipocytes in response to chronic exercise training.
2. Basal and catecholamine-induced lipolysis in VC and SC adipocytes will be reduced by chronic endurance training.
3. Alterations in lipolysis will be caused by distinct regulation of ATGL, HSL, CGI-58 and PLIN activity under conditions of chronic exercise training.

4. MATERIALS AND METHODS

4.1. Reagents

Isoproterenol, forskolin, FA-free bovine serum albumine (BSA), protease and phosphatase inhibitor cocktail, and free glycerol determination kit were obtained from Sigma. D-[U-^{14}C] glucose was from Amersham. Specific antibodies against p-HSL, HSL, ATGL and β-actin were from Cell Signaling Technology, Inc. (Beverly, MA, USA), PLIN antibody was purchased from American Research Products, Inc (Waltham, MA, USA), p-PLIN antibody from Vala Sciences (San Diego, CA, USA), and β_3-AR antibody from BioVision (Milpitas, CA, USA). All other materials used were of the best quality available.

4.2. Animals

Male albino rats (Wistar strain) weighing approximately 140-150g (upon commencement of the endurance training protocol) were housed at 22°C on a 12/12-hr light/dark cycle and fed standard laboratory chow *ad libitum* throughout the entire study. York University Animal Care Ethics Committee approved the experimental protocol.

4.3. Acclimatization, selection and exercise training process

Upon arrival, animals were permitted to acclimatize for one week followed by a one week selection process. For selection, animals were trained for up to 23

minutes per day for 4 consecutive days at a progressively increasing intensity (Appendix A). Inclusion into the study was based on the demonstration of willingness to run continuously beyond a minimum speed of 18 m/min. This approach has been extensively and successfully used previously in our lab. This was done to ensure that all animals participating were capable of completing the endurance exercise training protocol. The selected animals were then randomly divided into sedentary and exercise groups. The rats were endurance exercise-trained by running on treadmills (AccuScan Instruments, Inc., Columbus, OH, USA). Exercise was performed 5 days per week (Appendix B) with the duration of training gradually increasing to 65 minutes per session by the end of the second week. Intensity was progressively increased throughout each training session and over the course of the 6 week training period. Peak VO_2 was determined every 3 weeks by tests on treadmills connected to the Comprehensive Laboratory Animal Monitoring System (C.L.A.M.S.; Columbus Instruments, Inc., Columbus, OH, USA). This allowed for assessment of the training protocol efficacy and adjustment of training intensity as the animals' aerobic capacity improved.

4.4. Plasma analyses

Blood was collected by saphenous vein bleeding during the morning hours at rest and following sub-maximal exercise prior to initiation of the exercise

training protocol (week 0) and following the completion of training weeks 3 and 6. Submaximal intensity was determined by calculations based on the maximal speed animals achieved in a graded treadmill test. For testing of maximal speed, animals warmed up for 5 minutes at a speed of 10 m/min followed by an increase in speed at a rate of 1 m/min every minute until the animal stopped running or could no longer keep up with the treadmill. The interval prior to the speed at which animals could no longer run was taken as their maximum. Animals were not fasted prior to the collection of blood samples. After collection, samples were centrifuged for 5 min at 4°C and plasma stored at -80°C for subsequent analysis of NEFAs (Wako HR Series NEFA-HR, VWR, Radnor, PA, USA), catecholamines (2-CAT ELISA, Rocky Mountain Diagnostics, Inc., Colorado Springs, CO, USA), corticosterone (Rat Corticosterone ^{3}H Kit, MP Biomedicals, Solon, CO, USA) and lactate (Lactate Reagent, Trinity Biotech, Bray, Ireland) concentrations.

4.5. Experimental protocol and adipocyte isolation

In order to avoid the acute effect of exercise and assess the chronic effects of endurance training on adipose tissue metabolism, the animals were anesthetized (0.4 mg ketamine and 8 mg xylazine per 100 g body weight) 48 hours after the last training session. Previous experimentation has established 48 hours to be a sufficient amount of time for the reversal of metabolic changes induced by an acute exercise bout (85, 86). Inguinal and epididymal fat pads, the

former used as representative of SC and the latter of VC fat, were then extracted and weighed. A sample of each pad was taken to assess adipose tissue morphology and the remaining tissue used for adipocyte isolation as described previously (87-89). Briefly, the adipose tissue was finely minced in Krebs-Ringer Buffer (0.154M NaCl, 0.154M KCl, 0.11M $CaCl_2$, 0.154M $MgSO_4$, 0.154M KH_2PO_4, 0.154M $NaHCO_3$, pH 7.4) with 5.5mM glucose and 30 mM HEPES (KRBH) supplemented with type II collagenase (0.5 mg/ml). Adipocytes were then incubated at 37°C with gentle agitation (120 orbital strokes/min) for approximately 25-30 min. Digested tissue was strained using a nylon mesh and cells were transferred to 50 ml tubes to be carefully washed three times and resuspended in KRBH containing 3.5% BSA (KRBH-3.5% BSA). The height of lipocrit per 300 µl of cell suspension was determined and the average cell diameter was measured using a light microscope equipped with a micrometer ruler (90). This was done to determine the number of cells present in a given quantity of cell suspension (90) in order to ensure equal distribution of cells for each condition in the subsequent assays.

4.6. Assessment of adipose tissue morphology

Morphological analysis was performed using light microscopy as described by Gaidhu and colleagues with alterations (89). Upon extraction of the fat pads, a small sample (~100mg) was removed and fixed in 4%

paraformaldehyde, 0.1 M phosphate buffer solution (pH 7.4) for 24 hours at room temperature. After fixation, tissue samples were washed 3 times and stored in 70% ethanol. Samples were subsequently sent to the Toronto Centre for Phenogenomics (Toronto, ON, CA) where they were embedded in paraffin blocks, sectioned and processed for hemotoxylin and eosin (H&E) staining. Stained samples were viewed using a Nikon Eclipse TiE inverted microscope (Nikon Canada, Mississauga, ON, CA) under 20x magnification. Average adipocyte size was calculated by measuring the area of all adipocytes (minimum of 100) present in randomly selected fields of view. This was done to prevent the biased selection of cells for measurement. Area was determined by NIS-elements basic research imaging software (Nikon Canada, Mississauga, ON, CA) and images were captured with a digital Nikon DS-QI1Mc camera (Nikon Canada, Mississauga, ON, CA).

4.7. Determination of lipolysis

Lipolysis was measured by incubating adipocytes (2.5 x 10^5 cells) in the absence or presence of isoproterenol (10 μM), or forskolin (10 μM) in triplicate for 75 min at 37°C with gentle agitation (80 orbital strokes/min.). These agents were utilized in order to stimulate lipolysis by different mechanisms. Isoproterenol acts as a β-adrenergic agonist (91) while forskolin stimulates lipolysis by bypassing adrenergic receptors (92), activating adenylate cyclase directly (93). All

concentrations of lipolytic agents used in this study have been shown through previous experiments to be effective at stimulating lipolysis (41, 87, 88). After incubation, a 200 µl aliquot of media was taken and the glycerol concentration determined using a free glycerol determination kit (Sigma).

4.8. Incorporation of [U-^{14}C]D-glucose into TGs

As an indication of lipogenesis, glucose incorporation into TG was determined by incubating 0.5 ml cell suspension (~2.5x10^5 isolated adipocytes) in 0.5 ml KRBH-3.5% BSA containing D-[U-^{14}C] glucose (0.5 µCi/ml) with and without insulin (50 nM) for 1 hour at 37°C. Cells were subsequently lysed by the addition of 62.5 µl of H_2SO_4 (5N) terminating the processing of glucose into TG by the cells. Lipids were then extracted using Dole's Reagent (40:10:1 isopropylol:heptane: H_2SO_4 1N) and heptane to determine radioactivity in the total lipid fraction by radioactive counter (94).

4.9. Determination of protein content and phosphorylation

To prepare tissue homogenates for analysis, fat depots (EPI and ING) were excised and immediately frozen in liquid nitrogen. Tissues were later homogenized in lysis buffer containing 135 mM NaCl, 1mM $MgCl_2$, 2.7 mM KCl, 20 mM Tris (pH 8.0), protease and phosphatase inhibitors (0.5 mM Na_3VO_4, 0.2 mM PMSF, 1 mM pepstatin, protease and phosphatase inhibitor cocktail), 1%

Triton, and 10% glycerol. Homogenates were centrifuged (20 min, 13200 rpm) and the infranatant containing proteins of interest was collected.

Adipocyte lysates were also prepared in a similar manner. Isolated adipocytes were incubated in the presence or absence of isoproterenol (10 μM) or forskolin (10 μM) for 30 minutes. After the incubation period, the cells were gently centrifuged (30 seconds, 1000 rpm) and the buffer containing lipolytic agents removed. Adipocytes were subsequently lysed by vortexing for 1 min in the buffer described above. Samples were centrifuged (20 min, 13200 rpm) and the infranatant containing proteins of interest collected. Protein concentrations of the tissue homogenates and adipocyte lysates were determined by the Bradford method (95). Final samples were diluted 1:1 (v/v) with 2x laemmli sample buffer, then subjected to SDS-PAGE before being transferred to polyvinylidene diflouride membranes (Bio-Rad). Membranes were subsequently probed with antibodies specific for phospho-HSL Ser_{660}, Ser_{565}, and Ser_{563} to detect phosphorylation of HSL, as well as phospho-PLIN Ser_{517} to determine phosporylation of PLIN. Total levels of HSL, PLIN, ATGL, CGI-58 and β_3-AR were all evaluated similarly. All antibodies were diluted 1:1000 with the exception of phospho-PLIN (1:5000), PLIN (1:2000), CGI-58 (1:50,000) and β_3-AR (1:300). β-actin was used to confirm equal loading of samples.

4.10. Statistical analysis

Statistical significance was assessed by two-way analysis of variance (ANOVA) or two-way repeated-measures (RM) ANOVA with Bonferroni post-hoc test. Comparisons were also made using unpaired t-test. Statistical significance was set at $P<0.05$.

5. RESULTS

5.1. Effects of chronic endurance training on peak VO_2 and adiposity

Peak VO_2 was 19% greater in the exercise group after 6 weeks of treadmill running compared to the sedentary animals (3603 ± 189.90 ml/kg/hr vs. 4289 ± 106.20 ml/kg/hr for sedentary and exercise animals, respectively) (Fig. 3). There was no significant differences in peak VO_2 between groups at week 0 (5297 ± 352.30 ml/kg/hr in sedentary vs. 5392 ± 521.10 ml/kg/hr in exercise groups, not shown). There was an observed reduction in peak VO_2 from baseline to week 6 in both groups because the rate of change in VO_2 peak was not proportional to the more rapid rate of weight gain in the animals. Since VO_2 peak was normalized for body weight, the value decreased as the animals became larger.

To further characterize the training model, plasma corticosterone concentrations were assessed in samples obtained under resting conditions to ensure observed effects were not due to chronically elevated stress levels imposed by forced treadmill running. Corticosterone did not differ between groups at any measured time point (165.22 ± 48.13 ng/ml vs. 133.00 ± 71.65 ng/ml in week 0, 3.29 ± 0.52 ng/ml vs. 3.88 ± 0.58 ng/ml in week 3 and 18.23 ± 5.58 ng/ml vs. 20.27 ± 6.69 ng/ml in week 6 for sedentary and exercise animals, respectively) (Fig. 4). Furthermore, once animals were accustomed to handling (weeks 3 and 6), plasma concentrations of corticosterone were minimal and

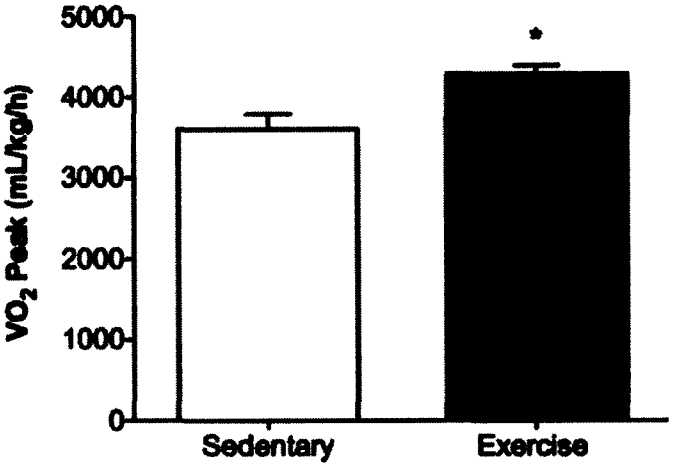

Figure 3. Peak VO_2 in sedentary and endurance exercise groups after six weeks of training by treadmill running. Values are expressed as mean ± SEM. N=10-14, *P<0.05 vs. sedentary (unpaired t-test).

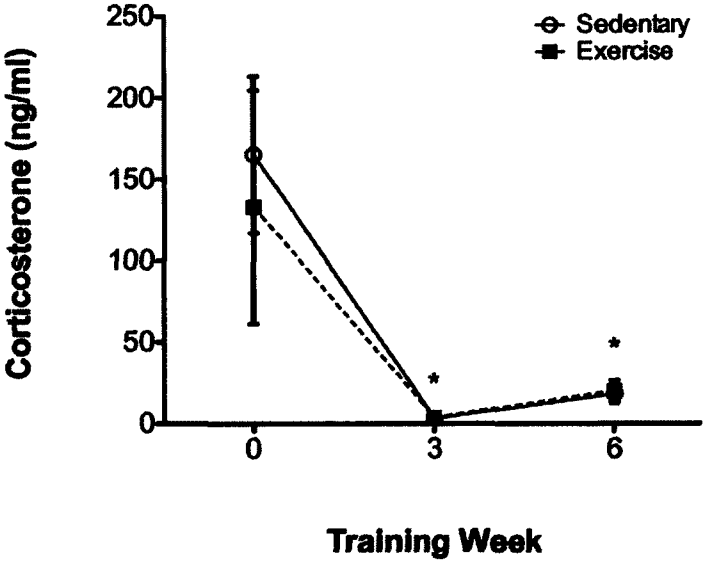

Figure 4. Plasma corticosterone concentration (ng/ml) from sedentary and exercise animals at rest during weeks 0, 3 and 6 of the exercise training protocol. N=7-8, *P<0.05 vs. respective week 0 (2-way ANOVA).

significantly less than levels at baseline. Corticosterone concentrations in weeks 3 and 6 were found to be similar to levels obtained in previous studies from resting male rats during the morning hours (96). This suggests that chronic elevation of stress was not induced by the forced running protocol.

The enhanced peak VO_2 observed in the exercise group during the last week of the training protocol was accompanied by a significantly lower (5%) body weight (399.10 ± 17.50 g in sedentary vs. 380.40 ± 21.80 g in exercise) (Fig. 5, A). This alteration in body weight could not be attributed to differences in food intake between the groups (Fig. 5, B). Changes in body weight were associated with alterations in adiposity (Fig. 6, A and B) as the EPI and ING adipose tissue depots were 18% and 10% smaller in trained animals versus sedentary controls (1.30 ± 0.03 g vs. 1.06 ± 0.03 g and 1.76 ± 0.04 g vs. 1.58 ± 0.03 g in EPI and ING depots, respectively).

5.2. Effects of chronic endurance training on adipose tissue morphology

While exercise training lead to a reduction in adiposity, the reduction cannot be fully accounted for by alterations in cell size (Fig. 7). Analysis of H&E stained adipose tissue revealed no significant differences in adipocyte size between sedentary and exercised animals (1037 ± 46.83 μm^2 vs. 955.8 ± 31.04 μm^2 in EPI and 1299 ± 24.22 μm^2 vs. 1223 ± 266.0 μm^2 in ING depots of sedentary and exercise animals, respectively). These results were confirmed by

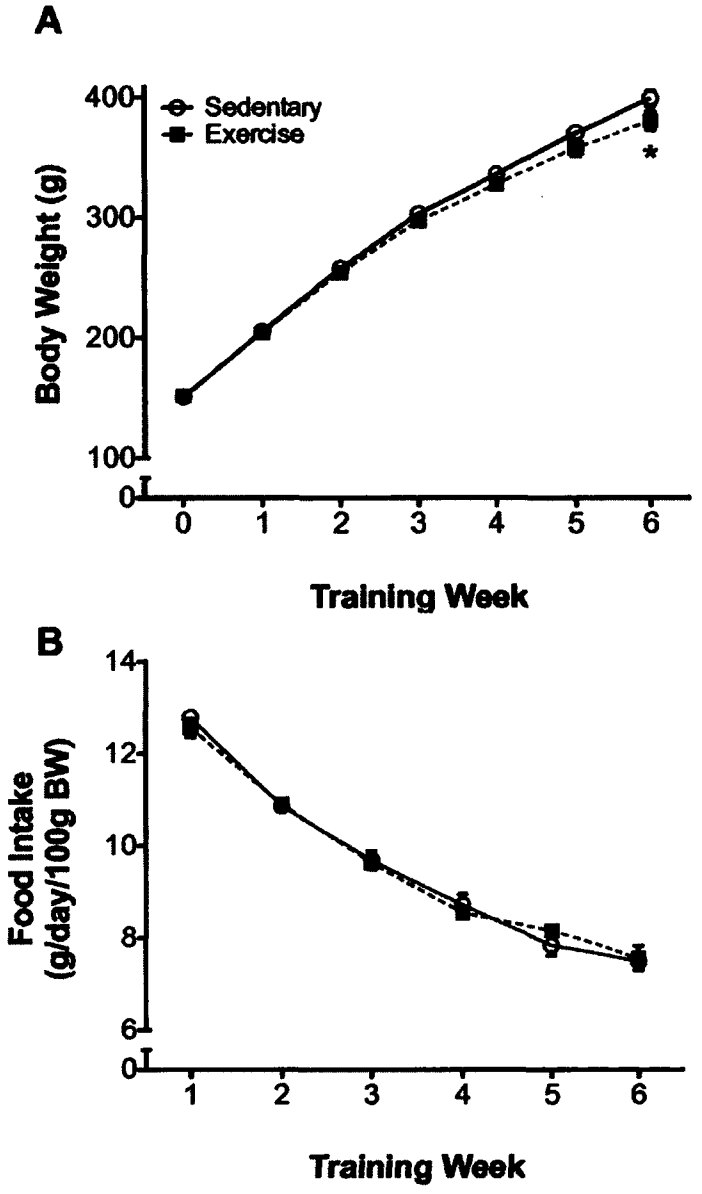

Figure. 5. Alterations in body weight (g) (A) could not be accounted for by differences in food intake (g/day/100g BW) (B) in sedentary and exercise-trained rats over 6 weeks of endurance exercise training. Body weight was taken on the last day of each week and food intake is the average daily intake calculated over the specified week of training normalized for body weight. Values are expressed as group mean ± SEM. N=8, *P<0.05 vs. sedentary (2-way ANOVA).

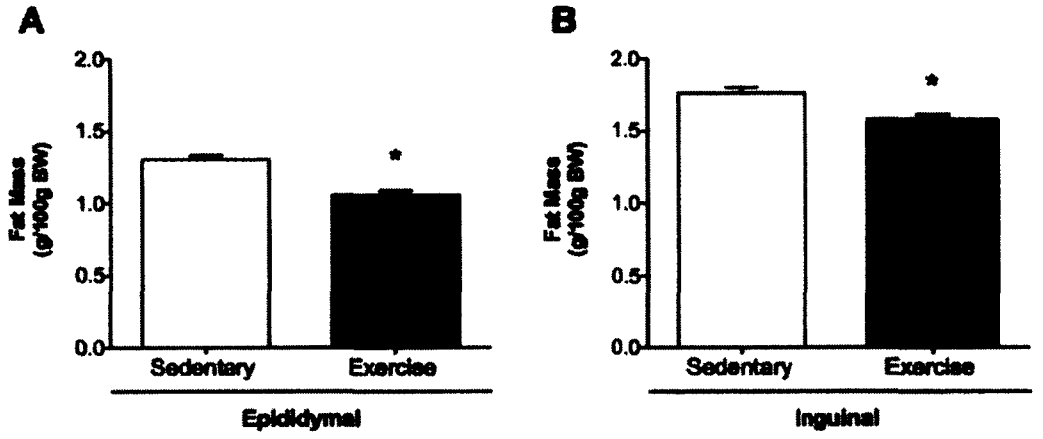

Figure. 6. EPI (A) and ING (B) fat mass (g/100g BW) of sedentary and 6 week endurance trained rats. Values are expressed as mean ± SEM. N=34-38, *P<0.05 vs. sedentary (unpaired t-test).

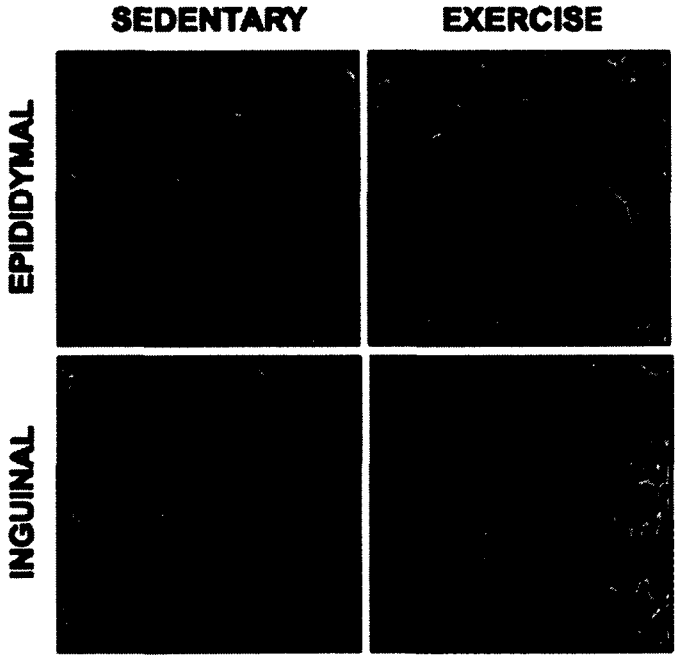

Figure 7. Representative H&E stained sections of adipose tissue (10x magnification) from EPI and ING depots in sedentary and exercise animals. No difference in average adipocyte area was noted, N=3.

measurements of isolated adipocyte diameter. No significant difference was noted in ING adipocyte diameter between sedentary and exercise groups (69.22 ± 1.28 μm in sedentary vs. 66.60 ± 1.56 μm in exercise groups) (Fig. 8, B). There was however an 8% reduction in the diameter of EPI adipocytes with exercise training (78.65 ± 1.49 μm vs. 72.46 ± 1.68 μm in sedentary and exercise groups, respectively) (Fig. 8, A). The disparity between measurements taken from tissue sections versus isolated adipocytes may be due to a small sample size of H&E stained tissue. In either case, the results support a small to insignificant difference in EPI adipocyte size with training that cannot fully account for the observed reduction in adiposity.

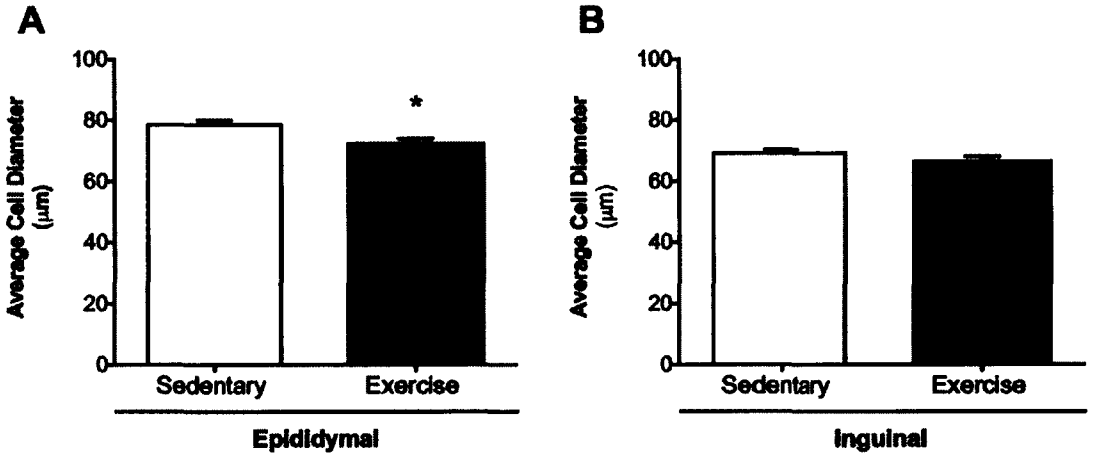

Figure 8. Average cell diameter as determined by the measurement of 100 EPI (A) and ING (B) isolated adipocytes per experiment in each group. Values are expressed as means ± SEM. N=23, *P<0.05 vs. sedentary (unpaired t-test).

5.3. Effects of chronic training on the response to submaximal exercise

Animals ran for 20 minutes at 70-80% of their maximal speed followed by a blood collection. While groups did not differ in submaximal running speed during baseline (29.40 ± 1.83 m/min in sedentary and 33.04 ± 2.38 m/min in exercise-trained), exercise animals ran at a 1.3- and 1.4-fold higher speed compared to sedentary animals in weeks 3 and 6, respectively to account for an increased exercise capacity that accompanied training (28.03 ± 2.25 m/min vs. 36.30 ± 1.63 m/min in week 3 and 25.39 ± 2.92 m/min vs. 35.44 ± 3.06 m/min in week 6) (Fig. 9, A). This was done to ensure that animals were running at a equivalent intensity prior to blood collection. To confirm that the intensity was comparable between groups, plasma lactate was measured and found to be similar (3.58 ± 0.22 mM, 3.98 ± 0.32 mM, 3.92 ± 0.30 mM in sedentary and 4.15 ± 0.32 mM, 3.08 ± 0.36 mM, 3.71 ± 0.50 mM in exercise-trained animals during weeks 0, 3 and 6 of training, respectively). In agreement with this finding, plasma adrenaline was not altered with training following the submaximal exercise bout (881.11 ± 121.21 pg/ml vs. 1056.98 ± 148.58 pg/ml in week 0, 1368.61 ± 201.10 pg/ml vs. 1290.62 ± 118.17 pg/ml in week 3, and 1282.21 ± 121.65 pg/ml vs. 1323.17 ± 221.03 pg/mL in week 6 in sedentary and exercised animals, respectively) (Fig. 9, C). Noradrenaline was also not different between groups (1315.05 ± 139.58 pg/ml, 1052.59 ± 57.16 pg/ml, 881.11 ± 73.66 pg/ml in sedentary and 1174.42 ± 206.74 pg/ml, 1242.12 ± 208.03 pg/ml, 1005.09 ±

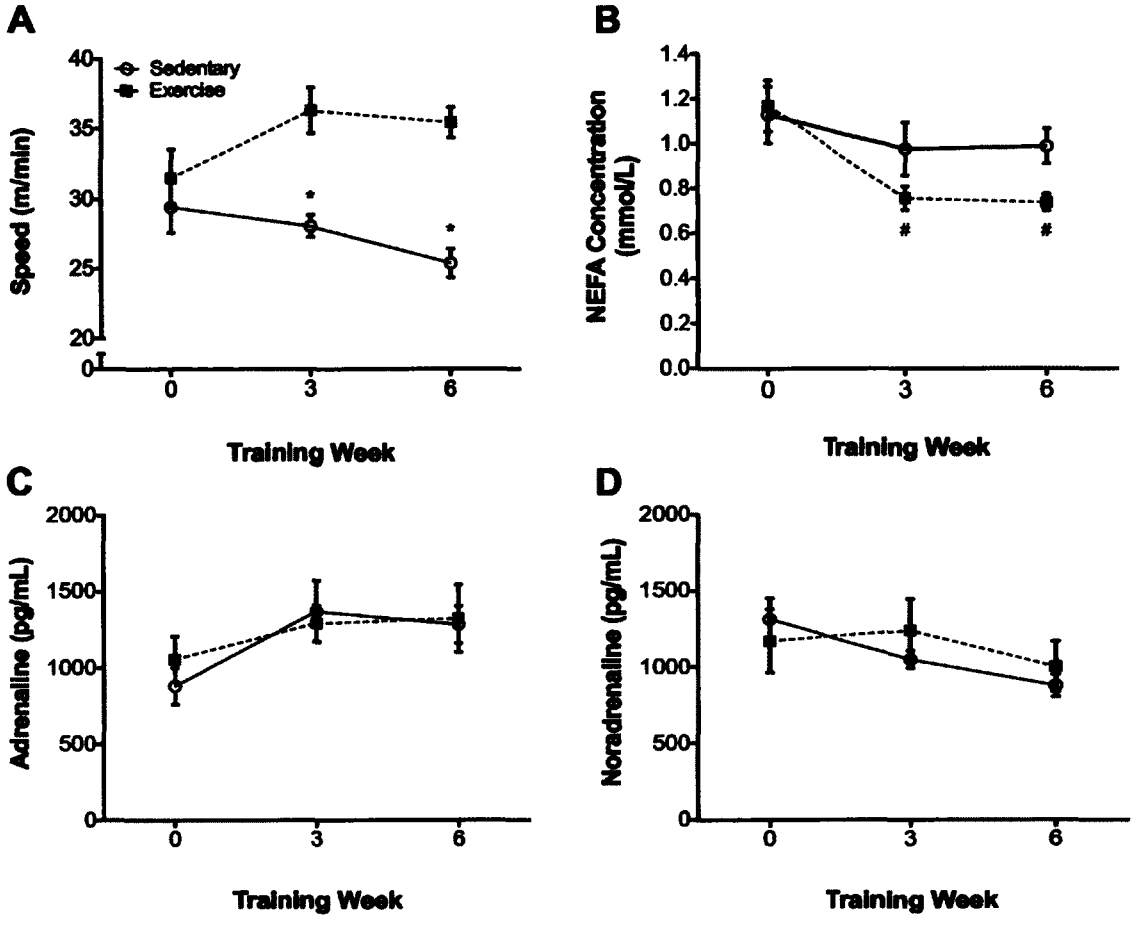

Figure 9. Profile of running speed (70-80% of maximal speed) (A) and plasma NEFA (B), adrenaline (C) and noradrenaline (D) concentration for a 25 minute exercise bout. Values are expressed as mean ± SEM. N=7-8, *P<0.05 vs. SED at respective time point, #P<0.05 vs. respective week 0 (2-way RM ANOVA).

165.73 pg/ml in exercise groups during weeks 0, 3 and 6, respectively) (Fig 9, D). With evidence that animals were running at a similar intensity, plasma NEFA concentrations were compared. There was a significant reduction from baseline to weeks 3 and 6 in trained animals following exercise but no difference was found between groups at any time point (1.13 ± 0.13 mM, 0.98 ± 0.12 mM, 0.99 ±

0.08 mM in sedentary and 1.17 ± 0.11 mM, 0.76 ± 0.05 mM, 0.74 ± 0.04 mM in exercise groups during weeks 0, 3 and 6, respectively) (Fig. 9, B). Since these parameters were evaluated in the plasma, several factors related to other metabolic systems may have contributed to the observed effects. In this context, an *in vitro* model of adipocyte metabolism was assessed.

5.4. Effects of chronic training on lipolysis in isolated adipocytes

With unaltered adipose tissue lipolysis as indicated by similar plasma NEFA concentrations following submaximal exercise, the rate of stimulated lipolysis in isolated adipocytes was measured. Lipolytic rate was assessed through the determination of glycerol release over a 75 minute incubation period with a number of agents. Under non-stimulated (basal) conditions, the rate of glycerol release in EPI and ING adipocytes did not differ between groups (0.95 ± 0.23 and 0.57 ± 0.19 nmol/75 min/2.5x10^5 cells in EPI and 0.75 ± 0.28 and 0.36 ± 0.15 nmol/75 min/2.5x10^5 cells in ING for sedentary and exercise groups, respectively). With isoproterenol (10μM) stimulation, sedentary animals had an expected 52- and 51-fold increase in glycerol release in EPI and ING adipocytes (49.42 ± 3.82 nmol/75 min/2.5x10^5 cells in EPI and 38.51 ± 3.77 nmol/75 min/ 2.5x10^5 cells in ING), respectively. Similarly, in exercise trained rats there was a 64- (EPI) and 79-fold (ING) increase in lipolysis over basal values with isoproterenol stimulation (36.37 ± 3.20 and 28.59 ± 2.89 nmol/75 min/2.5x10^5

cells in EPI and ING adipocytes, respectively). This equated to a 26% reduction in isoproterenol stimulated lipolysis in both EPI and ING adipocytes from exercise trained animals as compared to their sedentary counterparts (Fig. 10, A and B). In addition, sedentary animals demonstrated significantly elevated lipolysis in EPI versus ING cells with isoproterenol stimulation by 1.3-fold. Similarly, a 1.3-fold elevation in EPI compared to ING lipolysis was noted in exercise trained animals although the difference did not reach significance (P=0.07).

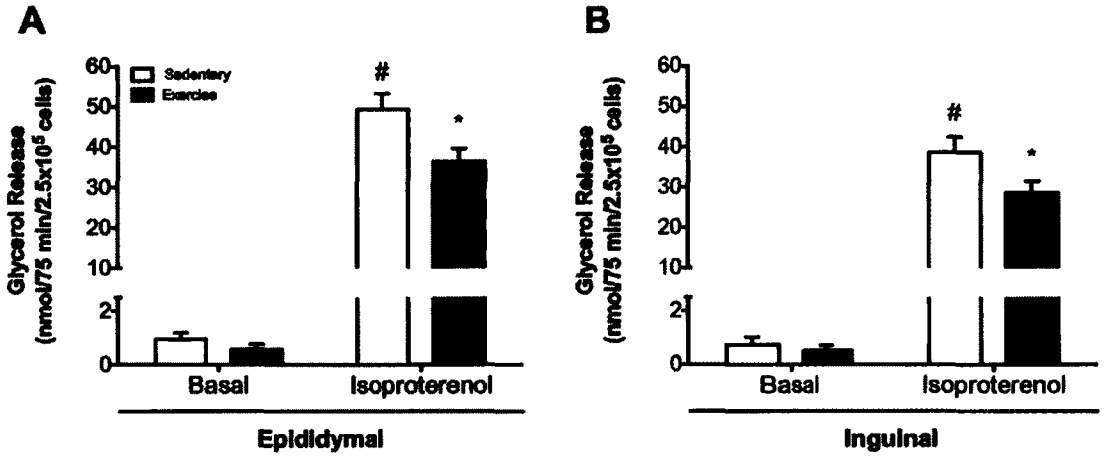

Figure 10. Glycerol release (nmol/75min/5x10^5 cells) by EPI (A) and ING (B) adipocytes from sedentary and endurance exercise-trained rats under basal (vehicle) and isoproterenol (10 μm) stimulated conditions. Measurements were taken in triplicate and values expressed as means ± SEM. N=12-13, *P<0.05, #P<0.05 vs basal condition (2-way ANOVA).

With a significant training induced reduction in lipolysis stimulated by a β-AR agonist, glycerol release was next assessed after adipocytes were incubated with forskolin (10 μm). Given that forskolin stimulates lipolysis bypassing the β-

AR, this was done to determine if a blunted lipolytic response was mediated by a post-receptor mechanism. As expected with forskolin stimulation, adipocytes from sedentary animals had a 21- and 17-fold increase in glycerol release per 75 minute incubation in EPI and ING adipocytes (from 1.34 ± 0.19 to 28.12 ± 5.98 nmol/75 min/2.5x10^5 cells in EPI and 1.57 ± 0.50 to 26.26 ± 7.32 nmol/75 min/ 2.5x10^5 cells in ING), respectively. In adipocytes from exercise trained rats there was a 19-fold (EPI) and 13-fold (ING) increase in lipolysis over basal values with forskolin stimulation (from 0.73 ± 0.16 to 13.79 ± 2.06 nmol/75 min/2.5x10^5 cells and from 1.00 ± 0.42 to 13.38 ± 3.43 nmol/75 min/2.5x10^5 cells in EPI and ING adipocytes, respectively). These values demonstrate a training induced reduction in lipolysis with forskolin stimulation by 51% in EPI adipocytes and a trend towards a 49% reduction in ING adipocytes (Fig. 11, A and B). This indicates that blunted lipolysis is, at least in part, mediated by a post-receptor mechanism in EPI adipocytes. There was no difference in lipolysis with forskolin stimulation between depots within a given group.

5.5. Assessment of molecular mechanisms regulating adipocyte lipolysis

With a reduction in forskolin stimulated lipolysis in exercise EPI adipocytes, various enzymes involved in the regulation of lipolysis were assessed for phosphorylation and/or content in tissue homogenates and adipocyte lysates. In EPI tissue, there was a 3.6- and 2.2-fold increase in ATGL

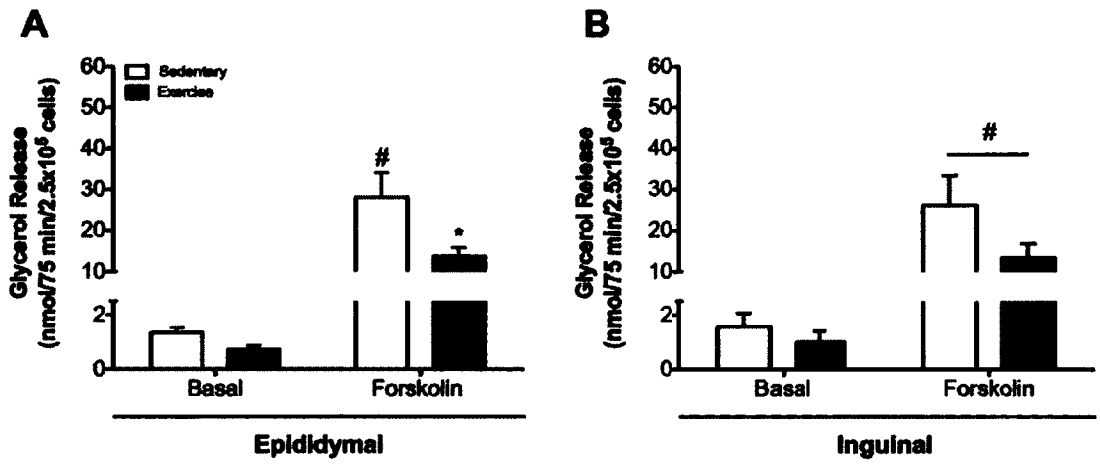

Figure 11. Glycerol release (nmol/75min/2.5x10^5 cells) by EPI (A) and ING (B) adipocytes from sedentary and endurance exercise-trained rats under basal (vehicle) and forskolin (10 µm) stimulated conditions. Measurements were taken in triplicate and values expressed as means ± SEM. N=4, *P<0.05, #P<0.05 vs. basal condition (2-way ANOVA).

and CGI-58 content, respectively (Fig. 12). This result would seem to indicate an elevated rate of lipolysis given that ATGL is a major lipase involved in TAG catabolism. Therefore, PLIN, an enzyme involved in the regulation of ATGL activity was assessed for content and phosphorylation at Ser_{517} in stimulated lysates (Fig. 13). There was no difference noted between PLIN phosphorylation of EPI adipocytes under isoproterenol or forskolin stimulated conditions. No changes in the content of ATGL or CGI-58 were noted in ING tissue homogenates nor was there a significant change in the phosphorylation of PLIN between groups under any condition in this depot.

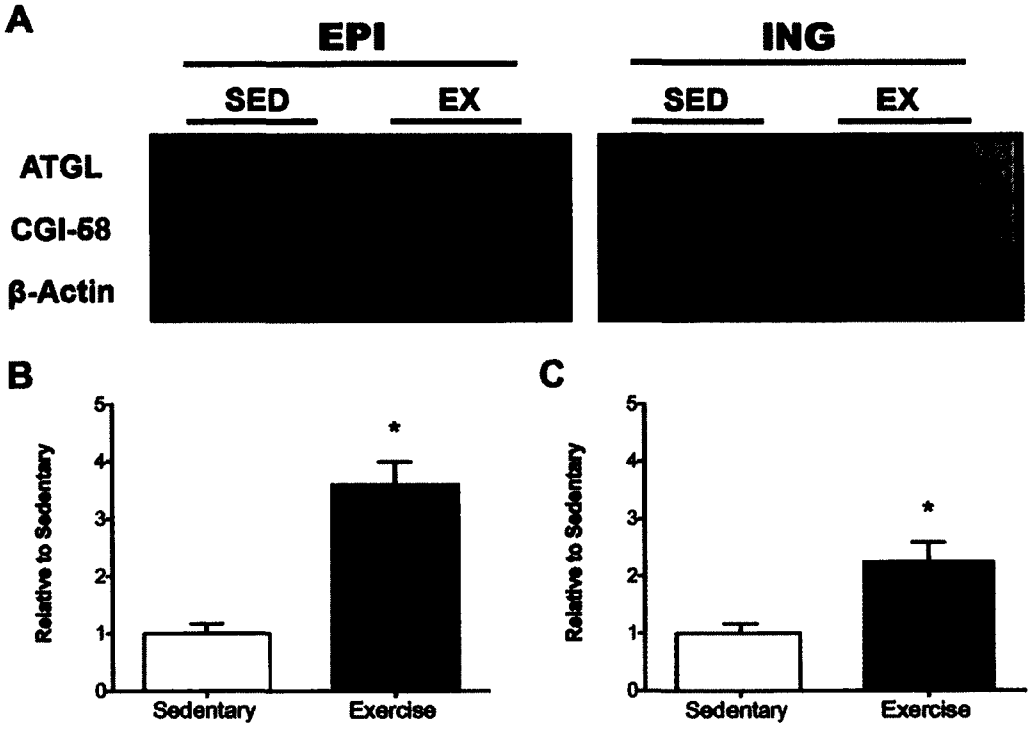

Figure 12. Representative western blots of ATGL and CGI-58 content in EPI and ING tissue homogenates from SED and EX animals (A). Quantification of ATGL (B) and CGI-58 (C) western blots from EPI homogenates corrected for β-Actin. Values are expressed relative to sedentary. N=7-9, *P<0.05 (unpaired t-test).

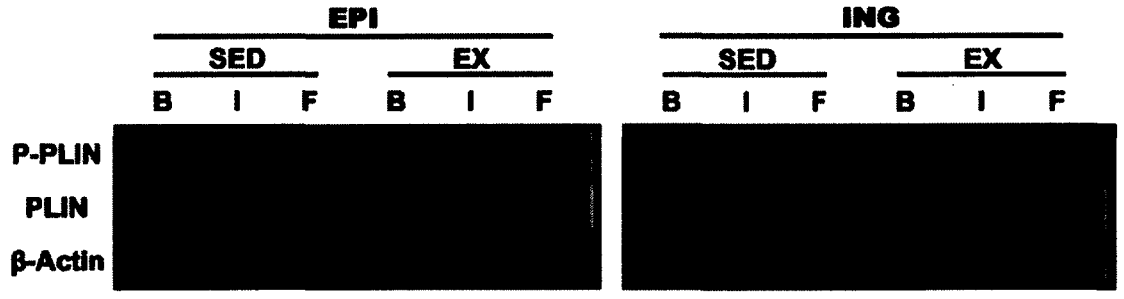

Figure 13. Representative western blot of total and phosphorylated (Ser_{517}) PLIN in adipocyte lysates under basal (B), isoproterenol (I) (10μM), and forskolin (F) (10μM) stimulated conditions in SED and EX rats. β-actin was used as a loading control, N=3.

The primary DAG hydrolase HSL was also evaluated for content and phosphorylation at the lipolytic Ser_{563} and Ser_{660} residues as well as the antilipolytic Ser_{565} residue in adipocyte lysates. As expected, there was a marked increase in phosphorylation of HSL at Ser_{563} and Ser_{660} over respective basal conditions with isoproterenol stimulation and no change in phosphorylation of HSL at Ser_{565}. No significant difference in content or phosphorylation of HSL in adipocyte lysates was found between sedentary and exercise groups (Fig. 14). Similarly, forskolin stimulation did not significantly alter phosphorylation of HSL at Ser_{565} but showed a marked increase in phosphorylation of Ser_{563} and Ser_{660} residues compared to respective basal conditions. There was no consistent difference in phosphorylation of any measured residue between sedentary and exercise animals in either depot (Fig. 15).

5.6. Glucose incorporation into TG

Since there was significantly less adiposity among exercise trained animals but a reduction in stimulated TG breakdown by adipocytes, the rate of lipogenesis was assessed to explore a potential explanation for the decrease in fat mass. TG synthesis was measured by evaluating the rate of glucose incorporation into TG in EPI and ING adipocytes after a 1 hour incubation in the presence and absence of insulin. After 6 weeks of endurance training, the incorporation of glucose into TG in EPI cells was reduced by 55% in basal and

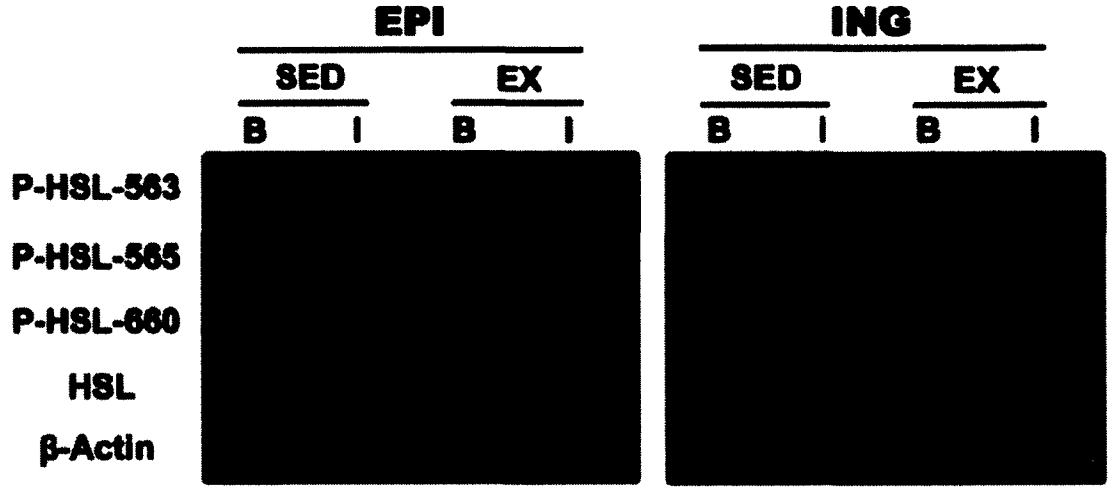

Figure 14. Representative western blots of HSL content and phosphorylation at Ser_{563}, Ser_{565} and Ser_{660} in adipocyte lysates from sedentary (SED) and exercise (EX) animals under basal (B) and isoproterenol (I) stimulation (10μM). β-actin was used as a loading control, N=4-5.

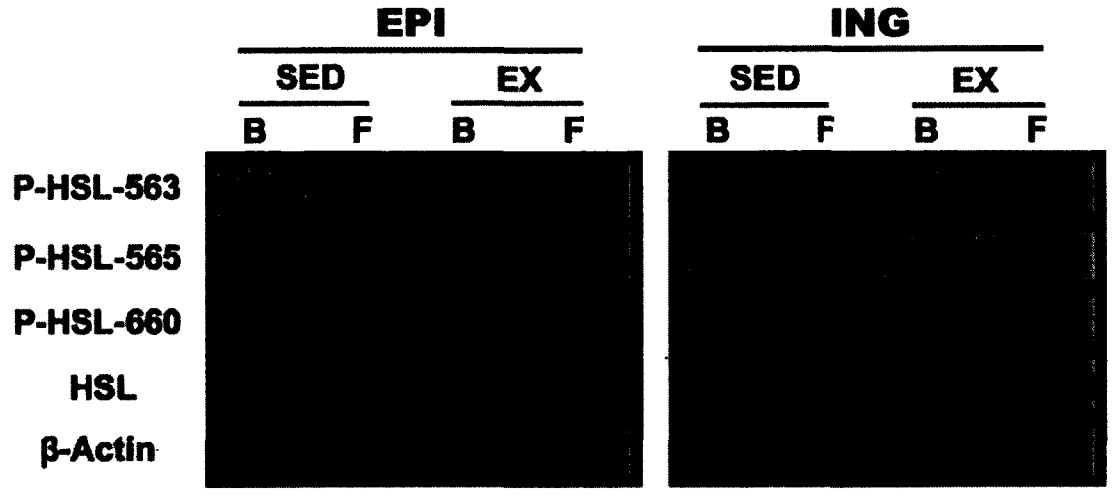

Figure 15. Representative western blots of HSL content and phosphorylation at Ser_{563}, Ser_{565} and Ser_{660} in adipocyte lysates from sedentary (SED) and exercise (EX) animals under basal (B) and forskolin (F) stimulation (10μM), β-actin was used as a loading control, N=3.

50% in insulin stimulated conditions when expressed relative to the sedentary basal condition. ING adipocytes also demonstrated a 48% reduction in glucose incorporation into TG under insulin stimulated conditions only (Fig. 16, A and B).

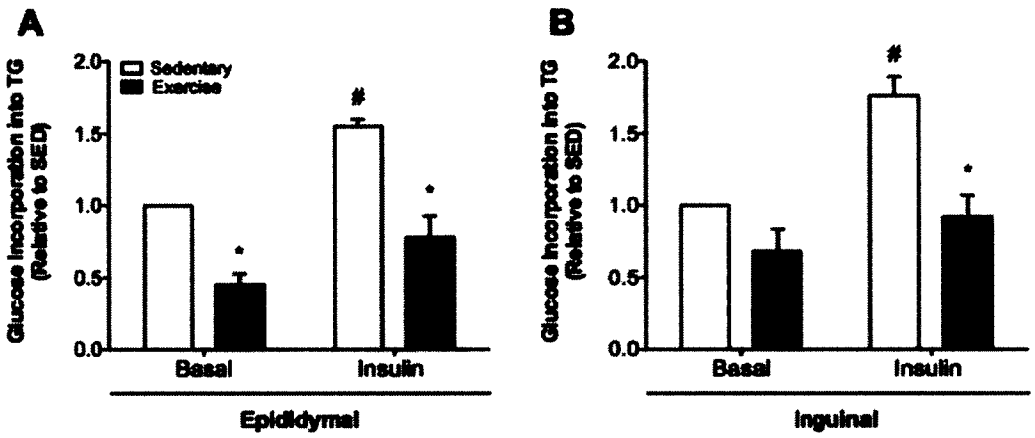

Figure 16. Incorporation of glucose into TG by EPI (A) and ING (B) adipocytes from sedentary and exercise-trained rats during a 1 hour incubation with and without insulin (50 nm). Measures were taken in triplicate and values expressed as mean relative to sedentary basal ± SEM. N=10-13, *P<0.05 vs. sedentary, #P<0.05 vs. respective basal condition (2-way ANOVA).

6. DISCUSSION

6.1. Chronic training effects on adipose tissue lipolysis

Previous studies have shown that after a period of chronic endurance exercise training there are reduced plasma catecholamine (80, 97) and NEFA (98) concentrations in response to exercise. In contrast to these findings, our study failed to detect a difference in these plasma parameters between sedentary and trained rats that exercised at the same relative submaximal intensity. The changes in NEFAs and catecholamines reported by previous studies may have been detected because measurements were taken after participants exercised at the same absolute workload during both pre and post training tests. This does not account for an increase in exercise capacity that develops with chronic endurance training. Therefore, reported reductions in catecholamine and NEFA release may simply be due to performing exercise at a lower intensity in the trained state. In the present study, changes in exercise capacity were accounted for as exercise trained animals ran at ~40% higher speed compared to the sedentary group during the final week of testing. Despite concentrations of plasma catecholamines and NEFAs not being significantly different between groups at any time point, we did find a decrease in NEFA levels over time in the exercise animals. This may be an indication of a reduction in adipose tissue lipolysis with training but there are numerous factors unrelated to adipose tissue metabolism that could have effected plasma NEFA

concentrations. That is why we went further to investigate if lipolysis was altered specifically in adipocytes.

Previous studies have reported that chronic exercise training enhances glycerol release from adipocytes in response to stimulation by various lipolytic agents (4-6, 75, 76). This seems reasonable when considering that exercise training increases FA utilization by skeletal muscles (2, 3). Presumably, an increased demand for FA by the periphery could be met by elevated adipose tissue lipolysis. Other experiments, however, have demonstrated that while exercise training increases skeletal muscle FA oxidation, it also enhances utilization of intramuscular TG pools with a proportionate reduction in reliance on adipose tissue derived FA (2, 3, 7, 74). Given this finding, enhanced mobilization of TG from adipocytes would be paradoxical. An increased release of FA from adipose tissue would then need to be accompanied by increased rates of re-esterification to remove elevated levels of un-utilized FA from the plasma. This does not fit with observations from our study, as rates of glucose incorporation into TG by adipocytes was suppressed in the exercise trained group. Our finding would indicate reduced TG esterification in the trained state and this has been confirmed by other researchers (83).

In contrast to the above mentioned research reporting enhanced stimulated lipolysis with training, the present study demonstrated a reduction in isoproterenol stimulated glycerol release from adipocytes of EPI and ING fat

pads. There was also a significant training induced attenuation of lipolysis in EPI adipocytes with forskolin stimulation. Even though glycerol release appeared to be lower in ING cells in response to forskolin as well, the reduction failed to reach significance but this may have been due to a small sample size. This evidence suggests that chronic endurance training elicits blunted stimulated lipolysis in adipose tissue that is regulated though changes in the adipocytes' ability to respond to lipolytic agents. The discrepancy between reports of enhanced lipolysis and our observation of reduced lipolysis with training may be due to methodological differences related to exercise protocol or the length of time between the cessation of activity and adipocyte extraction. Martin and colleagues demonstrated that stimulated lipolysis decreases in trained individuals progressively with a few days of inactivity (99). This shows that depending upon when lipolysis is assessed, an enhanced catecholamine response may be due to an acute rather than chronic effect of exercise (100). To address this issue in the current study, adipocytes were extracted 48 hours following the last exercise training session.

6.2. Mechanisms governing training induced adaptations in lipolysis

The complex orchestration of numerous enzymes and proteins is required to effectively breakdown TG through lipolysis. As such, there are many levels of regulation in this cascade that could potentially mediate the observed training

induced attenuation of glycerol release. With conflicting evidence in the literature regarding the overall lipolytic response to stimulation in the trained state, reports related to alterations in the β-adrenergic cascade are inconsistent. In one study, increased lipolysis in adipocytes from 9 week treadmill trained rats was observed and associated with elevated PKA and HSL activity (75). Similarly, others speculated that a training induced enhancement of lipolysis was mediated by a step distal to receptor binding and also likely due to increased PKA or lipase activity (4). There is currently little available information regarding the mechanism regulating training induced suppression of lipolysis. In one report by Chapados and colleagues, reduced stimulated lipolysis after training was found in high-fat-fed rats (8). The only conclusion drawn related to molecular regulation of the β-adrenergic cascade in this study was that the suppression in TG breakdown was not mediated by a change in PLIN content (8). The present study attempted to elucidate potential molecular mechanisms regulating the training induced blunting of lipolysis in adipocytes but the results were also inconclusive. A significant reduction in EPI glycerol release with forskolin stimulation was noted in adipocytes from exercise trained animals. A trend towards decreased TG breakdown was also observed in the ING cells of these animals with this agent. This suggests that attenuation of lipolysis with training may be mediated by a post-receptor mechanism. However, alterations in β-AR sensitivity or density contributing to attenuated lipolysis cannot be ruled out. Attempts were made to

quantify levels of β_3-AR present in adipose tissue extracts but the results were inconclusive and further investigation is required.

With the observed changes in forskolin stimulated lipolysis, evaluation of the major lipases ATGL and HSL, along with their associated regulatory proteins PLIN and CGI-58 was conducted via western blot. In EPI fat, analysis of tissue homogenates indicated an up-regulation of ATGL and CGI-58 content in the exercise trained group. Given that ATGL is a primary TAG lipase and CGI-58 activation of ATGL results in increased TG breakdown (58), up-regulation of these proteins would seem to imply enhanced rather than suppressed lipolysis. Therefore, we looked at PLIN as this enzyme is co-localized with CGI-58 in its unphosphorylated state (58), indirectly interfering with CGI-58 activation of ATGL. Phospho-PLIN Ser_{517} was also measured because phosphorylation at this residue is involved in releasing CGI-58 to allow for activation of ATGL (58, 70). No consistent difference in content or phosphorylation of PLIN between sedentary and exercise animals was noted, implying that ATGL would be activated to the same extent in both groups. The up-regulation of ATGL content in the EPI depot of exercise trained rats does not seem to necessarily correspond with increased activity of this protein. In ING tissue, there was no consistent change in ATGL, CGI-58 or PLIN between groups.

HSL, the prominent DAG lipase was also assessed for content and phosphorylation at multiple Serine residues. Assessment of isolated adipocytes

indicated there was a marked increase in phosphorylation at the lipolytic $Ser_{660/563}$ residues when stimulated by isoproterenol or forskolin. The drastic increase in phosphorylation of these sites correlates well with the elevation in lipolysis after incubation with stimulating agents. Even though it is apparent that HSL plays an important role in mediating lipolytic rate, there was no consistent change in phosphorylation of any evaluated residue between sedentary and exercise animals. While attenuation of lipolysis with training may not be mediated by changes in phosphorylation at $Ser_{563/565/660}$, another major lipolytic residue, Ser_{569}, could not be evaluated due to lack of a commercially available antibody. Therefore, a role for Ser_{569} in mediating lipolytic alterations with exercise training could not be ruled out.

Overall, determination of the underlying mechanism regulating training induced attenuation of lipolysis remains to be elucidated by the assessment methods employed in this study. This is because many of the proteins that were evaluated have some degree of association with the lipid droplet, making total amounts of these proteins difficult to extract. While attempts were made to obtain the highest protein concentration possible, we cannot guarantee that equal amounts of the proteins of interest were extracted from each sample. Furthermore, proteins such as PLIN and HSL associate with the surface of the lipid droplet in a complex manner depending on β-AR activation. Stimulation of the lipolytic cascade leads to phosphorylation and translocation of HSL from the

cytosol to the lipid droplet where it is thought to doc with PLIN to better access TG stores (66). Since HSL translocates to the lipid only when phosphorylated (101, 102), there is greater potential to underestimate levels of the protein in this state. Furthermore, it has been suggested that phosphorylation of PLIN increases its susceptibility to extraction by fractionation procedures (103). This implies that un-phosphorylated PLIN is more likely to be underestimated due to incomplete protein extraction from the lipid fraction. Given that un-phosphorylated PLIN has been shown to prevent lipases from accessing TG stores (68, 69), increased levels of un-phosphorylated PLIN may be a potential mechanism by which lipolysis is reduced in the trained state. Therefore, PLIN compartmentalization under stimulated conditions should be assessed in future studies.

6.3. Exercise mediated changes in adiposity

With a significant reduction in stimulated release of TG from adipose tissue, a reduction in adiposity seems counterintuitive. In an attempt to elucidate why animals in this study have significantly less EPI and ING fat tissue, we evaluated lipogenesis by measuring the incorporation of glucose into TG. Lipogenesis in EPI adipocytes was reduced with training under basal and insulin stimulated conditions and in ING adipocytes with insulin stimulation only. This indicates that alterations in adiposity with training may be facilitated by changes

in the ability of adipocytes to store TG in addition to the creation of a negative energy balance via an exercise induced increase in energy expenditure. Our finding is consistent with other research reporting reduced glucose incorporation into TG in EPI adipocytes of 6-week swim trained rats (83).

While down-regulation of lipogenesis in adipocytes after chronic training can account for a reduction in fat mass, the reduced adiposity would theoretically result from decreased TG storage and ultimately smaller adipocytes. In our study, changes in adiposity with exercise training could not be accounted for by changes in adipocyte size. There was only a moderate reduction in EPI and no difference in ING adipocyte diameter in the trained group. Therefore, the observed changes in adiposity could be due to a reduced cell number in both depots of exercise animals. In this case, there would be less cells to accommodate the storage of FA in these animals. This limited number of cells would need to compensate by taking up more FA, leading to a similar cell size as what is seen in sedentary animals. While the effects of training on adipogenesis were not evaluated in our study, previous work by Sakurai and colleagues showed that 9 weeks of endurance exercise in rats lead to inhibition of adipocyte differentiation in the adipose tissue stromal-vascular fraction. This resulted in a 20% reduction in cell number (84). Further investigation will need to be done in order to determine if changes in cell number are mediating reduced adiposity with training in our model.

7. CONCLUSIONS

In this study, we demonstrated that after 6 weeks of chronic endurance exercise, rats displayed well established training-induced adaptations. These included enhanced VO_2 peak, reduced body weight, and decreased adiposity in both visceral and subcutaneous depots. Using an *in vitro* approach, we also demonstrated that chronic endurance training leads to increased exercise capacity without altering sympathetic or lipolytic activity. This was shown when rats running at the same relative submaximal speed displayed similar plasma concentrations of NEFA and catecholamines (adrenaline and noradrenaline).

With respect to adipocyte metabolism, we determined that chronic exercise leads to blunted isoproterenol stimulated lipolysis in both EPI and ING depots. Furthermore, a training induced reduction in lipolysis was evident in EPI adipocytes with forskolin stimulation suggesting mediation of this response by a post-receptor mechanism. A similar trend with forskolin stimulation was present in the ING depot but the difference did not achieve significance. Attempts to elucidate the molecular mechanism underlying reduced stimulated lipolysis with chronic exercise were unsuccessful but determined that ATGL, CGI-58, HSL and PLIN are likely not responsible.

Lipogenesis was also found to be reduced in the EPI depot under both basal and insulin stimulated conditions. In the ING depot lipogenesis was only reduced with insulin stimulation only. This could at least partially explain the

significant reduction in fat mass observed with exercise training. Even though adiposity was reduced, only moderate to no reductions in cell size in both depots were found suggesting that chronic exercise training also led to a reduction in adipocyte cellularity.

Table 1. Summary of observations in epididymal and inguinal adipose tissue from 6 week endurance exercise trained animals compared to sedentary animals.

		EPIDIDYMAL	INGUINAL
Fat Mass		Reduced	Reduced
Cell Diameter		Reduced	Unchanged
Lipogenesis	- Basal	Reduced	Unchanged
	- Insulin	Reduced	Reduced
Lipolysis	- Basal	Unchanged	Unchanged
	- Isoproterenol	Reduced	Reduced
	- Forskolin	Reduced	Unchanged
ATGL Content		Increased	Unchanged
CGI-58 Content		Increased	Unchanged
P-PLIN (Ser_{517})		Unchanged	Unchanged
P-HSL ($Ser_{563/565/660}$)		Unchanged	Unchanged

8. FUTURE DIRECTIONS

8.1. Analysis of molecular mechanisms regulating lipolysis

Given that the current study was unsuccessful in identifying the regulatory step leading to reduced lipolysis in trained rats, further investigation to elucidate the responsible mechanism will be required. Of particular importance is the evaluation of adipose tissue adrenergic receptor density. Given that the reduction in forskolin stimulated lipolysis with chronic exercise failed to obtain significance in ING adipocytes, the training induced blunting of isoproterenol stimulated lipolysis may be mediated at the receptor level in this depot. Additionally, an alteration in β-AR density with training cannot be ruled out in EPI adipocytes. With inconclusive results obtained through western blot analysis of β_3-AR content, quantitative real time polymerase chain reaction (qPCR) analysis can be an alternative way to assess receptor differences between sedentary and exercise animals.

In addition to assessing the content and phosphorylation of various enzymes involved in the lipolytic cascade, the cellular compartmentalization of these proteins should be determined because the function of some enzymes such as HSL and PLIN is partially dependent on their cellular location. This can be attempted through immunohistochemisty.

8.2. Analysis of mechanisms regulating lipogenesis.

To determine which lipogenic pathway is contributing to reduced TG synthesis with exercise training, DNL and TG esterification must be separately evaluated. To do this, TG extracted from adipocytes after a period of incubation with labeled glucose must be fractionated so that glucose incorporation into FA and glycerol can be differentially assessed. If lower levels of glucose are incorporated into FAs in exercise versus sedentary animals, then it is evident that DNL plays a significant role in mediating the reduced adiposity observed in this group. In this case, evaluation of major enzymes regulating the production of FA from glucose including ACC1 and FAS is warranted. This can be done using western blotting and should utilize adipocyte samples under both basal and insulin stimulated conditions, since insulin was shown to differentially effect the rate of TG synthesis between groups in both depots. Without a significant alteration in glucose incorporation specifically into FA, there would be a clear indication that the ability of adipocytes from trained animals to either: 1) produce glyceride-glycerol, 2) take up FA and/or 3) esterify FA to G3P is mediating changes in TG synthesis. To identify regulatory mechanisms mediating differences in glyceride-glycerol production, G3PD can be evaluated. This enzyme is responsible for the production of G3P from glucose to form the glycerol backbone of the TG (24). In the case of the ability of adipocytes to import FA, FABP1 and FAT/CD36 act as important FA transport proteins (32, 34, 35) and

should be measured. A down-regulation of either one of these FA transporters may lead to reduced availability of FA in the cell for TG synthesis. Finally, to monitor changes in esterification of TG, enzymes responsible for the sequential esterification FAs to G3P can be evaluated include GPAT, AGPAT, PPH-1, lipin 1 and DGAT. All mentioned proteins involved in lipogenic pathways can be measured either through western blot or qPCR analysis.

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10. APPENDICES

10.1. Appendix A: Exercise protocol for animal selection

Table A1: Protocol utilized for baseline selection of animals into the study.

Time (min)	Speed (m/min)	Inclination (%)
0	10	0
5	14	5
7	16	10
9	18	10
11	20	10
13	22	10
15	24	10
17	26	10
19	28	10
21	30	10
23	—	—

10.2. Appendix B: Six week treadmill exercise protocol

Table A2: Protocol for daily treadmill training. Length of time (min) indicated for each speed (m/min). Inclination remained constant (10%) in each session.

Week	Day	Speed (m/min)												Total (min)
		10	12	14	16	18	20	22	24	26	28	30	32	
1	1	5	5	10	10	—	—	—	—	—	—	—	—	30
	2	5	5	10	10	—	—	—	—	—	—	—	—	30
	3	5	5	20	10	—	—	—	—	—	—	—	—	40
	4	5	5	20	15	—	—	—	—	—	—	—	—	45
	5	5	5	20	15	5	—	—	—	—	—	—	—	50
2	1	5	—	25	15	10	—	—	—	—	—	—	—	55
	2	5	—	15	15	15	5	—	—	—	—	—	—	55
	3	5	—	15	15	15	10	—	—	—	—	—	—	60
	4	5	—	15	15	15	15	—	—	—	—	—	—	65
	5	5	—	10	15	15	20	—	—	—	—	—	—	65
3	1	5	—	5	15	15	25	—	—	—	—	—	—	65
	2	5	—	5	15	15	20	5	—	—	—	—	—	65
	3	5	—	5	10	15	20	5	5	—	—	—	—	65
	4	5	—	5	10	10	20	10	5	—	—	—	—	65
	5	VO_2 PEAK / MAX SPEED TESTING												—
4	1	—	5	5	5	5	15	15	10	5	—	—	—	65
	2	—	5	5	5	5	15	15	10	5	—	—	—	65
	3	—	5	5	5	5	15	15	10	5	—	—	—	65
	4	—	5	5	5	5	10	10	10	10	5	—	—	65
	5	—	5	5	5	5	10	10	10	10	5	—	—	65
5	1	—	5	5	5	5	10	10	15	5	5	—	—	65
	2	—	5	5	5	5	10	10	10	10	5	—	—	65
	3	—	5	5	5	5	10	10	15	5	5	—	—	65
	4	—	5	5	5	5	10	10	15	5	5	—	—	65
	5	—	5	5	5	5	10	10	15	5	5	—	—	65
6	1	—	5	—	5	5	5	10	15	10	5	5	—	65
	2	—	5	—	5	5	5	10	10	10	10	5	—	65
	3	—	5	—	5	5	5	10	10	10	5	5	5	65
	4	—	5	—	5	5	5	10	10	10	5	5	5	65
	5	VO_2 PEAK / MAX SPEED TESTING												—