THE EFFECTS OF PHYSICAL ACTIVITY ON ADIPOSE TISSUE METABOLISM AND DNA METHYLATION

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The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

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Abbreviations

AL, *ad libitum*

cAMP, cyclic adenosine monophosphate

AMPK, 5' adenosine monophosphate-activated kinase

ATP, adenosine triphosphate

BMI, body mass index

bp, base pair

CDC, Center for Disease Control

C/EBPα, CCAAT/enhancer binding protein alpha

C/EBPβ, CCAAT/enhancer binding protein beta

C/EBPδ, CCAAT/enhancer binding protein delta

CpG, cytosine-guanine dinucleotide

CGI, CpG island

CVD, cardiovascular disease

db/db mice, leptin deficient mice

DNA, deoxyribonucleic acid

DEXA, dual-energy X-ray absorptiometry

DIO, diet induced obesity

Dnmt, DNA methyltrasferase

dsDNA, double stranded deoxyribonucleic acid

ECM, extracellular matrix

ER, endoplasmic reticulum

FAO, fatty acid oxidation

FAS, fatty acid synthase

FFA, free fatty acid

GPDH, glycerol-3-phosphate dehydrogenase

HbA1c, hemoglobin A1c

IL-6, interleukin-6

L-1, (end) light cycle minus 1 hours

L+5, (end) light cycle plus 5 hours

LPL, lipoprotein lipase

LETO, Long-Evans Tokushima Otsuka

MBD, methyl binding protein

MCP-1, monocyte chemoattractant protein-1

mtDNA, mitochondrial DNA

MMP, matrix metalloproteases

MeDIP, immunoprecipation of Methylated DNA

OLETF, Osaka Long Evans Transgenic Fatty

OB-R, leptin receptor

ob/ob mice, leptin receptor deficient mice

PF, pair fed

PPARγ, peroxisome proliferator-activated receptor gamma

PGC-1α, peroxisome proliferator-activated receptor gamma coactivator-1 alpha

PTP1B, protein tyrosine phosphatase-1B

RBP-4, retinol binding protein four

SREBP-1c, sterol regulatory element binding protein-1c

SVF, stromal vascular fraction

TAG, triacylglyceride

TZD, thiazolidinediones

TNF-α, tumor necrosis factor alpha

ABSTRACT

The increased prevalence of obesity and diabetes threatens both the real and economic health of western counties. Importantly, the physiological and pathological responses of adipose tissue (WAT) to excess energy intake and energy expenditure are critical to maintaining energy homeostasis. In peripheral tissues, such as skeletal muscle, liver and recently WAT, mitochondria dysfunction is associated with obesity and diabetes. Furthermore, although obesity and diabetes have a genetic component it is now also appreciated that they also have an epigenetic component, such as aberrant DNA methylation. There were two major goals of this dissertation, 1) If in response to inactivity and hyperphagia there is a decrease in fatty acid oxidation and mitochondria concentration in WAT, similar to skeletal muscle and liver and 2) Whether physical active and sedentary mice have differentially methylated DNA in skeletal muscle. First (chapter 2) I established that the increase in abdominal WAT during inactivity occurs even when food intake is restricted to the level of always-sedentary animals. A follow-up study (chapter 3) showed that the increase in WAT is not associated with a decrease in acid oxidation adipocytes fatty, which was increased compared to sedentary animals 5 and 173 hours following inactivity, but was associated with a decrease in skeletal muscle fatty acid oxidation. The changes in WAT fatty acid oxidation occurred independent of changes in markers of mitochondrial concentration and in order to test whether physical activity can prevent the reduced WAT mitochondria in obesity, a hyperphagic rat model was employed. Markers of WAT mitochondrial protein content (cytochrome c, COXIV-subunit I, and citrate synthase activity) significantly increased from 13 to 40 weeks in the wild type rats, were significantly attenuated in the hyperphagic sedentary rats, but were

partially restored to the wild type levels with wheel running in the hyperphagic rats. While decreases in mitochondrial concentration is not causal, it may be linked with aging, with future studies necessary to determine the mechanisms by which this is occurring. Although strong evidence suggest that differences in DNA methylation in physically active and sedentary animals can occur, I was unable to verify candidate genes selected based on initial microarrays. However, two novel physical activity responsive genes were found, *Dnmt3a* and *Pitx3*. Perhaps, the slow turnover combined with only a moderate phenotype in response to voluntary wheel running makes skeletal muscle less likely to have physical activity induced differences in DNA methylation. Clearly, future studies are needed. In summary, WAT oxidative phenotype is modified by aging and physical activity, while it remains unclear whether DNA methylation differences in skeletal muscle can occur.

Chapter 1: Introduction

In the past several decades the prevalence of obesity and type 2 diabetes has risen dramatically. In the United States the prevalence of overweight adults (25-30 BMI) has increased from 47% to 66% while the prevalence of obesity (>30 BMI) has increased from 15% to 32%, from 1976-2004 (204). Similarly, the prevalence of type 2 diabetes, or non-insulin-dependent diabetes mellitus in adults (>20 years of age) has increased from 5% of the United States population in 1960 to more than 10% of the population in 2007 (107). The increasing frequency of children with type 2 diabetes led to the removal of the descriptor "adult onset" and it is now predicted that the number of type 2 diabetic children will surpass the number of children with insulin dependent diabetes mellitus, or type 1 diabetes (6).

In 2004, diabetes was the sixth most common cause of death in the United States and obesity, due to inactivity and poor nutrition, the second most frequent cause of preventable death (190). Additionally, type 2 diabetes increases the risk of heart disease, high blood pressure, blindness, kidney disease, diseases of the nervous system, amputation, dental diseases, pregnancy complications, and immune system disorders according the U.S. National Institutes of Health (http://diabetes.niddk.nih.gov/complications/index.htm). Obesity also increases the risk of cardiovascular disease, osteoarthristis, urinary incompetence, sleep disorders, cancer, and gallbladder diseases (http://www.obesityresearch.nih.gov/health/public.htm#5). Not surprisingly, diabetes and BMI are tightly linked with 85% of diabetics being overweight,

64% of those obese (1). While these epidemics cause much suffering, the economic consequences are also significant including a projected 47 billion dollars in direct costs and an additional 109 billion dollars in indirect costs for type 2 diabetes alone (271). With both an increasing number of people affected by the disease and increasing costs, overcoming these "twin" epidemics of obesity and type 2 diabetes is critical to the long-term economic and real health of our nation.

Fortunately, a number of epidemiological studies suggest that prevention of type 2 diabetes is possible through lifestyle interventions. In the Finnish Diabetes Prevention Study, a 58% reduction in diabetes incidence occurred with lifestyle modifications in subjects with abnormal glucose tolerance (167). While in the U.S. Diabetes Prevention Program, lifestyle modifications resulted in a similar reduction of 58% in new type 2 diabetics despite only 58% of subjects adhering to the lifestyle treatment (156). This suggests lifestyle might be even more effective at preventing diabetes when actually adhered to. In fact, for every 6.9 subjects that adhere to a lifestyle intervention one case of diabetes is prevented, while pharmacological treatment of 13.9 subjects with Metformin was necessary to prevent one case of type 2 diabetes (156). In the Harvard Nurses study, poor lifestyle choices led to 91% of new diabetes diagnosis, again suggesting lifestyle changes can almost completely prevent type 2 diabetes (127). These studies defined lifestyle modifications as only 30 minutes of brisk walking a day as the physical activity component (156, 167). This amount of physical activity is also recommended by the American College of Sports Medicine and American Heart Association, but yet only 50% of Americans currently meet these activity guidelines, which undoubtedly has increased the prevalence of diabetes in the United States (117).

THE ROLE OF ADIPOSE TISSUE IN METABOLIC HOMEOSTASIS

Central to understanding the connection between obesity and how it increases the risk of developing diabetes and other metabolic diseases is understanding the physiology and pathophysiology of white adipose tissue (WAT) in response to a positive energy balance. WAT is the major organ for storage of excess energy in humans and individual adipocytes contain more than 80% lipid by mass (133). Although all cells contain lipid membranes, fatty acids, steroids, and isopretones, 90% of lipid in humans is stored as the neutral lipid triacylglyceride (TAG), in adipocytes (314). In times of positive energy balance WAT can respond by increasing cell size, by hypertrophy, or increasing cell number, by hyperplasia, and the formation of new adipocytes through adipogenesis of preadipocytes. Conversely, brown adipose tissue disposes of excess energy as heat through uncoupling energy utilization and ATP production, is only present in significant amounts in rodents and infants, and has an origin more similar to skeletal muscle than to WAT (253, 292). Although recent evidence suggests a maintenance of adipocyte cell number in human adults (272), this is still controversial since hyperplasia is routinely seen in rodents (68, 135, 170, 178, 183) and humans (3, 93, 305).

The “thrifty gene hypothesis” by Neel (198) articulated the selective advantages and importance of storing energy in WAT during periods of energy excess to improve survival during times of famine. In the Paleolithic hunter-gatherer environment, WAT was essential for free fatty acid (FFA) homeostasis balancing the release of FFAs, as fuel for extended bouts of physical activity, with FFA storage (23, 55). Since fatty acids are

toxic it is important to sequester them in the WAT as TAGs, a neutral lipid, to minimize their negative effects.

The hormone insulin is the primary regulator of glucose and fatty acid metabolism in WAT under fed conditions. Increased insulin, as in the fed state, increases glucose and fatty acid uptake into WAT (243), partly through increases in fatty acid synthase activity and expression (30, 42). Decreased insulin occurs during fasting or exercise allowing catecholamines such as epinephrine and norepinephrine to control lipolysis. High catecholamines increase lipolysis and the mobilization of FFAs through increasing the activity of lipases in WAT. Although insulin and catecholamines are the primary regulators of lipolysis, more recent studies indicate a more complex regulatory system. For example increased lipolysis by both atrial and brain natriuretic peptides, *in vivo* and *in vitro,* is independent of catecholamines (92, 194, 256). Additionally, increased glycerol concentration, a marker of lipolysis, occurs in response to small pulses of growth hormone in the femoral and abdominal WAT, although the physiological relevance in humans is questionable (105). Through inhibition of the anti-lipolytic effect of insulin, cytokines, such as tumor necrosis factor alpha (TNF-α), also increase lipolysis (96).

The two major lipase enzymes responsible for lipolysis of TAGs to FFA are hormone sensitivity lipase and WAT specific lipases. These enzymes are differentially regulated at rest and in response to catecholamines in human adipocytes (14). Additional mechanisms for increasing lipolysis and lipid mobilization occur through increases in lipid-droplet associated proteins, such a perilipin (106), adipocyte fatty acid-binding protein (267). Another lipid-droplet protein, fat-specific protein 27, conversely increases lipid storage and decreases lipolysis (229). Insulins anti-lipolytic action is mediated

through tyrosine kinase receptor signaling (207), while other anti-lipolytic molecules such as niacin (142), prostaglandin E2 (59), and neuropeptide Y (301) signal through a family of G_i receptors.

Failure to suppress lipolysis and/or respond to insulin signaling results in a failure of WAT to expand and excess circulating FFA. Increased circulating FFAs leads to the ectopic storage of fatty acids in tissues such as liver, skeletal muscle, and pancreas (101, 230, 257, 263, 296). This ectopic lipid storage, termed lipotoxicity, is linked with insulin resistance and abnormal glucose metabolism in skeletal muscle and liver (249). In support of this, acute infusion of FFAs is capable of causing insulin resistance in healthy individuals (78). Conversely, lowering of circulating fatty acids with the anti-lipolytic drug Acipimox partially increases peripheral glucose uptake in response to insulin (246). Furthermore the inability to store excess fatty acids in WAT of individuals with lipodystrophy is associated with insulin resistance and ectopic storage of lipid in skeletal muscle, despite individuals being of normal weight (82, 235). However, increased ectopic storage of neutral lipid species, TAG, in skeletal muscle of highly trained endurance athletes, suggests only certain lipid species such as ceramide, diacylglycerides, or specific fatty acids are responsible for insulin resistance in skeletal muscle (79, 161).

Lowering of FFAs in insulin resistance individuals does not fully reverse insulin resistance, indicating additional mechanisms responsible for insulin resistance must exists (246). One such mechanism is likely endoplasmic reticulum (ER) stress. In the WAT and liver of obese insulin-resistant individuals ER stress is caused by an increase in trafficking of misfolded proteins that exceeds the ERs capacity (36, 109). The increase in misfolded proteins is partially caused by an oversupply of nutrients and causes insulin

resistance by activating inflammatory pathways and increasing reactive oxygen species production (123, 345). Conversely markers of ER stress are reduced in the liver and WAT following gastric bypass surgery and a reduction in WAT mass (109).

ADIPOSE TISSUE DIFFERENTIATION

During a state of chronic positive energy balance maintaining energy homeostasis by the differentiation of preadipocytes into adipocytes through a process termed adipogenesis is critical. Preadipocytes are fibroblast-like cells that reside in the stromal vascular fraction (SVF) of WAT. In addition to preadipocytes the SVF contains smooth muscle cells, endothelial cells, monocytes, and macrophages (119). However it is the differentiation of preadipocytes that leads to the formation of mature adipocytes. Differentiation of preadipocytes is increased by insulin (100), gluccocoricoids (336), and angiotensin II (244). Conversely, inhibition of differentiation occurs in response to catecholamines (350) and cytokines (169). Importantly, increased differentiation of preadipocytes is inversely related to proliferation thus a population of preadipocytes is unable to differentiate and proliferate simultaneously.

Differentiation is initiated through a well-characterized transcriptional cascade of different transcription factors. Initially two basic loop helix transcription factors, CCAAT/enhancer binding protein beta and delta (C/EBPβ and C/EBPδ), increase causing an increase in the transcription factor peroxisome proliferator-activated receptor gamma (PPARγ) (63, 112). PPARγ is necessary for preadipocyte differentiation and directly increases CCAAT/enhancer binding protein alpha (C/EBPα), which helps maintains PPARγ expression and leads to the formation of a mature adipocyte. Mature adipocytes are characterized by both lipogenic responses to insulin and the expression of lipoprotein

lipase (LPL) and glycerol-3-phosphate dehydrogenase (GPDH) (111). Decreased lipogenesis and adipogenesis is present in the WAT of obese type 2 diabetics, revealing the importance of adipogenesis in maintenance of metabolic and FFA homeostasis. For example, the decreased expression of the adipogenic proteins sterol regulatory element binding protein-1c (SREBP-1c) and PPARγ (but not C/EBPα or C/EBPδ), occurs in obese type 2 diabetics, but not BMI matched non-diabetic individuals (80).

In addition to differentiation of preadipocytes, the other cells within the SVF such as endothelial, monocytes, and macrophages, play an important role in angiogenesis and extracellular matrix (ECM) remodeling during adipogenesis. During adipogenesis the ECM shifts from predominately collagen types I/III, fibronectin, and ß1-integrins to predominately collagen type IV and entactin (254, 273). Furthermore, breakdown of the ECM with the matrix metalloproteases (MMPs), 2 and 9, and the membrane-anchored MMP, is necessary for adipogenesis (45, 61). Likewise, blocking angiogenesis with a vascular endothelial growth factor antibody prevents adipocyte adipogenesis, and leads to adipocyte hypertrophy (200). The adipocyte hypertrophy further exacerbates the reduced adipogenesis by increasing adipocyte hypoxia, a known inhibitor of adipogenesis (151). This underscores the importance of how the process of ECM remodeling, angiogenesis, and adipogenesis are coordinated to expand WAT depots.

ADIPOSE TISSUE AS AN ENDOCRINE ORGAN

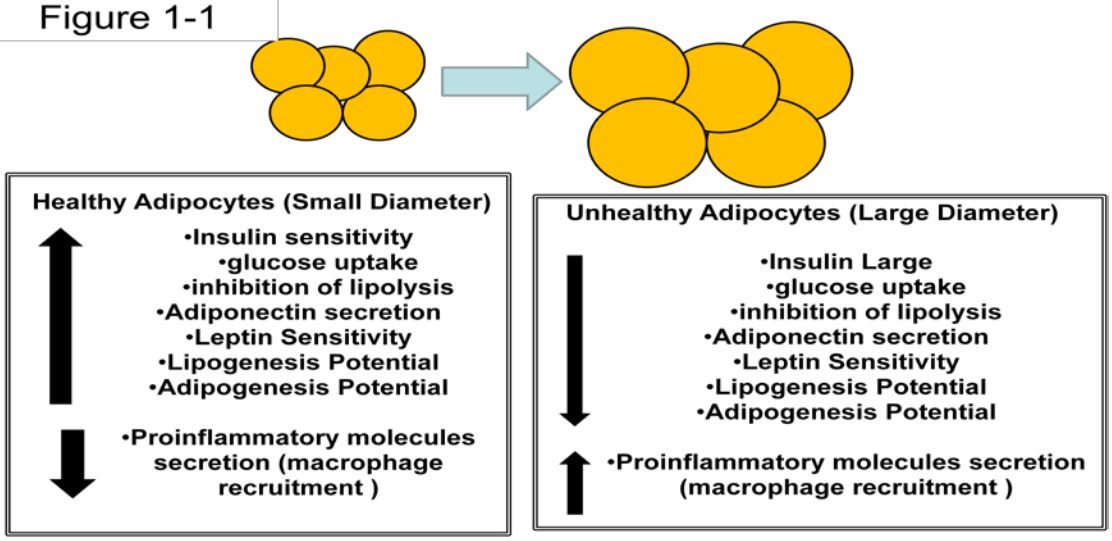
In order for the WAT to carry out such a wide range of physiological tasks adipocytes release many proteins that have significant endocrine, paracrine, and autocrine effects. The first protein discovered to be synthesized and secreted only by adipocytes was leptin in 1994 (346). Leptin is critical in the regulation of appetite and energy

balance. Since 1994, several proteins, now termed adipokines, were discovered to be exclusively or predominately expressed and secreted by adipocytes including: adiponectin (250), resistin (278), visfatin (91), apelin (44), and retinol binding protein four (RBP-4) (340). Of these known adipokines adiponectin is a well-established anti-inflammatory protein capable of improving insulin sensitivity, reducing cardiovascular disease, and is decreased in diabetic and obese individuals (306). Resistin is believed to antagonize insulins action and is also elevated in the serum of obese individuals. However, resistin is also high in endurance-trained individuals, making its true biological function unclear (219, 222). Visfatin is secreted to a greater extent by visceral than subcutaneous fat and is believed to lower glucose levels, although it is unclear the mechanism of action (91). Apelin, like adiponectin, has beneficially effects on the cardiovascular system and is capable of increasing heart contractility and potentially improves vascular relaxation (29, 348). While RBP-4 is a strong predictor type 2 diabetes and more highly secreted from visceral than subcutaneous WAT, the evidence of RBP-4 playing a casual role in insulin resistance is limited (104, 154). In addition to these adipokines adipocytes express many other proteins involved in matrix remodeling (226), angiogenesis (49), and energy homeostasis (10).

Adipocytes can increased secretion of pro-inflammatory proteins such as, interleukin-6 (IL-6), TNF-α, monocyte chemoattractant protein-1 (MCP-1), and interferon-γ-inducible protein (265), during hypertrophy. Similarly, adipocyte hypertrophy is associated with decreased secretion of the anti-inflammatory proteins IL-10 and adiponectin (265). The increase in pro-inflammatory molecules, especially MCP-1, from hypertrophied adipocytes leads to the recruitment of activated macrophages to

expanding WAT depots (286). Activated macrophages can then further increase the release of pro-inflammatory cytokines such as TNF-α, and IL-6 (322). Importantly, the macrophages, and other cells within the SVF fraction release much higher amounts of pro-inflammatory proteins than adipocytes (83). The local increased inflammation causes insulin resistance in the adipocytes causing an inability of insulin to suppress lipolysis and a subsequent elevation in circulating free fatty acids. Systemically, the increases in TNF-α and IL-6 originating from WAT depots may contribute to the low-grade

Figure 1-1



inflammation typical of both cardiovascular disease and insulin resistance (8). Prevention of macrophage recruitment by tissue specific receptor knock-outs or antibodies that block macrophage binding is sufficient to prevent macrophage infiltration and subsequent inflammation of WAT following high-fat feeding in rodents (287, 321). Furthermore, preventing hypertrophy of adipocytes by increasing adipogenesis (150), or reducing adipocyte size after bypass surgery and weight loss (48) also reverses the macrophage infiltration and inflammatory phenotype within WAT.

REGIONAL DIFFERENCES IN ADIPOSE TISSUE DEPOT PHENOTYPE

Just as different skeletal muscle fibers types have different phenotypes, different WAT depots have very different phenotypes. In 1954, Jean Vague noticed a sexual difference in regional adiposity, with male adiposity more pronounced in the abdominal area, and females adiposity more pronounced on the limbs (299). He also made the connection that abdominal adiposity regardless of gender was more highly correlated with diabetes (299). Today, the abdominal adiposity recognized by Jean Vague is considered a visceral WAT depot, while the "limb" adiposity is considered a subcutaneous adipose depot. Additionally, visceral WAT includes depots surrounding the vasculature (perivascular) and heart (epicardial), while subcutaneous WAT can be anatomically divided into the superficial-subcutaneous WAT, directly below the skin, and the deep-subcutaneous WAT (7). Perivascular WAT has a phenotype similar to visceral WAT (58), while deep-subcutaneous WAT has an intermediate phenotype between visceral and superficial subcutaneous WAT (310).

In addition to diabetes, high amounts of visceral WAT depots are independently associated with insulin resistance, cardiovascular disease (CVD), and systemic low-grade inflammation (27, 337). This is in part due to the differences in visceral and subcutaneous phenotypes. Intrinsic differences in metabolism, that include lower leptin/adiponectin release (196, 303) higher cytokine release (86, 90), higher lipolytic response to catecholamines (13), and lower differentiation capacities (304) in visceral than subcutaneous WAT. Visceral WAT also has more macrophage infiltration with obesity compared to subcutaneous WAT (47, 115).

The amount and distribution of visceral and subcutaneous WAT is pharmacologically manipulated by a class of drugs called thiazolidinediones (TZD). TZDs are PPARγ agonists that reduce visceral adiposity, while increasing subcutaneous adiposity (308). This adipose redistribution is associated with adipogenesis of hypertrophied subcutaneous adipocytes typically leading to a gain in WAT, but an increase in whole-body and peripheral insulin sensitivity (193, 308). An example of the metabolic benefits of adipose redistribution is the leptin receptor deficient (*ob/ob*) mice that also overexpress adiponectin (also a PPARγ agonist). These mice have twice as much adipose as regular *ob/ob* mice, but remain as insulin sensitive and are metabolically similar to wild type sedentary mice (150). Not surprisingly, these mice have massive subcutaneous depots but very little visceral WAT (150).

ADIPOSE TISSUE MITOCHONDRIA

As the primary site of ATP synthesis in most cell types, mitochondrial function and/or content is tightly linked to cellular function and health. Mitochondria form a reticulum in which individual mitochondria are constantly undergoing fission, fusion, and autophagy to maintain a population of healthy mitochondria (74). Skeletal muscle has tremendous plasticity in mitochondrial adaptation and is capable of increasing mitochondrial content proportionally to the metabolic stress of endurance training (85). Conversely, cessation of endurance training leads to a decrease in mitochondrial content (67). Interestingly, reductions in mitochondrial content and/or function in neurons, smooth muscle cells, endothelial cells, liver, skeletal muscle, or pancreatic-β cells is associated with a number of diseases, including; neurodegenerative disease, atherosclerosis, non-alcoholic fatty liver disease, and diabetes (217, 233, 258, 297).

Whether the reduction in mitochondria directly contributes to the cause or indirectly plays only an associative role in the development of these chronic diseases remains unknown (125, 155).

A reduction of mitochondria in WAT is also associated with type 2 diabetes and obesity (60, 239, 284, 300). Choo et al (60) showed that mitochondrial ATPase proteins, mitochondrial DNA (mtDNA), mitochondrial respiration, and fatty acid oxidation (FAO) in adipocytes were decreased in diabetic leptin deficient (*db/db*) mice and *ob/ob* mice. Others have shown that high-fat feeding reduces mitochondrial concentration, peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α), and FAO in WAT of mice (239) and rats (284). Conversely, reverses the reductions in WAT mitochondrial concentration is accomplished with PPARγ agonist treatment using TZDs in diabetic humans (37, 38), *db/db* mice, *ob/ob* mice, high-fat fed mice, and *in vitro* (239). However, PPARγ agonist treatment is ineffective in increasing WAT mitochondrial content in patients given antiviral therapies that cause mitochondria toxicity (181). Additionally, increased mitochondrial enzyme concentrations in adipocytes is accomplished with both pharmacological agents that increase cAMP, such as epinephrine or forskolin (38, 283), or physiological stimulus, like swimming, that also increase cAMP (275, 283).

One of the major purposes of mitochondria is to generate ATP used to carry out a variety of energy requiring processes. Although the ATP demands of adipocytes are less than in working skeletal muscle there are several metabolic and physiological processes within adipocytes that require significant ATP production. *De novo* lipogenesis in WAT requires acetyl-CoA, which is created in the mitochondria by the

oxidation of pyruvate (309). However, acetyl-CoA is unable to directly cross the mitochondrial membrane. Acetyl-CoA and oxaloacetate can combine via citrate synthase forming citrate, which is subsequently moved to the cytosol and simultaneously converted back to acetyl-CoA and oxaloacetate by ATP citrate lyase, which is located in the mitochondrial membrane (19). This shuttle provides the cytosolic acetyl-CoAs necessary for *de novo* lipogenesis and requires ATP at several steps. Alternatively, ATP production is increased during fatty acid cycling, which is characterized, by increased lipolysis and subsequent fatty acid re-esterification. Two examples of when fatty acid cycling occurs are experimentally increasing serum leptin with an adenovirus (262) and overexpression of desnutrin (4), both of which prevent obesity in response to a high fat diet. However, others have shown that leptin increases FAO of exogenous fatty acids over endogenously stored triacylglycerides, which does not result in fatty acid cycling (328). Future studies are needed to reconcile these differences in the literature.

In support of mitochondria playing an important role in lipogenesis Kaaman et al (139) showed that in humans mtDNA copy number in isolated subcutaneous adipocytes strongly correlates to basal and insulin stimulated *de novo* lipogenesis, but not lipolysis. They also showed a weak inverse association with mtDNA and age. Likewise, age-associated decreases in mitochondria have been previously shown in metabolically active tissues such as skeletal muscle and liver (46, 186). However, in wild type mice increasing in age from 0.5 years of age to 1.5 years of age resulted in a transient increase in epididymal WAT mitochondrial proteins. By 2.5 years of age WAT mitochondrial proteins fell to similar values as seen in the 0.5 year old mice. (143). Interestingly, unlike the wild type mice, in mice with a WAT specific knockout of the insulin receptor

mitochondrial protein concentration remained high at 2.5 years of age (143). However, these mice had reduced lipogenesis, suggesting that WAT mitochondria is not related to increased lipogenesis (143). Another potential role for WAT mitochondria is their importance in the synthesis of adipocyte specific proteins, exemplified by a recent report showing the necessity of mitochondria for adiponectin synthesis (19).

CHANGES IN ADIPOSE TISSUE WITH INCREASED PHYSICAL ACTIVITY

A negative energy balance is required for weight loss which can be achieved through increased energy expenditure or decreased energy intake. A meta analysis by Miller et al (187) in 1997 showed that exercise alone (74 studies) resulted in an average weight loss of 2.4 kg, diet alone 10.7 kg (224 studies), and diet plus exercise 11.0 kg (119 studies). However, since the average daily caloric restriction was 500-1000 kcal/day, while the increase in energy expenditure only reached 142-214 kcal/day (1000-1500 kcal/week) this finding is not unexpected.

For weight maintenance only 4 of 14 random clinical trials reviewed by Catenacci and Wyatt (53) showed exercise to be significantly better at weight maintenance than no intervention. However, this was not surprising considering 42-72% of subjects reported to have discontinued exercise in three studies (218, 302, 332), and the 58% of individuals who did exercise averaged were only able to average 42 minutes/week of walking in one study (218). Strong evidence suggests that if subjects maintained high exercise levels weight maintenance is possible. Indeed, in cross sectional studies, the National Weight Control Registry (153) and others (116, 145) report that 90% of people averaging greater than 2,500 kcal/week of energy expenditure through exercise maintain signigicant weight loss. Similarly, using the gold standard (double-labeled water) for energy expenditure

studies, women needed 77-80 minutes of exercise/day for successful maintenance of weight loss (252, 320). The STRRIDE study used multiple doses of exercise to show that the equivalent of jogging 20 miles/week was necessary for reductions in abdominal adiposity, but only the equivalent of 12 miles/week could prevent gains in abdominal adiposity (266).

Various exercise modalities can decrease adiposity in numerous obesity paradigms in rodents. Voluntary wheel running is sufficient to prevent weight gain in hyperphagic Osaka Long Evans Transgenic Fatty (OLETF) rats (195, 232). Eight weeks of treadmill training can reverse increases in visceral and subcutaneous adiposity in wild type rats after eight weeks of a high fat diet (98). Swimming rats swimming either 3 x 30 minutes/day or a continuous 90 minutes/day reduces visceral adiposity on a high-fat diet (255). Wheel running following caloric restriction, maintains a body weight and adiposity similar to rats never caloric restricted, while rats without access to running wheels became heavier than the always *ad libitum* fed control group (175). In summary both human and rodent data indicate that increases in physical activity are an effective treatment and prevention measure against obesity.

INCREASED ADIPOSE TISSUE WITH PHYSICAL INACTIVITY

In cross-sectional studies reductions in leisure-time physical activity, or increases in physical inactivity, are correlated with significant weight gain (102, 251, 330). This also occurs in highly trained individuals such as female swimmers whom, two months following the cessation of training had a 4.3 kg increase in fat mass, an amount equivalent to the reduction in calories expended through training (9). Likewise, after the end of their competitive season male competitive cyclists gain 5% absolute body fat in 2

months (99). A large prospective study showed inactivity increases in abdominal fat mass in male and female runners in a dose-dependent manner (329). Finally, when males decreased compliance to resistance exercise to only 14% fat mass increased about 6 kg or 20% during the 24 months following a 6-month period utilizing resistance training for weight maintenance (42).

Rodent models show similar changes in adiposity following physical inactivity. In high-fat fed rats cessation of 6 weeks of treadmill training for 2 weeks, resulted in an increase in food intake, retroperitoneal, and epididymal WAT mass to the level of the non-trained rats (12). Adults rats fed low fat chow and given access to running wheels for 8 weeks show an increase in epididymal fat mass, adipocyte diameter, and adipocyte number, during 1 week of inactivity. A second week of inactivity that did not further increase any of the adiposity markers (170). In lean and obese rats, given access to a running wheel for 3 hours/day as rat pups during weaning and then 24 hours/day until 8 weeks of age, fat mass was reduced, but subsequently surpassed that of control rats after 8 weeks of no access to running wheels (279). Following 3 weeks of voluntary wheel running Kump and Booth (163) saw a 25% and 48% increase in epididymal and omental WAT mass during just 53 hours of inactivity, respectively.

The rapid increases in fat mass observed with the onset of physical inactivity maybe explained by several mechanisms. Following the cessation of physical activity most studies have reported a significant increase in food intake relative to always-sedentary animals (12, 163, 279). In 1 week of inactivity increase fatty acid delivery to WAT occurs through a 155% increase in WAT tissue LPL activity, and may contribute to the increased WAT mass (170). Furthermore, cessation of wheel running for 4, but not

9 days enhances insulin-stimulated glucose uptake and likely lipogenesis, in isolated adipocytes (69). In agreement the lipogenic enzymes GPDH, citrate cleavage enzyme, and malic enzyme all increased in a 2 week period of sedentary cage activity that followed 6 weeks of treadmill training in rats (76). A shorter length of inactivity shows that while 5 hours after the cessation of voluntary wheel running palmitate incorporation into TAG is reduced 73% relative to sedentary animals, it increases 14-fold, and to a level above that of sedentary animals by 10 hours (163). This rapid increase is in part due to an increase in mitochondrial glycerol-3-phosphate acyltransferase-1 activity and protein concentration (164). Together these studies demonstrate a rapid upregulation of the enzymes necessary for storage and conversion of FFAs to TAG in WAT following the cessation of physical activity.

ANIMAL MODELS OF PHYSICAL ACTIVITY, INACTIVITY, AND OBESITY

Three main models of physical activity in rodents are used; voluntary wheel running, treadmill running, and swimming. Forced treadmill and swimming training are capable of rapidly and robustly increasing skeletal muscle mitochondrial content (17, 85). However, this increase is in part due to the larger stress response forced exercise elicits and may not be as representative of how humans historically engaged in physical activity (55). More similar to hunter-gatherer human type physical activity is voluntary wheel running which is intermittent over a period of 12 hours (55, 65). A limitation of voluntary wheel running is that while total duration and distance exceeds that of treadmill-trained rats, classical exercise adaptation phenotypes are relatively modest, with both only a 20-30% increase in mitochondrial enzymes and no increase in treadmill tested maximal oxygen consumption (162, 170). Thus, voluntary wheel running is a

good model of increased physical activity (akin to increased leisure time activity), but not exercise training. Preventing access to voluntary wheels is done to transiently study inactivity. This differs from a classical detraining study, during which cessation of swim or treadmill training occurs.

A variety of obesity models in rodents exist. The three most commonly utilized models in mice are the *ob/ob* mice, which lack the long form of the leptin receptor, the *db/db* mice, which lacks the leptin gene, and diet induced obesity (DIO) in C57/BL6 mice (236). *ob/ob* and *db/db* mice both show disruptions in glucose homeostasis at ~3 weeks of age and develop diabetes at the relatively young age of ~10 weeks. In C57/BL6 mice, which are sensitive to DIO, feeding a relatively high-fat diet (40-65% calories from fat) for as short as 4 weeks increases WAT mass and circulating insulin levels, which full diabetes at a much later age (40, 57). DIO allows the ability to study adult mice, but disadvantages such as the heterogeneity of response and strain differences exist (282). Importantly, most models of rodent obesity exhibit severely reduced voluntary wheel running activity (138, 279).

The OLETF rat is unique because it is has normal voluntary wheel running activity, but is also a monogenetic model of obesity (195, 232). Discovered in a Long Evans colony in the 1980s in Japan, the OLETF rat lacks the cholecystokinin A receptor 1 in the brain, making it hyperphagic and predisposed to develop obesity (144). OLETF rats progress to hyperglycemia by 12 weeks of age and clinical diabetes by 40 weeks of age, making it an excellent model for studying the progression of diabetes in adult animals (192). Unlike other rodent models of obesity, such as Zucker rat, the OLETF rat will use a running wheel. Voluntary wheel running in OLETFs prevents non-alcoholic

fatty liver and the progression to diabetes, but through a mechanism that fails to significantly alter skeletal muscle phenotype (188, 195, 232).

THE EPIGENOME

Francis Collins has stated that genes alone do not tell the whole story; increases in chronic diseases cannot be due to major shifts in the human gene pool as those changes take much more time to occur. Rather, they must be due to environmental changes, including diet and physical activity, which may produce disease in genetically predisposed persons (64).

Unlike genetic mutations or single nucleotide polymorphisms, epigenetic modifications do not result in changes in the DNA sequence. Instead DNA packing or confirmation is indirectly modified by histone modifications (e.g. acetylation or methylation) or directly modified by methylation of the cytosine base in CpG dinucleotides (28). There are over a 100 known histone modifications, some of which exert their effect by recruiting specific transcription factors or modifying chromatin to allow an increase (chromatin opening) or decreasing (chromatin compaction) in gene expression (158). DNA methylation only occurs at cytosines followed by guanines dinucleotides (CpG) within the entire mammalian genome. Importantly, CpG dinucleotides are underrepresented in the genome compared to what is expected by chance (94). However, some regions exhibit a much higher frequency of CpGs and are termed CpG islands (CGIs). CGIs are arbitrarily defined by length (500bp-3000bp), cytosine/guanine content (>50%), and CpG frequency (>60%) (94). Importantly, these CGI are found in an estimated 60-72% of promoters and first exons and are key sites where CpG methylation varies in cancer (84, 247).

MOLECULAR MECHANISMS CONTROLLING CPG METHYLATION AND GENE EXPRESSION

Methylation of cytosines in CpGs is catalyzed by the DNA methyltransferase class of enzymes which transfer a methyl group to the carbon 5 position of cytosine to produce 5-methyl cytosine (295). Methylation of CpGs can be divided into two basic types, either maintenance of methylation that occurs exclusively on the daughter strand during DNA replication, or *de novo* methylation of DNA in non-dividing cells. During the replication of cells DNA methyltransferase 1 (*Dnmt1*) has a 7-21 fold greater preference to methylate hemimethylated dsDNA rather than unmethylated DNA, suggesting a role in methylation maintenance (228). However, each replication of artificially methylated DNA in mouse cells losses 5% of the CpG methylation indicating the fidelity of Dnmt1 is not perfect (327). Furthermore, the $Dnmt1^{-/-}$ mouse has normal methylation of 80% of its CpGs indicating other DNA methyltransferase are clearly involved in methylation maintenances (234). Responsible for *de novo* methylation the DNA methyltranferases, *Dnmt3a* and *Dnmt3b*, were discovered in 1998 by Okano et al (208); who showed they contain non-overlapping functions, and are upregulated during development.

The mechanisms through which the DNA methyltranferases target particular CpGs is poorly understood. Evidence suggests repeated sequences within the DNA seem preferentially targeted for *de novo* methylation (95), as are CpGs within CGI (118, 206). Targeting specific CpGs for *de novo* methylation by recruitment of *Dnmt3a* may occur through histone modifications, such as dimethylation of lysine three on histone four (347). Conversely, a decrease in CpG methylation does occur in rat myoblasts although such a putative demethylase enzyme has not been characterized in mammals (323).

Furthermore, demethylase activity of, methyl binding protein 2 (MBD2), Dnmt3a, and Dnmt3b, is present *in vitro*, but this has yet to be verified *in vivo* (30).

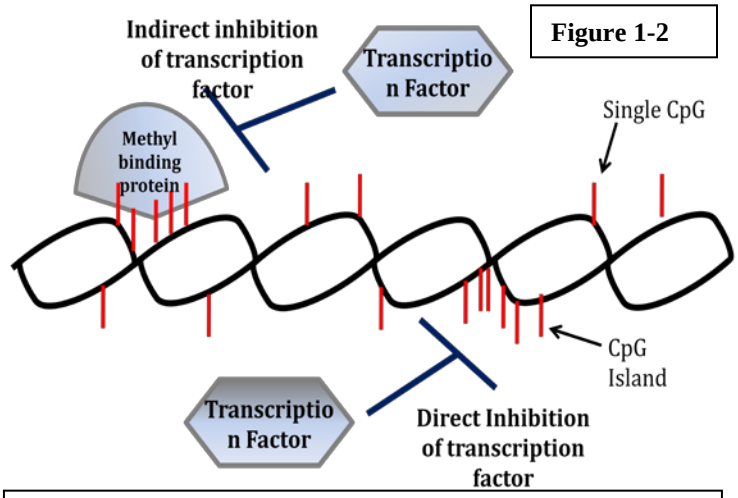


Figure 1-2

Figure 1-2 Dense methylation at CpG Islands can inhibit binding of transcription factors and mRNA trnslation directly and indirectly.

Generally, methylation of CpGs represses mRNA transcription by two main mechanisms; 1) directly inhibiting the binding of certain proteins, or 2) indirectly recruiting additional proteins to the promoter that block the binding of transcription factors (Figure 1-2). An example of the former is CpG methylation at the *H19/Igf2* locus that blocks the vertebrae enhancer protein CTCF from binding the DNA, leading to decreased transcription of *Igf2* (25). Additionally, binding of methylated DNA by the proteins MBD1, MBD2, MBD3, and MeCP2 can both recruit co-repressor complexes to the methylated DNA and block transcription factors that enhance transcription from binding (31).

CPG METHYLATION IN CANCER AND CHRONIC DISEASES

In tumorgenesis the entire genome is generally hypomethylated, except the promoters of tumor suppressor genes, which are hypermethylated, and whose expression is consequently repressed (22). Interestingly, this hypomethylation of the genome also seems to play a role in the development of cancer, as the hypomethylation present in the Dnmt1$^{-/-}$ mouse is associated with an increased incidence of T cell lymphomas (97). On

the other hand, hypermethylation of tumor suppressor genes, such as *p16*^{INK4a}, is one of the earliest changes in cancer cells that prevents normal cell growth checkpoints both in cell culture models of cancer (87) and human carcinomas (24). An insight into how increases in CGI methylation progress spatially was accomplished by overexpressing dnmt3a. Overexpression of dnmt3a increases *de novo* methylation of the E-cadherin promoter first occurs at either end and then progresses towards the middle of the CGI, eventually covering the entire island (103). An increase in DNA methylation in the promoter of the genes that are methylated in colon cancer also occurs with aging, suggesting an environmental role in DNA methylation (5, 134).

A landmark study by Fraga et al (88) comparing monozygous twins provided substantial indirect evidence that environmental factors are capable of changing DNA methylation patterns. The authors compared total DNA methylation content and variability in circulating leukocytes of monozygous twins ranging from 3-74 years old, with a mean age of 30.6 years old. The older the twin pair, the greater difference in total methylated DNA and methylation variability. Differences in the amount of DNA methylation between different aged twins were confirmed with both bisulfite sequencing of specific genes and a global technique using a methylation-sensitive endonuclease (88). Another group, showed age-dependent increases in promoter DNA methylation of the estrogen receptor alpha gene in the right atrium of humans, veins, and arteries; as well as greater methylation within atheroscleric plaque regions of vessels relative to non-diseased area in the same individual studying the promoter (225). Increased promoter methylation of estrogen receptor beta decreases gene expression following repeated passages of smooth muscle cells, but is prevented by pharmacological inhibition of DNA methylation

(148). Interestingly, increases in DNA methylation in leukocytes of subjects with chronic kidney disease positively correlates with cardiovascular disease risk (277). Collectively these studies indicate that differences in DNA methylation can arise from environmental factors and play a role in disease development.

EPIGENETIC CHANGES DUE TO ENVIRONMENTAL CHANGES

There are several examples of epigenetic changes due to the environmental differences in developing mammals worth noting. For example, an increase in the behavior of pup licking by mothers leads to reduced methylation of the growth hormone receptor promoter in pup mice (318). While feeding pregnant (A(vy)/a) mice a methyl-supplemented diet leads to hypermethylation of the agouti gene in and yellow coat color in offspring (333). Although exercise has not been shown to cause changes in CpG methylation, it has been shown to change histone modifications. Acute exercise in rats results in a two-fold increase in acetylation of histone 3 allowing the transcription factor MEF2A to bind the promoter of the GLUT4 gene and increase mRNA expression (269).

SUMMARY OF RATIONALE AND HYPOTHESES FOR ALL EXPERIMENTS

WAT phenotype has long been known to influence chronic disease such as diabetes and cardiovascular disease, while a role of aberrant DNA methylation is just now beginning to be appreciated. The overall rationale for this dissertation was to determine the effects of changes in physical activity levels on established and potential risk factors known for chronic diseases.

Chapter 2: *Rationale*: During the cessation of physical activity, an increase in food intake is observed above that of always-sedentary animals in concert with an increase in WAT. The question of whether the increase in WAT during physical

inactivity is due to inactivity or elevated food intake remains to be answered. *Hypothesis*: Pair feeding of rats during a period of 173 hours of inactivity to that of sedentary rats will prevent the gain in WAT mass normally seen with cessation of voluntary wheel running.

Chapter 3: *Rationale*: During the cessation of physical activity a reduction in fatty acid catabolism in skeletal muscle occurs and may contribute to the increase in WAT mass. The question of how quickly following inactivity FAO falls in skeletal muscle, and whether it also falls in liver and WAT remains unknown. *Hypothesis*: FAO and markers of mitochondrial activity will fall to sedentary levels in skeletal muscle, liver, and WAT following 173 hours of inactivity.

Chapter 4: *Rationale*: In rodents that become obese, mitochondrial content in WAT is reduced. Swimming exercise increases mitochondrial content in WAT in healthy rats, but it remains unknown whether voluntary wheel running can prevent the reduction of mitochondrial content in obese rodents. How WAT mitochondrial content during the progression to diabetes changes remains unknown. *Hypothesis*: Voluntary wheel running will prevent a decrease in WAT mitochondrial concentration in hyperphagic rats and aging will differentially affect healthy and obese rats white WAT mitochondrial content.

Chapter 5: *Rationale*: Evidence suggests that changes in environment are likely to cause differences in DNA methylation that are associated with chronic diseases. Increased physical activity reduces chronic diseases, but it remains unknown if physical activity can change DNA methylation. *Hypothesis:* Voluntary wheel running and

sedentary mice will have different global DNA methylation patterns, which lead to differences in mRNA expression of differentially methylated genes.

Chapter 2: Inactivity Induces Increases in Abdominal Fat[1]

ABSTRACT

Previously, inducing inactivity for 53 h after 21 days of voluntary running resulted in a 25 and 48% increase in epididymal and omental fat pad weights, respectively, while rats continued to eat more than a group that never had access to a running wheel. We wanted to test the hypothesis that inactivity, independent of excessive caloric intake, could induce an increase in fat pad mass. Twenty-one-day-old rats were given access to voluntary running wheels for 42–43 days so that they were running ~9 km/day in the last week of running, after which wheels were locked for 5, 53, or 173 h (WL5, WL53, WL173) before the rats were killed. During the 53 and 173 h of inactivity, one group of animals was pair fed (PF) to match sedentary controls, whereas the other continued to eat ad libitum (AL). Epididymal and retroperitoneal fat masses were significantly increased in the WL173-PF vs. the WL5 group, whereas epididymal, perirenal, and retroperitoneal fat masses were all significantly increased in the WL173-AL group compared with the WL5 group. Additionally, hyperplasia, and not hypertrophy, of the epididymal fat mass was responsible for the increase at WL173-AL as demonstrated by a significant increase in cell number vs. WL5, with no change in cell diameter or volume. Thus two important findings have been elucidated: *1*) increases in measured abdominal fat masses occur in both AL and PF groups at WL173, and *2*) adipocyte expansion via hyperplasia occurred with an ad libitum diet following cessation of voluntary running.

[1] This chapter is presented exactly as published by Laye et al *JAP,* 2007

INTRODUCTION

The Centers for Disease Control (CDC) has classified tobacco, poor diet, and physical inactivity as "actual causes" of premature death, distinguishing them from heart disease and malignant neoplasm as "leading causes" of death (189). The CDC defines physical inactivity as, "not engaging in any regular pattern of physical activity beyond daily functioning" (http://www.cdc.gov/nccdphp/dnpa/physical/terms/index.htm.2006); the model of inactivity employed here ceases voluntary running of rats with only regular cage activity remaining. Of the three major actual causes of death, physical inactivity has received the least attention. Having previously studied physical inactivity in rodents as a further reduction in activity from sedentary or caged conditions by physical restraint (41, 288), we have altered our approach by developing a model that would more closely approximate comparison between innate and voluntary physical activity with more sedentary but ambulatory conditions. It is known that the provision of running wheels to "caged" rats and mice results in their running 2–15 km per night, depending on the strain (72, 238); thus animals provided access to running wheels are naturally more physically active than caged animals. In conjunction with the CDC classification of physical inactivity as an actual cause of premature death, we speculated that allowing rats to have access to running wheels and then systematically locking the wheels would provide insight into processes important during the transition from the natural state of physical activity to a more sedentary existence in caged animals.

Upon locking the wheels of rats to induce physical inactivity, we unexpectedly observed a rapid (53 h) increase in epididymal and omental WAT masses (163). This

observation could be important because human visceral obesity is associated with higher incidence of premature death from the cardiometabolic syndrome (34, 73). However, in the "wheel-lock" (WL) model employed, rats ate ad libitum and consumed more food than sedentary rats during both the voluntary running and the 53 h of wheel lock (inactivity) period, compared with age-matched rats that never had access to running wheels (163). Therefore, it remained uncertain whether the increase in WAT was solely due to excess food consumption rather than decreased physical activity. The present study was designed to specifically test the hypothesis that excess food intake was exclusively responsible for the increase in abdominal fat by feeding one group of rats ad libitum (AL) while limiting food intake of a second group ["pair fed" (PF)] to that of age-matched, always sedentary rats. The basis for selecting the three time points for AL and PF groups after locking wheels was determined from our laboratory's previous findings and are as follows: *1*) 5 h (WL5) because the acute exercise effects on basal glucose uptake into epitrochlearis muscle had disappeared (162); *2*) 53 h (WL53) because enhanced insulin sensitivity had returned to sedentary levels (162) and epididymal and omental fat masses increased (163); and *3*) 173 h (WL173) to ensure that the 53-h increase in fat mass was not a transient effect and that the period of inactivity was of sufficient length to test the hypothesis.

METHODS

Materials. Nylon mesh was from Sefar America (Kansas City, MO). All other reagents were either from Sigma or Fisher.

Animal protocol. The animal protocol was approved by the Institutional Animal Care and Use Committee at the University of Missouri-Columbia. Thirty-six, male

Fischer 344 x Brown Norway F1 hybrid rats (Harlan) were obtained in the fourth week of life. Animals were randomly separated into single cages with access to running wheels (WL) and those without access to the wheels (SED). Rats assigned to running groups were immediately housed (at the age of 21 days) in cages equipped with a voluntary running wheels outfitted with a Sigma Sport BC 800 bicycle computer (Cherry Creek Cyclery, Foster Falls, VA) for measuring daily running activity. Voluntary running was selected to approximate the more natural activity state of the animal. The selection of the age of the rats was based on our laboratory's previous observation that the first night's running distance in voluntary wheels by rats in the fifth week of life was threefold longer than rats in the eighth week of life (6 vs. 1.5 km) (163, 164). Therefore, we hypothesized rats in their fourth week of life would run further total distances than those in their fifth week. Cages were in temperature-controlled animal quarters (21°C) with a 0600–1800 light:1800–0600 dark cycle that was maintained throughout the experimental period. Two cohorts of equal numbers were obtained that had initial body weights of 38.8 ± 1.0 and 33.0 ± 0.9 g and who ran an average of 9.49 ± 1.00 and 8.16 ± 0.74 km daily, respectively, during the last week of running.

All animals were provided 200 g of standard rodent chow (Formulab 5008, Purina Mills, St. Louis, MO) in new cages at the beginning of each week when cages were changed and body weights obtained between 0800 and 1000. Animal cages were changed 7 days before the rats were killed for each group based on a dip in food intake that was noted the day after changing cages (see *days –9* and *–2* in Figure 2-2A). Running activity (for groups with wheel access) was obtained every day of running between 0800 and 1000. Body mass and food intake were measured daily during the fifth and sixth week of

running and following locking of the wheels. Rats in running groups had access to wheels and food and water ad libitum for 42 or 43 days, at which time (0600) wheels were locked for all groups (Figure 2-2). One group of rats was anesthetized [ketamine (80 mg/kg), xylazine (10 mg/kg), and acepromazine (4 mg/kg)] and killed by exsanguination by removal of the heart 5 h (1100; WL5) after locking of wheels; a group of sedentary rats were killed at the same time. The remaining four groups of rats were divided into two main groups (either fed AL or PF to the predicted amount of food being eaten per gram of body weight by an age-matched group that never had access to running wheels). These two main groups of rats were further assigned so that they underwent 53 h (WL53-AL and WL53-PF) or 173 h (WL173-AL and WL173-PF) of wheel lock (designated "inactivity"); these groups were killed after being anesthetized (as above) at 1100 after either 53 or 173 h of inactivity. All animals had access to food until the day of death, when food was removed at 0600.

Increases in upper body fat in humans are reported to worsen metabolic risk factors, but whether this incremental effect is due to abdominal subcutaneous or intraperitoneal fat is disputed (307). Selection of fat depots was based on body cavity; epididymal (intraperitoneal), perirenal (intraperitoneal), and retroperitoneal (extraperitoneal) WATs were removed from exsanguinated animals and weighed. Epididymal fat was divided and placed into osmium tetraoxide or blocked in paraffin following an overnight fix in formalin for microscopic examination. Epididymal fat was selected because of its well-defined anatomic boundaries in the intraperitoneal cavity.

Adipocyte isolation. Adipocytes from epididymal fat pads were isolated by a modification of the Rodbell method (237) as modified by our laboratory (163).

Adipocyte size and number. Preparation of epididymal WAT for determination of cell size and number was performed essentially as described by Cartwright (50), as previously delineated by our laboratory (163). Cell volumes were directly measured with a Coulter counter, and the total number of cells was then calculated from the average cell volume and the weight of the entire epididymal fat pad. For morphometric determinations, sections were stained with hematoxylin and eosin. Thirty random adipocytes from two different areas of the microscopic section for each sample to verify Coulter counter data were measured at a x20 magnification using Image Pro (Silver Spring, MD) imaging software.

Statistics. For each outcome measure, a one-way analysis of variance was done using the MIXED procedure (SAS, Carry, NC). Pairwise comparisons were done using least squares means (SAS) but only for the five pairs of interest (WL5 vs. WL53-AL; WL5 vs. WL53-PF; WL5 vs. WL173-AL; WL5 vs. WL173-PF; and WL173-AL vs. WL173-PF). The 25 *P* values obtained from these comparisons were then used in the PROC MULTTEST (the procedure approaches the multiple testing problem by adjusting the *p*-values from a family of hypothesis tests, SAS) to obtain the set of comparisons that met an approximate 0.05 false discovery rate adjustment (26). Significance for all tests was set at $P < 0.05$. All data are presented as means ± SE.

RESULTS

In the final week of running, rats ran ~9 km/day (Figure 2-1B). On the final day of 42 or 43 days of running, the absolute food intakes of WL rats (23.5 ± 0.5 g/day) was 23% more ($P < 0.001$) than SED rats (19.1 ± 0.7 g/day) (Figure 2-2A). In the first 24 h, when wheels were locked, there was an immediate drop in food intake of 11 and 24% for

the WL-AL and WL-PF groups, respectively (Figure 2-2B); WL-PF rats consumed what SED rats were eating per gram of body weight (Figure 2-2B/C). The WL-AL group consumed significantly more food during *days 1–3* of wheel lock ($P < 0.005$) (Figure 2-2B). However, by *day 4* of wheel lock, this statistical difference no longer existed as determined by repeated-measures ANOVA ($P = 0.06–0.08$ for *days 4–6*, $P = 0.57$ for *day 7* (Figure 2-2B).

Body weights (g) at death were not statistically different between groups (Table 2-1). During the 7 days of WL the WL173-AL and WL173-PF groups gained 9.14 ± 4.2 and 1.4 ± 2.6 g of body weight, respectively. In the first cohort ($n = 5$ for Sed, $n = 2–3$ for other groups) of animals, muscle weights for soleus and plantaris were taken and determined to not differ among groups (data not shown).

Figure 2-1

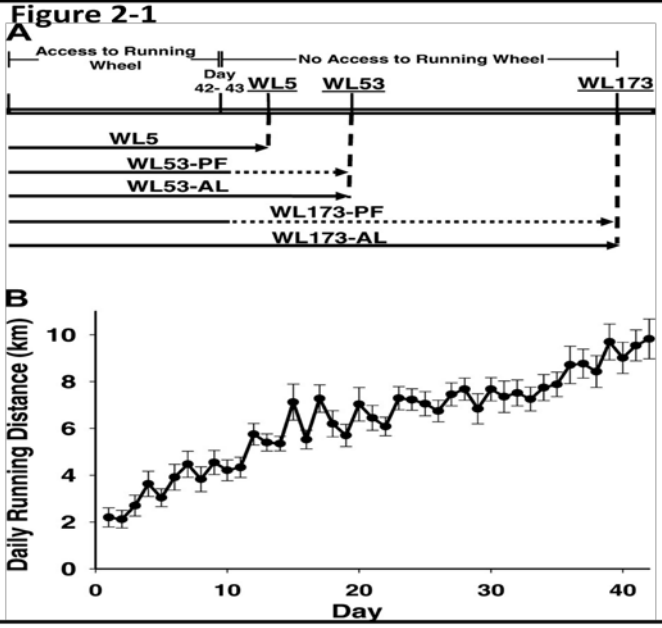


Fig.2-1. Experimental groups and distances run. *A*: depictions of the 5 experimental groups with access to running wheels (WL-5, WL-53, and WL-173, where WL-"X" refers to the hours without access to running wheel after 42–43 days of running). A sixth sedentary group (Sed) without access to running wheels during the entire 42–43 days of running was killed at the same time as the WL5 group (not shown). Arrow tip, time when rats were killed for the running groups; solid line, access to food ad libitum (AL); dashed line, when the pair fed (PF) groups were pair fed per gram of body weight the amount of food being eaten by the Sed group (see METHODS for details); thus PF groups were ad libitum fed during running (solid line) and pair fed (dashed line) during inactivity. *B*: daily distances run. Values are means ± SE; $n = 26$ rats.

Figure 2-2

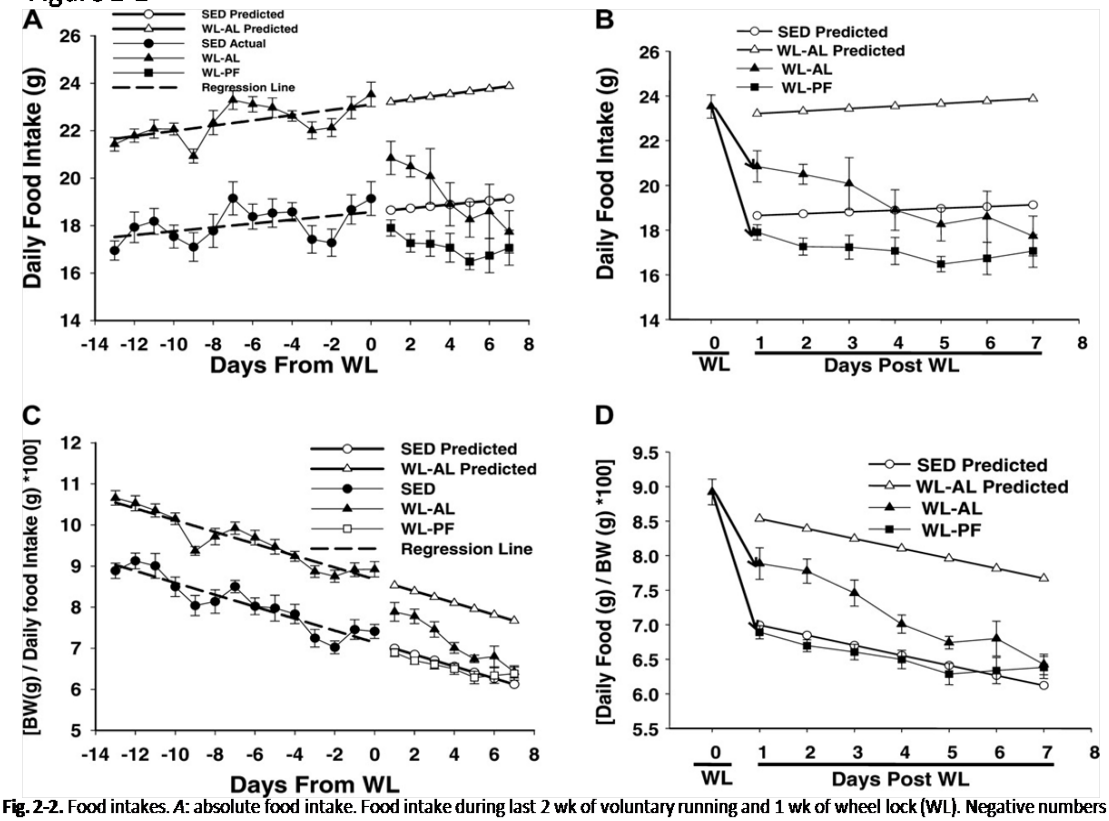


Fig. 2-2. Food intakes. *A*: absolute food intake. Food intake during last 2 wk of voluntary running and 1 wk of wheel lock (WL). Negative numbers correspond to the running days preceding WL and positive numbers the days following WL. *Day 0* represents the end of the preceding 24-h period before locking the wheels at 0600 for all WL animals; therefore, all food intakes on *day 0* include the last night of running. Regression lines (solid lines) were drawn using the 2 wk of daily food intake preceding locking of wheels (dashed lines) to predict the food intake of Sed after wheels were locked. Sed group never had access to wheels and were killed with WL5 group. All animals are fed ad libitum (AL) up until wheels were locked, at which time half the animals were pair fed (PF) and the other half continued ad libitum feeding. *B*: data in *A* after wheels were locked (*day 0WL* on the *x*-axis) are enlarged (*days 1–7* post WL). At 0WL, the former runners were consuming significantly more calories than the Sed group ($p < 0.001$). The last day of mean food intake for the Sed group is at 0WL (*day 0*) and is indicated by ● On *day 1*, there was an immediate drop in food intake of 11 and 24% for the WL-AL and WL-PF groups respectively. The predicted Sed values (○) indicate that the WL-PF group (■) was indeed fed an amount not significantly different from Sed rats throughout WL. Furthermore, although the WL-AL group consumed significantly more food during *days 1–3* ($P < 0.005$), by *day 4* this statistical difference no longer existed ($P = 0.06–0.08$ for *days 4–6*, $P = 0.57$ for *day 7*). Values are means ± SE; $n = 5$ rats for WL5, WL53-PF, WL53-AL, or WL173-AL; $n = 6$ for WL173-PF; $n = 9$ rats for Sed. *C* and *D*: relative food intakes. Food intake is expressed on the *y*-axis relative to body weight (BW) during the last 2 wk of voluntary running and 1 wk of WL. Because body weights did not differ among groups, similar statistical results for relative food intakes as for absolute food intakes were found; thus the statistics for relative food intakes are not given for *C* and *D*. See *A* and *B* for further description.

Table 2-1

Determination	Experimental Groups					
	WL5	WL53-PF	WL53-AL	WL173-PF	WL173-AL	Sed
Final body weight, g	265±9.3	256±10	261±10	268±8.8	276±8.8	256±5.6
Epididymal fat mass, g	1.40±0.11	1.46±0.06	1.54±0.11	1.87±0.08*	2.1±0.17*	1.66±0.12
Perirenal fat mass, g	0.16±0.02	0.21±0.04	0.18±0.02	0.25±0.03	0.30±0.05*	0.25±0.02
Retroperitoneal fat mass, g	0.43±0.25	0.50±0.07	0.41±0.03	0.82±0.03*	0.86±0.18*	0.76±0.06
Epididymal adipocyte volume, pl	202.1±12.1	208.3±22.3	208.9±13.9	214.4±12.2	218.1±15.7	218.0±16.1
Epididymal adipocyte diameter, µm	68.2±1.2	69.8±2.7	68.2±1.2	71.1±1.5	72.5±1.9	71.4±1.5
Epididymal adipocyte number x 10^6	7.63±0.64	8.00±0.81	8.06±0.37	9.66±0.65	10.76±1.06*	8.94±0.90

Values are means ± SE. *n* = 5 rats for WL5, WL53-PF, WL53-AL, or WL173-AL; *n* = 6 rats for WL173-PF; and *n* = 9 rats for SED for all determinations except retroperitoneal fat mass where *n* = 3 rats for WL5, WL53-AL, WL173-PF, and WL173-AL; *n* = 2 rats for WL53-PF; and *n* = 4 rats for Sed. WL5, WL53, WL173, rats given access to voluntary running wheels for 42–43 days after which wheels were locked for 5, 53, or 173 h, respectively; PF, pair fed; AL, ad libitum fed; Sed, sedentary

Figure 2-3

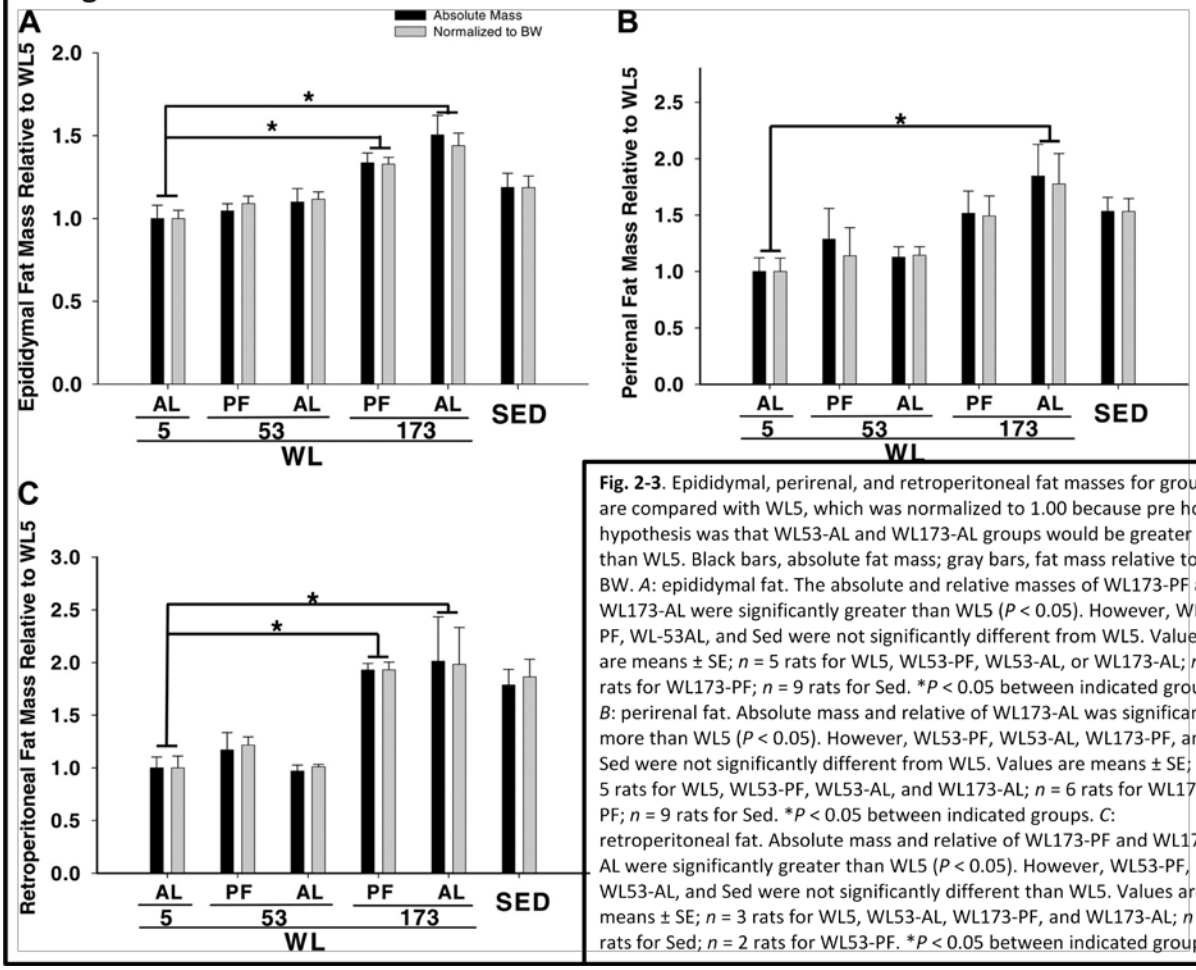


Fig. 2-3. Epididymal, perirenal, and retroperitoneal fat masses for groups are compared with WL5, which was normalized to 1.00 because pre hoc hypothesis was that WL53-AL and WL173-AL groups would be greater than WL5. Black bars, absolute fat mass; gray bars, fat mass relative to BW. *A*: epididymal fat. The absolute and relative masses of WL173-PF and WL173-AL were significantly greater than WL5 ($P < 0.05$). However, WL53-PF, WL-53AL, and Sed were not significantly different from WL5. Values are means ± SE; *n* = 5 rats for WL5, WL53-PF, WL53-AL, or WL173-AL; *n* = 6 rats for WL173-PF; *n* = 9 rats for Sed. *$P < 0.05$ between indicated groups. *B*: perirenal fat. Absolute mass and relative of WL173-AL was significantly more than WL5 ($P < 0.05$). However, WL53-PF, WL53-AL, WL173-PF, and Sed were not significantly different from WL5. Values are means ± SE; *n* = 5 rats for WL5, WL53-PF, WL53-AL, and WL173-AL; *n* = 6 rats for WL173-PF; *n* = 9 rats for Sed. *$P < 0.05$ between indicated groups. *C*: retroperitoneal fat. Absolute mass and relative of WL173-PF and WL173-AL were significantly greater than WL5 ($P < 0.05$). However, WL53-PF, WL53-AL, and Sed were not significantly different than WL5. Values are means ± SE; *n* = 3 rats for WL5, WL53-AL, WL173-PF, and WL173-AL; *n* = 4 rats for Sed; *n* = 2 rats for WL53-PF. *$P < 0.05$ between indicated groups.

Figure 2-4

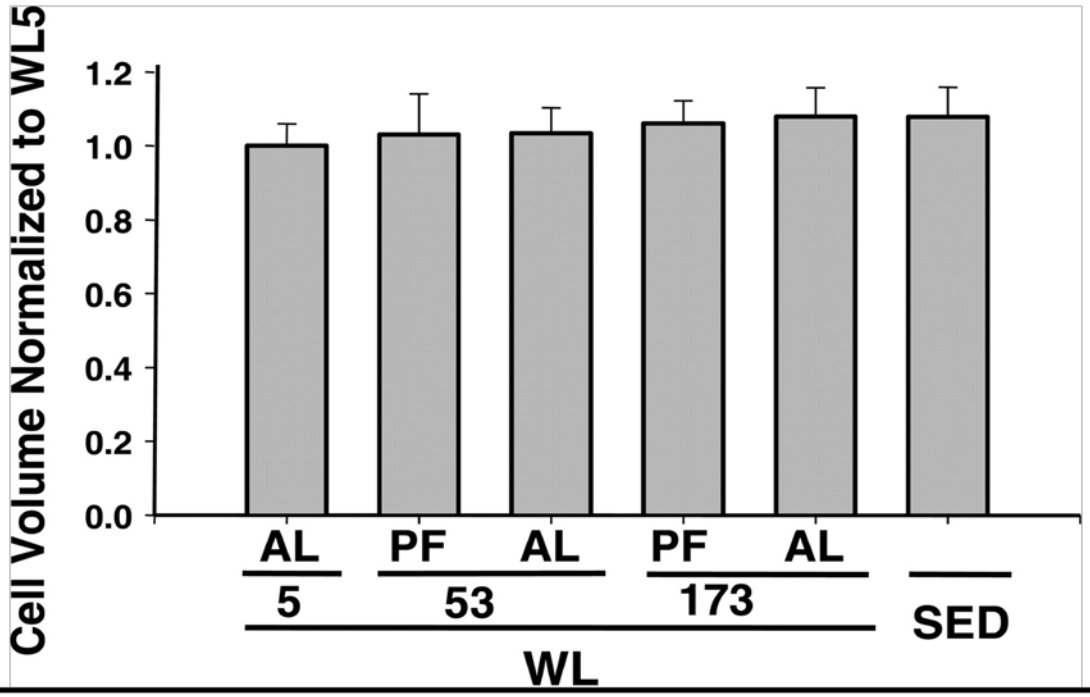


Fig. 2-4. Average epididymal adipose cell volume (pl) for groups are expressed relative to WL5, which was normalized to 1.00. No significant differences were found between WL5 and any group as determined with by a Coulter counter. Values are means ± SE; *n* = 5 rats for WL5, WL53-PF, WL53-AL, and WL173-AL; *n* = 6 rats for WL173-PF; *n* = 9 rats for Sed.

Figure 2-5

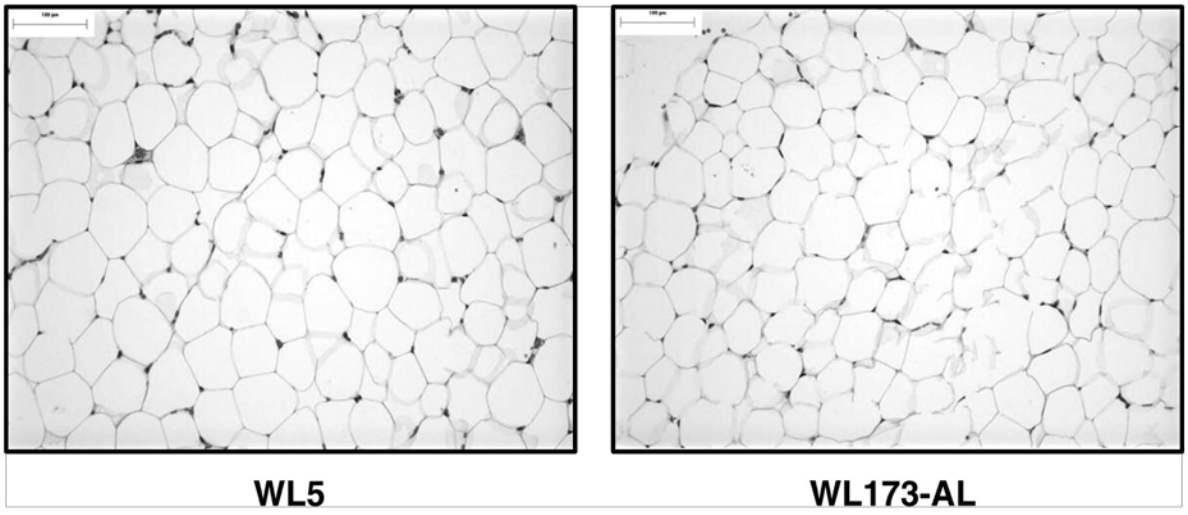


Fig.2-5. Morphometric comparisons of average adipocyte diameters. Representative cross sections with hematoxylin and eosin staining of paraffin-fixed, epididymal adipose tissue is shown. Mean cell diameters of the WL5 (*left*) and WL173-AL (*right*) groups (*n* = 5) were not significantly different. Scale bar in *top left* corner = 100 µm.

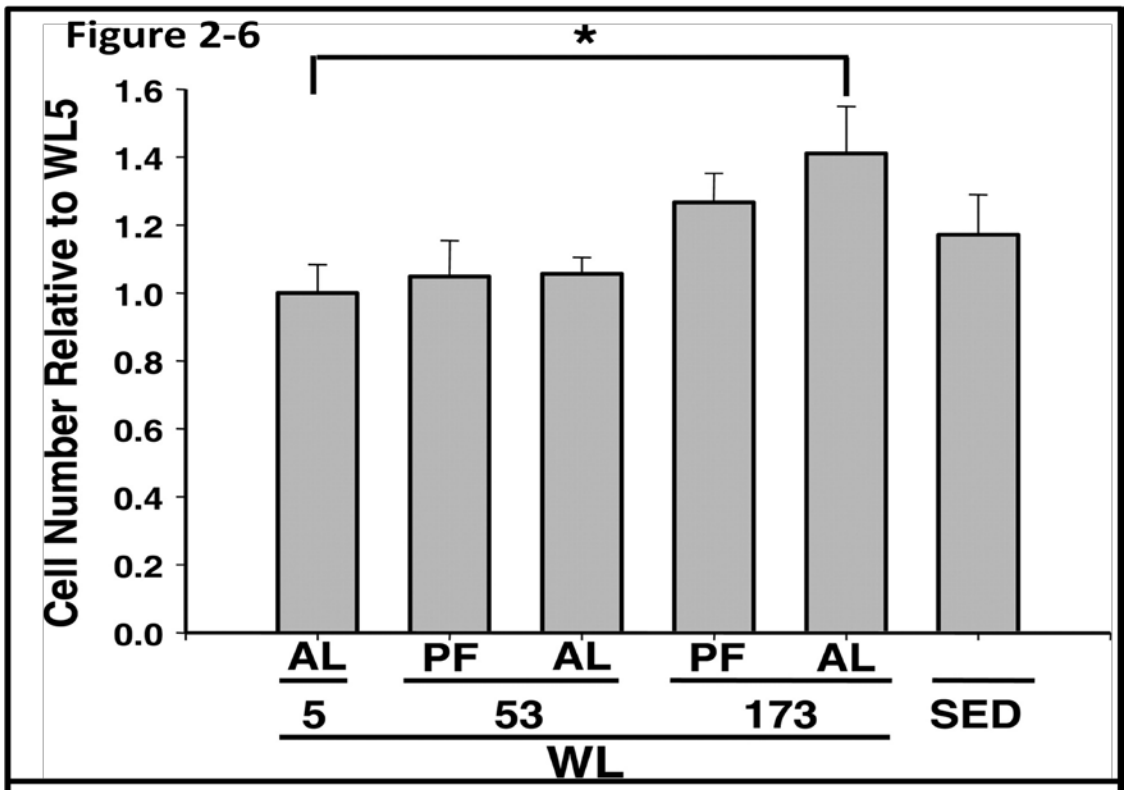


Fig. 2-6. Average adipocyte cell number for groups are expressed relative to WL5, which was normalized to 1.00 (nonnormalized means ± SE for adipose cell numbers are given in RESULTS). Adipocyte cell number is significantly higher in epididymal fat of the WL173-AL group than it is in the WL5 group. No other groups were significantly different from WL5. Values are means ± SE; *n* = 5 rats for WL5, WL53-PF, WL53-AL, and WL173-AL; *n* = 6 rats for WL173-PF; *n* = 9 rats for Sed. **P* < 0.05 between indicated groups.

Epididymal fat masses (g) of WL173-PF and WL173-AL were both significantly ($P < 0.05$) greater than WL5 (Figure 2-3A, Table 2-1). However, WL5 was not different from WL53-PF, WL-53AL, and SED. Perirenal fat mass (g) of WL173-AL was significantly greater ($P < 0.05$) than WL5 (Figure 2-3B, Table 2-1). However, WL5 was not different from WL53-PF, WL53-AL, WL173-PF, or SED. Retroperitoneal fat masses (g) of WL173-PF and WL173-AL were both significantly ($P < 0.05$) greater than WL5 (Figure 2-3C, Table 2-1). However, WL5 was not different than WL53-PF, WL53-AL, or SED. WL173-PF tended to have slightly, nonsignificantly, less fat gains than WL173-AL for all three fat masses, which could be related to WL173-AL consuming more food on

the first 3 days of inactivity (Figure 2-2). Altogether then, the hypothesis that excess food intake was exclusively responsible for the increase in abdominal fat from WL5 to WL173 is deemed not to be held for two of the three determinations; i.e., epididymal and retroperitoneal fat increased at WL173 in the PF condition.

Epididymal adipose cell volume did not significantly differ between WL5 and any group as determined with a Coulter counter (see methods for details) (Figure 2-4, Table 2-1). No differences in adipocyte diameters were observed between WL5 and WL173-AL (Figure 2-5). Only diameters (μm) from WL5 and WL173-AL were calculated via microscope method to verify Coulter counter data. The values were 71.1 ± 1.99, and 74.4 ± 3.45 for WL5 and WL173-AL, respectively. The diameters (μm) from the Coulter counter were not different between WL5 and other groups (Table 2-1). The coulter counter and microscopic (Figure 2-5) methods were in agreement that no difference existed for adipocyte cell size. Both methods gave a 4.6%, nonsignificant, higher value for cell diameter in WL173-AL than in WL5.

Adipocyte number ($x10^6$) in the epididymal fat pad was significantly greater ($P < 0.05$) in WL173-AL (10.76 ± 1.06) than WL5 (7.63 ± 0.64) (Figure 2-6, Table 2-1). Adipocyte numbers per epididymal fat pads for remaining groups were not different from WL5.

DISCUSSION

The sole purpose of the present study was to test whether pair-feeding (food intake levels provided at levels of age-matched rats never having access to running wheels) would entirely prevent the increase abdominal fat that occurs in ad libitum eating upon locking running wheels. A major finding of the present study is that pair feeding

during inactivity did not prevent epididymal and retroperitoneal, but it did prevent the perirenal, fat masses from increasing in size after rats ceased 42–43 days of daily voluntary running. Therefore, the hypothesis that excess food intake was exclusively responsible for the increase in abdominal fat is rejected; the alternative hypothesis that excess food is not exclusively responsible is thus accepted. A new hypothesis generated from these observations is that physical inactivity likely plays some role in increased abdominal fat in the model employed in the present experiments. Another major finding is that adipocyte hyperplasia was present at the seventh day after ceasing voluntary running in those rats ad libitum fed.

Pair feeding during inactivity did not totally prevent the gain in mass of epididymal and retroperitoneal fat masses at the seventh day of inactivity. When comparing the 173rd with 5th h of inactivity after ending 42 nights of voluntary running, PF and AL groups had increases in epididymal fat mass of 34 and 50% and in retroperitoneal fat mass of 93 and 101% (Figure 2-3 A and C, black bars). Importantly, body weights only increased by 0.5 and 3.5% during the 7 days of inactivity, in the WL173-PF and WL173-AL, respectively. Thus body growth alone cannot fully account for the increases in fat mass. For perirenal fat mass, an increase of 85% occurred from the 5th to the 173rd h of inactivity for the AL-ed group. However, the difference from the 5th to the 173rd h of inactivity was 52% in the pair-fed group ($P = 0.19$). No significant differences existed at the 173rd h of inactivity between PF and AL groups for any of the three fat masses. Similar results were obtained when specific fat masses were normalized to body weight (Figure 2-3, A–C, gray bars), suggesting that increases in fat masses during inactivity were in large part independent of body mass. These observations suggest

that inactivity after voluntary running, independent of excess caloric intake, leads to the enlargement of abdominal fat. The major limitation to the interpretation is the absence of indirect calorimetry to more precisely balance caloric intake with expenditure in the inactivity period; therefore, our interpretations are based on pair feeding. An alternative, but not contradictory, interpretation is that physical activity performed voluntarily slowed the growth of abdominal WAT, and the ensuing physical inactivity permitted catch-up growth. Nonetheless, whatever the interpretation, the rapid growth of abdominal WAT opens a future opportunity to dissect mechanisms by which reductions in the caloric expenditure of physical activity increases the partition of calories to fat masses.

Three observations from our laboratory's earlier study (163) provide notions as to how maladaptations to physical inactivity may have played some role in the increased epididymal fat mass, which are *1*) inactivity would omit the nightly 80% suppression of palmitate incorporation into TAG and increase in lipolysis; *2*) palmitate incorporation into triacylglycerol of epididymal fat overshot sedentary values by fourfold at the 10th h of the light cycle (i.e., the 10th h after the wheels were locked to prevent further running); and *3*) a three- to fourfold overshot in palmitate incorporation into triacylglycerol was maintained at the 29th and 53rd h of inactivity after the last night (in contrast to the 80% suppression seen 24 and 48 h earlier at the 5th h after the last night of running). The overshoot of palmitate incorporation into TAG at the 29th and 53rd h after running did not occur after only a single night of running (164), suggesting a potential enzymatic adaptation, such as mitochondrial glycerol-3-phosphate acyltransferase enzyme activity, an enzyme that catalyzes the first committed step in TAG and phospholipid biosynthesis, was 48, 45, and 58% higher than sedentary values at 10, 29, and 53 h of wheel lock,

respectively (164). Because the percent increase in palmitate incorporation into TAG was many fold greater than the increase in glycerol-3-phosphate acyltransferase activity, increases TAG hydrolysis (lipoprotein lipase), free fatty acid transport (fatty acid translocase/CD36), or other potential adaptations could also enhance triacylglycerol stores.

These findings may apply to the gains in fat mass found in human subjects. For example, Yanovski et al. (341) observed almost 10% of subjects gained ≥2.3 kg in an ~6-wk period; the greatest gain being 4.07 kg. The laboratory of Bouchard et al. (224) reported that human fat mass increased an average of 14.3% over a 22-day period; the largest gain was 3.2 kg. The two major differences between the above-mentioned human studies and the present rodent study are the percent increases and rate of increase in fat mass gained in humans are less than rats. Potential explanations are the larger relative metabolic rate seen in rats and/or shorter life span of the rat compared with humans. Additionally, in human studies total fat mass rather than depot specific masses were measured, which may mask gains in a few specific fat depots.

Some (69, 76, 164, 170), but not all (11), studies have reported increases in abdominal fat following the cessation of physical activity in rats. For example, there is a 41% increase in epididymal fat mass (76), a 53% increase in parametrial adipocyte volume (69), a 23% increase in epididymal adipocyte diameter, and 25 and 18% increases in epididymal mass and adipocyte volume (163), respectively, 14, 4, 7, and 2 days, respectively, following the cessation of exercise training. In the latter study, our laboratory used a model of inactivity where wheels are locked after voluntary running; the wheel locked (former runners) continued to consume ~20% more food for the first 2

days of wheel lock than age-matched sedentary (163), raising the concern that the 25% gain in epididymal fat mass was because of higher food consumption and because of not physical inactivity.

The enlargement of fat masses raises the question of whether the increase is associated with adipocyte hypertrophy or hyperplasia, or both. In our laboratory's previous study (163), no adipocyte hyperplasia was observed at the 53rd h of inactivity. In the present study, an unanticipated 41% increase in epididymal fat pad cell number was observed at the 173rd h of inactivity in WL173-AL with no change in cell size. However, in WL173-PF with a 33% increase in epididymal mass, neither the 26% increase in cell number ($P = 0.16$) nor the 6% increase in cell volume ($P = 0.48$) was statistically significant. The mechanism for the increase in epididymal fat mass in WL173-PF is likely to be hyperplasia, but a larger number of observations are necessary to reach statistical significance. A power calculation based on the standard deviations and means of the cell number data for the PF group, as well as the assumption that a difference of 50% would be clinically meaningful, found that nine per group would give a power of $\geq$80% in the WL173-PF group.

The presence of adipocyte hyperplasia in 7 days of physical inactivity in ad libitum-fed rats as employed here is somewhat faster than that occurring in a diet-induced obesity model, described next. At 6 wk of age, rats given a 60% fat diet for 3 wk quadrupled epididymal fat mass (178). However, increases in adipocyte size reached an essential plateau after the first week, which Li et al. (178) interpreted to imply that any subsequent increase in epididymal wet weight from 1 to 3 wk of the high-fat diet was largely the result of hyperplasia. Similarly, human subjects overfed for 8 wk gain 3–4 kg

in fat mass, which according to Levine et al. (176) is likely due to hyperplasia as mean adipocyte size in subcutaneous WAT did not increase. Therefore, there is evidence that humans can undergo adipocyte hyperplasia in the relatively short duration of weeks similar to rats when in a positive-calorie state.

Previous studies have shown adipocyte hyperplasia, but these studies compared separate groups of exercising and sedentary animals and also were not designed to measure durations of inactivity as short as in the present study. Sedentary rats, either pair weighted to the exercising group or ad libitum fed, had 28 or 54%, respectively, more adipocytes per epididymal fat pad than a group of rats participating in a 14- to 16-wk swim training program (211). Craig et al. (69) showed that sedentary rats had 108% more epididymal adipocytes than rats that had voluntarily ran in wheel from 6 to 12 mo of age (and subjected to an ~8% food restriction for approximately the last 2 mo). In addition, we have observed that 87-wk-old sedentary female rats had 123% greater number of adipocytes in the ovarian fat pad than age-matched rats that had access to running wheels beginning at 4 wk of age (D. S. Kump and F. W. Booth, unpublished observation; $n = 3$ per group). Taken together, our results extend these observations to show that it takes only 7 days of inactivity for adipocyte hyperplasia to occur in ad libitum-fed rats. Future studies need to determine whether known inducers of adipocyte hyperplasia (108) occur between inactivity days 2–7 because our laboratory's earlier paper (163) found no increases in peroxisome proliferator-activated receptor-γ and CCAAT/enhancer binding protein-α protein levels in epididymal fat at 53 h of inactivity after 21 days of voluntary running.

Disproportional greater rates of fat deposition relative to lean tissue are well documented in infants born small for gestational age and/or whose growth faltered during infancy and childhood, but who show subsequent catch-up growth (210), and in adults (81) recovering body weight after weight loss due to a variety of conditions (war-related famine, poverty-related undernutrition, experimental starvation, anorexia nervosa, and other clinical hypermetabolic conditions such as cancer, septic shock, and acquired immunodeficiency syndrome). The rapid increase in abdominal fat encountered after running ceases in young rats may be have similar biochemical drives as the aforementioned examples.

Differences in outcomes for adipocyte hypertrophy have been noted between the present and our laboratory's previous study (163); that may be, in part, due to the two experimental designs used. Rats in the present and previous reports (162-164) were given access to running wheels in the fourth and fifth week of life, respectively, ran 2 and 6 km the first night, respectively, and had access to running wheels for 6 and 3 wk, respectively. The two designs produced remarkably different outcomes. Rats in the present and our laboratory's previous reports (162-164) ran distances of 9 and 5 km/day during the last week of running, respectively, and they had body masses, skeletal muscles, and epididymal adipocyte volumes that were similar in size and larger, respectively.

Attention to the specificity of the model needs to be made. Inactivity (and the lagging attenuation in caloric expenditure) follows a period of caloric intake and expenditure that are greater than in rats never having access to voluntary running. Thus it is inactivity coming after a chronic period of daily running that produces the enlargement of fat pads. A potential speculation is that daily activity inhibits adipocyte proliferation in

young animals and that the induction of physical inactivity allows for adipocyte hyperplasia. Because very young rats were used in the study, a future study needs to examine more mature rats.

In summary, physical inactivity, independent of excess caloric intake, is associated with rapid increases abdominal WAT masses. Furthermore, adipocyte hyperplasia in epididymal fat was present by the seventh day of inactivity in the *ad libitum*-eating group.

Chapter 3: Cessation of daily wheel running differentially alters fat oxidation capacity in liver, muscle, and adipose tissue[2]

ABSTRACT

Physical inactivity is associated with the increased risk of developing chronic metabolic diseases. To understand early alterations caused by physical inactivity, we utilize an animal model in which rats are transitioned from daily voluntary wheel running to a sedentary condition. In the hours and days following this transition, WAT mass rapidly increases, due in part to increased lipogenesis. However, whether a concurrent decrease in fatty acid oxidative capacity (FAO) in skeletal muscle, liver, and WAT occurs during this period is unknown. Following six weeks of access to voluntary running wheels (average distance of ~6 km a night), rats were rapidly transitioned to a sedentary state by locking the wheels for 5 hours (WL5) or 173 hours (WL173). Complete (^{14}C palmitate oxidation to $^{14}CO^2$) and incomplete (^{14}C palmitate oxidation to ^{14}C labeled acid soluble metabolites) was determined in isolated mitochondrial and whole homogenate preparations from skeletal muscle and liver and in isolated adipocytes. Strikingly, the elevated complete FAO in the red gastrocnemius at WL5 fell to that of rats that never ran (SED) by WL173. In contrast, hepatic FAO was elevated at WL173 above both WL5 and SED groups; while in isolated adipocytes, FAO remained higher in both running groups (WL5 and WL173) compared to the SED group. The alterations in muscle and liver fat oxidation were associated with changes in carnitine palmitoyl transferase-1 activity and inhibition, but not significant changes in other mitochondrial enzyme activities. In addition, PGC-1α mRNA levels that were higher in both skeletal

[2] Chapter is published exactly as by Laye et al *JAP,* 2009

muscle and liver at WL5 fell to SED levels at WL173. This study is the first to demonstrate that the transition from high to low daily physical activity causes rapid, tissue-specific changes in FAO.

INTRODUCTION

Alterations in our current environment, including physical inactivity and the excessive consumption of high calorie/fat foods, are root causes for the rapid rise of obesity and diabetes in humans (16, 55, 128, 242). Although many investigations have detailed the role of over-nutrition following changes in substrate utilization and storage, the effects of a transition from high to low physical activity remain largely understudied and underappreciated.

Our research group utilizes "the rodent wheel lock model" to study acute transitions from high to low levels of daily physical activity (162-164, 173, 195, 232). After days or weeks of daily voluntary wheel running (2-15 km/night), wheels are locked for progressive periods of time (from 5 to 173 hours) providing an acute window of time to study the metabolic alterations that ensue. While resulting in only modest increases in mitochondrial enzymes compared to forced treadmill training (85, 162), voluntary wheel running (5-15 km/night) can increase energy expenditure far greater than treadmill training (~2 km/60 min treadmill bout). Thus, it is a good model to study the transition from high to low daily physical activity, but an inappropriate model to study detraining. In our previous studies, the sudden cessation of daily running caused rapid and robust increases in the weight of retroperitoneal and omental fat pads after 53 and 173 hours of wheel lock whose causes are beginning to be explained by intrinsic changes in substrate partitioning and not simply a positive energy balance due to decreased daily energy

expenditure (163, 173). For example, rates of key enzymes in WAT *de novo* lipogenesis and palmitate incorporation into adipose triglycerides remain significantly higher in the hours and days immediately following wheel lock (163, 164) and after the cessation of forced treadmill running (76). Although, enhanced *de novo* lipogenesis could partially explain the rapid increases in fat pad weight after the cessation of activity, we also speculated that rapid decreases in fatty acid oxidative capacity (FAO) of skeletal muscle, liver, and WAT also may play a significant role.

Decreased FAO in metabolic tissues has been strongly associated with chronic disease(s) (33, 121, 126, 130, 146, 149, 264). Though findings are not universal (113, 161), extremely obese and type 2 diabetic subjects display reduced systemic and skeletal muscle FAO, which has been linked to increased adiposity, increased intramuscular lipid storage, and skeletal muscle insulin resistance (33, 126, 149, 290). Decreased hepatic FAO directly causes or increases susceptibility for fatty liver and steatosis (131, 232) and also has been linked to increased susceptibility to diet-induced obesity (136). In addition to skeletal muscle and liver FAO in adipocytes has become an important area of study. FAO is decreased in the WAT of *db/db* mice (60) and mitochondrial markers are also reduced in the adipocytes of several animal models of type 2 diabetes and obesity (71, 300). However, while it is well known that increased physical activity decreases the risks of type 2 diabetes and obesity it remains unknown whether physical activity modifies adipocyte FAO.

The aim of this study was to determine if the cessation of voluntary wheel running leads to concurrent alterations in tissue fatty acid catabolism, carnitine palmitoyl transferase-1 (CPT-1) activity and regulation, and mitochondrial enzyme activity. Since

the transition from high to low physical activity is likely to cause systemic changes, we assessed FAO and mitochondrial function in WAT, liver, and mixed skeletal muscle in hopes of better understanding the integrative response(s) of an organism to reductions in energy expenditure and rapid gains in adiposity. We hypothesized, that the transition from high to low physical activity would lead to a coincident reduction in FAO and mitochondrial enzyme activity in skeletal muscle, liver, and adipocytes.

METHODS

Animal protocol. The animal protocol was approved by the Institutional Animal Care and Use Committee at the University of Missouri-Columbia. Thirty-six, male Fischer 344 x Brown Norway F1 hybrid rats (Harlan) were obtained at 21 days of age. Animals were randomly separated into those with access to running wheels (WL) and those without access to the wheels (Sed). Rats assigned to running groups were immediately housed (at the age of 21 days) in cages equipped with a voluntary running wheels outfitted with a Sigma Sport BC 800 bicycle computer (Cherry Creek Cyclery, Foster Falls, VA) for measuring daily running activity. Voluntary running was selected to approximate the more natural physical activity level of the animal. Rats were maintained on a 12-h light, 12-h dark cycle that ran from 0600 to 1800. Running activity was obtained every day of running between 0800 and 1000, and rats in the running group had access to wheels and food and water ad libitum until ~9 wk of age, at which time (0600) wheels were locked. Rats were anesthetized [ketamine (80 $mg \cdot kg^{-1}$), xylazine (10 $mg \cdot kg^{-1}$), and acepromazine (4 $mg \cdot kg^{-1}$)] and killed by exsanguination by removal of the heart either 5 h (WL5), or 173 h (WL173) after locking of wheels; the sedentary rats (SED) were sacrificed at the same time. All animals were fasted for 5 hours prior to sacrifice.

Dual-energy X-ray absorptiometry (DEXA). One week prior to and the day of sacrifice animals were anesthetized with isoflurane (one week prior) or ketamine (80 $mg \cdot kg^{-1}$), xylazine (10 $mg \cdot kg^{-1}$), and acepromazine (4 $mg \cdot kg^{-1}$) (day of sacrifice) and whole-body composition was measured using a Hologic QDR-1000/w DEXA machine calibrated for rats.

Serum measures. Plasma glucose (Sigma), triglycerides (Sigma), and free fatty acids (FFAs; Wako Chemicals, Richmond, VA) were measured with commercially available kits according to the manufacturer's instructions.

Adipocyte isolation. Adipocytes from epididymal fat pads were isolated by the method of Rodbell (237) and modified by us (163). Fresh triplicate 30 μL aliquots of packed adipocytes were used for FAO and a single 30 μL aliquot frozen at -80° C for DNA content, with the remainder lysed in Cell Lytic (Sigma, USA) for enzyme activity assays.

Adipocyte DNA content. DNA content in isolated adipocytes was determined by the flurometric assay described by Labarca and Paigen (168). Briefly, packed adipocytes were lysed in 2M NaCl, 50 mM $NaPO_4$ (pH 7.4) and 20 ul added to 80 μL of working solution (0.04 μg/mL of Hoeschst #33258, 10 mM Tris-HCl, 1mM EDTA, 200 mM NaCl, pH 7.4) in a 96-well plate. Samples were excited at 356 nm resulting in an emission at 458 nm. Calf thymus DNA (Sigma, USA) was used to generate a standard curve.

Tissue homogenization procedure. Red gastrocnemius and livers were quickly excised from anesthetized rats and placed in ice-cold isolation buffer (100 mM KCl, 40 mM Tris·HCl, 10 mM Tris-base, 5 mM $MgCl_2 \cdot 6H_2O$, 1 mM EDTA, and 1 mM ATP; pH

7.4). For fresh acid oxidation assays, ~50–100 mg of tissue was thoroughly minced with scissors in 200 µl SET buffer (250 mM sucrose, 1 mM EDTA, 10 mM Tris·HCl, and 2 mM ATP; pH 7.4) and then the buffer volume was brought up to yield a 20-fold (wt/vol) diluted sample. This was transferred to a 3-ml Potter-Elvehjem glass homogenization vessel. Tissue suspensions were mechanically homogenized on ice with a Teflon pestle at 10 passes over the course of 30 s at 1,200 rpm. Homogenates were kept on ice until oxidation experiments were performed.

Mitochondrial isolation from red gastrocnemius skeletal muscle and liver. Mitochondrial suspensions were prepared according to modified methods of Koves et al. (159, 160). A portion of the red gastrocnemius and liver was quickly excised from unconscious rats and placed in 5 ml of fresh *buffer A* (100 mM KCl, 50 mM MOPS, 5 mM MgSO4, 1 mM EGTA, 1 mM ATP, pH 7.4). The tissue was then minced and suspended 7-fold (wt/vol) in *buffer A*, and homogenized for 15 s. The homogenate was then centrifuged at 800 *g* for 10 min at 4°C and the supernatant was filtered through gauze and set aside. The remaining pellet was resuspended in *buffer A*, homogenized, and the homogenate was then centrifuged at 800 g for 10 min at 4°C. The remaining supernatant was added to the previously set aside supernatant after filtering through gauze. The pooled supernatants were centrifuged at 9,000 *g* to pellet the mitochondria. The pellet was resuspended in *buffer B* (*buffer A*, 0.2% BSA, pH 7.4), and centrifuged at 8,000 *g* before the pellet was washed and resuspended in *buffer C* (100 mM KCl, 50 mM MOPS, 0.5 mM EGTA, pH 7.4) and centrifuged for 10 min at 7,000 *g*. The final pellet was resuspended in 0.5 ml of sucrose EDTA (SET) buffer and protein content was determined.

Fatty acid oxidation. FAO was measured with radiolabeled [1-^{14}C]palmitate (American Radiochemicals) in fresh red gastrocnemius skeletal muscle and liver homogenates and isolated mitochondria using modified methods of Dohm et al. (77, 232). The reaction buffer used for whole homogenates utilized a cold palmitate concentration of 200 µM while the reaction buffer used for isolated mitochondria utilized a concentration of 50 µM. For isolated adipocytes, 30 µl of freshly isolated adipocytes/reaction was added to 120 µl reaction buffer in a trapping device with an adjacent chamber containing 100 µl of NaOH that captured the $^{14}CO_2$, representing the complete oxidation radiolabeled palmitate, when 100 µl of perchloric acid was added to the reaction following a two-hour incubation at 37º C. The ^{14}C labeled acid soluble metabolites, representing incomplete FAO were also collected.

Citrate synthase, beta-hydroxyacyl-CoA dehydrogenase (β-HAD), and cytochrome c oxidase activity. Citrate synthase and β-HAD activities were determined in red gastrocnemius, liver, and adipocyte homogenates using the methods of Srere et al (274) and Bass et al (21), respectively, and as previously described by our group (232). β-HAD activity was undetectable in the adipocyte fraction. Cytochrome c oxidase activity was determined by a commercially available kit (Sigma, St. Louis, MO, USA) in all three tissues as previously described by our group (232). All assays were performed at 37˚C.

CPT-1 assay CPT-1 activity and inhibition by malonyl-CoA using modified methods of Koves et al. (161). 90 µl of reaction buffer (117 mM Tris HCl, 0.28 mM reduced Glutathione, 4,4 mM ATP, 50 uM Palmitoyl-CoA, 4 mg/ml Rotenone, 0.1% BSA) containing 0.2 mM [^{3}H]-Carnitine and either 0 or 10 µM malonyl-CoA was added

to 10 µl of isolated mitochondria of red gastrocnemius skeletal muscle and liver homogenate for 6 min at 37^{o}C. [^{3}H]palmitoyl-carnitine formed was extracted with 350 µl of water-saturated butanol, quantified by liquid-scintillation counting, and normalized to protein content.

Statistics. Each outcome measure was examined in 6-8 animals. For each outcome measure, a one-way analysis of variance was performed (Sigma Stat). However, if normality of the data set failed, a Kruskal-Wallis ANOVA on ranks was performed, with Dunn's method comparison (control group = WL5) as the post-hoc test. A significant main effect ($p<0.05$) was followed-up with Student-Newman-Kuel post-hoc comparisons. Values are reported as means ± standard error of the mean (SE), and a $P < 0.05$ denotes a statistically significant difference.

RESULTS

Body Composition. DEXA measures were taken 7 days prior to, and on the day of sacrifice to capture any changes leading to sacrifice. In the 173-hour wheel lock period, the WL173 group increased percent body fat and total body fat mass by 45% and 46%, respectively, ($p < 0.05$), essentially returning body fat levels to those measured in the SED group (Table 3-1). No changes in percent or total body fat mass occurred in the 7 days prior to sacrifice in the WL5 or SED groups. Intriguingly total lean mass showed a different pattern than total fat mass (Table 3-1). Total lean mass increased in WL5 and SED in the 7 days prior to sacrifice suggesting a positive protein balance ($p < 0.05$). In contrast the WL173 group did not show a net increase in lean body mass during the 7 days of wheel lock prior to sacrifice, suggesting different rates of lean mass catabolism

and/or anabolism during the 7 days following the cessation of running. All groups gained equal body mass in the 7 days prior to sacrifice.

Table 3-1

DEXA Measurements		Group WL5	WL173	SED
Body Fat %	7 Days Prior to Sac	4.1±0.5^{A}	4.7±0.6^{A}	6.6±0.4^{B}
	Day of Sac	4.5±0.4^{A}	6.8±0.4^{B}	6.8±0.7^{B}
	Δ	0.4±0.4	2.1±0.8$^{\#}$	0.2±0.6
Total Body Fat (g)	7 Days Prior to Sac	9.1±1.0^{A}	10.7±1.7^{AB}	13.8±1.1^{B}
	Day of Sac	10.6±0.8^{A}	15.6±1.2^{B}	15.6±2.1^{B}
	Δ	1.5±0.8	4.9±1.7$^{\#}$	1.8±1.3
Total Lean Mass (g)	7 Days Prior to Sac	214.7±4.6	211.1±8.5	193.4±8.4
	Day of Sac	223.6±4.1	212.1±7.4	208.3±7.1
	Δ	8.9±2.8$^{\#}$	0.9±3.1	14.9±1.5$^{\#}$
Total Body Mass (g)	7 Days Prior to Sac	223.9±4.4	221.8±9.5	207.2±9.2
	Day of Sac	234.1±4.0	227.7±8.0	223.8±8.6
	Δ	10.3±2.4$^{\#}$	5.9±2.0$^{\#}$	16.6±1.5$^{\#}$
Ave Daily Food Intake (g)	Week -2 to -1	18.3±0.8^{A}	18.8±0.6^{A}	13.7±0.3^{B}
	Week -1 to 0	19.3±0.3^{A}	16.6±0.3$^{\#B}$	15.2±0.6$^{\#B}$

Table 3-1. Body composition and food intakes. Body anthropometric measurements were taken 7 days prior to sacrifice and on the day of sacrifice by small animal DEXA. Values are means ± SE (n=7-8/group). Δ, is the difference between the two measures. Weekly food intakes were made for the 2 weeks preceding sacrifice. Different letters indicate that values are significantly different between groups at 7 days prior to, or on the day of sacrifice ($p<0.05$). $^{\#}$ indicates a significant increase during the seven-day period within the same group ($p<0.05$). Daily food intake was averaged for the week prior to sacrifice (week -1 to 0) in WL5 and SED, and from two weeks prior to sacrifice to one week prior to sacrifice (week -2 to -1) in WL173, allowing comparison of pre WL food intake to post WL in the WL173 group. Values are means ± SE. WL5, WL173, and SED groups 7-8 animals.group.

Food Intake and Running Distances. Average daily food intakes during the last week of life for WL5 and WL173 were 27% and 37%, respectively, higher than SED ($p<0.05$) (Table 3-1). This is consistent with our previous findings that voluntary wheel running rats have greater food intakes than sedentary counterparts in this strain, age and gender of rat (162, 173). During the 7 days of wheel lock, the WL173 group decreased their average daily food intake by 12% ($p<0.05$) as compared to the previous week; at the same time, food intake by the SED group increased 11% ($p<0.05$). Therefore, any differences detected were not due to overeating by the WL173 group since they ate a similar level to the SED during the 173 hrs of WL.

Neither total 6-wk nor average daily running distances (6.12 vs. 5.43 km/day, $p=0.42$) differed between WL5 and WL173 groups. The average daily running distance in the last week of running was 8.87 km and 9.73 km in WL5 and WL173 groups, respectively ($p=0.68$).

Fat Pad and Serum Characteristics. Absolute epididymal fat masses did not differ among groups ($p=0.086$), but when normalized to total mass, WL5 were lower than WL173 by 25% ($p<0.05$). Serum triglycerides ($p=0.146$), FFAs ($p=0.188$), and glucose ($p=0.147$) did not statistically differ among WL5, WL173, and SED groups (Table 3-2).

Table 3-2

Epididymal Fat Pad Characteristics	WL5	WL173	SED
Epididymal Fat Pad (g/g of BW*100)	5.13±0.24^{A}	6.84±0.38^{B}	6.63±0.42^{B}
Epididymal Fat Pad (g)	1.20±0.06	1.57±0.13	1.50±0.15
Serum Measurements	**WL5**	**WL173**	**SED**
Triacylglycerol (mg/mL)	34.3±2.4	44.8±3.3	38.3±4.2
Free fatty acids (mmol/L)	146.2±9.7	168.6±9.4	176.3±13.7
Glucose (mg/dL)	470.9±10.6	462.8±19.7	429.9±13.7

Table 3-2. Epididymal adipose and serum measurements. Values are means ± SE (n=6-8/group. Different letters indicate that values are significantly different between groups ($p<0.05$). Values are means ± SE.[3]

Fatty Acid Oxidation and Mitochondrial Enzyme Activities. Complete [$^{14}CO_2$] and incomplete [^{14}C labeled acid soluble metabolites (ASM)] capacity for palmitate oxidation was determined in isolated mitochondria or whole tissue homogenates from red gastrocnemius muscle and liver. In addition, complete and incomplete FAO was also measured in isolated adipocytes from epididymal fat pads. For whole red gastrocnemius homogenate, palmitate oxidation at WL5 was 33% and 50% higher than WL173 and SED, respectively, ($p=0.059$; Figure 3-1A). For isolated mitochondria from the red gastrocnemius muscle, complete palmitate oxidation to CO_2 was 91% higher at WL5 compared with SED ($p<0.05$, Figure 3-1B) but did not differ from WL173 ($p=0.24$).

[3] Serum measurements data collected by RS Rector and lab of JP Thyfault

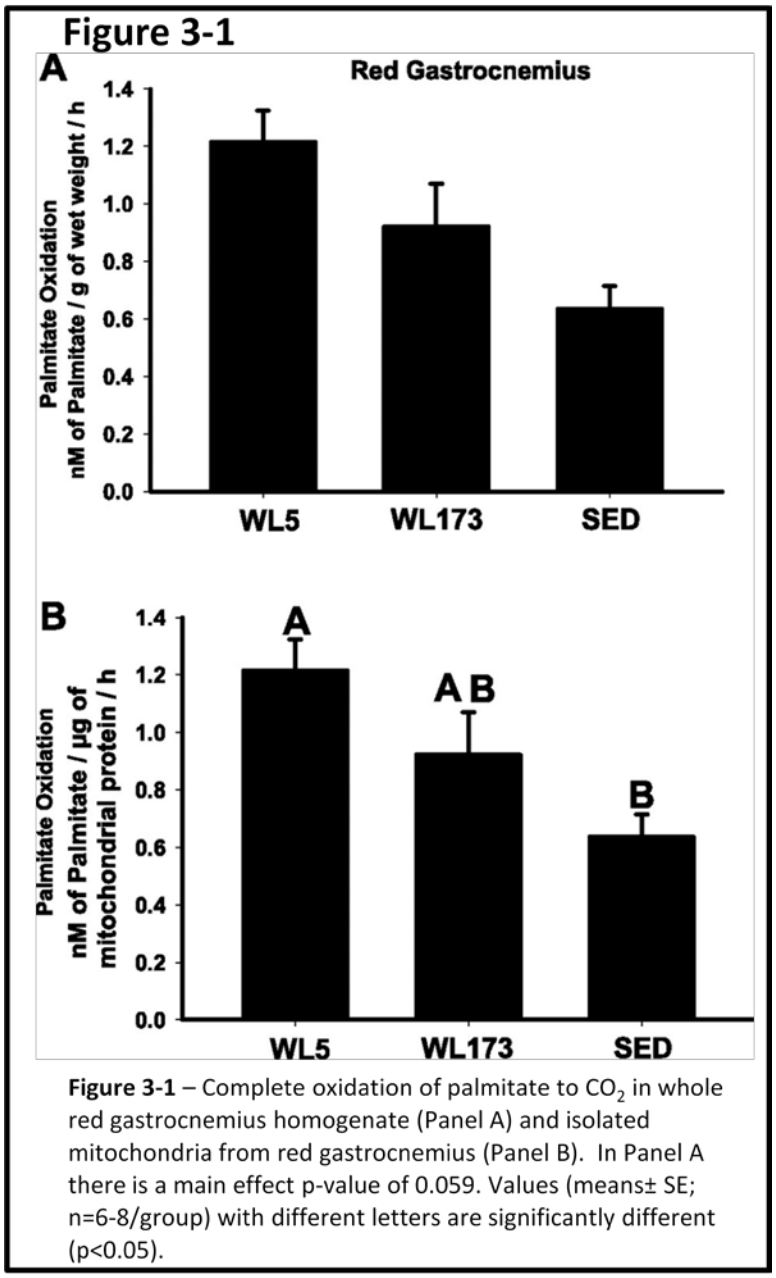


Figure 3-1 – Complete oxidation of palmitate to CO_2 in whole red gastrocnemius homogenate (Panel A) and isolated mitochondria from red gastrocnemius (Panel B). In Panel A there is a main effect p-value of 0.059. Values (means± SE; n=6-8/group) with different letters are significantly different ($p<0.05$).

[4]

In a contrast to what was found in the red gastrocnemius skeletal muscle, complete hepatic FAO increased after the transition to physical inactivity. Whole liver homogenates from the WL173 group completely oxidized 65% and 46% more palmitate than WL5 and SED groups respectively ($p<0.05$; Figure 3-2A). Total palmitate oxidation

[4] Data collected by lab of JP Thyfault

(CO_2 + ASMs) in liver did not differ among groups, an effect potentially in part due to the contribution of extra-mitochondrial peroxisomal activity. In addition, complete oxidation in the isolated liver mitochondria was not statistically different among groups, but showed a similar trend as the whole homogenate (Figure 3-2B).

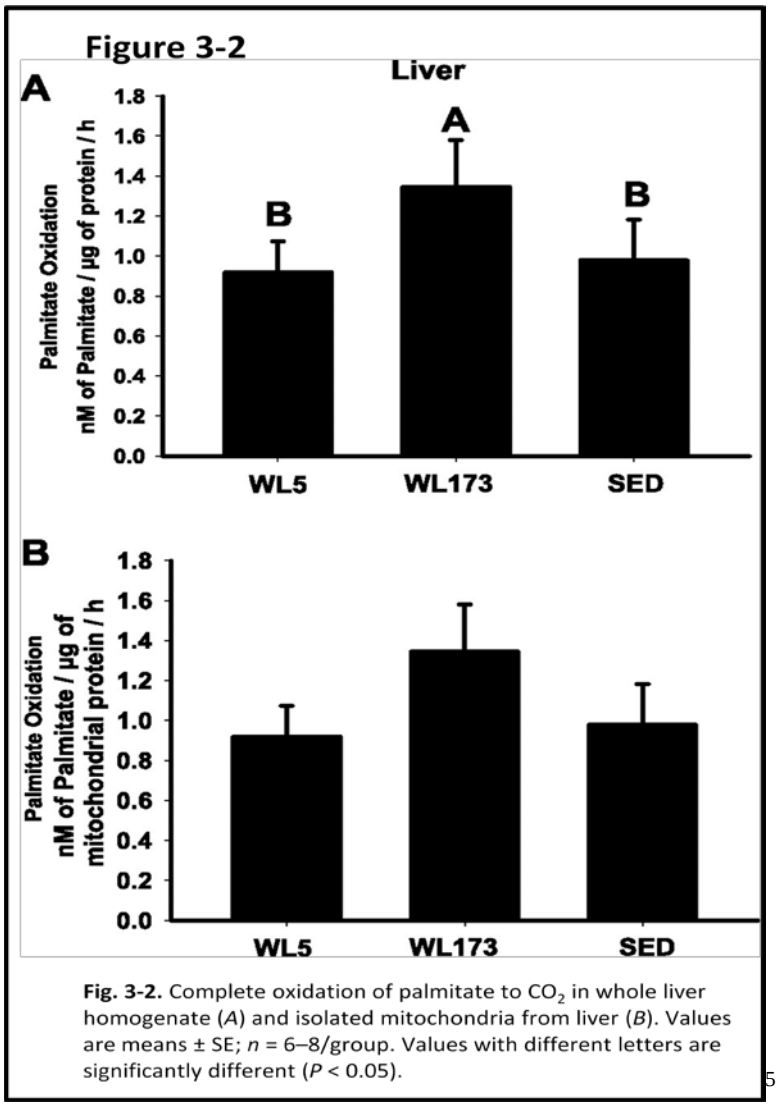


Fig. 3-2. Complete oxidation of palmitate to CO_2 in whole liver homogenate (*A*) and isolated mitochondria from liver (*B*). Values are means ± SE; *n* = 6–8/group. Values with different letters are significantly different ($P < 0.05$).[5]

[5] Data for figure collected by RS Rector

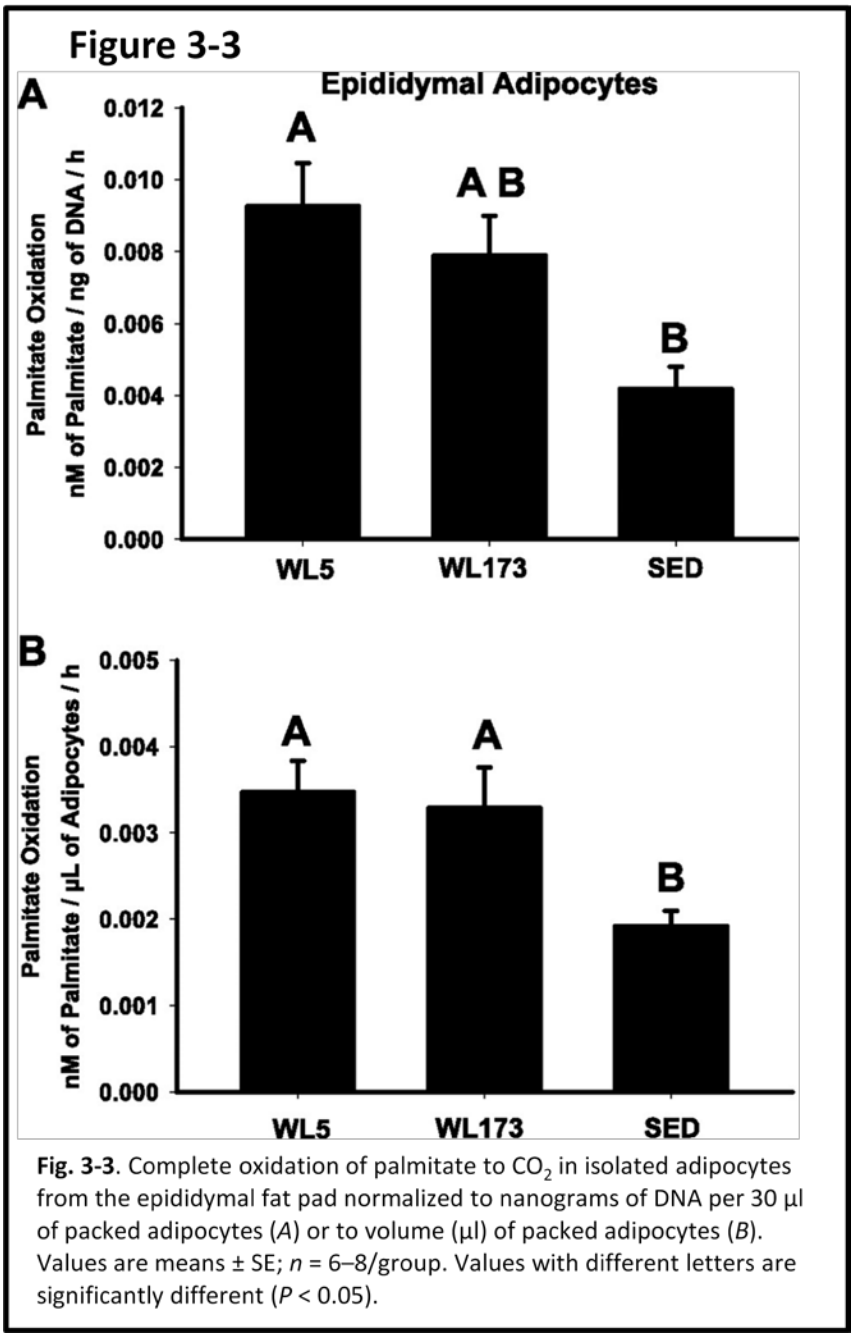


Fig. 3-3. Complete oxidation of palmitate to CO_2 in isolated adipocytes from the epididymal fat pad normalized to nanograms of DNA per 30 µl of packed adipocytes (*A*) or to volume (µl) of packed adipocytes (*B*). Values are means ± SE; *n* = 6–8/group. Values with different letters are significantly different ($P < 0.05$).

In isolated epididymal adipocytes, complete palmitate oxidation was 81% higher in the WL5 compared to SED ($p<0.05$) and 71% higher in the WL173 compared to SED ($p<0.05$), but not different between WL5 and WL173 when normalized to the volume of packed adipocytes (Figure 3-3A). However, when complete palmitate oxidation was normalized to DNA content (ng of DNA/30µL of packed adipocytes) WL173 no longer

remained significantly higher than SED group (Figure 3-3B). Total palmitate oxidation (CO_2 + ASMs) in the adipocytes did not differ among groups.

Table 3-3		WL5	WL173	SED
Red Gastrocnemius				
β-HAD	(nmol/min/ug)	26.5±3.7	26.6±2.6	23.1±2.0
Citrate synthase	(nmol/min/ug)	1786±184	1908±270	1342±141
Cytochrome c oxidase	(Units/min/g)	75.1±6.9	78.8±8.5	65.5±10.3
Liver				
β-HAD	(nmol/min/ug)	16.6±1.2	18.3±1.7	16.7±0.7
Citrate synthase	(nmol/min/ug)	408±25	376±32	374±15
Cytochrome c oxidase	(Units/min/g)	46.6±1.9	42.7±2.6	43.7±1.4
Epididymal Adipose				
β-HAD	(nmol/min/ug)	U.D.	U.D.	U.D.
Citrate synthase	(nmol/min/ug)	21±7	20±4	21±6
Cytochrome c oxidase	(Units/min/g)	0.7±0.1	1.0±0.2	0.9±0.2

Table 3-3 – Enzyme activities in red gastrocnemius, liver, and epididymal adipocytes. Values are means ± SE (n= 6-8/group). No significant differences between groups were detected. β-HAD = β-hydroxyacyl CoA dehydrogenase; U.D. = Undetectable.

[6]

No statistically significant differences in β-hydroxy-acetyl-CoA dehydrogenase, citrate synthase, or cytochrome c oxidase activities in red gastrocnemius, liver, or isolated adipocytes were detected among groups (Table 3-3).

CPT-1 activity. In isolated mitochondria from red gastrocnemius muscle, the WL5 group had 41% higher baseline CPT-1 activity than the WL173 group ($p<0.05$) but WL5 did not differ from SED. As expected, the addition of 10 μM malonyl-CoA caused decreases in CPT-1 activity in all three groups (Figure 3-4). Unexpectedly, the change in CPT-1 activity from baseline to 10 μM malonyl-CoA was nearly twice as much (delta) in the WL5 group compared to the WL173 and SED groups ($p<0.05$), suggesting a greater

[6] Data for Red Gastrocnemius collected by JP Thyfault lab, for liver by RS Rector

CPT-1 sensitivity to malonyl-CoA in a physically active condition (WL5) that was lost after 7 days of lowered physical activity (WL173). No differences in the CPT-1 activity of isolated mitochondria from liver existed among groups for 0 or 10 μM malonyl-CoA concentrations.

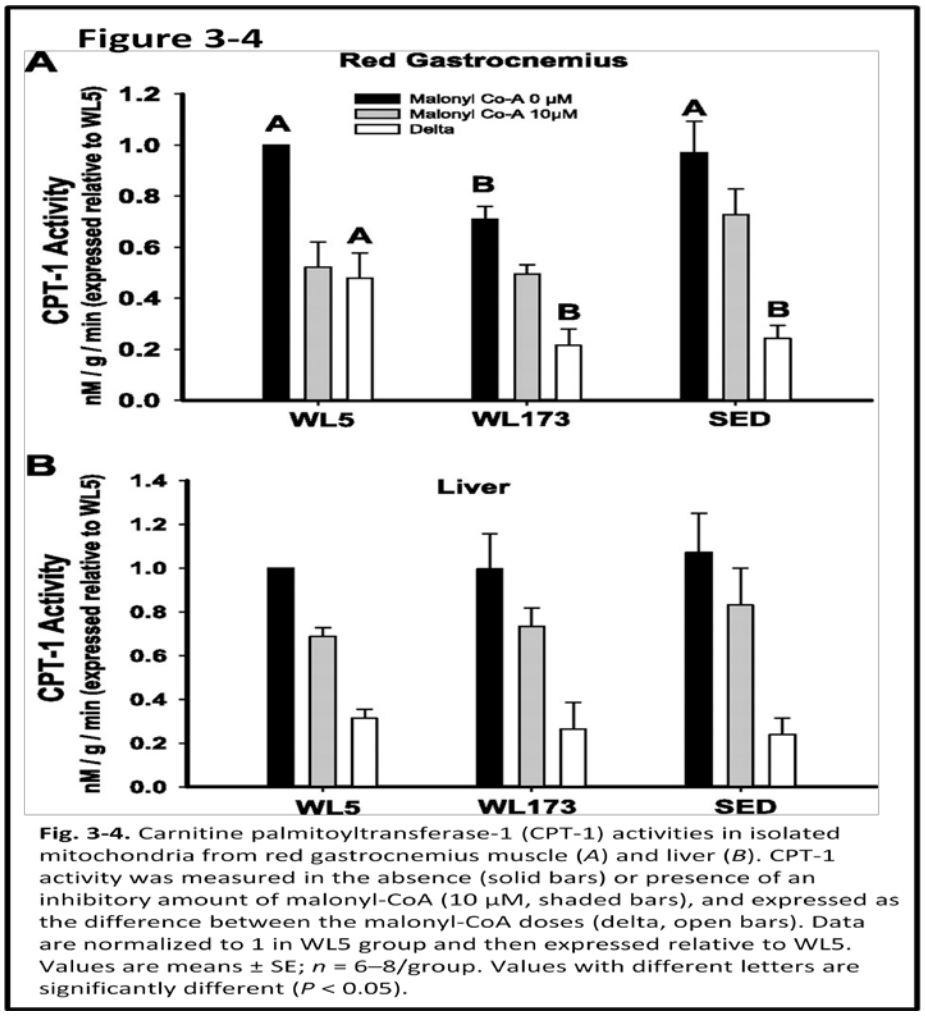


Fig. 3-4. Carnitine palmitoyltransferase-1 (CPT-1) activities in isolated mitochondria from red gastrocnemius muscle (*A*) and liver (*B*). CPT-1 activity was measured in the absence (solid bars) or presence of an inhibitory amount of malonyl-CoA (10 μM, shaded bars), and expressed as the difference between the malonyl-CoA doses (delta, open bars). Data are normalized to 1 in WL5 group and then expressed relative to WL5. Values are means ± SE; n = 6–8/group. Values with different letters are significantly different ($P < 0.05$).

[7]

[7] Data Collected by JP Thyfault lab

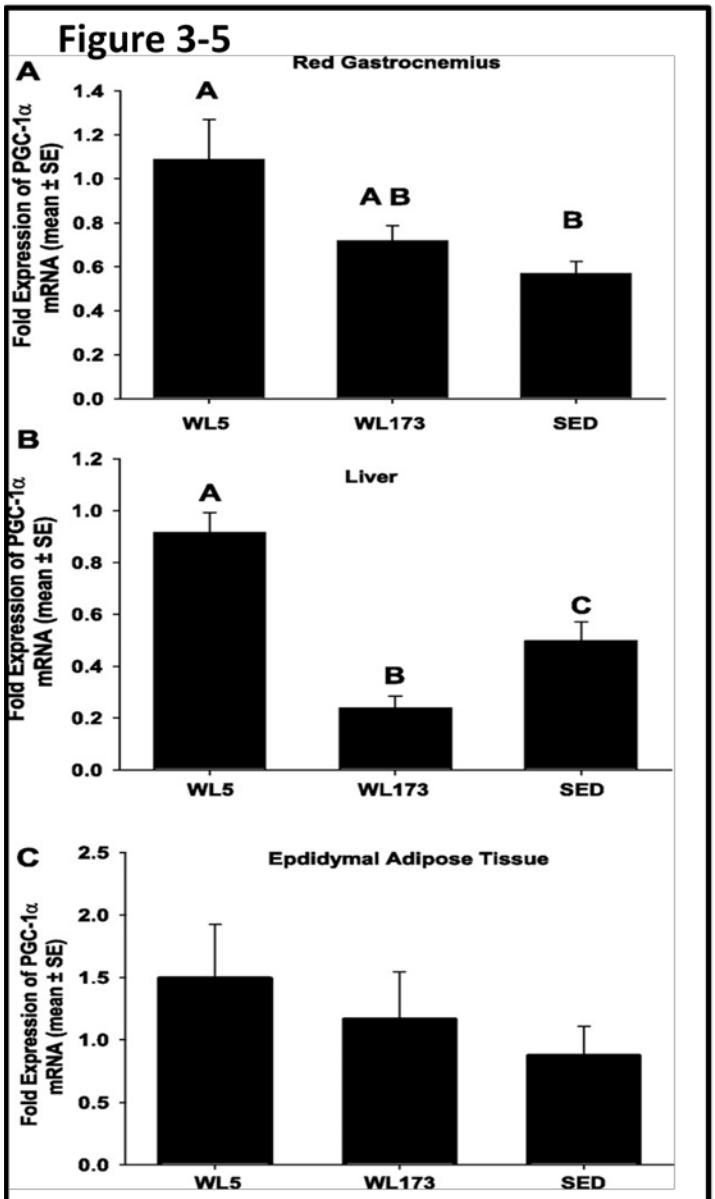


Fig. 3-5. Peroxisome proliferator-activated receptor coactivator-1 (PGC-1) mRNA in red gastrocnemius (*A*), liver (*B*), and epididymal adipose tissue (*C*). Data are normalized to 18S expression and expressed in fold difference. Values are means ± SE; *n* = 6–8/group. Values with different letters are significantly different ($P < 0.05$).[8]

PGC-1α mRNA Levels. PGC-1α mRNA levels were significantly higher at WL5 compared with the SED group in both red gastrocnemius and liver (Figure 3-5A, 3-5B). The transition from WL5 to WL173 led to a 35% and 68% decrease in red gastrocnemius

[8] Data for Red Gastrocnemius (A) collected by JP Thyfault lab, for Liver (B) by RS Rector

($p = 0.05$) and liver ($p < 0.05$) PGC-1α mRNA levels, respectively. Consequently, WL173 and SED groups PGC-1α mRNA did not differ in red gastrocnemius (Figure 3-5A). Remarkably in the liver, WL173 PGC-1α mRNA fell to levels significantly less than the SED group (Figure 3-5B). In epididymal WAT, PGC-1α mRNA did not differ among groups (Figure 3-5C).

DISCUSSION

The transition from high to low physical activity in our rat model caused: 1) dissimilar responses for FAO in skeletal muscle (decreasing from its elevated value in daily running), liver (increasing from its unchanged value in daily running), and WAT (maintenance of its increased value in daily running); 2) decreased CPT-1 activity and increased CPT-1 sensitivity to malonyl-CoA inhibition in muscle; 3) reduced PGC-1α mRNA in both skeletal muscle and liver; and 4) impaired growth of lean body mass compared to animals that sustain wheel running or the sedentary condition.

The transition from high to low physical activity likely decreases FAO in skeletal muscle by decreasing cellular energy requirements and decreasing PGC-1α expression, a critical transcriptional co-activator that coordinates increases in FAO enzyme, mitochondrial biogenesis, and a general increase in oxidative capacity. Observing similar trends in both homogenate and mitochondrial fraction leads us to speculate that the changes in FAO are not entirely due to a mass action effect, but also in part to an inherent change in mitochondrial "quality", which may be related to PGC-1α expression.

PGC-1α, as a co-activator, putatively increases FAO by increasing the promoter activity and thus expression of pyruvate dehydrogenase kinase-4 and CPT-1 (180, 325). This results in more acetyl CoA being derived from fatty acids and increased fatty acid

entry into the mitochondria (159). Unlike muscle, where energy derived from FAO fuels muscle contraction, an increase in hepatic PGC-1α mediated FAO provides a key source of ATP for ketogenesis and gluconeogenesis during fasting conditions (342). A limitation of the current study is that we did not measure oxidation of substrates, malate or pyruvate, that are commonly used to assess mitochondrial function.

Exercise causes an acute and transient increase in skeletal muscle PGC-1α gene expression (17, 223). Conversely, we show here that the cessation of daily physical activity reduces skeletal muscle PGC-1α expression in 173 h supporting earlier work in which more extreme forms of physical inactivity reduced skeletal muscle PGC-1α in humans (291). We also witnessed that CPT-1 activity, a downstream target of PGC-1α, decreased in red gastrocnemius skeletal muscle after the cessation of daily running. Reduced PGC-1α expression in skeletal muscle has been linked to insulin resistance in sedentary subjects (191, 216), leading us to speculate that a physical inactivity induced drop in PGC-1α is linked to skeletal muscle insulin resistance.

To our knowledge, this is the first report to measure changes in hepatic PGC-1α expression after chronic voluntary exercise or after a transition to inactivity. We speculate that daily exercise increases hepatic PGC-1α in a similar manner to fasting as both stimuli rely on increased hepatic glucose output to restore or sustain plasma glucose homeostasis. Interestingly, in contrast to what was found in muscle, the reduction in hepatic PGC-1α expression found after wheel lock was associated with increased complete hepatic FAO and no change in CPT-1 enzyme activity. Further research is needed to fully understand hepatic PGC-1α gene expression, its regulation by exercise, and its role in controlling hepatic FAO.

Intracellular accumulation of malonyl-CoA reduces fat and increases glucose utilization by the mitochondria (20, 184). The current findings show a greater reduction in CPT-1 activity in the presence of 10 uM malonyl-CoA in the WL5 group suggesting that skeletal muscle mitochondrial fatty acid entry may be more tightly regulated in wheel running rats, than SED rats. Potentially, this would allow for skeletal muscle to be more responsive in switching substrate utilization to match current energy demands. In addition, the CPT-1 responses to 10 uM malonyl CoA were similar between SED and WL173 skeletal muscle, suggesting that the enhanced exercise-induced regulation of CPT-1 by malonyl CoA is quickly lost if daily physical activity is not maintained. A lack of malonyl CoA inhibition is also noted with type 2 diabetes, where isolated skeletal muscle mitochondria have greater FAO than healthy controls despite having elevated malonyl CoA levels (20). Collectively, these observations suggest that physical activity modulates plasticity for malonyl-CoA's effects on skeletal muscle CPT-1 activity.

We speculate that the observed increase in complete FAO in liver during 7-day periods of decreased physical activity, in addition to the expansion of WAT, may help prevent excess lipid storage in lean animals on a low-fat diet. The findings that complete hepatic FAO is increased after the cessation of wheel running is opposite to our previous findings in hyperphagic, obese OLETF rats in which the cessation of wheel running for 7 days reduced hepatic palmitate oxidation to sedentary levels (232). These differences may be due to the larger body fat stores and /or older age in the OLETF rat. It is important to emphasize that the witnessed changes were for complete FAO (^{14}C palmitate to $^{14}CO_2$) or the complete breakdown of fatty acids through the TCA cycle, rather than the incomplete oxidation of fatty acids (^{14}C palmitate to ^{14}C labeled acid soluble

metabolites) in which fatty acids are only processed through β-oxidation. The difference between complete and incomplete FAO is significant for two reasons, 1) only complete FAO is coupled to significant ATP production, and 2) as suggested by Koves et al (161) complete FAO is less likely to produce intermediate metabolites that may negatively impact metabolic processes. In addition, our FAO measurements represent the maximal capacity or potential to oxidize fatty acids, and because they are not in-vivo measures they should be interpreted cautiously.

Unlike skeletal muscle and liver, adipocyte FAO remained elevated over the SED group with 7 days of physical inactivity. Mitochondrial DNA number (mtDNA) from human subcutaneous fat is positively associated with increased lipogenic enzymes (139), and we previously have demonstrated that cessation of voluntary wheel running increased protein and enzyme activity for mitochondrial glycerol-3-phosphate acyltransferase, a rate-limiting lipogenic enzyme, in WAT in rats (164). In addition, although our unpublished preliminary work has found no significant change in red gastrocnemius or liver triglyceride content, there is a significant increase in adipocyte triglyceride deposition after the cessation of wheel running in wild-type rats on a low-fat diet (163, 173). The exercise-induced increase in adipose FAO in WAT with ceased running may partially supply ATP for free fatty acid reesterfication, an energy costly process, or the increased ATP may be used to synthesize adipokines like adiponectin, a process shown to require functional mitochondria (157).

To our knowledge this is the first evidence that the cessation of voluntary running acutely (for at least 173 h) attenuates lean body mass growth. We have also recently reported that lean body mass significantly decreases in young healthy men who decrease

their daily walking from 10,501 to 1,344 steps for 2 wks (209). The apparent change in lean mass in both studies leads us to question if the attenuated growth or loss of muscle mass in both studies is linked to a repartitioning of glucose and amino acids away from skeletal muscle (loss of insulin sensitivity) and/or decreased FAO. Because of the importance of lean body mass in metabolic health, future research is needed to study these interactions.

In conclusion, we show that FAO in skeletal muscle, liver, and adipocytes display strikingly different responses to a 1-wk transition from high to low daily physical activity, a period in which fat pad mass increases rapidly. In addition, we found that two factors associated with metabolic disease, skeletal muscle FAO and PGC-1 α, were both reduced after the cessation of daily activity. These results support our overall hypothesis that the cessation of daily physical activity quickly alters metabolic function in a manner that likely precedes the later development of chronic disease should physical inactivity continue.

Chapter 4: Changes in visceral adipose tissue mitochondrial content with type 2 diabetes and daily voluntary wheel running in OLETF rats[9]

ABSTRACT

Using the hyperphagic, obese, Otsuka Long-Evans Tokushima Fatty (OLETF) rat, we sought to determine if progression to type 2 diabetes alters visceral white adipose tissue (WAT) mitochondrial content and if these changes are modified through prevention of type 2 diabetes with daily exercise. At four wks of age, OLETF rats began voluntary wheel running (OLETF-EX) while additional OLETF rats (OLETF-SED) and Long-Evans Tokushima Otsuka (LETO-SED) rats served as obese and lean, sedentary controls, respectively for 13, 20, and 40 weeks of age (n=6-8 for each group at each age). OLETF-SED animals displayed insulin resistance at 13 and 20 weeks and type 2 diabetes by 40 weeks. OLETF-SED animals gained significantly ($p<0.001$) more weight and omental fat mass compared with OLETF-EX and LETO-SED. Markers of WAT mitochondrial protein content (cytochrome c, COXIV-subunit I, and citrate synthase activity) significantly increased ($p<0.05$) from 13 to 40 weeks in the LETO-SED, but was significantly attenuated in the OLETF-SED rats. Daily exercise normalized WAT cytochrome c and COXIV-subunit I protein content in the OLETF-EX to the healthy LETO-SED animals. In conclusion, increases in omental WAT mitochondrial content between 20 and 40 weeks of age in LETO control animals are attenuated in the hyperphagic, obese OLETF rat. These alterations occurred in conjunction with the progression from insulin resistance to type 2 diabetes and were prevented with daily exercise. Reduced ability to increase WAT mitochondrial content does not appear to be a

[9] Chapter is written exactly as submitted to *J.Physio* by Laye et al (submitted March 2009)

primary cause of insulin resistance, but may play a key role in the worsening of the disease condition.

INTRODUCTION

Poor dietary choices and sedentary lifestyles are leading to an obesity epidemic in Westernized societies, and recent epidemiological studies suggest an increased risk of cardiovascular disease and type 2 diabetes in overweight and obese individuals. WAT is now recognized as more than just an energy reservoir for lipid storage; in fact, it is considered an active endocrine organ and an important regulator in glucose homeostasis. Overexpression of GLUT4 selectively in WAT has been shown to prevent insulin resistance and type 2 diabetes in mice lacking skeletal muscle GLUT4 (51). High amounts of visceral white WAT (WAT) is more closely associated with insulin resistance, cardiovascular disease, type 2 diabetes, and systemic low grade inflammation than subcutaneous WAT (337). In addition, reducing the accumulation of WAT is considered an important component in preventing the development of type 2 diabetes. This is due in part to intrinsic differences in metabolism that include lower anti-inflammatory leptin and adiponectin release (196, 303), higher pro-inflammatory cytokine release (90), higher lipolytic response to catecholamines (13), and lower adipocyte proliferation capacities (304) in visceral versus subcutaneous WAT.

Decreases in mitochondrial content are noted in various tissues and often associated with disease states (46, 233, 297). Decreased rates of mitochondrial protein synthesis (240) and mitochondrial autophagy (43) leading to an overall decrease in mitochondrial volume and ATP generation per given mitochondria (182) are observed in aging skeletal muscle. Furthermore, diabetic humans have reduced skeletal muscle

mitochondria (120) that fail to increase ATP production in response to insulin infusion (15). While a prominent role for mitochondrial function is more apparent in skeletal muscle, little is known about the physiological relevance of mitochondria in WAT. Mitochondria in WAT are necessary for adiponectin synthesis and positively correlated with lipogenic potential in human subcutaneous WAT (139, 157). In addition, reductions in WAT mitochondrial content, mitochondrial DNA, and mitochondrial function recently have been observed in the epididymal fat of type 2 diabetic mice (60), suggesting a potential role for the disruption of WAT mitochondrial content and function in type 2 diabetes.

Increasing physical activity has been shown to increase or prevent age-related loss in mitochondrial content and function in skeletal muscle and liver (46, 232), but the mechanism through which this occurs remains largely unknown. Furthermore, there is limited evidence that swimming exercise increases WAT mitochondrial content (283) and voluntary wheel running increases adipocyte FAO (172) in healthy animals.

The Otsuka Long-Evans Tokushima Fatty (OLETF) rats are a commonly studied model of obesity and type 2 diabetes (144). Selectively bred for null expression of the cholecystokinin-1 receptor, OLETF rats exhibit a within meal feedback defect for satiety, resulting in hyperphagia and obesity, and in the spontaneous development of insulin resistance and type 2 diabetes (192). When allowed to exercise daily on voluntary running wheels, the development of insulin resistance and type 2 diabetes is prevented in OLETF animals (261). In addition, we have previously shown that daily exercise prevents the accumulation of WAT and also increases hepatic mitochondrial function in OLETF rats (232). However, changes in WAT mitochondrial content and its relationship

to glucose homeostasis under sedentary and exercise conditions have yet to be examined in omental WAT.

Many of the previous reports studied a single age and none have examined prospectively the changes in WAT mitochondria content in a rodent model throughout the development and progression of insulin resistance and type 2 diabetes. In addition, recent findings indicate that epididymal adipocyte mitochondrial content is reduced in 33 week old OLETF animals (60), but these reductions in the context of progression from insulin resistance to type 2 diabetes was not examined. Therefore, the purpose of the study was to examine changes in omental WAT mitochondrial content in a hyperphagic, obese animal model of type 2 diabetes and gain mechanistic insight into the potential role these changes may have on the initiation and progression of the disease. In addition, as anti-diabetic therapies have shown promise in altering WAT mitochondrial concentration in type 2 diabetic mice (60), we sought to determine the impact of daily physical activity on WAT mitochondrial content in a diabetic model.

METHODS

Animal protocol. The animal protocol was approved by the Institutional Animal Care and Use Committee at the University of Missouri. OLETF and LETO male rats at 4 wks of age were kindly supplied by the Tokushima Research Institute, Otsuka Pharmaceutical (Tokushima, Japan). The OLETF-runners (OLETF-EX) were immediately housed (at the age of 28 days) in cages equipped with voluntary running wheels outfitted with a Sigma Sport BC 606 bicycle computer (Cherry Creek Cyclery, Foster Falls, VA, USA) for measuring daily OLETF running activity. Voluntary running was selected to approximate the more natural activity state of the animal. Cages were in

temperature-controlled animal quarters (21°C) with a 06.00–18.00 h light: 18.00–06.00 h dark cycle that was maintained throughout the experimental period. All animals were provided standard rodent chow (Formulab 5008, Purina Mills, St Louis, MO, USA) in new cages at the beginning of each week when cages were changed and body weights obtained between 08.00 and 10.00 h. Body mass and food intake were measured weekly throughout the investigation. Running activity was obtained daily between 08.00 and 10.00 h, and rats in the running groups had daily access to wheels and food and water ad libitum until designated time of sacrifice (13, 20, or 40 wks of age). Age-matched, sedentary OLETF (OLETF-SED) and LETO (LETO-SED) rats did not have access to running wheels throughout the duration of the study. Rat chow was removed on the day of sacrifice at 0600 h. At 13, 20, and 40 weeks of age, rats were anesthetized with sodium pentobarbital (100 $mg \cdot kg^{-1}$) and killed by exsanguination 53 h after locking of wheels; the sedentary rats (OLETF-SED and LETO-SED) were sacrificed at the same time. The timing of the last exercise bout was designed to mirror our previous study (232) and also to examine the effects of chronic exercise, not the acute effects, of the last bout of exercise. All animals were fasted for 5 hours prior to sacrifice.

Dual-energy X-ray absorptiometry (DEXA). Whole-body composition was measured using a Hologic QDR-1000/w DEXA machine calibrated for rats.

Western blotting. Western blot analyses were performed for the determination of the protein content of cytochrome oxidase IV-subunit I (COX anti-complex IV- subunit I; Molecular Probes, Eugene, OR, USA), total cytochrome c (Cell Signaling, Beverly, MA, USA), fatty acid synthase (FAS H-300, Santa Cruz Biotechnology, Santa Cruz, CA),

FIS1 (Alexis Biochemical, San Diego, CA), and SREBP1c (Santa Cruz Biotechnology, Santa Cruz, CA), as previously described (231, 232).

Fat pad collection. Omental WAT fat pads were removed from exsanguinated animals and weighed. A portion was fixed in formalin, paraffin embedded, stained with hematoxylin and eosin, and analyzed for cell diameter and cell number as previously described (173). At least 3 fields of views were used to size >300 adipocytes/animal using Image Pro (Silver Spring, MD) imaging software. An additional portion was frozen at -80°C for enzyme activity analyses and western blotting.

Serum assays. Serum glucose (Sigma, St. Louis, MO, USA), triglycerides (TG) (Sigma, St. Louis, MO, USA), free fatty acids (FFA; Wako Chemicals, Richmond, VA, USA), insulin (Linco Research, St. Charles, MO, USA), and leptin (Linco Research, St. Charles, MO, USA) were measured using commercially available kits according to the manufacturer's instructions. Hemoglobin A1c (HbA1c) concentrations were determined in EDTA-whole blood by the Diabetes Diagnostics Lab at the University of Missouri using a boronate-affinity HPLC method (Primus Diagnostics, Kansas City, MO).

Citrate synthase, Beta-hydroxyacyl-CoA dehydrogenase (β-HAD), and cytochrome c oxidase activities. Citrate synthase and β-HAD activities were determined in omental fat homogenate using the methods of Srere et al. (274) and Bass et al. (21), respectively, and cytochrome c oxidase activity by a commercially available kit (Sigma, St. Louis, MO, USA) as previously described by our group (172, 231, 232). Both β-HAD and cytochrome c oxidase activity was undetectable in omental WAT homogenates.

Statistics. Each outcome measure was examined in 5-8 animals/group. For each outcome measure, a one-way analysis of variance was performed (SPSS/15.0, SPSS,

Chicago, IL, USA). A significant main effect ($p<0.05$) was followed-up with Student-Newman-Kuel post-hoc comparisons. Values are reported as means ± standard error of the mean (SE), and a P value less than 0.05 denotes a statistically significant difference.

RESULTS

Animal characteristics. Average daily running distance and running time for the OLETF-EX groups are shown in Figure 1A and Figure 1B, respectively. As we have previously reported (195), initial running distance at 4 weeks of age averaged ~4 km/d (~150 min/d). Peak distances of ~12 km/d (~275 min/d) were achieved between 8 and 11 weeks of age, declining to ~7 km/d (~200 min/d) at 20 wk of age, and ~4 km/d (~150 min/d) by 40 weeks of age. It remains unknown as to why the OLETF animals exhibited decreased voluntary wheel running as they age, but perhaps the OLETFs appropriately mirror the human condition in which aging is often associated with reduced physical activity.

Consistent with our previous reports (231, 232), body mass and body fat percent, determined by small animal DEXA, was significantly greater in the OLETF-SED animals compared with LETO-SED and OLETF-EX at all ages studied ($p<0.001$, Figure 1C and Table 1). A marker of exercise training, higher heart weight to body weight ratio, was found in all OLETF-EX animals compared with OLETF-SED and LETO-SED (Table 1).

Absolute weekly food consumption was significantly greater ($P < 0.05$) in OLETF-SED and OLETF-EX rats at 13, 20, and 40 wk compared with non-hyperphagic, LETO-SED (Table 1). While absolute weekly food consumption only differed at 40 wk between OLETF-EX and OLETF-SED, food consumption relative to body weight was significantly greater in the OLETF-EX animals at 13 and 20 weeks of age (Table 1),

suggesting that observations in OLETF-EX animals are not due to consuming fewer calories.

Table 4-1. [10]

Groups	Age	% body fat (g)	Food consumption (g/wk)	Food consumption (g per wk/g BW)	HW/BW (mg/g)
LETO-SED	13 wk	10.0 ± 0.5^{a}	155.2 ± 2.6^{a}	0.43 ± 0.01^{a}	3.37 ± 0.13^{a}
	20 wk	15.4 ± 1.1^{b}	159.1 ± 1.5^{b}	0.33 ± 0.01^{b}	2.92 ± 0.06^{b}
	40 wk	21.9 ± 1.3^{c}	164.7 ± 5.6^{b}	0.30 ± 0.01^{b}	2.58 ± 0.03^{c}
OLETF-EX	13 wk	7.2 ± 0.7^{a}	$247.1\pm6.61^{*}$	$0.67\pm0.02^{a*}$	$4.00\pm0.05^{a*}$
	20 wk	11.7 ± 2.0^{b}	$242.1\pm4.5^{*}$	$0.56\pm0.02^{b*}$	$3.57\pm0.10^{b*}$
	40 wk	18.0 ± 1.8^{c}	$231.5\pm8.8^{*}$	$0.43\pm0.01^{c*}$	$3.05\pm0.06^{c*}$
OLETF-SED	13 wk	$21.4\pm1.8^{a*}$	$214.2\pm10.2^{a*}$	0.41 ± 0.01^{a}	3.11 ± 0.05^{a}
	20 wk	$30.2\pm0.9^{b*}$	$219.8\pm10.9^{a*}$	0.36 ± 0.01^{b}	2.67 ± 0.07^{b}
	40 wk	$30.6\pm4.5^{b*}$	$306.2\pm21.4^{b*\#}$	$0.45\pm0.05^{a*}$	2.71 ± 0.14^{b}

Table 4-1: Animal characteristics .Values are means ± SE (n = 6-8). Values with different letter superscripts within each animal group are significantly different ($p < 0.05$). *Significantly different than other animal groups without symbol at respective age ($p<0.01$). #Significantly different than OLETF-EX at respective age ($p<0.01$). HW/BW = heart weight to body weight ratio.

Serum characteristics. OLETF-SED animals exhibited age-associated increases in serum TG and FFAs and these concentrations were significantly higher than OLETF-EX and LETO-SED at all ages studied (Table 2). OLETF-SED animals displayed insulin resistance by 13 and 20 weeks, demonstrated by elevated levels of insulin and glucose compared with OLETF-EX and LETO-SED, and developed overt diabetes mellitus by 40

[10] Data collected and analyzed by RS Rector

weeks with a ~50% drop in serum insulin and 2-fold increase in HbA1c (Table 2); whereas, OLETF-EX animals remarkably maintained glycemic control to the level of the healthy LETO-SED animals at all ages studied (Table 2).

Omental Fat Pad Characteristics. Omental fat pad mass significantly increased from 13 through 40 weeks in all groups ($p<0.05$) (Figure 2). However, omental fat pad mass increased significantly less in the OLETF-EX and LETO-SED animals at each age compared with OLETF-SED (Figure 2). Mean fat cell diameter increased dramatically by 54% in the OLETF-SED from 13 to 20 weeks, corresponding with a drop in cell number. However, by 40 weeks of age, fat cell diameter had returned to 13 week levels in the OLETF-SED group, while cell number had increased dramatically (Figure 2). Serum leptin, a marker of fat mass, followed a similar pattern as fat cell diameter, with a dramatic rise from 13 to 20 weeks followed by a precipitous fall from 20 to 40 weeks (Figure 2). The increase in cell size followed by a subsequent increase in cell number is similar to the progression of obesity and diabetes previously reported in high-fat fed mice (280). In addition, while the LETO-SED showed an increase in adipocyte cell number at 20 wks of age, the OLETF-EX did not have a similar increase until 40 weeks of age.

Table 4-2 [11]

Groups	Age	Serum TG (mg/dL)	Serum FFAs (μmol/L)	Serum glucose (mg/dL)	Serum insulin (ng/ml)	HbA1c (%)
LETO-SED	13 wk	38.5±5.1	175.2±47.0	253.8±25.7	7.7±0.9	4.7±0.1
	20 wk	45.2±4.0	172.9±27.1	348.1±39.2	9.2±0.8	4.6±0.1
	40 wk	41.5±3.6	179.6±25.1	276.0±16.8	11.1±0.7	4.6±0.1
OLETF-EX	13 wk	53.7±6.6	98.9±5.0	260.4±34.6	8.6±1.5	4.8±0.1
	20 wk	71.2±12.5	127.5±30.3	350.5±52.4	9.9±1.2	4.7±0.1
	40 wk	81.2±11.2	153.1±20.4	302.1±19.2	11.4±0.8	4.8±0.1
OLETF-SED	13 wk	$128.2\pm14.0^{a*}$	$222.4\pm10.7^{a*}$	$414.3\pm32.1^{a*}$	10.0 ± 1.3^{a}	5.4 ± 0.1^{a}
	20 wk	$172.4\pm28.8^{b*}$	$306.1\pm54.0^{b*}$	$548.5\pm67.8^{b*}$	$12.2\pm0.7^{a*}$	5.3 ± 0.1^{a}
	40 wk	$265.0\pm35.4^{c*}$	$325.6\pm62.8^{b*}$	$683.7\pm43.6^{c*}$	$5.0\pm1.3^{b*}$	$8.8\pm0.8^{b*}$

Table 4-2: Serum characteristics. Values are means ± SE (n=5-8). Values with different letter superscripts within each animal group are significantly different ($p < 0.05$). No significant differences existed within the LETO-SED and OLETF-EX groups for any of the measured parameters. *Significantly different than other animal groups without symbol at respective ages ($p<0.01$).

[11] Data collected and analyzed by RS Rector

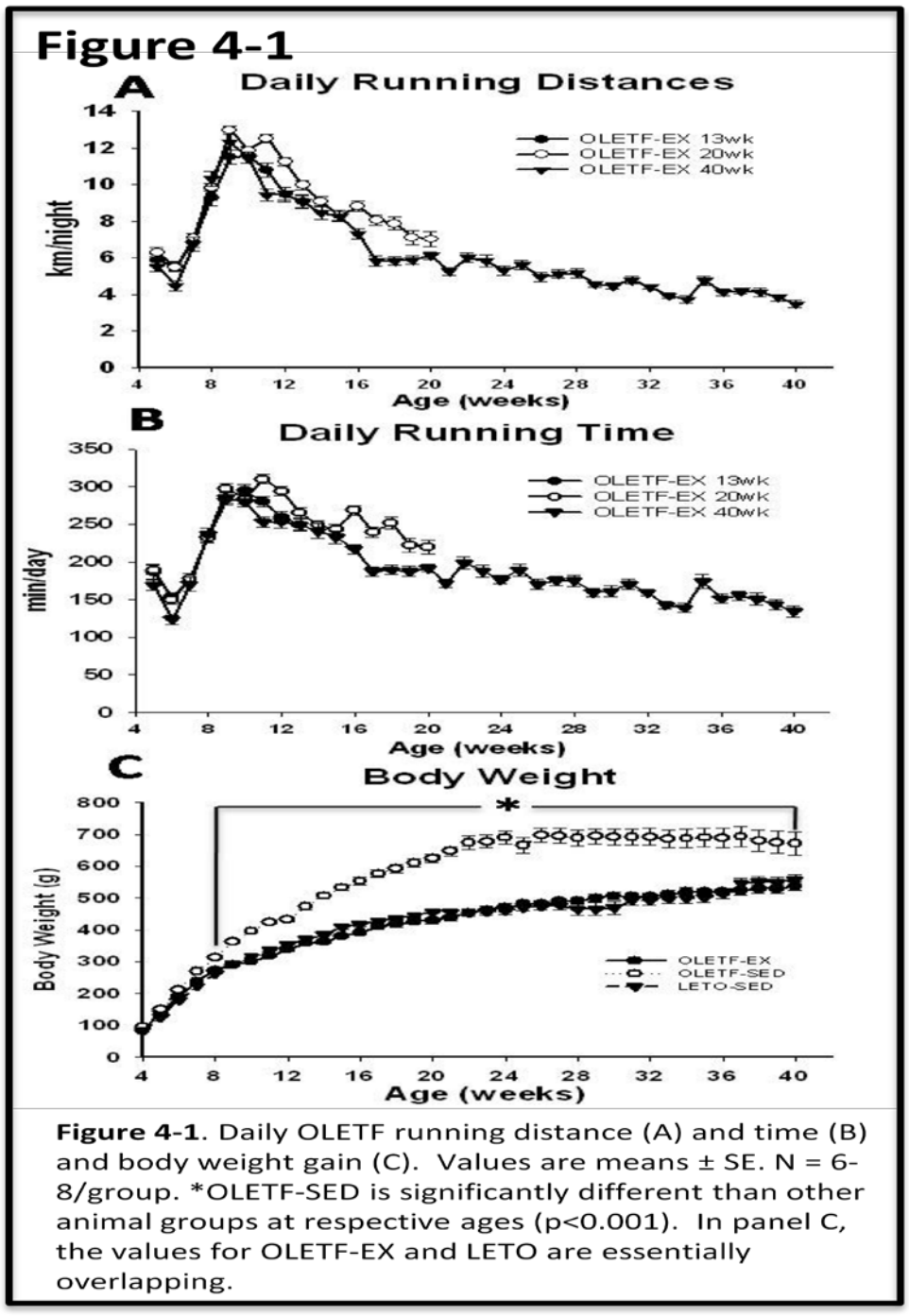


Figure 4-1. Daily OLETF running distance (A) and time (B) and body weight gain (C). Values are means ± SE. N = 6-8/group. *OLETF-SED is significantly different than other animal groups at respective ages ($p<0.001$). In panel C, the values for OLETF-EX and LETO are essentially overlapping.

[12]

[12] Data collected and analyzed by RS Rector

Omental WAT mitochondrial content. Total cytochrome c protein content, a nuclear DNA encoded mitochondrial protein, was significantly increased by 40 weeks in the LETO-SED and OLETF-EX animals but not in the OLETF-SED rats (Figure 4A). Similarly, protein expression for the mitochondrial DNA encoded protein COXIV-subunit I was significantly increased at 40 weeks in the LETO-SED and OLETF-EX groups, but not the OLETF-SED animals (Figure 4B). FIS1 protein, a marker of mitochondrial fission, did not differ among groups, but did display a dramatic age-associated reduction in all groups (Figure 4C). Citrate synthase activity in omental WAT tended to be elevated in the OLETF-EX animals compared with OLETF-SED at 13 and 20 weeks of age ($p=0.07$; Figure 4D). LETO-SED and OLETF-SED groups showed an age-associated increase from 20 to 40 weeks, with LETO values significantly higher than OLETF-EX or OLETF-SED by 40 weeks. Mitofusion 2, a measure of mitochondrial fusion, also was assessed, but blots resulted in extensive non-specific binding combined with low signal (data not shown). In addition, cytochrome c oxidase and β-HAD activities were below detectable levels, highlighting the relatively low levels of mitochondria in omental WAT (data not shown).

Lipogenic Proteins in omental WAT. The OLETF-EX rats exhibited significantly elevated FAS protein content compared with LETO-SED and OLETF-SED rats at 13 weeks of age (Figure 5). Although there was an age-associated drop in FAS content in all groups, the elevated levels in the OLETF-EX group persisted at 20 and 40 weeks of age. SREBP1c did not differ among groups regardless of age (representative western blot in Figure 5).

Figure 4-2

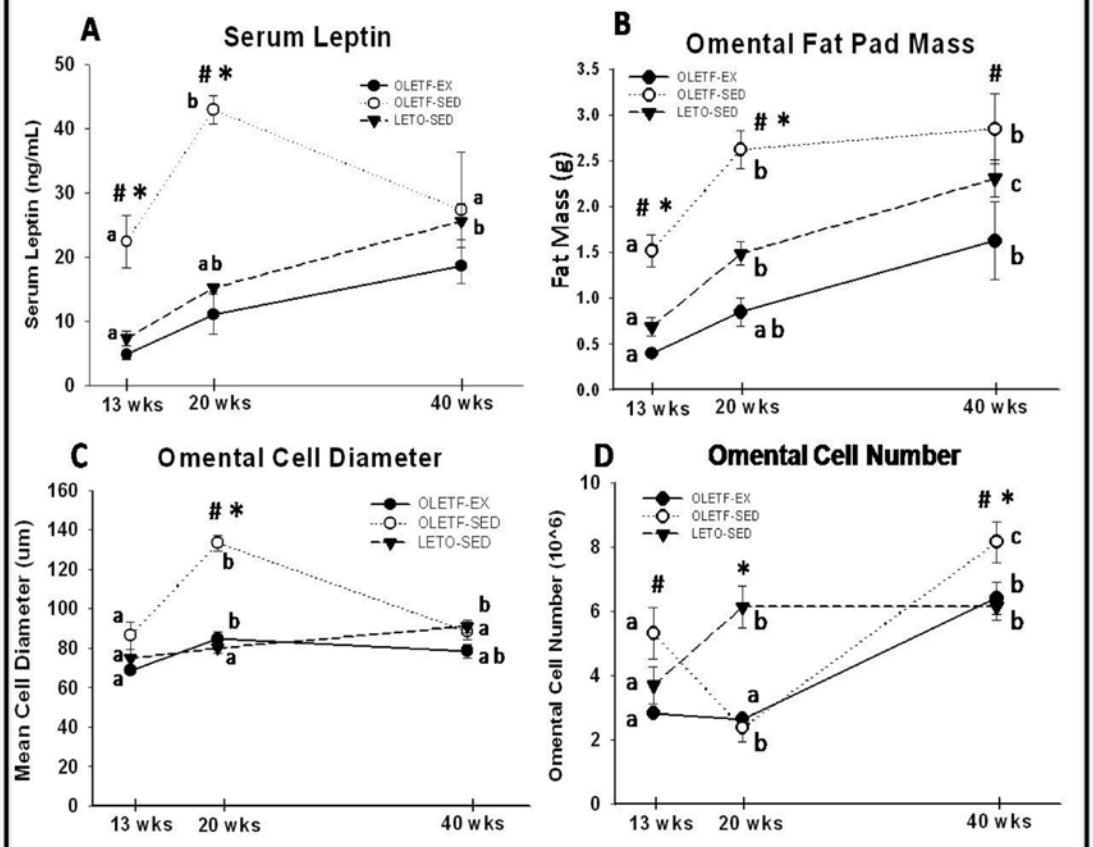


Figure 4-2. Changes in serum leptin (A), omental fat mass (B), omental fat cell diameter (adipocyte hypertrophy); C), and omental fat cell number (adipocyte hyperplasia); D). Values (means ± SE, n = 5-8) within each animal group with different letter superscripts are significantly different ($p<0.05$). Significant differences ($p<0.05$) between groups at a particular age are denoted by *LETO-SED vs. OLETF-SED and #OLETF-EX vs. OLETF-SED.

Figure 4-4

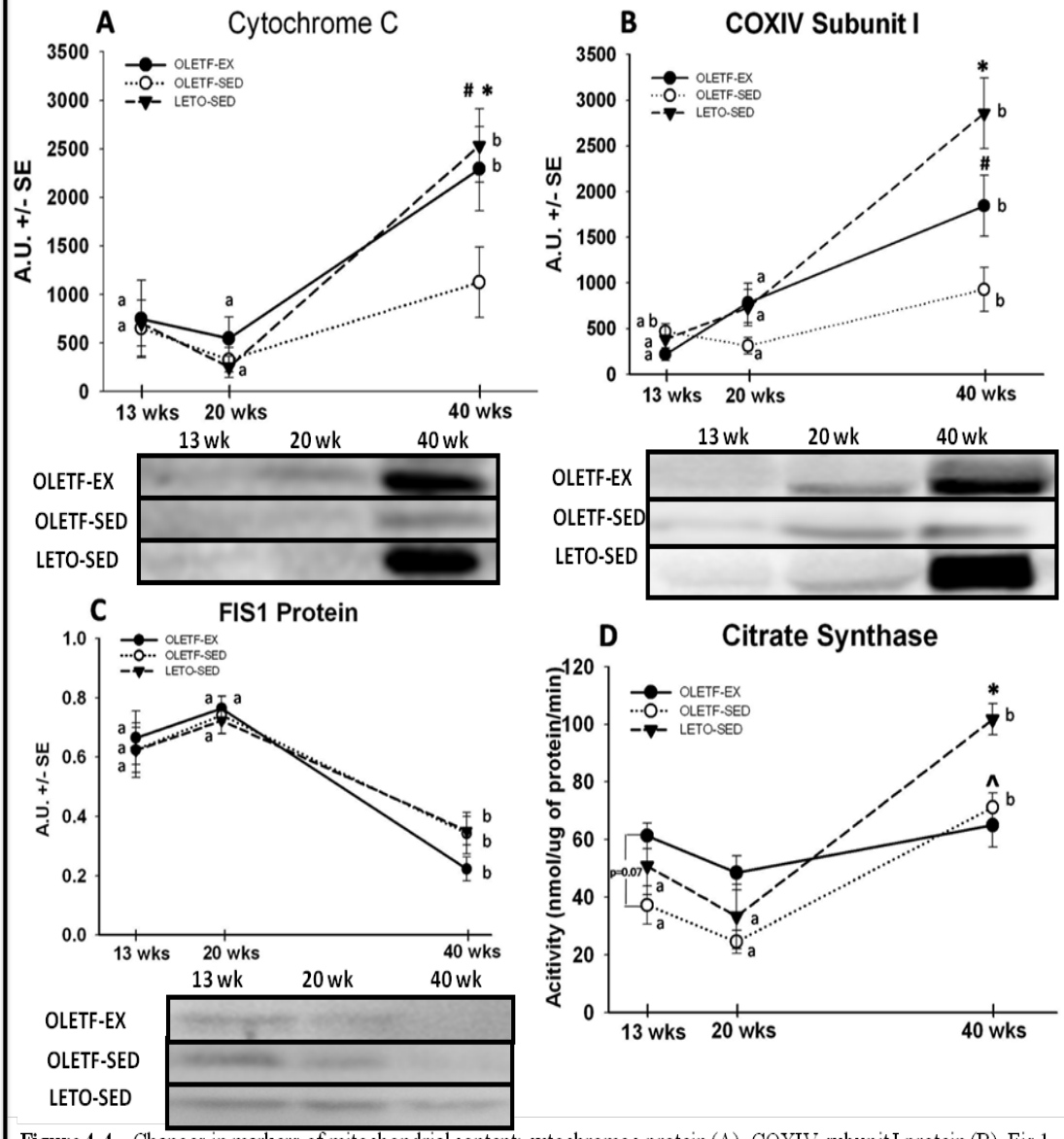


Figure 4-4. Changes in markers of mitochondrial content; cytochromec protein (A), COXIV-subunit I protein (B), Fis 1 protein (C), and citrate synthase activity (D) in omental fat. Values (means ± SE, n = 5-8) within each animal group with different letter superscripts are significantly different ($p<0.05$). Significant differences ($p<0.05$) between groups at a particular age are denoted by *LETO-SED vs. OLETF-SED, #OLETF-EX vs. OLETF-SED, and ^OLETF-EX vs. LETO-SED.

[13]

[13] Data partially collected by, RS Rector, and SH Warner

Figure 4-3

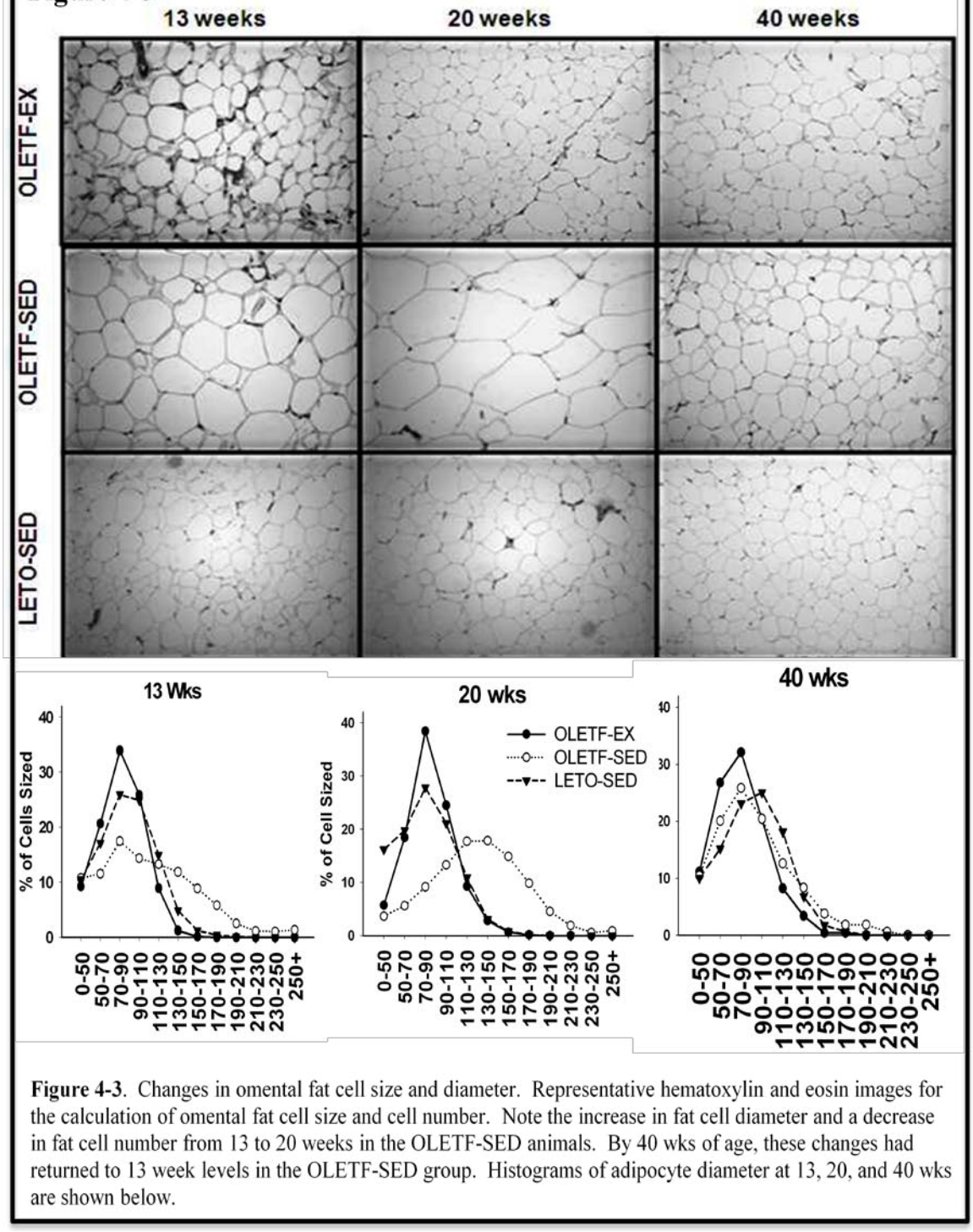


Figure 4-3. Changes in omental fat cell size and diameter. Representative hematoxylin and eosin images for the calculation of omental fat cell size and cell number. Note the increase in fat cell diameter and a decrease in fat cell number from 13 to 20 weeks in the OLETF-SED animals. By 40 wks of age, these changes had returned to 13 week levels in the OLETF-SED group. Histograms of adipocyte diameter at 13, 20, and 40 wks are shown below.

Figure 4-5

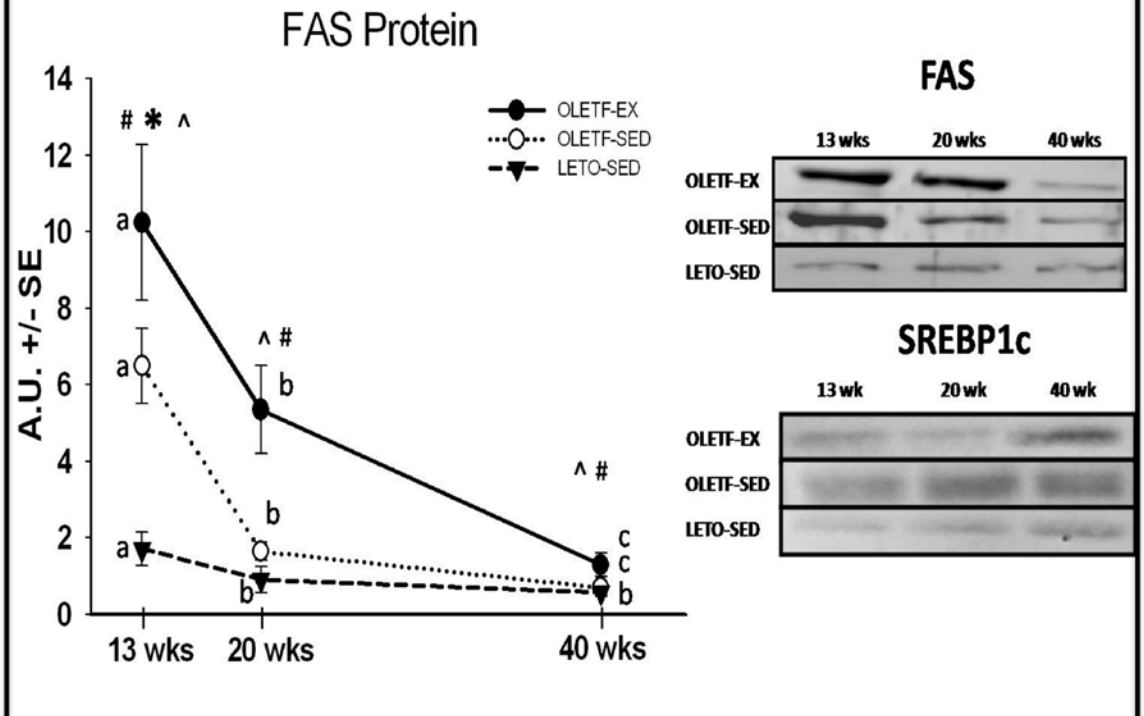


Figure 4-5. Changes in protein expression of FAS and SREBP1c in omental fat. Representative blots are shown. Values (means ± SE, n = 5-8). Within each animal group, different letter superscripts are significantly different ($p<0.05$). Significant differences ($p<0.05$) between groups at a particular age are denoted by *LETO-SED vs. OLETF-SED, #OLETF-EX vs. OLETF-SED, and ^OLETF-EX vs. LETO-SED.

DISCUSSION

WAT is becoming widely recognized as an active endocrine organ and an important regulator in whole body glucose metabolism. Limited data suggests that type 2 diabetes and obesity may be associated with lower epididymal/subcutaneous mitochondria content and an increased accumulation of visceral WAT stores (60, 284). Here we provide novel mechanistic insight into WAT mitochondrial content changes during the transition from insulin resistance (13 weeks) to type 2 diabetes (40 weeks) in a well characterized rodent model of hyperphagia-induced obesity and type 2 diabetes. We also examined the changes in WAT mitochondria in animals protected from type 2 diabetes by daily exercise. During the progression of type 2 diabetes, WAT

mitochondrial content failed to increase in the OLETF-SED animals to the same magnitude as in lean, healthy LETO controls. In addition, daily exercise normalized changes in mitochondrial content to the level of the healthy controls. We also demonstrate that FAS protein content decreases from 13 to 40 weeks of age, but is sustained at a higher level with daily exercise in the absence of a concurrent increase in fat mass. Lastly, we demonstrate that in the OLETF-SED rats there is a pattern of omental WAT expansion with adipocyte hypertrophy and a decrease in cell number which is followed by a return of adipocyte diameter and increase in cell number, a response that is similar to that previously reported in high-fat fed mice (280).

There appears to be an age-associated reduction in mitochondrial content in skeletal muscle and liver (46, 186); however, here we demonstrate that omental WAT mitochondrial content increased from 20 to 40 weeks of age in the lean, sedentary LETO rats. Similarly, Katic and colleagues (143) found that wild type and adipose tissue-specific insulin receptor knockout (FIRKO) mice showed a dramatic increase in mitochondrial proteins from age 0.5 years to 1.5 years in epididymal WAT. However, by 2.5 years of age, mitochondrial protein in epididymal WAT of wild type mice had fallen to the 0.5-year level, but remained elevated in the FIRKO mice, which the authors postulate might contribute to their extended lifespan. In this context, our results in omental WAT at 40 weeks of age are in agreement with their observations between 0.5 and 1.5-years of age. Whether the LETO-SED or OLETF-EX would maintain an elevated WAT mitochondrial content and how this might contribute to the lifespan in OLETF rats remain unknown but warrants further study.

Recent reports suggest that mitochondrial dysfunction exists in epididymal adipocytes (a reproductive-associated adipose depot) in type 2 diabetic mice (60). We present similar findings of suppressed mitochondrial protein content in omental WAT (a non-reproductive adipose depot) in the type 2 diabetic OLETF rat. However, based on the current observations, differences in WAT mitochondrial content were not present until overt diabetes is present, suggesting these changes may not play a causal role in the worsening of insulin resistance. This is in support of previous observations where a decrease in WAT mitochondrial content was subsequent to impaired glucose homeostasis following high-fat feeding (284).

To our knowledge, we are the first to demonstrate that increased daily physical activity allows for the upregulation of WAT mitochondrial content in the hyperphagic OLETF rat, findings not seen in the sedentary OLETF animals. PPARγ agonists have been reported to reverse reductions in WAT mitochondria content and function in obese rodents in a manner similar to the effects of physical activity reported here in (60, 171). Although we did not observe a change in total PPARγ protein in WAT of young wild-type rats undergoing voluntary wheel running (163), others have shown an increase in PPARγ DNA binding activity with wheel running at a similar age (220). We also observed an age-associated decrease in FAS, another PPARγ-responsive protein, suggesting that increased PPARγ activity may not be playing a role in the OLETF rat used in the current study. Interestingly, Sutherland and colleagues (283) recently demonstrated that 2 hours of swimming a day in rats increases mitochondrial protein content in both epididymal and retroperitoneal adipose depots. The authors attributed their findings to increases in circulating catecholamines (283). However, voluntary

wheel running likely does not increase catecholamines as much as swimming (122) and we did not observe an increase in mitochondrial content relative to healthy controls as did Sutherland and colleagues (283). These findings collectively emphasize the need for future studies addressing the mechanism responsible for omental WAT mitochondrial biogenesis and the role physical activity may play in its regulation.

Mitochondria are dynamic organelles that are consistently undergoing fission and fusion to modify their reticulum. The increase in mitochondrial content in omental WAT led us to ask whether a marker of mitochondria fission, FIS1, may have been a contributing factor. FIS1 protein content decreased with age but did not differ among groups, suggesting that the differences in mitochondrial content observed in the current study are not likely due to FIS1. Inhibition of FIS1 has been shown to increase a senescence-like phenotype of increased elongated mitochondria (174), while decreasing the release of cytochrome c and subsequent apoptosis from mitochondria (89). Future studies to assess whether FIS1 protein expression reflects its biological activity in adipocytes, as it does in other tissues (343), are clearly needed.

Because insulin-stimulated lipogenesis is positively correlated to human subcutaneous adipose mtDNA copy number (139), we measured FAS and SREBP1c, two markers of lipogenesis. Unexpectedly, SREBP1c protein content did not differ among groups; however, in agreement with other studies examining epididymal WAT (201, 349), we observed an age-associated decrease in FAS expression in omental WAT. Although leptin is known to directly downregulate both FAS and SREBP1c (202), alternative pathways may be involved since circulating leptin in our animals did not inversely correlate with FAS or SREBP1c protein content. Furthermore, unlike in human

subcutaneous WAT (139), we did not observe a positive correlation between FAS protein content and markers of mitochondrial content in omental WAT. However, daily exercise resulted in significantly higher FAS protein expression at all ages studied compared with sedentary animals. It has been suggested that loss of mitochondrial content may reduce the ability for glucose utilization for lipogenesis in WAT (60). Thus, suppressed mitochondrial content in combination with reduced FAS and a decreased ability to convert circulating glucose into fat in the OLETF-SED animals may lead to elevated blood glucose levels, exacerbating the diabetic condition. In addition, the ability of exercise to increase FAS in WAT may prevent the accumulation of malonyl-CoA in a hyperphagic environment, reduce inhibition of carnitine palmitoyltransferase-1, and increase FAO (129).

The progression of adipocyte hypertrophy is associated with an increase in macrophage infiltration and subsequent reduction in both adipocyte number and size (280). Consistent with these findings, adipocyte size in the OLETF-SED group peaked at 20 wks, corresponding with reduced cell number. By 40 wks of age, adipocyte size was smaller, but omental fat mass was maintained due to adipocyte hyperplasia exceeding the cell number at 13 wks of age, all of which was prevented by daily exercise. Circulating serum leptin in OLETF-SED followed a similar pattern as fat cell diameter, with a dramatic rise from 13 to 20 weeks followed by a precipitous fall from 20 to 40 weeks. In the 40 week OLETF-SED animals, serum leptin was negatively correlated with HbA1c and positively correlated with % body fat, suggesting that the inability to continually increase the expansion of adipocytes during hyperphagic conditions may negatively impact glycemic control. These findings warrant future investigation.

In conclusion, we demonstrate for the first time that increases in omental WAT (index of upper visceral WAT) mitochondrial content are attenuated in the hyperphagic, obese OLETF-SED rat through 40 weeks of age. These alterations occurred in conjunction with the progression from insulin resistance to type 2 diabetes. Remarkably, these changes were prevented in hyperphagic OLETF-EX animals, implying that the changes were independent of dietary energy intake. The mechanism responsible for the increase in mitochondrial content appears to be unrelated to changes in FIS1 and lipogenic proteins, and likely does not play a causal role in the development of insulin resistance. However, the failure of WAT mitochondrial content to increase may play a key role in the worsening of the type 2 diabetic condition.

Chapter 5: Differential DNA Methylation in Skeletal Muscle with Physical Activity

ABSTRACT

Epigenetic modifications can occur either through histone modifications or DNA methylation. Although such modifications do not altering DNA sequence they are capable of altering gene expression. DNA hypermethylation of promoters is associated with cancer and atherosclerosis. Recently several reports suggest that DNA methylation can change in response to environmental factors. However, while the prevalence of atherosclerosis and other chronic diseases can be reduced with increased physical activity, whether physical activity can change DNA methylation remains unknown. We tested the hypothesis that physical activity would result in differential DNA methylation between physically active and sedentary mice on a low or high-fat diet. Initial screening for differential methylation utilized a NimbelGen array. From this candidate genes were screened for mRNA differences. Of the candidate genes, *Pitx3* and *Elovl3*, had significant differences in mRNA levels and an attempt to verify the differential methylation was done with two different follow-up techniques. However, neither technique was able to verify differences in methylation. Thus, although *Pitx3* and *Elovl3*, are novel physical activity regulated genes, they do not show differential methylation in response to physical activity. In conclusion, additional candidate genes need to be screened or alterations in the experimental design are needed to determine differentially methylated DNA due to physical activity.

INTRODUCTION

Unlike genetic mutations or single nucleotide polymorphisms, epigenetic modifications do not result in changes in the DNA sequence. Instead DNA structure is indirectly modified by histone modifications (e.g. acetylation or methylation) or directly modified by methylation of cytosines in CpG dinucleotides (28).

Although, CpG dinucleotides only occur at 25% of the frequency expected throughout most of the human genome, some regions exhibit a much higher frequency. These regions, CGIs are somewhat arbitrarily defined by length (200bp-3000bp), cytosine/guanine content (>50%), and CpG frequency (>60% expected) (11). Furthermore, these CGI are found in an estimated 60-72% of promoters and are key sites where CpG methylation can vary (84, 247). Importantly, current dogma suggests that epigenetic changes via DNA methylation are relatively stable, inheritable, and modifiable not only during development, but throughout life, e.g., cancer, causing changes in gene expression (124). Typically, methylation of CGIs located within a promoter of a gene leads to 1) the binding of methyl-binding proteins, which interact with histone deacetylases or other proteins to indirectly inhibit transcription, or 2) directly inhibiting transcription factors from binding DNA (30, 114).

DNA methylation is essential for normal development and is involved in a number of normal physiological processes such as inactivating the X chromosome, regulating chromatin structure, allowing tissue specific gene expression, and helping control cell differentiation (30, 177). Conversely, aberrant hypermethylation, occurring with cancer is mainly focused on the mechanisms by which hypermethylation of promoters in tumor suppressor genes, such as *p16*, *SFRPs*, and *GATA-4*, silence gene

expression (137). Importantly, CGI hypermethylation occurring early in life also increases the risk for later life development of chronic diseases such as type 2 diabetes and CVD (315). During fetal development in rats maternal environmental conditions, such as maternal protein restriction, caloric restriction, high-fat feeding, insufficient-uterine blood flow, and high levels of glucocorticoids, can all increase the likelihood that the offspring will develop a chronic disease in adulthood (212). A role for DNA hypermethylation due to the fetal environment, termed developmental programming, has recently been established in both embryonic and infancy conditions (268, 316). For instance subtle nutritional differences *in utero* results in differential methylation of the insulin growth factor-2 promoter, providing an attractive mechanistic link between low birth weight and later-life diabetes risk (316).

Compelling evidence for the existence of alterations in the epigenome throughout life comes from a monozygous twin study (88). While, monozygotic twins are epigenetically indistinguishable during the early years of life, older monozygotic twins exhibited remarkable differences in their overall content and genomic distribution methylated DNA (88). Furthermore, even within the same individual different blood vessels can have different levels of DNA methylation of the promoter of the estrogen related receptor-α gene and estrogen related receptor-β gene (148, 225). Interestingly, those vessels with visual signs of disease had higher methylation than those without disease within the same individual, suggesting the local environment of a tissue can result in differences in DNA methylation. Together, these studies indicate that increased DNA methylation is associated with specific diseases and is likely in part caused by both local (cellular niche) and systemic environmental factors such as smoking habits, physical

activity, and/or diet.

High levels of physical activity are a reduces the risk of developing many chronic diseases. However, the molecular mechanisms underlying this connection remain understudied and relatively unknown. In response to physical activity the plasticity of skeletal muscle results in significant biochemical, molecular, and physiological adaptations (221). In addition, more recently acute exercise has been shown to be capable of modifying histone in skeletal muscle (185). However, it remains unknown whether acute or chronic exercise can change DNA methylation. Although, it is known that exercise can reduce whole-body inflammation; and both aging and inflammation are associated with increased DNA methylation (35, 277). Thus, it is plausible that increased physical activity may alter inflammation and aging associated differences in DNA methylation.

The purpose of this study was to determine whether epigenetic changes in DNA methylation, occur in skeletal muscle following increases in physical activity on a low or high-fat diet. I hypothesized that sedentary and physically active skeletal muscle would have differences in DNA methylation of specific genes. I utilized several microarrays to generate lists of candidate genes, which were subsequently screened for differences in mRNA levels. Follow-up techniques to verify the differences in methylation were done on the candidate gene *Pitx3*.

METHODS

Animal protocol: Experiment 1 - Non-pregnant female and male C57/BL6 mice were purchased from Harlan (Indianapolis, IN) and bred so that multiple litters of offspring were born. Within a litter pair, each sex was randomly split and housed

individually in cages at 3 weeks of age with or without a 4.5" running wheel (L+5 LF RUN), outfitted with a Sigma Sport BC 800 bicycle computer (Cherry Creek Cyclery, Foster Falls, VA) to record daily running distances. Although voluntary wheel running results in distances exceeding those of treadmill trained animals (~7 km/night for low-fat and ~17 km/night for high-fat wheel running mice vs. ~1-2 km/treadmill session) mitochondrial content only increases ~25% compared to ~100% with treadmill running (Figure 5-2 and 5-3) (85, 162). However, VO_2 in wheel running mice is doubled resting values the duration of the dark cycle, but not during the light cycle (Laye unpublished observations). The other paired mouse (L+5 LF SED) was limited to normal cage activity. Mice had *ad libitum* access to a low-fat diet (Formulab 5008, 6.5% energy from fat) and water. The animal room was maintained on a 12 hour light:dark cycle.

Experiment 2: Female mice were purchased from Harlan at 9 weeks of age and divided into cages with and without a wheel. Mice had *ad libitum* access to a high-fat diet (Diet 58G8, 45% fat, TestDiet, Richmond, IN) and water for 10 weeks before sacrifice. Mice were anesthetized [ketamine (80 mg/kg), xylazine (10 mg/kg), and acepromazine (4 mg/kg)] and killed by exsanguinations, either five hours after the light cycle begun, L+5 HF (avoid acute exercise effects), or 1 hour before the light cycle begun, L-1 HF, (study acute exercise).

Tissue Collection: Between 0900-1100 hours (~5 hours after dark cycle ended, L+5 groups) or 0600–0715 hours (~1 hour before dark ended, L-1 groups) whole gastrocnemius were harvested bilaterally, frozen in liquid nitrogen, and stored in the -80°C freezer until further processing. Retroperitoneal adipose depots and whole hearts were removed, weighed, and also flash frozen in liquid nitrogen.

DNA Extraction: DNA was extracted from muscle with overnight digestion in buffer (100 mM NaCl, 10 mM Tris pH 8, 25 mM EDTA pH 8, 0.5% SDS, 0.1mg/mL Proteinase K) followed by extraction with Phenol:Chloroform:Isoamyl Alcohol 25:24:1(pH 8.0) and ethanol precipitation. Concentration was determined by absorbance at 260 and relative purity by 260/280 absorbance ratio. Integrity of genomic DNA was assessed by Southern blot.

Global Methylation: Total methylation was measured with the Methylamp Global DNA Methylation Quantification Ultra Kit (Epigentek, NY). The kit utilizes an ELISA-like reaction to detect as little as 0.4ng of methylation DNA.

Immunoprecipitation of Methylated DNA (MeDIP): MeDIP was performed essentially as described by Weber et al (319). Briefly, high quality DNA is reduced to 200-1000 bp fragments by *MseI* (New England Biolabs, Ipswich, MA) enzymatic digestion (low-fat fed mice) or sonication (5 x 10 sec, power ~4 Watts) (high-fat fed mice). A portion of DNA is set aside as input DNA at this time. 3μg of DNA is immunoprecipitated (IP) with 3 μg of 5-methycytosine antibody (Diagenode, Sparta, NJ) at 4°C overnight with gentle agitation in IP buffer. The antibody is bound to Protein A agrose beads (Invitrogen, Carlsbad, CA), released by Proteinase K digestion and the enriched fraction of DNA is isolated by ethanol precipitation.

MethylCollector Kit: The MethylCollector kit (Active Motif, Carlsbad, CA) was used as a second approach to collect methylation enriched DNA. This kit utilizes a methyl-binding protein (MBD2) with a H-tag to bind DNA fragments containing ≥6 methylated CpG. The MBD2 with bound DNA is then captured with anti-h tag antibody coated metal beads which is used with a magnetic rack to easily and accurately elute the

methylation enriched DNA. Starting with 1µg of DNA an enriched fraction containing ~50ng of DNA was obtained and concentrated to ~5ng/µL using a Miniprep PCR clean up column (Qiagen, Valencia, CA). The intra-assay coefficient of variation was 13.27% for 5 samples run in parallel, and the interassay variability was 15-30%. This variability is in part due to the low concentration of DNA (3-5ng/µL), which approaches the lower detection level of quantification on the nanodrop (2ng/µL). Enriched and input fractions can then be processed for microarray and/or used immediately for PCR.

DNA Preparation for NimbleGen Microarray: 10 ng of methylation enriched and 10 ng of input DNA were amplified using a WGA2 whole genome amplification kit (Sigma, St. Louis, MO). Initially 10 cycles were done to minimize the potential for amplification bias, but if less than 4 µg of DNA were obtained additional cycles were done until the needed 4-6 µg of high quality (260:280 ration >1.8, 260:230 ratio >2.0) DNA for the microarray was obtained. DNA labeling with Cy5 (methylation enriched DNA) and CY3 (input DNA) dyes, followed by co-hybridization, two color scanning (532nm = Cy3, 635nm = Cy5), and data analysis as per manufactures instructions was done at the University of Missouri DNA Core facilities. Each NimbleGen array (2007-02-27 MM8 CpG Promoter, Roche NimbleGen, Madison, WI) contained 385,000 probes of 50-75 bp in length covering all known genes from 1300 bp upstream to 500 bp downstream of the transcription start site. Additionally probes covered all known CGI, the smaller (<300 bp) of which, were extended with probes on either end by 200 bp. Microarrays were done on a low-fat fed mice (pooled 2 samples for each group) where DNA was prepared with MeDIP protocol, high-fat fed mice (pooled 2 samples for each group) prepared from the MeDIP protocol, and high-fat fed mice (pooled 4 samples for

each group) prepared in duplicate from the MethylCollector Kit. The $\log_2$-ratio p-value, assigned to each probe based on the ratio of IP signal to input signal, was used for visually (Signal Map program, Nimbelgen, Madison, WI) determining candidate genes. Genes were considered likely candidates for differential methylation if they, 1) had multiple probes within a single CGI at significance $p < 0.01$ indicating significantly greater methylation than the rest of the genome, 2) these probes were not significant in the opposite group, and 3) in the high fat fed animals each set of arrays (MeDIP prepared versus MethylCollector prepared) had similar directional changes for the same groups, although the magnitude of the changes was not always the same (Figure 5-7).

RNA Extraction: Gastrocnemius muscle RNA was extracted as previously done with RNeasy kit (Qiagen, Valencia, CA) (164, 172). Verification of RNA purity and quality was done with a nanodrop (260:280 ratio, 260:230 ratio) and visualization of 18S and 28S bands by electrophoresis. cDNA synthesis is performed with equal amounts of RNA using ABI cDNA high-capacity synthesis kit.

RT-PCR: RT-PCR was done as described previously (25;26). Briefly, primers were designed with Primer Express (ABI, Foster City, CA) and ordered from IDT (Des Moines, IA), and a standard curve was run to ensure appropriate efficiencies (90-110%) and absence of primer-dimers. Sequences are found in Table 5-1. Duplicate PCR reactions each containing 25 ng of cDNA was performed with the ABI 7000 Sequence Detection System (ABI) and Syber Green chemistry (ABI) in a final volume of 25 μL. Differences in gene expression were calculated using relative quantification to the control gene 18S (which did not differ, $p = 0.631$ and 0.463, for low-fat fed and high-fat fed mice respectively) by the comparative $\Delta\Delta$CT method (User Bulletin no. 2 ABI PRISM7700

Sequence Detection System).

Table 5-1 **Primer Sequences**

Mitochondrial DNA	*Sec16a* mRNA
Fwd 5' TGATCAACGGACCAAGTTACC 3'	Fwd 5' TTCCAGCCTCACTCCATTTTCT 3'
Rev 5' CGTTGAACAAACGAACCATTAA 3'	Rev 5' ACGGAAAGCTCGGAGCAAA 3'
Myoglobin mRNA	*TGFb2* mRNA
Fwd 5' ATCACACGCCACCAAGCAC 3'	Fwd 5' CTGTACCTTCGTGCCGTCTAATAA 3'
Rev 5' GTCCCCGGAATGTCTCTTCTTCA 3'	Rev 5' TGCCATCAATACCTGCAAATCT 3'
HKII mRNA	*Elovl3* mRNA
Fwd 5' CTGTCTACAAGAAACATCCCCATTT 3'	Fwd 5' GGACCTGATGCAACCCTATGA 3'
Rev 5' CACCGCCGTCACCATAGC 3'	Rev 5' GAGCTTACCCAGTACTCCTCCAAA 3'
MDB1 mRNA	*Pitx 3* mRNA
Fwd 5' TGGCCTTACCCCAGGTGAA 3'	Fwd 5'ACGGCTCTCTGAAGAAGAAGCA 3'
Rev 5' GGAAGTCAGGAAGGTCGTGACT 3'	Rev 5'TCATGTCAGGGTAGCGATTCC 3'
MBD2 (*MBD2a* & *MBD2b*) mRNA	*Arh28* mRNA
Fwd 5' GCTGTGGCCAGTGCTTTACA 3'	Fwd 5' TCGAGGAATCAGGTCTGGAATC 3'
Rev 5' CCACGGCAGCAGAGACTTG 3'	Rev 5' TTCTTCGCGGTACTGCTTGA 3'
MBD3 mRNA	*Telo2* mRNA
Fwd 5' TGCTGGAGGAAGTTTCTGGAA 3'	Fwd 5'GAACCTGGTGGTGAAGAATAAGAAA 3'
Rev 5' GACCCTGGCCTGTAATAGCCTTA 3'	Rev 5'TGGGTGTCGTGTGCTGGTA 3'
MeCP2 mRNA (variants 1-2)	*FgF12* mRNA (variant 1)
Fwd 5' GCCCCCTCCAAAATCCAT 3'	Fwd 5'AGGCGAGGGAGTCCAACAG 3'
Rev 5' TGGCTGCACTATTCTACAGATCTCA 3'	Rev 5'GAGGACGTGCCTCTCACACA 3'
Dnmt1 mRNA	*Dact2* mRNA
Fwd 5' CGTGAGTGTTCGGGAATGTG 3'	Fwd 5' TGAAACCTCTCCCGTGAAGCT 3'
Rev 5' TGCCGAAGAACCGGTAGCT 3'	Rev 5' TCGGGACTTTCTCACTAACTCTTCTT 3'
Dnmt3a mRNA (2 isoforms)	*Suv420h1* mRNA
Fwd 5' CACCCGTTTCTTCCAGCAA 3'	Fwd 5' GGTCTTGAAGGGTGTTTCTGCTT 3'
Rev 5' CTTTCCCAGTCTGCTCAAGGA 3'	Rev 5' CCGGGAATGGCGAGTTTC 3'
Dnmt3b mRNA (all 4 isoforms)	*Zbtb22* mRNA
Fwd 5' CCCTCCCCCATCCATAGTG 3'	Fwd 5' TGGGAAGGCTTTCTCACATAAGA 3'
Rev 5' TGTCTGCTGTCTCCCTTCATTG 3'	Rev 5' GGTCGCAGGTTGAGATGCAT 3'
Idh1 mRNA (variant 1)	*Api5* mRNA
Fwd 5' TCACACGTCACTACCGCATGT 3'	Fwd 5' GCAAGTGGGCCAGCATAAA 3'
Rev 5' GGCAAAAATGGAAGCAATGG 3'	Rev 5' CTGCTAATCGCTTTTCCTTGGT 3'
Pbx3 mRNA	*Mkl1* mRNA (Variant 1 only)
Fwd 5' CCCCGAAAGAGATCGAAAGG 3'	Fwd 5' AAGGGTGGAGAGTTGATGTCTTC 3'
Rev 5' CTTGAGCTGCATCTGAATGGAA 3	Rev 5' CCACAGCAAGCTTTACCAATTC 3'
Gtrgeo22 mRNA	*Fbxl10* mRNA (Variant 2 only)
Fwd 5' TGGGACCTGCAAACTGATGTC 3'	Fwd 5' CGAGGACGACGACTATGAATCG 3'
Rev 5' GCTGGTCCTGCAAAGTCCAA 3'	Rev 5' GGGCCCAACTTGCCCTTAG 3'

Bisulfite Treatment of DNA: The ABI kit methylSEQr per manufactures instructions was used to convert all unmethylated cytosines to uracils in DNA. Major advantages of this kit are that it requires significantly less input DNA (300 ng) versus standard bisulfite treatment (~2-10 ug) and the bisulfite-converted DNA is stable at 4ºC for 2+ years versus freezing or immediately using the sample as is the case with traditional bisulfite treatments.

Combined Bisulfite Restriction Assay (COBRA). Primers for the *Pitx3* candidate gene spanning 165bp of the 3'UTR (1131 – 1305 of gene) containing two Taq1 recognition cut sites (Forward 3' GTTTGAAAGGGGTGTTAGAGTATT 5', and Reverse 3' ACCAAAAATAAATTAACCCTAAAAA 5') of the bisulfite treated DNA

were designed on MethylPrimerExpress (ABI). 1µL of bisulfite treated DNA undergoes two rounds of PCR (12.5 µL of Amplitec Gold DNA Polymerase master mix, 1.25 µL of 10mM forward and reverse primers, 9 µL of H_2O) during which a 5°C reduction in Tm (from 60°C to 55°C) occurs in the first 5 cycles (1°C/cycle). After clean up of the PCR reaction with Qiaquick PCR clean-up (Qiagen), the purified product was digested with Taq1 restriction enzyme (New England Biolabs) overnight at 65°C. The digested product was run on a 3% agrose gel containing ethidium bromide, visualized under a ultraviolet light, and bands quantified using Kodak Imaging Software. Fragment sizes of 24, 46, 70, 95, 116, and 165 bp were possible.

Pyrosequencing. 1 µg of untreated high quality gDNA (n=5 for each L+5 HF RUN and L+5 HF SED) were sent to EpigenDk (Worcester, MA), where they underwent bisulfite treatment (Zymo Research EZ DNA methylation kit). EpigenDk designed one set of primers for a 365 bp region of *Pitx3* covering from exon 4 into the 3'UTR containing 28 unique CpG dinucleotides. Additionally, 4 separate sequencing primers were designed to cover the entire 365 bp amplicon product. A standard curve containing 0, 5, 10, 25, 50, 75, and 100% methylated DNA generated a mean $r^2 = 0.96$ when averaged across all 28 CpGs. Individual CpG standard curves ranged from r = 0.93 to r = 0.98.

Statistical Analysis. The study was designed to determine if methylation differed with voluntary wheel running, and therefore comparisons were only made within each diet group and not between diets. For the low-fat fed animals a Two-Way ANOVA comparing activity (LF-RUN v LF-SED) and sex (MALE v FEMALE) was done only on animal characteristics, while sexes were combined and only t-tests were done for other

outcomes with a $p<0.05$ significance level for all measures. Post-hoc analysis, when necessary, was done with a Student-Newman-Keuls test. For high-fat fed animals either t-tests between RUN and SED were performed unless a Two-Way ANOVA (HF-RUN vs. HF-SED, L-1 v L+5) could be performed with data from both L-1 and L+5 groups was available. All tests were performed with SimgaStat software (SysStat Software, Germany).

RESULTS

Animal characteristics. Body weights, retroperitoneal adipose mass, heart mass, heart mass/BW ratio, and food intakes for L+5 LF mice are given in Table 5-2. Body weights, lean and fat mass, retroperitoneal adipose mass of HF-fed mice (both L+5 and L-1) are given in table 5-3. Over 10 weeks low-fat fed mice averaged 6.94 ± 0.73 km/night, while the L+5 HF-fed mice averaged 17.52 ± 0.85 km/night (Table 5-3). L-1 HF RUN mice averaged the same distance ran on the night of sacrifice as they did throughout the 10 wks (14.74 km/night ± 0.74 vs. 15.71 ± 1.71 on last night). L-1 HF RUN and L-1 HF SED mice did not differ in body weight from their L+5 HF counterparts (Table 5-3).

Table 5-2

Parameter	All RUN	All SED	MRUN	MSED	FRUN	FSED	Activity	Sex
n	20	18	10	9	10	9		
BW (g)	25.63 ± 0.39	25.60 ± 0.58	26.78 ± 0.35	27.29 ± 0.71	24.48 ± 0.47	23.92 ± 0.48	no	p<0.05
RP Adipose (mg)	100.00 ± 9.02	147.12 ± 17.3	81.46 ± 6.52	164.68 ± 33.04	118.53 ± 14.99	129.57 ± 9.95	p<0.05	p<0.05
Heart (mg)	126.93 ± 2.54	119.83 ± 3.35	133.21 ± 3.25	127.44 ± 4.10	120.64 ± 2.80	112.22 ± 4.07	p<0.05	p<0.05
Heart/BW	4.96 ± 0.09	4.68 ± 0.09	4.98 ± 0.14	4.68 ± 0.13	4.93 ± 0.11	4.69 ± 0.13	p<0.05	no
n	7	6						
Food (g/day)	6.08 ± 0.23	5.09 ± 0.25	p<0.05					

Table 5-2. Low fat-fed mice body characteristics. A Two-Way ANOVA was performed for. Main effects are as indicated, no significant interactions were present. For food intake all data was combined due to the low n and a t-test was performed. MRUN, Male Runner, MSED, Male Sedentary, FRUN, Female Runner, FSED, Female Sedentary All data is presented as the Mean ± SEM.

Table 5-3

	n	Body Weight (g)	Fat Mass (g)	Lean Mass (g)	% Body Fat	RP Fat
L+5 HF RUN	10	24.55 ± 0.49 *	4.3 ± 0.3 *	19.8 ± 0.4 *	17.9 ± 1.1 *	0.242 ± 0.02 *
L+5 HF SED	12	30.44 ± 1.64	13.6 ± 1.8	16.7 ± 0.4	41.8 ± 3.7	0.958 ± 0.13
L-1 HF RUN	6	25.06 ± 1.72				
L-1 HF SED	6	31.16 ± 1.89				

	Wheel Running (km/night)	
	Daily Ave	Last Night
L+5 HF Run	17.52 ± 0.85	N/A
L-1 HF Run	14.74 ± 0.74	15.71 ± 1.71

Table 5-3. HF-FED mice characteristics. A t-test was performed and * indicates a $p < 0.05$ between the RUN and SED group. All data is presented as the Mean ± SEM.

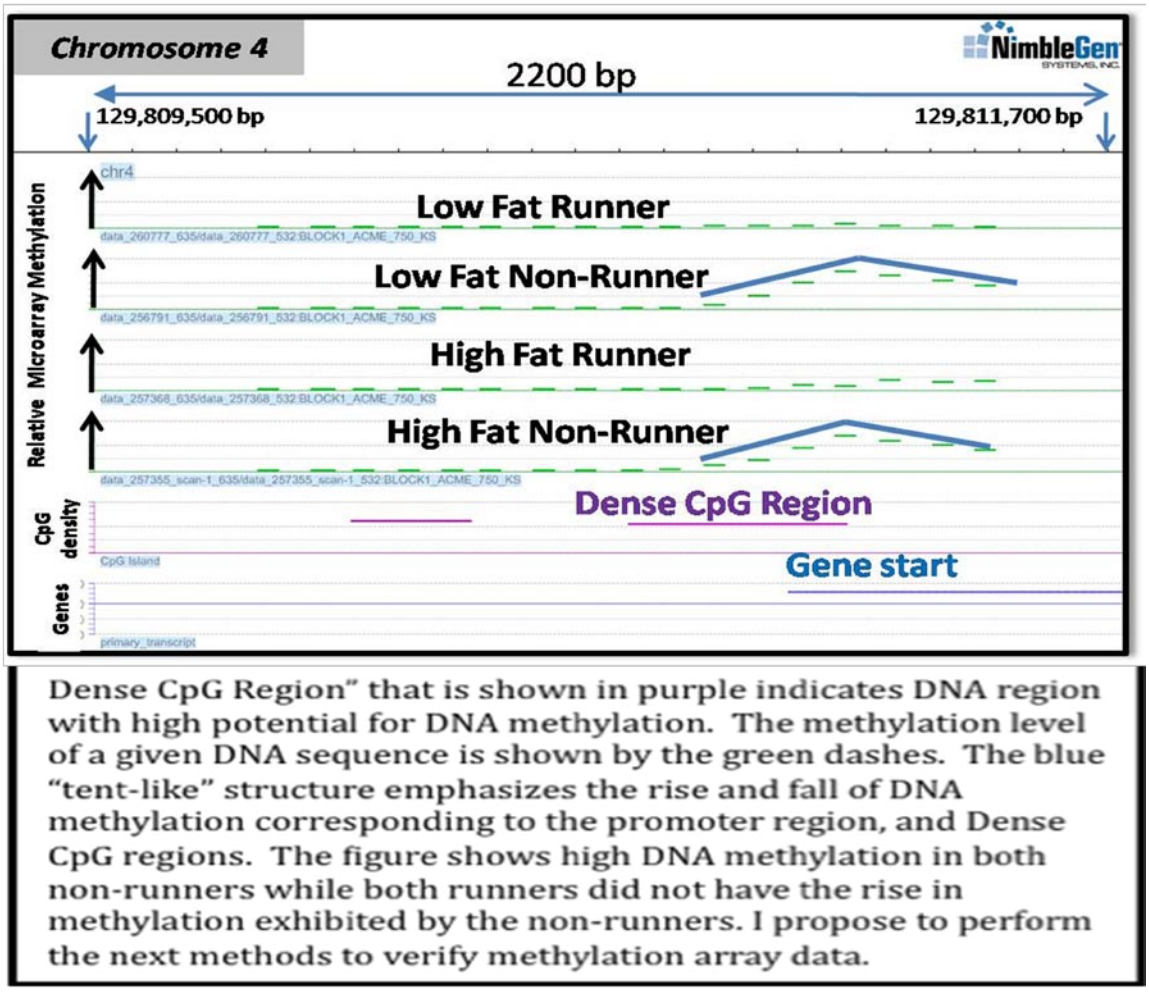
Training adaptations. In the low-fat fed mice a significant increase in heart/BW ratio (Table 5-2), myogloblin mRNA, and trends for HKII mRNA ($p=0.18$) and relative mtDNA copy number ($p=0.56$) (Figure 5-2) consistent with the relatively moderate training effects seen with voluntary wheel running (163). In the HF-fed mice there were surprisingly no differences in HKII mRNA, myogloblin mRNA, and relative mtDNA number existed (Figure 5-3A). However, citrate synthase activity was significantly elevated ($p<0.05$) in the gastrocnemius of the L+5 HF RUN vs. L+5 HF SED (t-test) (Figure 5-3B).

Methyl Binding Proteins and Methyltransferases. The mRNA levels of several proteins that bind methylated CpGs (*MBD2*, *MBD3*, and *MeCP2*) were unchanged in any groups (data not shown). DNA methyltransferase 1 (*Dnmt1*) remained unchanged in any groups (data not shown). However, DNA methyltransferase 3A (*Dnmt3a*) mRNA showed both a physical activity ($p<0.05$, RUN vs. SED group) and a time of day effect ($p<0.05$, L-1 vs. the L+5 group) (Figure 5-3). *Dnmt3b* showed a time of day effect ($p<0.05$, L-1 HF vs. L+5 HF), and showed a trend towards a physical activity effect ($p=0.09$) in the HF-RUN vs. HF-SED group) (Figure 5-3).

Global DNA Methylation. Global DNA methylation did not differ between and groups (Table 5-4).

Candidate genes based on Nimbelgen microarray. In an attempt to maximize the likelihood of finding differences in methylation between wheel running and sedentary animals I initially focused on genes that were similarly differentially methylated in both the low-fat and high-fat fed mice. *FABP3* is an example of a gene meeting the selection criteria (Figure 5-1). The following genes were also initially selected to analyze; *MUSK, Kelch22, Rcor, Mtm1B, Mtnr1B, SREBP1f, Edgf6, Pitx3, Pip5k3, SEC16A, Pbx3, Ireb2, Hdac11, Fxbl 10, Ankrd17,kcnd3, IL11ra2,Tgfb2, Htatip2, Phgdh* and *DAP3*. In addition to trying to find similar genes between the low-fat and high-fat RUN and SED groups, I collected a methylated enriched fraction of DNA using the MethylCollector kit from this same cohort of high fat fed animals. I then compared the arrays utilizing the two different techniques to collect methylated DNA (MeDIP vs. MethylCollector), although the 5-methylcytosine antibody is (used in MeDIP) and MBD 2 (used in MethylCollector) likely to have a different affinity for methylated-cytosines than the MBD2. The following additional candidate genes were found comparing these two sets of arrays; *Suv420h1, Dact2, Arh28, Telo2, Ztb22, Api5,MKL1-1* and *Fgf12*. In several cases there was a second gene close to the primary gene of interest and the expression of that gene was also measured.

Figure 5-1



Dense CpG Region" that is shown in purple indicates DNA region with high potential for DNA methylation. The methylation level of a given DNA sequence is shown by the green dashes. The blue "tent-like" structure emphasizes the rise and fall of DNA methylation corresponding to the promoter region, and Dense CpG regions. The figure shows high DNA methylation in both non-runners while both runners did not have the rise in methylation exhibited by the non-runners. I propose to perform the next methods to verify methylation array data.

Verification of Candidate Genes. Since we were interested in genes where the mRNA levels changed due to differences in methylation I decided to screen candidate genes (and when necessary nearby genes) for mRNA differences before proceeding to verify methylation differences. In the L+5 LF group I screened the following genes; *Idh, Ireb2, Pitx3, Sec16a, kcnd3, Ankrd17, Hdac11, Elovl3, Tgfb2* for differences in methylation. Several looked promising (*SEC16A, Gtgreo22, Idh, Pbx3, Pitx3, Elolv3, Tgfb2*) and I decided to increase the n from L+5 LF RUN n=3 and L+5 LF SED n=5, to 9 and 7 respectively. After the increase in n, only *Pitx3* had a trend (p=0.10) to be different

(Figure 5-4). In the L+5 HF groups I screened all of the same genes in the L+5 LF group plus the following *Arh28, Telo2, Fgf12, Dact2, Suv420h1, Ztb22, Api5,* and *Mkl1* (Figure 5-5). *Pitx3*, *Idh*, and *Elovl 3* significantly differed between the L+5 HF RUN and L+5 HF SED, while *pbx3* showed a trend (p=0.056) toward being different.

With very little of the candidate genes showing mRNA differences I decided to 1) focus on the high-fat animals since their phenotypes differ more than the low-fat fed animals, and 2) add the L-1 HF RUN and L-1 HF SED groups. The L-1 groups were added based on the rationale that a gene that is differentially methylated between runners and sedentary mice might only show a difference in mRNA when the animals are actively using the running wheel. I screened *Idh, Elovl 3, Pitx3, Arh28, Telo2, Fgf12, Dact2, SUV420h1, Api5, Mkl1,* and *Fbx1 10* for differences in mRNA levels between the L-1 HF RUN and L-1 HF SED groups. While most genes showed changes from the L+5 to L-1 (*Idh, Elovl 3, Pitx3, Telo2, Fgf12, SUV420h1, Mkl1*) (Figure 5-6), no significant differences between the two L-1 groups occurred.

Since *Pitx3,* and *Elovl3* share the same 3'UTR, where the CGI that was putatively methylated is located(Figure 5-7), and the expression of both genes was lower in the L+5 HF SED group relative to the L+5 HF RUN group this gene was an attractive target. Additionally, several different regulatory mechanisms controlling *Pitx3* expression in muscle, including an alternative promoter (66) and mi-RNA (147), further encouraged the pursuit to verify the differences found in the microarray methylation of the *Pitx3* gene.

Pyrosequencing and COBRA of Pitx3 3'UTR CpG Island. Both pyrosequencing and the COBRA assay utilize bisulfite treatment to "lock-in" the methylation status of DNA by converting unmethylated to uracils, while methylated cytosines are unaffected. The COBRA digests yielded results 4 bands of 165, 119, 95, and 46 bp, and faint band at 70 bp. The positive control showed all 5 bands as predicted (Figure 5-8). No differences in band intensity were determined, suggesting no differences in methylation at the CpGs within the restriction site (Figure 5-8). Since COBRA is limited in sensitivity and only measured the methylation status of the 2 individual CpGs located at the cut sites for TaqI pyrosequencing was simultaneously done in an attempt to detect smaller differences in methylation at each of the individual 28 CpGs within the 3'UTR. Of the 28 CpG dinucleotides analyzed, 10 had greater than 10% methylation (Figure 5-X). Of these 10, methylation was consistently 10% higher (absolute difference of 1%) in the L+5 HF SED group compared to L+5 HF RUN (Figure 5-9). However, a one-way ANOVA revealed no significant main effect of physical activity across all 28 CpGs. While, some individual CpGs were statistically different when using a t-test, the 1% absolute difference is below the sensitivity of the assay and should be interpreted with caution (personal communication, Dr. Yan, EpigenDK Inc.).

Figure 5-2

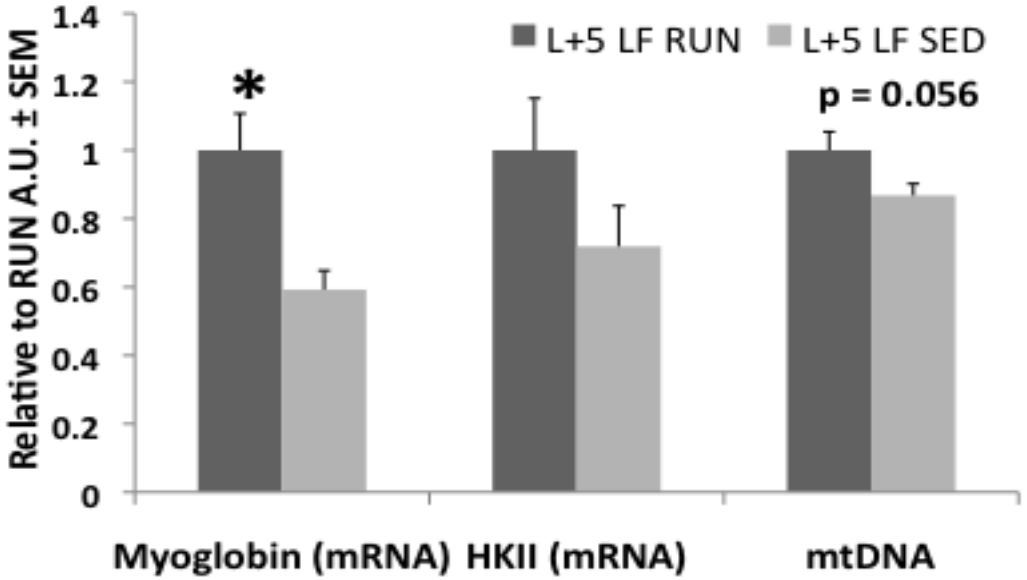


Figure 5-2. Whole gastrocnemius gene expression of training adaptation genes (n=6-9) and mtDNA content (n=10-12). * indicates p <0.05. Data expressed as Mean ± SEM, relative to L+5 LF SED group

Figure 5-3

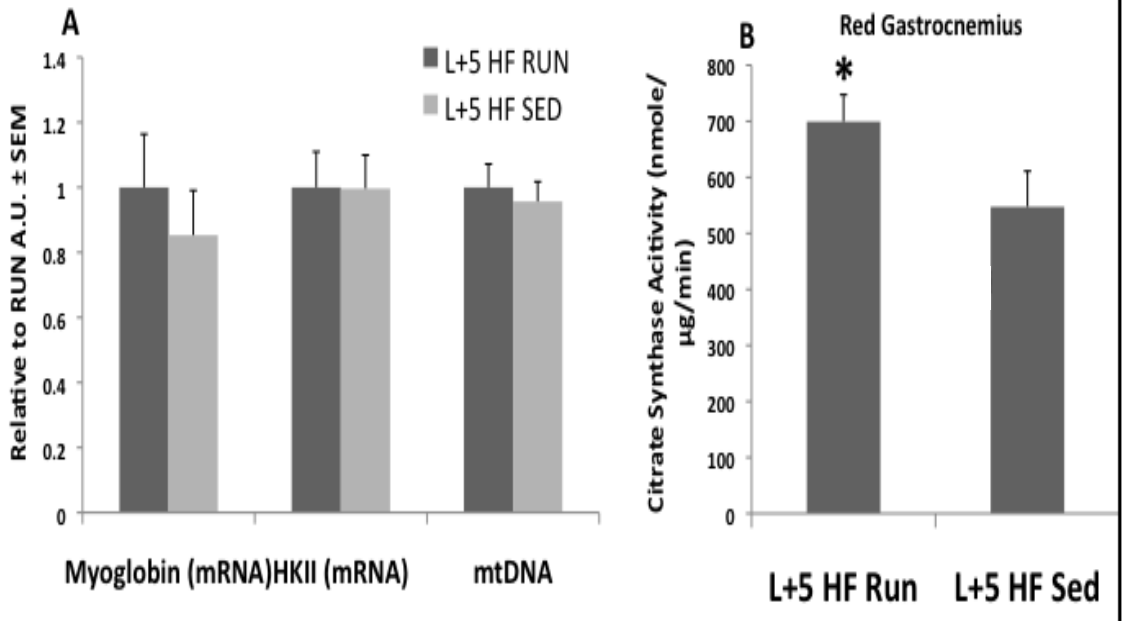


Figure 5-3. Panel A: Whole gastrocnemius gene expression of training adaptation genes (n=8) and mtDNA content (n=7-8). Panell B: Citrate synthase activity in red gasctronemius * indicates p <0.05. Data expressed as Mean ± SEM, relative to L+5 LF SED group

Figure 5-4

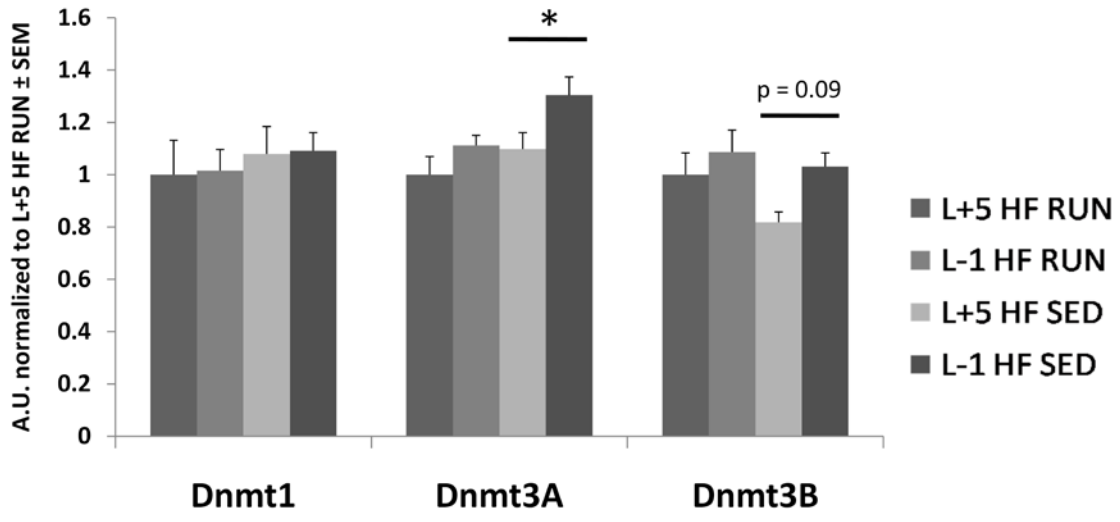


Figure 5-3. mRNA of various methyltransferase enzymes in the gastrocnemius of mice sacrificed 5 hours after the light cycle (L+5), and 1 hour prior to light cycle coming on (L-1). All mice were fed a high-fat diet, half were given access to a voluntary running wheel (RUN) and half were limited to cage activity (SED). N=8-9. Values are normalized to L+5 HF RUN and expressed as means ± SEM. * indicates significance of $p<0.05$ using a 2-Way ANOVA

Figure 5-5

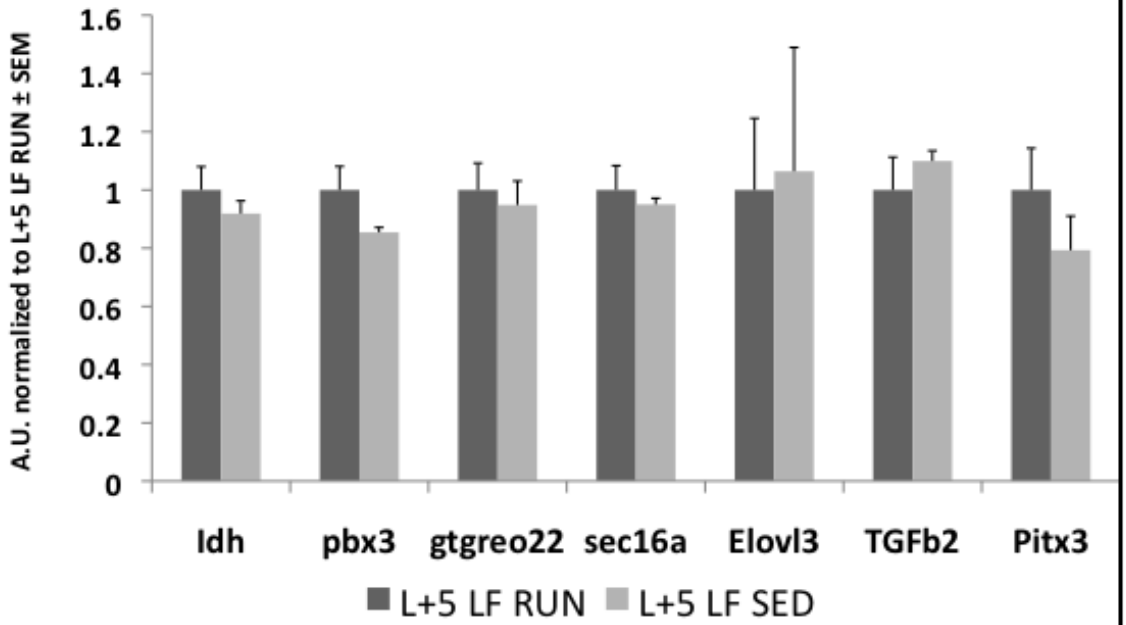


Figure 5-5 mRNA levels of genes that appeared to be differentially methylation on the Nimbelgen microarray. Values are normalized to L+5 LF RUN and expressed as mean ± SEM

Figure 5-6

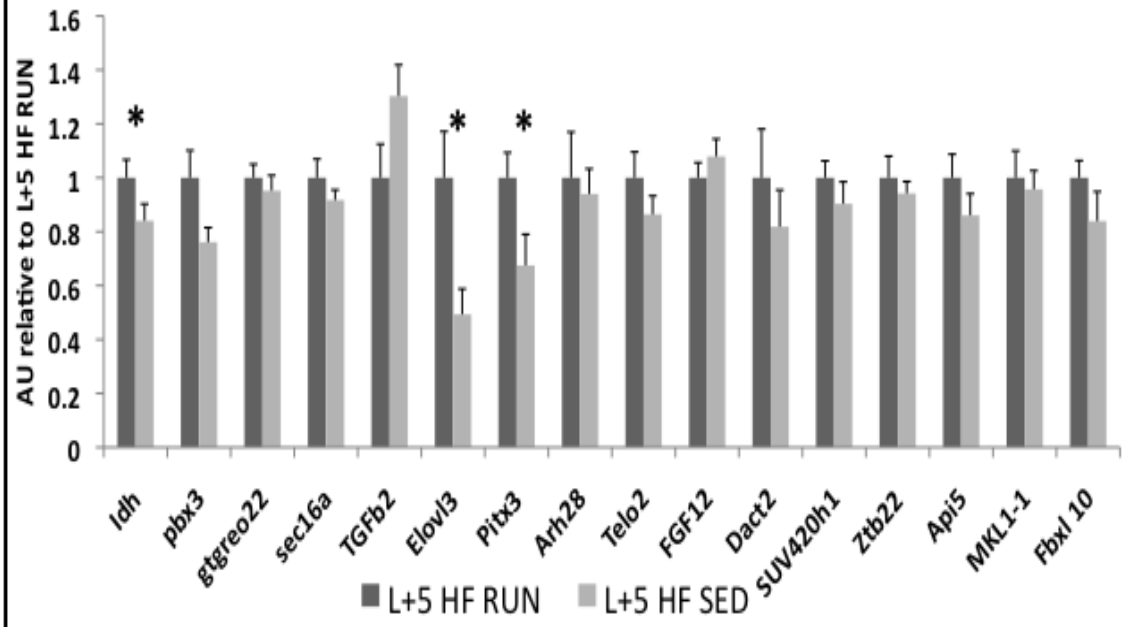


Figure 5-6 mRNA levels of genes that appeared to be differentially methylation on the Nimbelgen microarray. * indicates that L+5 HF RUN and L+5 HF SED are significantly different p<0.05. Values are normalized to L+5 LF RUN and expressed as mean ± SEM

Figure 5-7

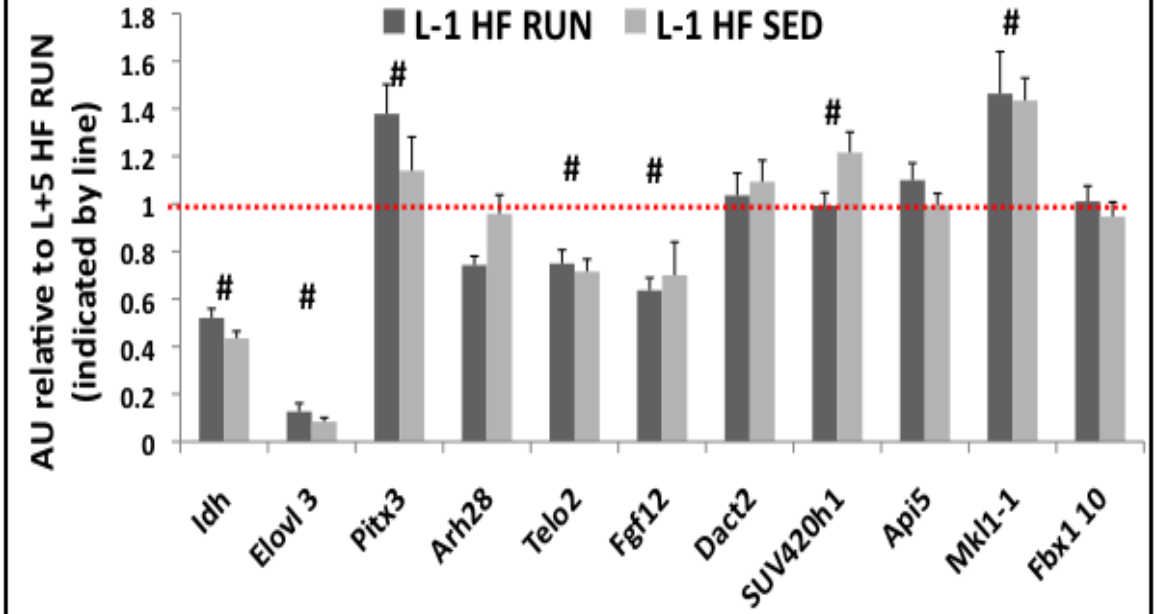


Figure 5-7 mRNA levels of genes that appeared to be differentially methylation on the Nimbelgen microarray. The dotted line indicates the levels of expression of L+5 HF RUN group. # indicates that L+5 and L-1 time points are significantly different p<0.05. Values are normalized to L+5 LF RUN and expressed as mean ± SEM

Figure 5-8

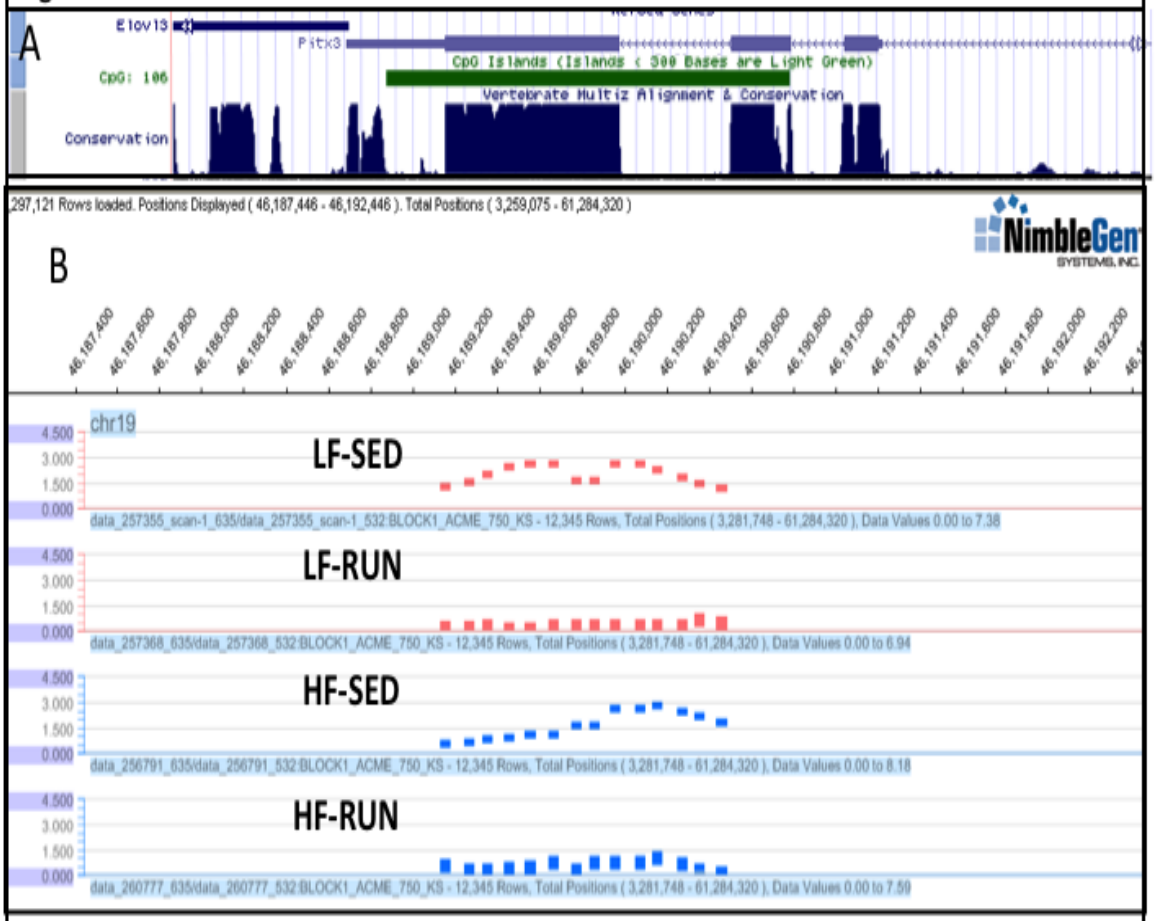


Figure 5-8. Panel A. Screenshot of the layout of the *Pitx3* and *Elovl3* genes on the UC Santa Cruz genome browser (http://genome.ucsc.edu/). Note that the 3'UTR, just outside the CpG island (green) of each gene almost overlaps in a region of high conservation. The CGI of interest is A is lined up with the same region on the Nimbelgen microarray in Panel B. Notice that each the LF-SED and HF-SED a number of probes above 2 on the $\log_2$-ratio p-value, indicating a significance of P<0.01.

Table 5-4

	n	Methylated DNA (ng/ug)
L+5 LFRUN	4	18.10 ± 0.36
L+5 LFSED	4	18.57 ± 0.23
L+5 HFSED	3	17.97 ± 0.92
L+5 HFRUN	3	18.30 ± 1.16

Figure 5-9

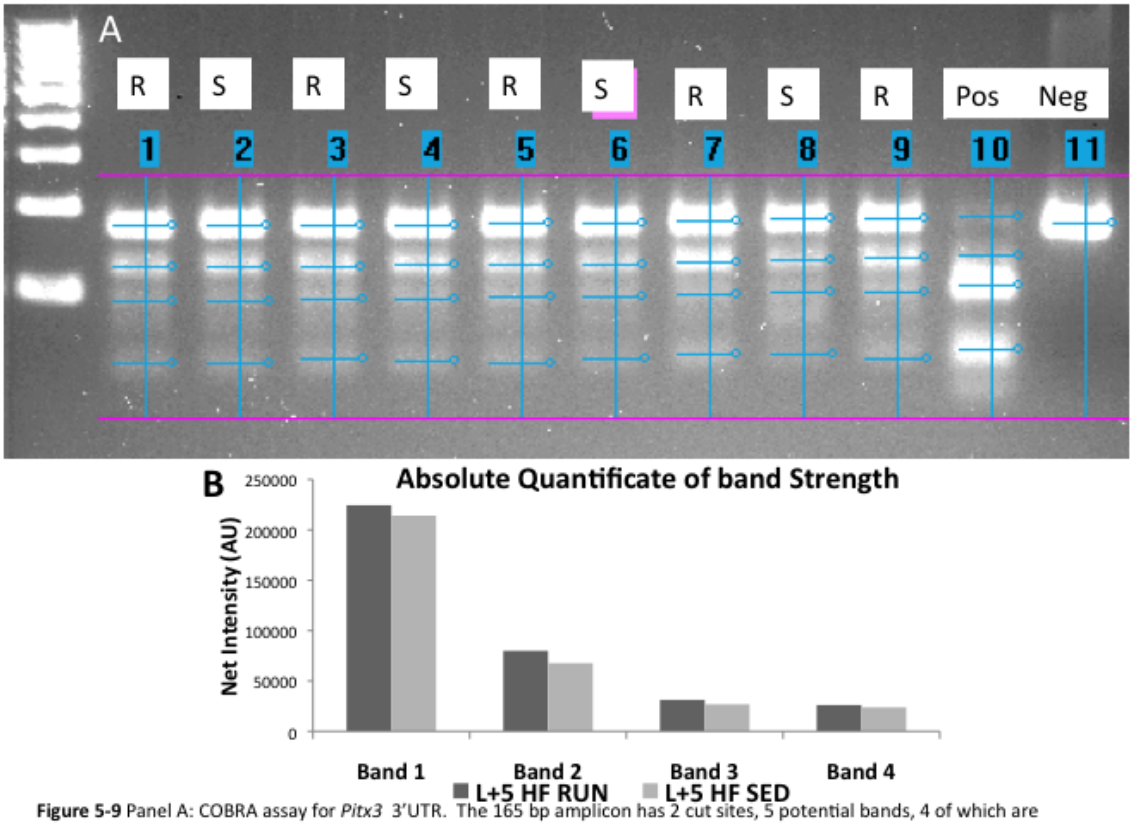


Figure 5-9 Panel A: COBRA assay for *Pitx3* 3'UTR. The 165 bp amplicon has 2 cut sites, 5 potential bands, 4 of which are quantified in Panel B. **R** = L+5 HF RUN, **S**= L+5 HF SED. 100 bp ladder on far left lane. Pos = Positive control, that should be fully digested. Neg = amplicon not subjected to digest. Data is mean only, SEM omitted for simplicity.

Figure 5-10

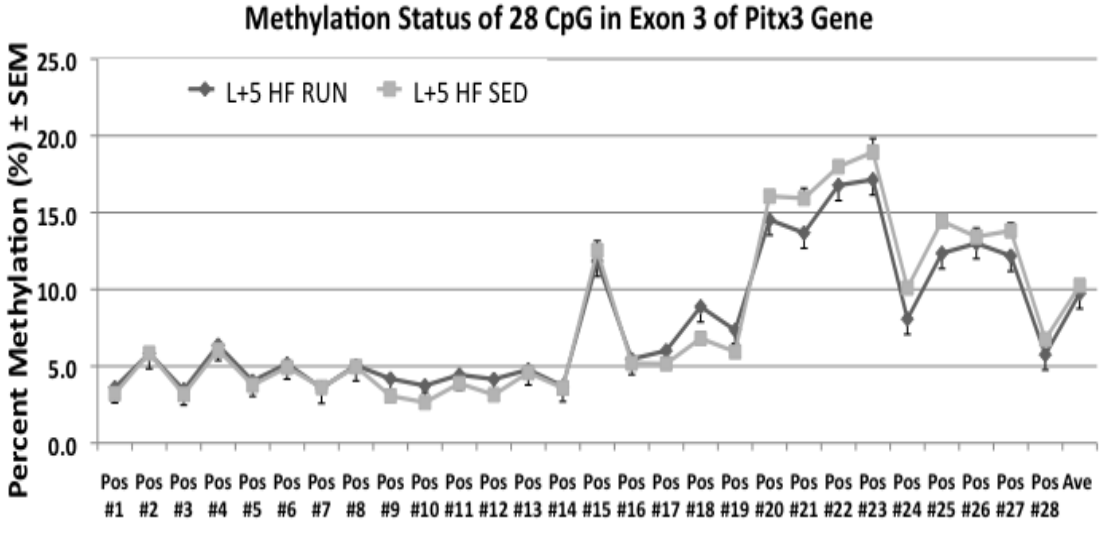


Figure 5-10. Pryosequencing of 28 CpG sites within the last exon and 3'UTR region of *Pitx3* gene. N=5/group. Data expressed as % methylation ± SEM

DISCUSSION

In this study I attempted to find differentially DNA methylation of specific genes in skeletal muscle in response to wheel running. To do this I used a microarray approach, initially identifying 21 candidate genes that differed independent of diet (low-fat vs. high-fat). Eight additional candidate genes were determined by comparison of how the enriched fraction of methylated DNA was isolated (MeDIP vs. MethylCollector magnetic beads). Candidate genes were initially screened for differences in mRNA (Figures 5-4 and 5-5). In order to exclude the possibility that gene expression of candidate genes only differed while the animals were actively using the running wheels, a group of animals sacrificed one hour prior to the end of the dark cycle was also used to screen the mRNA of candidate genes (figure 5-6). Additionally, to see if physical activity may alter the mRNA level of any genes involved in DNA methylation both DNA methyltranferases and methyl-binding proteins mRNA were examined. Interestingly, the *de novo* methyltransferase *Dnmt3a* was increased in the HF-SED versus the HF-RUN group. Of the candidate genes *Pitx3* mRNA was reduced in the HF-SED versus HF-RUN and was selected for further methylation verification by pyrosequencing and COBRA analysis. However, neither analysis verified the differences found in the microarrays, and thus it is unlikely that *Pitx3* methylation differs between voluntary wheel running and sedentary mice. At this time, I have been unable to verify differences in methylation of candidate genes, suggesting potential limitations with the experimental design, methods or both (see discussion below).

Although differences in DNA methylation of *Pitx3* between the HF-RUN and HF-SED groups were unable to be verified, *Pitx3* is still a novel exercise-induced gene. *Pitx3*

expression was thought to be limited to dopaminergic neurons in the brain (203), but recently *Pitx3* expression was also found in developing skeletal muscle (166). In skeletal muscle the first exon of *Pitx3* differs from dopaminergic neurons and is under the transcriptional control of an alternative promoter (66). This alternative promoter for *Pitx3* contains binding motifs for the transcription factors myogenin and MyoD, which are critical to the myogenic program (66). Interestingly, previous work has shown CpG hypermethylation in the brain-specific *Pitx3* promoter in breast cancer tissue (75). However, my microarray screen did not find differences in the promoter methylation, but in a CpG island spanning much of the last exon and 3'UTR (Figure 5-7). The 3'UTR was recently shown to be a binding site for microRNA 133b, which decrease *Pitx3* expression in dopaminergic neurons (147). It is tempting to speculate that differences in miR133b expression or activity might contribute to the differences in *Pitx3* mRNA seen with physical activity, since I was unable to verify methylation differences.

Another novel finding was that expression of the *de novo* methyltransferase, *Dnmt3a*, was decreased in the HF-RUN versus the HF-SED groups. Expression of *Dnmt3a* in old fibroblasts decreased compared to young fibroblasts; while in cancer cells expression increased (52). While differences in *de novo* methylation that maybe expected with differences in *Dnmt3a* mRNA methyltransferase-activity independent effects for *Dnmt3a* have been reported, including directly interacting with p53 to inhibit transactivation of p21 and inhibit progression of the cell cycle (313). However, lower expression of *Dnmt3a* can be caused by both estrogen and progesterone (339). Thus, I cannot rule out differences in the estrous cycle between groups contributing to the differences in *Dnmt3a* mRNA levels.

Limitations of MeDIP/MethyCollector procedure. Both MeDIP and MethyCollector result in 200-1000bp fragments of methylation enriched DNA. A limitation of this approach is that it only provides a general "roadmap" of where differences in methylation are occurring and not the specific CpGs where differences in methylation are. The MethylCollector kit states, "The provided His-MBD2b protein binds to all methylated cytosines and the MethylCollector method enriches for DNA fragments that contain six or more methylated cytosines" (http://www.activemotif.com/documents/149.pdf). Thus, the MethylCollector kit is unable to distinguish between subtle variations in CpG methylation (i.e. 6 versus 8) and is most effective at determining substantial differences in CpG methylation over 200-1000 bp fragments of DNA. Although this is useful to detecting large differences between cancer and non-cancer cells or between different cell types, differential methylation at a single CpG can influence gene expression (199). No data about the sensitivity of the 5-methylcytosine for methylated CpGs is currently available, making it difficult to compare the sensitivity of the two methods.

Limitations of pyrosequencing. Bisulfite treatment of DNA keeps all methylated CpGs as CpGs, while unmethylated CpGs are converted to UpGs. Several sequencing techniques can exploit this difference in sequence to find differences in methylation. Although bisulfite treatment followed by sequencing is considered the gold standard for detecting methylation differences significant limitations still exist. For instance, direct sequencing of PCR products can lead to superimposed signals from primer-dimers or secondary products, while samples with mixed methylation at each CpGs (as in a tissue with multiple cell types in it) will migrate differently over 400 bp frequently causing

misalignment. Furthermore, using direct sequencing differences in absolute methylation of less than 10% are not reliably detected, a high number of T's (typically >9 in a row) in an amplicon can increase the likelihood of PCR slippage, and misalignment of signal (58). Alternatively, clone sequencing clones the PCR product into a plasmid, grows up the plasmids and sequence individual clone, eliminating mixed methylation and some potential misalignment problems. Limitations with this technique also exist. Two major problems are PCR bias, where unmethylated DNA is amplified more efficiently than methylated DNA, and ensuring that a sufficient number of clones (>10) are sequenced so a confident interpretation of the methylation status can be made (59). On the other hand, pyrosequencing is considered highly quantitative and more sensitive than direct sequencing, without the major drawbacks of sequencing clone (293). However, the sensitivity is still somewhat questionable. Thus, although there was a consistent 10% relative difference in methylation over a section of 8 CpGs in the 3'UTR of *Pitx3*, an absolute difference of 1% methylation is unreliable.

Limitations of the Experimental Design. The selected intervention for this study, physical activity and diet, does not produce large phenotypic differences over the time, 10 weeks, studied. For instance, the differences in phenotype of skeletal muscle between mice given access to voluntary wheel running and those not, are as drastic as the differences between cancers and non-cancer cells. In addition the age of the mice at the time of sacrifice, 13 weeks for low-fat and 18-20 weeks for high-fat, is the equivalent to a young adult in humans, while most differences in CpG methylation in humans do not seem to arise until middle to old age (88). While, voluntary wheel running is protective against aging related oxidative damage, 10 weeks is unlikely to be long enough for aging

related oxidative stress in skeletal muscle to be significant (70, 88). Furthermore, physical activity reduces local inflammation, which appears to be a key initiator of DNA methylation (225, 277, 298). However, inflammation of skeletal muscle following the current intervention is unlikely as high as in cancer or atherosclerotic plaques. Potentially the local inflammation in WAT following high-fat diet induced macrophage infiltration might cause differences in DNA methylation (322). A tissue with a more dramatic phenotypic in response to voluntary wheel running is might be more likely to show a difference in DNA methylation as even the metabolic adaptations to voluntary wheel running are nuanced in the gastrocnemius (Figure 5-2 and 5-3). For example, the hyperphagic obese OLETF rat, voluntary wheel running dramatically improves the liver phenotype, but not the skeletal muscle, and may be a better candidate to detect differences in CpG methylation In (195, 231).

In conclusion differences in the genes *Pitx3* and *Dnmt3a* are novel exercise induced genes and require further examination. However, I was unable to verify microarray differences in DNA methylation of *Pitx3* between voluntary wheel running and sedentary mice on a low or high-fat chow diet in skeletal muscle. Changes in the experimental design, such as using an alternative model or examining different tissues, may also be required to maximize differences in methylation between physically active and sedentary animals.

Chapter 6: Discussion

Overall, the major theme of this dissertation has been to determine how physical activity and inactivity modify WAT phenotype. In this document I have provided novel evidence that 1) physical inactivity is sufficient to cause increases in intra-abdominal WAT in the absence of caloric overconsumption, 2) voluntary wheel running increases FAO in isolated epididymal adipocytes. The increased FAO is maintained following 173 hours of inactivity, unlike skeletal muscle and liver, and is independent of changes in mitochondrial enzyme concentration, 3) Maturation (from 20 to 40 wks) increases omental mitochondrial concentration in wild type sedentary rats, but not hyperphagic sedentary rats. Physically active hyperphagic rats have an omental mitochondrial concentration between hyperphagic sedentary and wild type sedentary rats. In addition we compared skeletal muscle DNA methylation between physically active and sedentary mice.

PHYSIOLOGICAL GROWTH OF ADIPOSE TISSUE IN RESPONSE TO INACTIVITY

There is a growing appreciation for the role of WAT in maintaining energy homeostasis and acting as an endocrine organ. Understanding the physiology and pathology of WAT is essential to developing effective preventions and treatments for obesity and diabetes (324). In chapter 2 have shown that daily voluntary wheel running in rats reduces WAT, and is a similar physical activity pattern as Paleolithic-era hunter-gathering humans (55). Rats wheel running 8-10km/night eat ~25% more food than sedentary rats, and continue to do so several days after they cease wheel running (173). By pair feeding rats to the level of sedentary rats during the wheel lock period we

believed we could prevent the gain in WAT mass. Thus, it was surprising to find that pair feeding rats, failed to prevent the regain of adipose mass (173). This finding was opposite of the original hypothesis and suggests a strong physiological preference for the storage of WAT during even when caloric intake is restricted.

I speculate that daily physical activity requiring increased caloric intake leads to metabolic adaptations favoring expanding WAT over increasing lean mass. Both the sedentary and WL5 groups gained similar amounts of lean mass in the one week prior to sacrifice (Chapter 3, Table 3-2). However, one week of inactivity prevented any gain in lean mass, even in rats allowed *ad libitum*. The prevention of gain in lean mass is similar during both a paradigm of cessation of voluntary wheel running and the cessation of caloric restriction. For example, following caloric restriction pair feeding rats to the level of normal *ad libitum* fed rats also preferentially increases WAT, similar to what occurs during the cessation of voluntary wheel running (54).

The preferential increase in WAT is likely evolutionarily advantageous. In support of this, females with reduced WAT often have amenorrhea and are unable to reproduce with low WAT (56). Amenorrhea is then prevented if exogenous leptin is given to "fool" the body into thinking a higher amount of WAT is present (56). More body fat also leads to a longer breeding season and more reproductive success than less body fat in male mandrills (326). Thus, the predisposition to increase fat mass over lean mass may at times be beneficial for the reproductive success of the animal in multiple ways. Compensatory mechanisms to reduced WAT also exist in humans. For example at 10% loss in weight increases metabolic efficiency, which is reversed by "fooling" the body by injecting exogenous leptin (241). Although evolutionarily conserving energy

during periods of reduced WAT was likely beneficial to our ancestors, in our modern environment this is undesirable when sustained weight loss is desired.

A majority of obese individuals are not insulin resistant (64% in ref (73)) and likely do not have many metabolic abnormalities. Whether metabolic abnormalities were associated with the increase in WAT following 173 hours of inactivity in unknown. Several measurements indicated maintenance of metabolic health during 173 hours of inactivity. For instance, after 173 hours of inactivity no differences in 5 hour fasting blood glucose, insulin, or free fatty acids were found (Chapter 2, Chapter 3 and (172, 173)). An additional experiment to test this hypothesis was insulin tolerance tests on WL5, WL173, and SED animals. This experiment sought to expand a finding that following just 53 hours of inactivity there was reduced glucose uptake into isolated skeletal muscle following a sub-maximal dose of insulin (162). However, no differences in whole body insulin sensitivity were found (Appendix Figure A-1). These contradictory results were likely due to 1) a supramaximal intraperitoneal injection of insulin during the insulin tolerance test, and/or 2) increases in glucose uptake by WAT that compensates for decrease uptake in skeletal muscle. Changes in GLUT4 content would help support hypothesis number 2. While, no change in GLUT4 concentration was found, the increase in WAT mass would result in an increase in the total amount of GLUT4 content in WAT (Appendix Figure A-2).

Another major determinate of metabolic health is the size of the adipocytes present. Initially, I hypothesized that the increase in WAT mass during inactivity would occur through adipocyte hypertrophy and not hyperplasia. Adipocyte hypertrophy would also be accompanied by an increase in infiltrating macrophages. Contrary to this

hypothesis WAT mass increased via adipocyte hyperplasia and not hypertrophy. Thus, the finding that infiltrating macrophages did not differ on either a low-fat or high-fat diet existed was consistent with previous literature (Appendix Figure A-3). In summary, in rats, the increases in WAT following 173 hours of reduced activity are physiological responses that may initially help maintain metabolic homeostasis and possibly improved reproductive success rather than pathological responses associated with metabolic abnormalities.

One of the surprising findings in chapter 2 was the observation that adipocyte hyperplasia was occurring within 173 hours of the cessation of voluntary wheel running. Following up this observation was the measurement of the protein levels of key transcription factors (PPARγ, C/EBPα, and C/EBPβ) involved in adipogenesis. However, none of them differed between groups (Appendix B-1). Potential explanations include 1) protein increases that are very transient, 2) to few cells were differentiating to significantly change protein expression in the entire adipose depot, and/or 3) changes in the functionality of the protein (cytosolic versus nuclear, increased DNA binding) without a change in protein concentration. Consistent with my findings and hypothesis 3, voluntary wheel running increases DNA binding activity of PPARγ, without increasing total protein in WAT (220). Thus, measurements of transcriptional activity of key adipogenic proteins during inactivity would be prudent in feature experiments.

FATTY ACID OXIDATION IN ADIPOSE TISSUE OF HEALTHY RATS

I hypothesized that decreases in FAO in skeletal muscle, liver, and adipocytes would be associated with the increase in WAT during inactivity. Although the pattern of FAO in skeletal muscle was similar to that hypothesis, in both liver and adipocytes the

pattern differed. FAO in isolated adipocytes was elevated at both WL5 and WL173 relative to sedentary animals (Figure 3-3). In addition to being essential for adipogenesis the transcription factor PPARγ helps coordinate upregulation of the genes involved in FAO. For example, in WAT giving a PPARγ agonist, such as a TZD, increases FAO in primary isolated adipocytes (60, 171). Again, the increase in FAO at the WL173 might be due to an increase in the DNA binding activity of PPARγ (171, 220). If PPARγ DNA binding activity were increased the transcription of genes involved in mitochondrial biogenesis would be expected to increase. However, no such increase in markers of mitochondrial content in the WAT with voluntary wheel running occurred (chapter 3, table 3-3) (172).

An alternative mechanism is that the increase in FAO observed at WL5 and WL173 is mediated by increased leptin sensitivity in adipocytes. Leptin can increase FAO through the activation and phosphorylation of 5' adenosine monophosphate-activated kinase (AMPK). Leptin sensitivity is indirectly measured multiple ways. One such measurement is the phosphorylation of downstream targets in response to a leptin stimulation, such as phosphorylation of AMPK or signal transducer and activator of transcription 3 (STAT3). More physiological measurement of leptin sensitivity includes the circulating leptin to body fat ratio, a reduction of food intake following a leptin injection, or increased FAO in response to leptin injection. Leptin resistance can occur following high-fat feeing in the central nervous system and peripheral tissues, while leptin sensitivity is enhances following exercise training (248, 276). For instance high-fat feeing reduces skeletal muscle FAO in response to leptin, but treadmill training prevents this reduction. Furthermore, lower circulating leptin to body weight ratios are present in

voluntary wheel running mice on either a high or low-fat diet (Appendix C-1). In response to an acute bout of swimming a subsequent leptin injection enhances hypothalamic STAT3 phosphorylation and increases the food reduction, suggesting increased leptin sensitivity. Lastly, circulating leptin to body fat ratio is maintained following a 173 hours of wheel lock indicating maintenance of leptin sensitivity during 7 days of inactivity. Thus, in summary the enhanced leptin sensitivity due to voluntary wheel running that is maintained following 173 hours of inactivity may partially explain the increased FAO in isolated adipocytes.

Increased signaling may occur through increased downstream signaling or increased receptor content. Leptin signals though the gp130 cytokine receptor family (285). Although six leptin receptor (OB-R) isoforms exist, it is believed that only two (Ob-Rb and Ob-Ra) are capable of intracellular signaling, which is thought to occur by tyrosine kinase induced phosphorylation and activation of various intracellular pathways (285). However, no differences in receptor isoforms Ob-Rb, Ob-Ra, and the soluble leptin receptor Ob-Re at the mRNA level existed between any groups (Appendix C-2). However, the full length receptor was significantly higher at the protein level in whole epididymal WAT of the SED versus WL5 and WL173 groups (Appendix C-3). Interestingly, this observation does not suggest that increased leptin receptor content is responsible for the increased FAO in WL5 and WL173 groups relative to the SED group. Alternative explanations are that the signaling occurs in different receptor isoforms and is highly specific and sensitive in regulating AMPK and/or FAO. Also receptor level inhibition by suppressor of cytokine signaling-3 or protein tyrosine phosphatase-1B (PTP1B) may be elevated in the SED rats compared to the WL5 and WL173 groups (311,

344). Interestingly, PTP1B is significantly elevated following 53 hours of wheel lock compared to the WL5 group in skeletal muscle (162). Thus, a similar change in PTP1B may account the discrepancies in leptin receptor content and FAO in WAT of my animals.

In a tissue that is designed to primarily store fatty acids the role of FAO in adipocytes has been understudied and is also not well understood. In adipocytes it is estimated only 0.2% of endogenous fatty acids are oxidized, while 50.1% are released and 49.7% are re-esterified in low-fat *ad libitum* fed rats (312). However, several studies suggest an increase in FAO can be coupled with increases in fatty acid release and fatty acid re-esterification, resulting in a futile cycle of fatty acid release and storage, with the net result of ATP consumption (4, 141). Additionally, PPARγ agonist treatment increasing esterification and storage of circulating fatty acids in subcutaneous WAT, and increases FAO in visceral WAT (171). Although PPARγ agonist can double the FAO, whether such a small absolute increase in FAO is directly involved in the prevention of weight gain or metabolic dysfunction within adipocytes remains unknown. Several studies have shown that increases in adipocyte FAO are associated with improved metabolic health of the adipocyte, although more mechanistic studies are needed (4, 60, 171).

To test the effects of modifying only FAO in adipocytes several approaches could be taken. One approach would be knocking down or overexpressing genes that are only involved in FAO, such as Cpt1 or fatty acid binding proteins, while simultaneously measuring metabolic processes such as lipogenesis, lipolysis, and/or esterification of radiolabeled fatty acids. This approach would allow insight into the cellular processes

that adipocyte FAO is required for. Another approach is to inhibit FAO with the addition of malonyl CoA, however this would also indirectly increase lipogenesis which itself increases energy utilization (139).

MITOCHONDRIAL CONTENT IN ADIPOSE TISSUE DURING THE PROGRESSION OF DIABETES

The young sedentary wild type rats used in chapters 2 and 3 are still metabolically healthy, insulin sensitive, and not obese. In chapter 4, a well-characterized rat model of obesity, the OLETF rat, was used to examine changes in markers of mitochondrial concentration in WAT during the progression of diabetes and with a physical activity intervention. Interestingly, at the younger ages (13, 20 wks) markers of mitochondrial concentration only differed slightly between groups (Chapter 4, Figure 4-3). At these ages the sedentary OLETF rats had abnormal glucose homeostasis, but the OLETF-RUN and LETO-SED did not. Not until the OLETF-SED animals were fully diabetic at 40 wks of age did mitochondrial content differ between them and the OLETF-RUN and LETO-SED groups. Surprisingly, this difference was not because of a decrease in mitochondrial concentration in the OLETF-SED group, but due to an *increase* in the OLETF-RUN and LETO-SED groups. However, voluntary wheel running in wild type rats does not increase adipose mitochondrial concentration (172). Thus, in the OLETFs it seems that voluntary wheel running allows mitochondrial content to increase by a similar mechanism as occurs with natural aging in wild type sedentary LETO rats.

The surprising increase in omental mitochondrial concentration of the LETO-SED/OLETF-RUN rather than a decrease in OLETF-SED was unexpected. Previous studies have interpreted the lower mitochondrial content between obese/diabetic and lean mice as a result from a decrease in mitochondrial content of the obese and/or diabetic

mice (60, 239, 260). However, my observations suggest that the age at which animals are examined seems critical, and multiple ages are needed to determine the directional change in WAT mitochondria concentration. An example of the usefulness of using multiple ages is a study by Sutherland et al (284). This group demonstrated a decrease in WAT mitochondrial following 6, but not 4 or 2 weeks of high fat feeding. Interestingly the decrease in WAT did not occur until after reductions in whole-body glucose disposal in response to a glucose tolerance test occurred, suggesting that decreases in WAT mitochondria are not casual. Indeed, my observations also agree that differences in WAT mitochondria could not be causal in insulin resistance in the OLETF animals.

While it seems clear that WAT mitochondria are not casual in diabetes, the increase with maturation is puzzling. A potential explanation is that WAT, like skeletal muscle, may increase mitochondrial content in response to insulin signaling, which is reduced in type 2 diabetics (281). In support of this explanation there is an increase in circulating insulin at 40 weeks of age (Table 4-2) that might be sufficient to increase mitochondria in healthy animals like the OLETF-RUN and LETO-SED groups, but not unhealthy ones like the OLETF-SED. Insulin stimulation of adipocytes also increases GLUT4 expression and translocation to the plasma membrane and total GLUT4 content in adipocytes is positively associated with whole-body insulin sensitivity (2, 259). Caloric restriction like voluntary wheel running increases whole body insulin sensitivity and prevents diabetes in OLETF rats (213). Furthermore, caloric restriction significantly increases WAT GLUT4 versus *ad libitum* fed OLETF rats, without changing in skeletal muscle and liver GLUT4 (213). Importantly, GLUT4 expression is positively associated with mitochondrial content in WAT of mice, going up when mitochondrial content is

increased and down when decreased (227). Insulin also increases PPARγ expression, with in turn can increase mitochondrial proteins in differentiating insulin sensitive adipocytes (37, 60, 171, 227, 331). Together these observations lead to speculation that the increase in omental mitochondrial content is related to the maintenance of insulin sensitivity in the OLETF-RUN and LETO-SED groups at 40 weeks of age. An alternative explanation for the increase in mitochondrial concentration is that an increase in the proportion of mature adipocytes relative to SFV cell number is occurring. Mature adipocytes contain more mitochondria than preadipocytes (331). Thus although no change in individual adipocyte mitochondrial content may occur, a shift in cell population could lead to an increase in mitochondrial concentration of the entire adipose depot. Isolation of mature primary adipocytes could be used to test this hypothesis.

The role of mitochondria in adipose tissue is not well understood. While it has long been recognized the mitochondria are necessary for generating acetyl-CoA used for fatty acid synthesis, the interactions between lipolysis, re-esterification, lipogenesis, and WAT mitochondrial content are only partially understood (110, 214, 270). One way to gain insight into this question is partial blockage of ATP production in isolated adipocytes with concurrent measures of re-esterification of endogenous and exogenous fatty acids, lipolysis, and lipogenesis. Decreasing mitochondrial content by ~20% adipocytes through knockdown of mitochondrial transcription factor A, a transcription factor important in mitochondrial biogenesis, is an additional approach for studying the physiological consequences of reduced mitochondria in adipocytes (260).

ALTERNATIVE APPROACHES TO DETECTING PHYSICAL ACTIVITY-INDUCED DIFFERENCES IN DNA METHYLATION

Recently, the association between CpG methylation in the promoter of specific genes and the progression of chronic diseases has been recognized as a potentially important interaction (277, 317). While increases in physical activity can prevent or delay the progression of these chronic diseases, a connection between increased levels of physical activity and changes in CpG methylation has not yet been established. I compared DNA methylation data with mRNA expression in skeletal muscle of physically active and sedentary mice. In addition, using pyrosequencing and COBRA analysis I was unable to verify DNA methylation differences between groups for the *Pitx3* gene that were seen initially on the microarray. There are likely several reasons why methylation in my model is unlikely to be detected and/or unlikely to occur.

In comparison to the cell types where changes in CpG methylation are most commonly found (epithelial, endothelial cells) skeletal muscle has a generally slow turnover. Once *de novo* methylation of DNA has occurred the methylation is passed on to daughter cells during meiosis (234). Thus, cell types that proliferate more rapidly following *de novo* methylation will increase DNA methylation of the entire cell population to a greater extent than cell types that proliferate more slowly or are terminally differentiated. While epithelial and endothelial cells have the potential to proliferate, skeletal myocytes are terminal differentiated and unable to further divide. Thus, *de novo* methylation is not passed on to any daughter cells in skeletal myocytes. However, CpG methylation could be propagated in skeletal muscle if *de novo* methylation in satellite cells occurs. Increases satellite cell proliferation and subsequent incorporation into skeletal muscle myocytes occur during the regrowth and recovery of

skeletal muscle following damage or an injury (140). Thus, only if *de novo* methylation of satellite cell DNA and subsequent proliferation and recruitment of satellite cells occurs can DNA methylation increase in myocytes. However, muscle damage and activate satellite cells occurs in response to a single acute bout of voluntary wheel running; but repeated bouts of running attenuate the activation of satellite cells (245, 294). Thus, it is possible that following a 10-week voluntary wheel running intervention (as I have used) skeletal muscle turnover and satellite cell recruitment is too low for major differences in CpG methylation to appear throughout the muscle.

Skeletal muscle consists of many different cell types including endothelial, fibroblasts, and vascular smooth muscle cells. The different cells type respond to increased physical activity differently (152). Furthermore, different cell types contain differences in methylation. Thus, using whole skeletal muscle, the mRNA and DNA contained nucleotides from many different cell types. A consequence of having multiple cell types is that any differences in DNA methylation or mRNA expression that occurred in specific cell types may have been diluted. To minimize this dilution effect in future studies isolating single muscle fibers or arterioles for DNA and mRNA extraction could help (335). Similarly isolation of endothelial cells, fibroblasts, or smooth muscle cells through cell culture techniques or flow cytometry could be done (62, 132, 338). An alternative approach could be to use a tissue such as liver, or WAT might be more likely to show differences in DNA methylation due to an increased turnover in response to voluntary wheel running

Another difficulty in studying CpG methylation is that the rate that differences in methylation arise is relatively slow, requiring many years in humans (165) or multiple

passages in cell culture (18). For example only 8-10% of two distinct subject populations showed a change after 11 or 16 years in global methylation of greater than 20%, while a 5% change was seen in 40-63% of the subjects (32). Interestingly, in addition to a slow rate this study (32) and others (39) find a wide between-subject variability in DNA methylation. This variability between subjects would make it difficult to use cross sectional studies or make between group comparisons to detect differences in DNA methylation. A better design to detect differences in DNA methylation would be a longitudinal study where multiple measurements were taken over a long period of time in the same individual. Furthermore, the high inter-individual variability could lead to a large number of false-positives on a microarray when a relatively small number of pooled samples are used, which may help explain my observations thus far. Several alternative approaches could be used. First, in skeletal muscle treatment of the limb on one side of the body that allows use of the contra-lateral non-treated limb to act as a control within the same individual eliminates the difficulty of high inter-individual variability. Second, repeated measurements, using circulating white blood cells or muscle biopsies, that can be taken throughout an intervention reduces inter-individual comparisons, rendering the population variability less important.

A different exercise and/or experimental design could also be employed in the future to maximize the possibility of determining an exercise-induced difference in DNA methylation. If voluntary wheel running remains the exercise modality of choice, extending the duration of the experiment from 10 weeks to 1-2 years, when mice are middle to old aged, might provide enough time for differences in DNA methylation in skeletal muscle to arise (46). Alternatively, a more specific and robust exercise

intervention might be used to increase skeletal muscle adaptation. One such experimental tool is electrical stimulation results in dramatic phenotypic changes that can be compared between the stimulated and the non-stimulated leg (179, 334). Lastly, a cell culture design that mimics exercise by pulsing either electrical stimulation (197), or caffeine into the media (205) may allow for more genetic manipulation, cell specific adaptations, and mechanistic studies, although it is less physiologically relevant.

Although, my initial experiments have yet to definitively provide evidence of differentially methylated genes with exercise important lessons have been learned with regards to the design of future experiments. Future experimental designs should consider the following. First, I would continue to use voluntary wheel running, but use an intervention period of a minimum of 18 months. Second, I would isolate single fibers and skeletal muscle arterioles for all my measurements. Third, I would make blood draws every 3-4 months and isolate white blood cell DNA for within individual comparisons. Finally, I would continue to collect multiple tissues (liver, WAT, colon, hypothalamus, heart, vessels) from sacrificed mice and use both MeDIP and bisulfite treatment methods to detect DNA methylation differences. I believe this approach is more likely to detect exercise-induced differences in DNA methylation.

RELEVANCE TO HUMAN HEALTH

An often overlooked aspect of the studies conducted in chapters 2-4 is that all of the animals used began voluntary wheel running at a young age (3-4 weeks of age). These young adults are thus always active, never undergoing periods of inactivity, until their wheels are locked or they are sacrificed. For instance, young rats (4 week old) undergoing 3 weeks of wheel running was sufficient to prevent obesity after an additional

10 weeks of high-fat diet (215). However, only 2 weeks of voluntary wheel running could not prevent obesity, suggesting a minimum amount of increased physical activity exists to alter long-term energy balance (215). Additionally, long term alteration of metabolic phenotype in skeletal muscle of humans following the cessation of endurance training has also been reported. Adult endurance athletes (30 years of age) who had trained on average for 10 years, maintained elevated capillarity, and oxidation enzyme concentration after cessation of training for 84, suggesting a phenotype "memory" (67).

Also important is whether beginning an exercise program as an adult will be as beneficial as when started in adolescence. Current dogma suggests adult adipocyte number is fixed (272). Thus, if excess adipocytes are obtained an adolescent, they would remain as adult. Of course weight loss would be possible, but evidence suggests only through a reduction in adipocyte cell size and not a reduction in cell adipocyte number. Small adipocytes can activate compensatory mechanisms to increase energy intake and prevent further weight loss. Thus, it is critical to maintain a "normal" weight to prevent increased adult adipocyte cell number. These examples along with the data presented in chapters 2-4) suggest that starting and maintaining an exercise (or physical activity) program throughout adolescence may be beneficial. However, future studies examining the mechanisms and extent of such benefits are clearly needed. It is intriguing to speculate that the long-term maintenance of adolescence phenotype may be mediated by DNA methylation since methylation is relatively stable and maintained.

CONCLUSIONS

Although the importance of WAT in whole body metabolic homeostasis has been a major focus in the past decade the role of physical activity in regulating DNA

methylation changes during aging and chronic disease development is in it is infancy. This document summarizes the contributions I have made to each of these fields. Still, studies on the specific physiological role of WAT mitochondria in both healthy and disease states are needed. Similarly, future studies focusing on how environmental perturbations, such as physical activity, interact with our epigenomes are critical to understanding the importance of epigenetics and chronic diseases.

Appendix

A – METABOLIC HEALTH OF ADIPOCYTES IN RESPONSE TO INACTIVITY

METHODS

Insulin tolerance tests were done as described in figure legend A-1. Briefly animals were locally anesthetized with topical lidocaine and restrained while a caudal catheter was placed. An initial blood draw was taken for baseline glucose and animals were injected with 0.75 U/kg of body weight of insulin. 200-400 µL of blood was drawn every 10 minutes. Dose of insulin was based on Thule et al (289).

Western blots were performed as done as described in chapter 4. Briefly, adipose tissue was homogenized in fat homogenization buffer (50 mM Tris-HCl, 225 mM sucrose, 1 mM EDTA·Na_4, pH 7.4) with protease and phosphotase inhibitors (Sigma, MO). Protein concentrations determined by Bradford Assay (BioRad). Equal amounts of protein in sample buffer (4X: 62.5 mM Tris, pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.05% bromophenol blue) are loaded on an SDS-Page gel in running buffer (25mM Tris, 192 mM glycine, pH 8.3, 0.1% SDS) at 150V for 70-90 minutes. Transfer of proteins to nitrocellulose was done either overnight at 30V or for 1 hour at 100V in 25 mM Tris, 192 mM Glycine, 20% Methanol, pH 9.3). Equal loading of protein is verified by ponceau staining. Primary antibodies were used at a concentration of 1:200-1000 and secondary at 1:5000-50000 (Pierce) and diluted in Tris-Buffer-Saline with 0.1% tween (TBS-T) and 5% BSA (for primary antibody) or non-fat milk (for secondary antibody). All washes (5 X 5 minutes) are done with TBS-T. Enhanced

chemiluminescent was used as instructed by the manufacturer (Pierce) and visualized with a Kodak Imaging system.

The animals used to looks at macrophage infiltration are the same as those used in Chapter 2. Briefly, Fischer brown Norway 344 rats were obtained at 21 days of age (Harlan, IN). They were given access to running wheels for 6 weeks, after which wheels were locked for 5, 53, or 173 hours. In second group of rats underwent the same experimental design except they were fed a high fat diet (35% fat by calories) throughout the experimental. A piece of WAT was places in formalin and embedded in paraffin. Slides of WAT were hydrated in progressively higher ethanol concentration, followed by an antigen retrieval step at 90°C for 5 minutes. Excess hydrogen peroxide was quenched by immersion in 3% fresh hydrogen peroxide. Application of an Avidin/Biotin block for 15 minutes, protein block for 10 minutes, and overnight incubation with an anti-ED1 (rat specific macrophage marker, Serotec) at 4°C were performed with washes in TBS-T in between each step. The next morning application of the secondary antibody, label (streptavindin), and color reagent (DAB-Chromogen) was applied with washes in TBS-T in between each step. Once color reached the appropriate intensity (10 minutes) slides were rinsed in ddH2O, and briefly counter stained with hematoxylin and eosin. All reagents were purchased from DAKO (LSAB + Kit, K0679). Image J Pro was used to quantify the number of ED1 positive nuclei, by first identifying the number of nuclei that co-stained for ED1 and hematoxylin and then determining the total number of nuclei in the field of view.

RESULTS

No differences in the insulin tolerance tests (Figure A-1), GLUT4 protein (Figure A-2), or percent ED1 positive nuclei (Figure A-3) were found. For more information please see the Discussion (Chapter 6).

Figure A-1

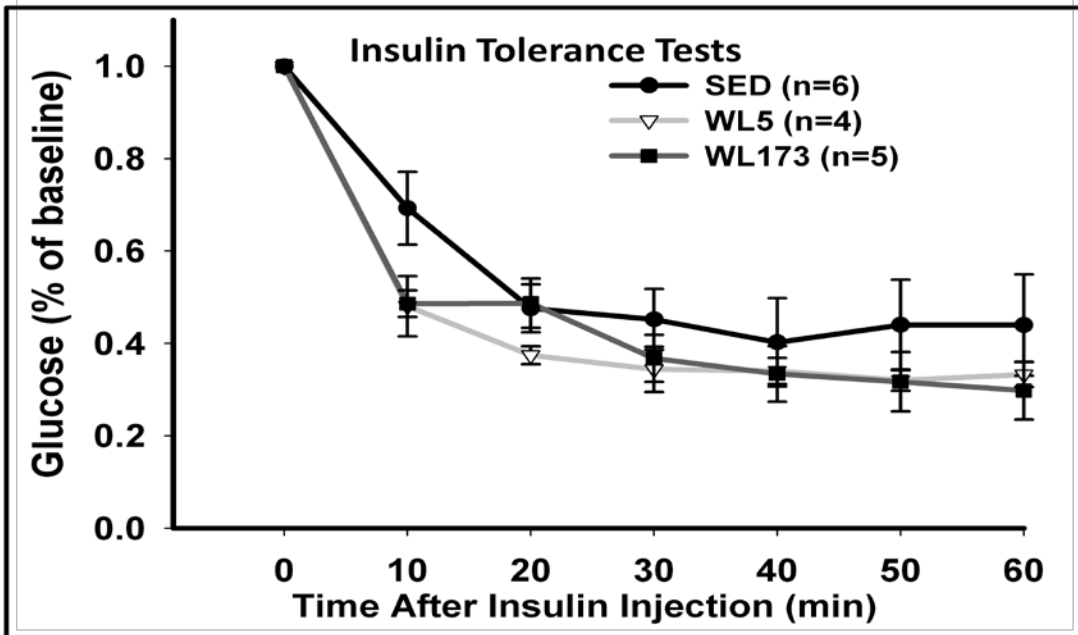


Figure A-1: Following a 5 hour fast rats received an intraperitoneal injection of 0.75 U/kg of body weight of insulin. Blood was drawn through a catheter in the caudal artery and glucose concentration measured with a Beckman glucose analyzer. The decrease in blood glucose is expressed as the percentage of starting value, mean ± SEM.

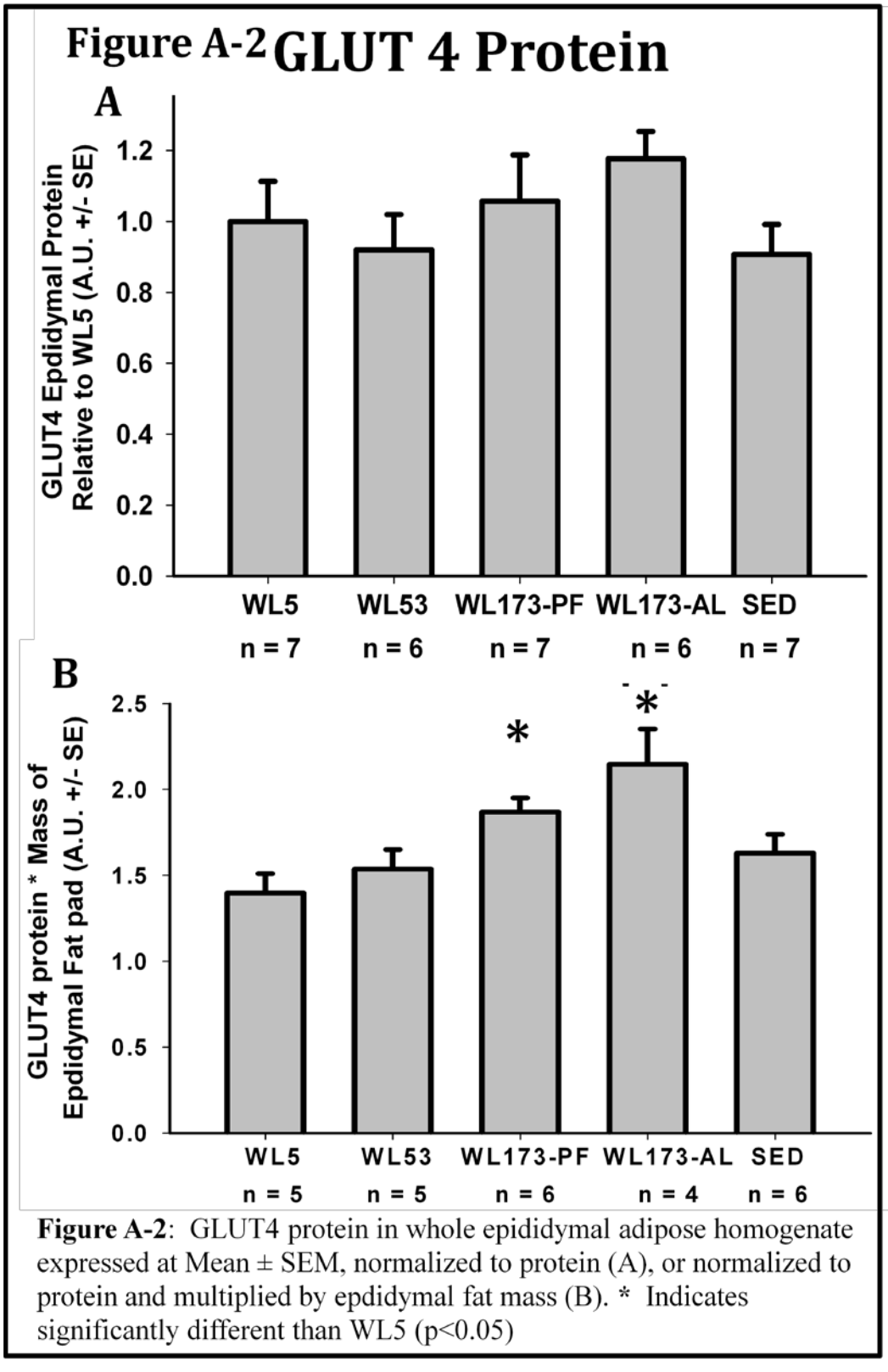


Figure A-2: GLUT4 protein in whole epididymal adipose homogenate expressed at Mean ± SEM, normalized to protein (A), or normalized to protein and multiplied by epdidymal fat mass (B). * Indicates significantly different than WL5 ($p<0.05$)

Figure A-3

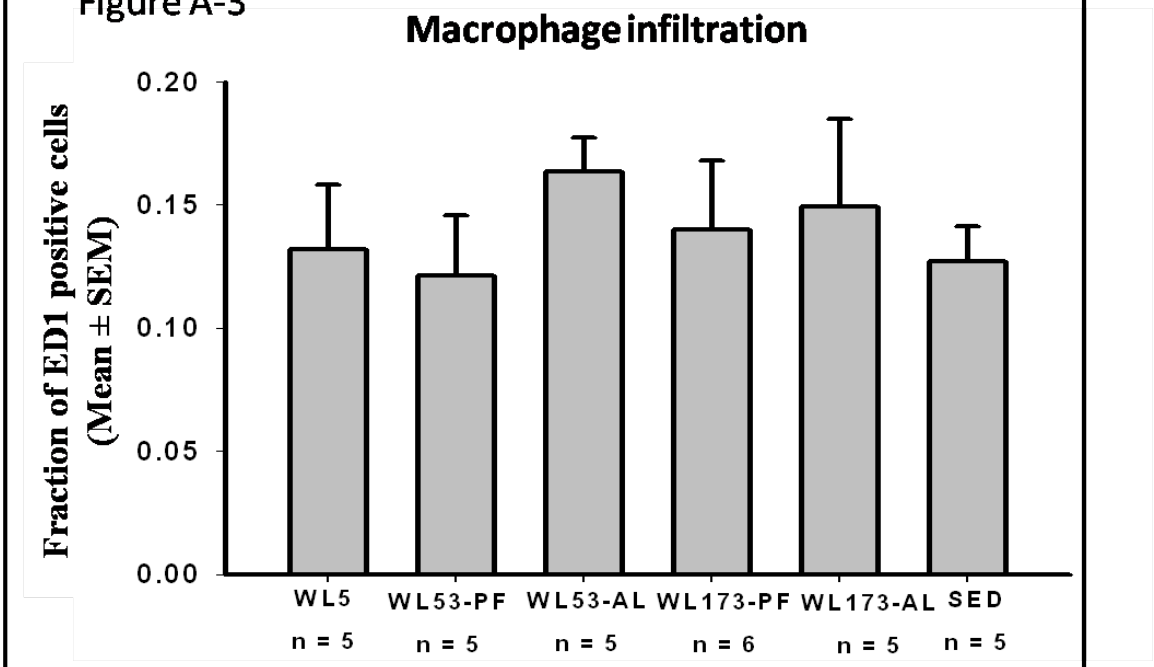


Figure A-3: No significant differences in the percent of ED1 positive cell versus total cells in sections of epdidymal adipose tissue existed.

B – CONCENTRATIONS OF ADIPOGENIC PROTEINS FOLLOWING ADIPOCYTE HYPERPLASIA

METHODS

See chapter 2 for a description of the animals used. Western blots were performed as done as described in chapter 4 and appendix A. Anti-PPARγ (Cell Signaling), anti- C/EBPα (Affinity BioReagents), and anti-C/EBPβ (Santa Cruz) were all used at 1:1000 concentrations.

RESULTS

No significant differences in protein concentration of PPARγ, C/EBPα, and C/EBPβ in whole WAT or PPARγ in stromal vascular fraction was determined. Additionally, C/EBPα was detected only in whole WAT and not the stromal vascular fraction. For more information see the Chapter 6 (Discussion).

Figure B-1

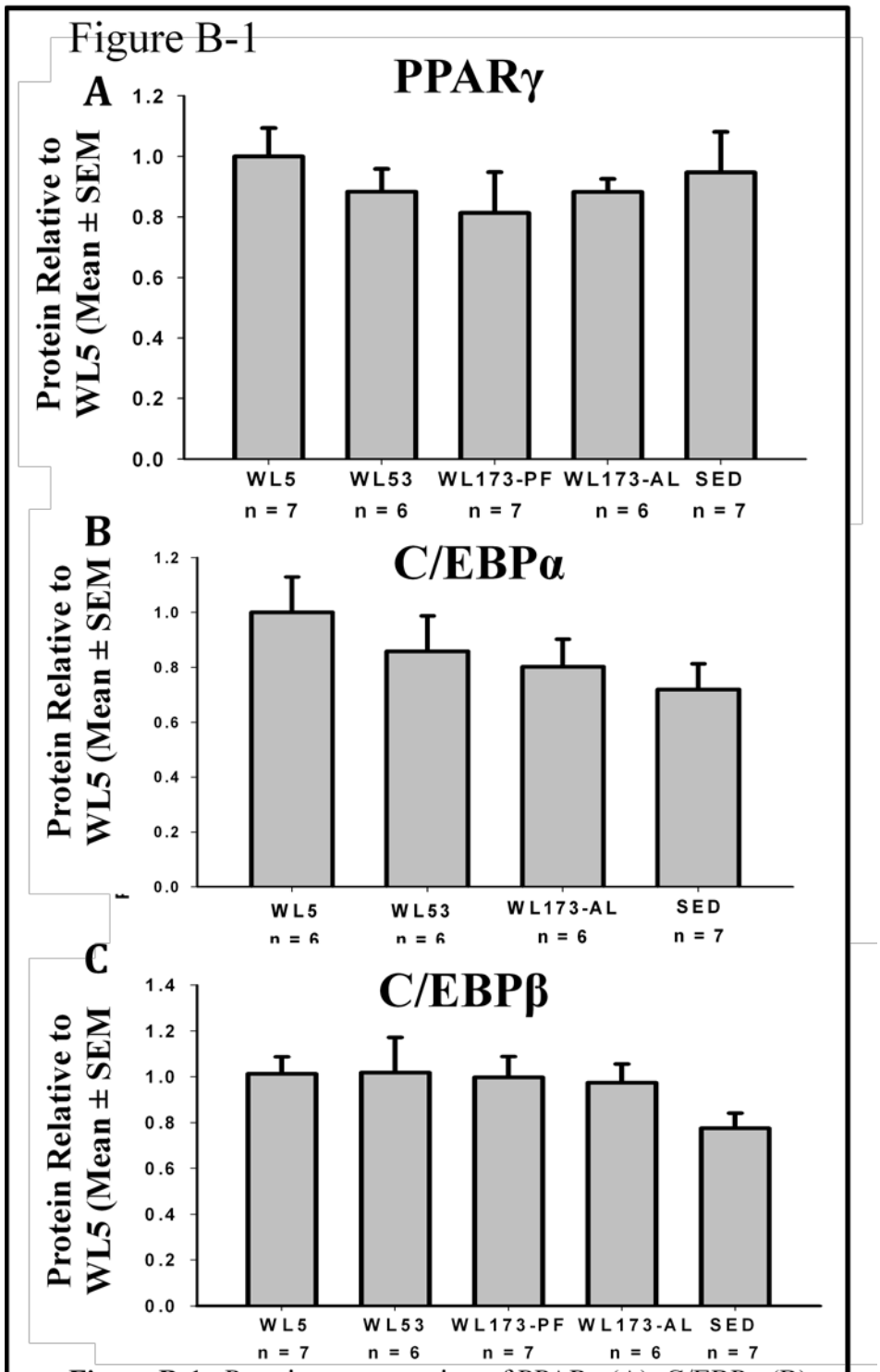
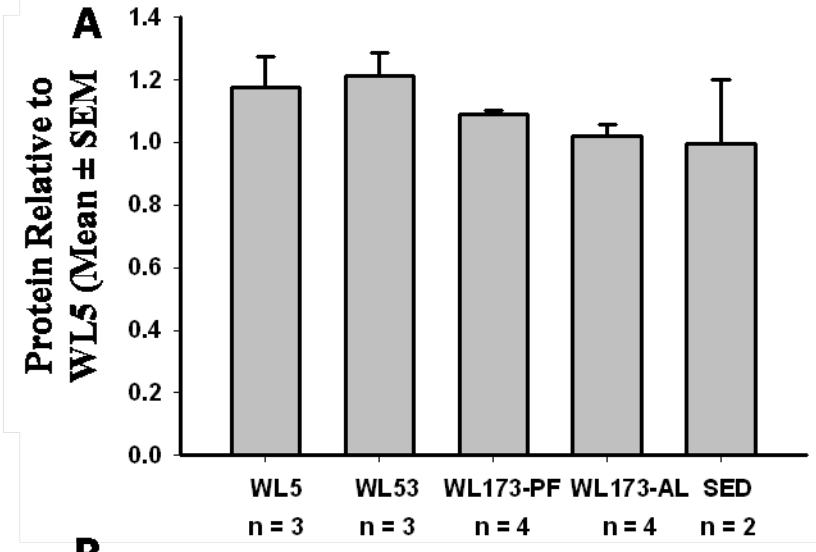


Figure B-1. Protein concentration of PPARγ (A), C/EBPα (B), and C/EBPβ (C) did not differ between any groups.

Figure B-2



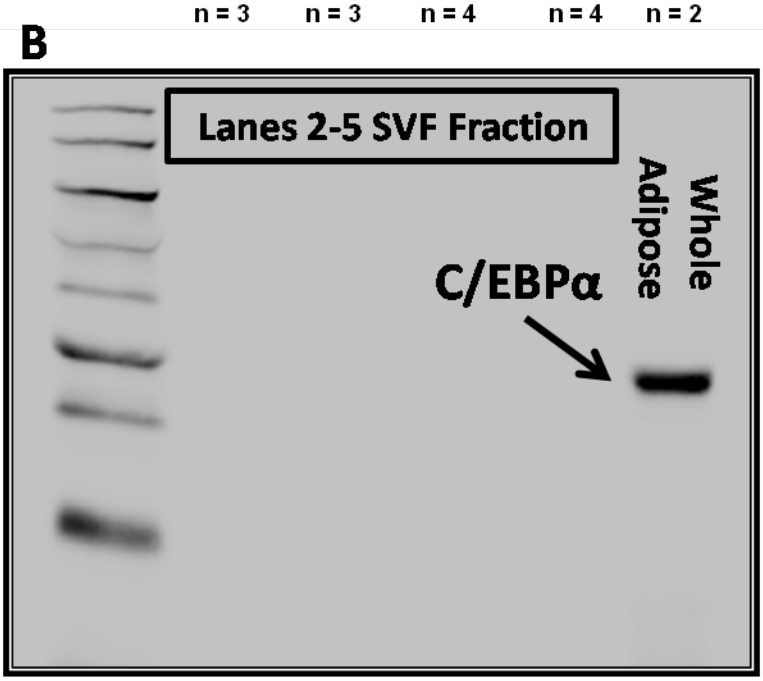


Figure B-2: SVF PPARγ protein expression did not differ between groups (A) and C/EBPα expression was absent (B) from the SVF fraction (lanes 2-5) as expected. Whole adipose includes as positive control.

C – LEPTIN AND WHEEL RUNNING

METHODS

The cohort of animals used is the same as in chapter 2. Serum leptin was measured with ELISA kit (Linco, MO). A standard curve was generated $r^2 = 0.99$, and samples were run in duplicate and with a CV < 10% .

mRNA was isolated, primers designed, and RT-PCR performed as described elsewhere (Chapters 3 and 5) on the isoforms of the leptin receptors; OB-Rb, OB-Re, and OB-Ra.

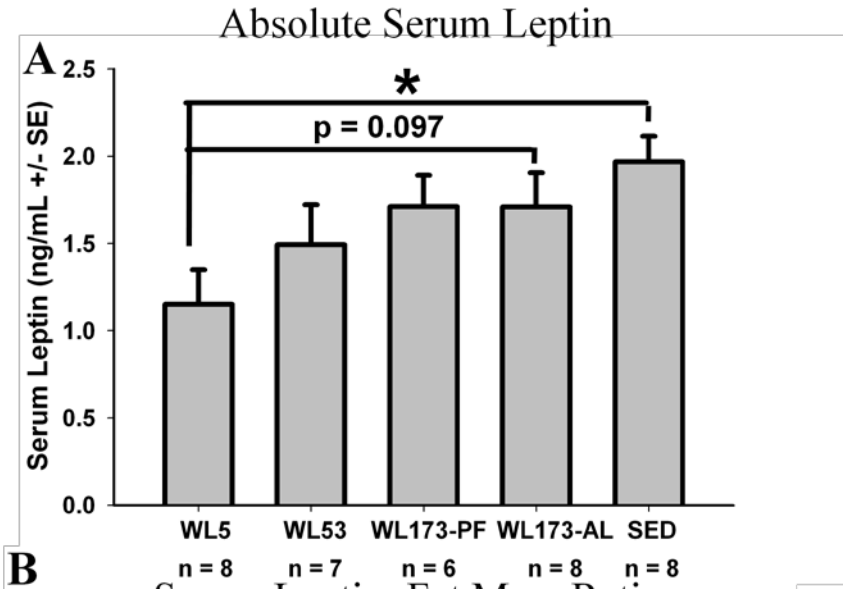
Immunobloting for the full length leptin receptor (Santa Cruz Antibodies, CA) was performed as described elsewhere (Appendix A). The molecular weight of the quantified band was 120 kDa, as expected.

RESULTS

Absolute serum leptin significantly decreased at WL5 compared to the SED group and following 173 hours of inactivity in the *ad libitum* fed group, and trended to in the pair-fed group (Figure C-1A). When corrected for epididymal WAT mass, the SED group has a significantly higher ratio than all other groups (Figure C-1b).

However, these changes in serum leptin where not associated with any changes in the mRNA of the long form of the leptin receptor (OB-Rb), short form (OB-Ra), or the secreted form (OB-Re) (Figure C-2). Conversely, although no change in mRNA in OB-Rb was detected a large increase in the protein levels of the receptor in the epididymal adipose depot of the SED group compared to both the WL5 and WL173-AL group (Figure C-3). This suggests a post translational regulation difference between the groups.

Figure C-1



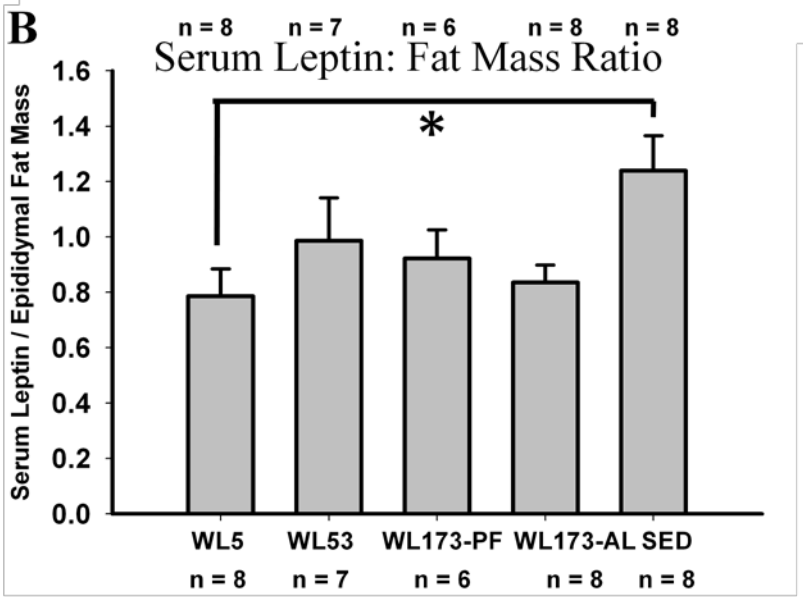


Figure C-1: Serum leptin both expressed as absolute values (A), and normalized to epididymal fat pad mass (B). * indicates significance from groups between two lines. Means ± SEM

Figure C-2

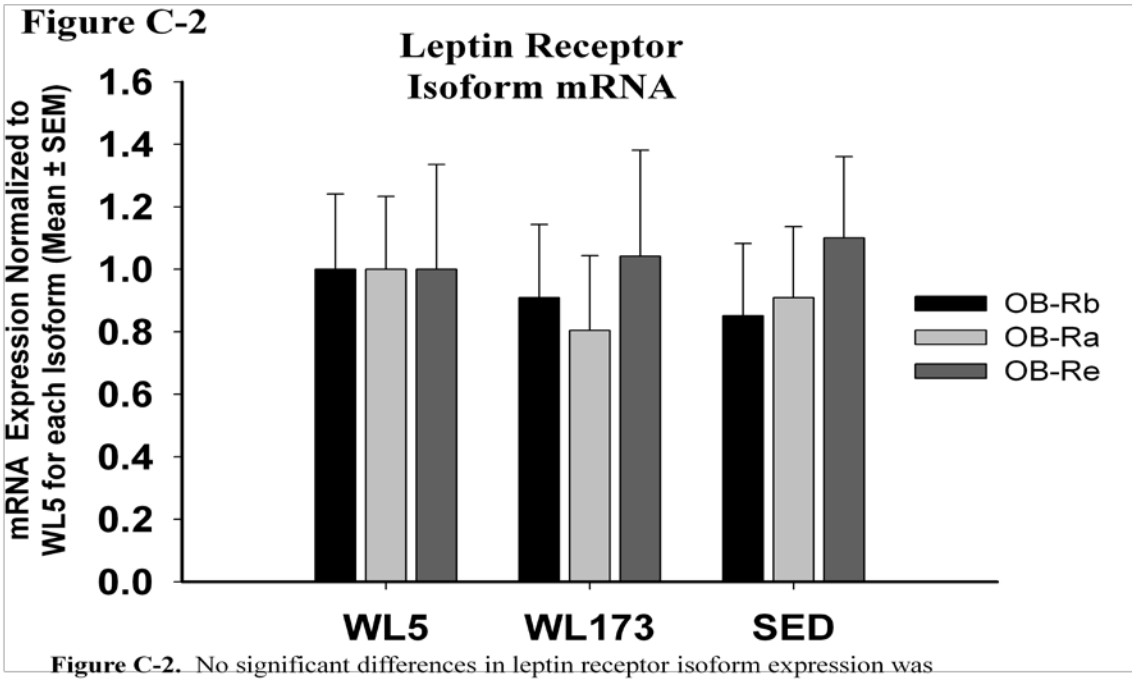


Figure C-2. No significant differences in leptin receptor isoform expression was found. Value for each isoform are normalized to WL5 and expressed as Mean ± SEM, n=7-8/group.

Figure C-3

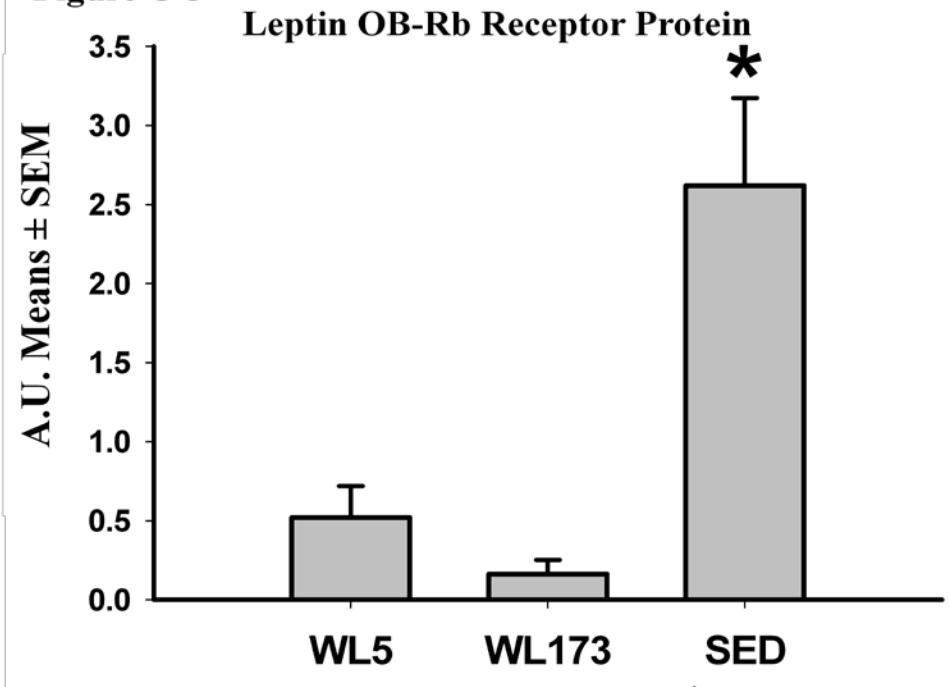


Figure C-3. Protein leptin of full length leptin receptor OB-Rb. * indicates significantly different than all groups. n=4-6/group, data expressed as Mean ± SEM

References

1. Prevalence of overweight and obesity among adults with diagnosed diabetes--United States, 1988-1994 and 1999-2002. *MMWR Morb Mortal Wkly Rep* 53: 1066-1068, 2004.
2. **Abel ED, Peroni O, Kim JK, Kim YB, Boss O, Hadro E, Minnemann T, Shulman GI, and Kahn BB**. Adipose-selective targeting of the GLUT4 gene impairs insulin action in muscle and liver. *Nature* 409: 729-733, 2001.
3. **Adams M, Montague CT, Prins JB, Holder JC, Smith SA, Sanders L, Digby JE, Sewter CP, Lazar MA, Chatterjee VK, and O'Rahilly S**. Activators of peroxisome proliferator-activated receptor gamma have depot-specific effects on human preadipocyte differentiation. *J Clin Invest* 100: 3149-3153, 1997.
4. **Ahmadian M, Duncan RE, Varady KA, Frasson D, Hellerstein MK, Birkenfeld AL, Samuel VT, Shulman G, Wang Y, Kang C, and Sul HS**. Adipose Overexpression of Desnutrin Promotes Fatty Acid Utilization and Attenuates Diet-induced Obesity. *Diabetes* 2009.
5. **Ahuja N, Li Q, Mohan AL, Baylin SB, and Issa JP**. Aging and DNA methylation in colorectal mucosa and cancer. *Cancer Res* 58: 5489-5494, 1998.
6. **Alberti G, Zimmet P, Shaw J, Bloomgarden Z, Kaufman F, and Silink M**. Type 2 diabetes in the young: the evolving epidemic: the international diabetes federation consensus workshop. *Diabetes Care* 27: 1798-1811, 2004.
7. **Alexander HG, and Dugdale AE**. Fascial planes within subcutaneous fat in humans. *Eur J Clin Nutr* 46: 903-906, 1992.
8. **Alexandraki K, Piperi C, Kalofoutis C, Singh J, Alaveras A, and Kalofoutis A**. Inflammatory process in type 2 diabetes: The role of cytokines. *Ann N Y Acad Sci* 1084: 89-117, 2006.
9. **Almeras N, Lemieux S, Bouchard C, and Tremblay A**. Fat gain in female swimmers. *Physiol Behav* 61: 811-817, 1997.
10. **Anghel SI, Bedu E, Vivier CD, Descombes P, Desvergne B, and Wahli W**. Adipose tissue integrity as a prerequisite for systemic energy balance: a critical role for peroxisome proliferator-activated receptor gamma. *J Biol Chem* 282: 29946-29957, 2007.
11. **Applegate EA, and Stern JS**. Exercise termination effects on food intake, plasma insulin, and adipose lipoprotein lipase activity in the Osborne-Mendel rat. *Metabolism* 36: 709-714, 1987.
12. **Applegate EA, Upton DE, and Stern JS**. Exercise and detraining: effect on food intake, adiposity and lipogenesis in Osborne-Mendel rats made obese by a high fat diet. *J Nutr* 114: 447-459, 1984.
13. **Arner P**. Differences in lipolysis between human subcutaneous and omental adipose tissues. *Ann Med* 27: 435-438, 1995.
14. **Arner P, and Langin D**. The role of neutral lipases in human adipose tissue lipolysis. *Curr Opin Lipidol* 18: 246-250, 2007.
15. **Asmann YW, Stump CS, Short KR, Coenen-Schimke JM, Guo Z, Bigelow ML, and Nair KS**. Skeletal muscle mitochondrial functions, mitochondrial DNA copy numbers, and gene transcript profiles in type 2 diabetic and nondiabetic subjects at equal levels of low or high insulin and euglycemia. *Diabetes* 55: 3309-3319, 2006.
16. **Astrup A**. Healthy lifestyles in Europe: prevention of obesity and type II diabetes by diet and physical activity. *Public HealthNutr* 4: 499-515, 2001.
17. **Baar K, Wende AR, Jones TE, Marison M, Nolte LA, Chen M, Kelly DP, and Holloszy JO**. Adaptations of skeletal muscle to exercise: rapid increase in the transcriptional coactivator PGC-1. *FASEB J* 16: 1879-1886, 2002.

18. **Bachman KE, Park BH, Rhee I, Rajagopalan H, Herman JG, Baylin SB, Kinzler KW, and Vogelstein B**. Histone modifications and silencing prior to DNA methylation of a tumor suppressor gene. *Cancer Cell* 3: 89-95, 2003.
19. **Ballard FJ, and Hanson RW**. The citrate cleavage pathway and lipogenesis in rat adipose tissue: replenishment of oxaloacetate. *J Lipid Res* 8: 73-79, 1967.
20. **Bandyopadhyay GK, Yu JG, Ofrecio J, and Olefsky JM**. Increased Malonyl-CoA Levels in Muscle From Obese and Type 2 Diabetic Subjects Lead to Decreased Fatty Acid Oxidation and Increased Lipogenesis; Thiazolidinedione Treatment Reverses These Defects. *Diabetes* 55: 2277-2285, 2006.
21. **Bass A, Brdiczka D, Eyer P, Hofer S, and Pette D**. Metabolic differentiation of distinct muscle types at the level of enzymatic organization. *EurJ Biochem* 10: 198-206, 1969.
22. **Baylin SB, Esteller M, Rountree MR, Bachman KE, Schuebel K, and Herman JG**. Aberrant patterns of DNA methylation, chromatin formation and gene expression in cancer. *Hum Mol Genet* 10: 687-692, 2001.
23. **Bays H, Mandarino L, and DeFronzo RA**. Role of the adipocyte, free fatty acids, and ectopic fat in pathogenesis of type 2 diabetes mellitus: peroxisomal proliferator-activated receptor agonists provide a rational therapeutic approach. *J Clin Endocrinol Metab* 89: 463-478, 2004.
24. **Belinsky SA, Nikula KJ, Palmisano WA, Michels R, Saccomanno G, Gabrielson E, Baylin SB, and Herman JG**. Aberrant methylation of p16(INK4a) is an early event in lung cancer and a potential biomarker for early diagnosis. *Proc Natl Acad Sci U S A* 95: 11891-11896, 1998.
25. **Bell AC, and Felsenfeld G**. Methylation of a CTCF-dependent boundary controls imprinted expression of the Igf2 gene. *Nature* 405: 482-485, 2000.
26. **Benjamini Y, and Hochberg Y**. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society Series B (Methodological)* 57: 289-300, 1995.
27. **Berg AH, and Scherer PE**. Adipose tissue, inflammation, and cardiovascular disease. *Circ Res* 96: 939-949, 2005.
28. **Bernstein BE, Meissner A, and Lander ES**. The mammalian epigenome. *Cell* 128: 669-681, 2007.
29. **Berry MF, Pirolli TJ, Jayasankar V, Burdick J, Morine KJ, Gardner TJ, and Woo YJ**. Apelin has in vivo inotropic effects on normal and failing hearts. *Circulation* 110: II187-193, 2004.
30. **Bird A**. DNA methylation patterns and epigenetic memory. *Genes Dev* 16: 6-21, 2002.
31. **Bird AP, and Wolffe AP**. Methylation-induced repression--belts, braces, and chromatin. *Cell* 99: 451-454, 1999.
32. **Bjornsson HT, Sigurdsson MI, Fallin MD, Irizarry RA, Aspelund T, Cui H, Yu W, Rongione MA, Ekstrom TJ, Harris TB, Launer LJ, Eiriksdottir G, Leppert MF, Sapienza C, Gudnason V, and Feinberg AP**. Intra-individual change over time in DNA methylation with familial clustering. *JAMA* 299: 2877-2883, 2008.
33. **Blaak EE, van Aggel-Leijssen DP, Wagenmakers AJ, Saris WH, and van Baak MA**. Impaired oxidation of plasma-derived fatty acids in type 2 diabetic subjects during moderate-intensity exercise. *Diabetes* 49: 2102-2107, 2000.
34. **Blackburn GL, and Waltman BA**. Pharmacotherapy to reduce visceral fat. *Clin Cornerstone* 7: 52-60, 2005.
35. **Blanco D, Vicent S, Fraga MF, Fernandez-Garcia I, Freire J, Lujambio A, Esteller M, Ortiz-de-Solorzano C, Pio R, Lecanda F, and Montuenga LM**. Molecular analysis of a multistep lung cancer model induced by chronic inflammation reveals epigenetic regulation of p16 and activation of the DNA damage response pathway. *Neoplasia* 9: 840-852, 2007.

36. **Boden G, Duan X, Homko C, Molina EJ, Song W, Perez O, Cheung P, and Merali S**. Increase in endoplasmic reticulum stress-related proteins and genes in adipose tissue of obese, insulin-resistant individuals. *Diabetes* 57: 2438-2444, 2008.
37. **Bogacka I, Ukropcova B, McNeil M, Gimble JM, and Smith SR**. Structural and functional consequences of mitochondrial biogenesis in human adipocytes in vitro. *J Clin Endocrinol Metab* 90: 6650-6656, 2005.
38. **Bogacka I, Xie H, Bray GA, and Smith SR**. Pioglitazone induces mitochondrial biogenesis in human subcutaneous adipose tissue in vivo. *Diabetes* 54: 1392-1399, 2005.
39. **Bollati V, Schwartz J, Wright R, Litonjua A, Tarantini L, Suh H, Sparrow D, Vokonas P, and Baccarelli A**. Decline in genomic DNA methylation through aging in a cohort of elderly subjects. *Mechanisms of Ageing and Development* 130: 234-239, 2009.
40. **Bonnard C, Durand A, Peyrol S, Chanseaume E, Chauvin MA, Morio B, Vidal H, and Rieusset J**. Mitochondrial dysfunction results from oxidative stress in the skeletal muscle of diet-induced insulin-resistant mice. *J Clin Invest* 118: 789-800, 2008.
41. **Booth FW**. Effect of limb immobilization on skeletal muscle. *J Appl Physiol* 52: 1113-1118, 1982.
42. **Borg P, Kukkonen-Harjula K, Fogelholm M, and Pasanen M**. Effects of walking or resistance training on weight loss maintenance in obese, middle-aged men: a randomized trial. *Int J Obes Relat Metab Disord* 26: 676-683, 2002.
43. **Bota DA, Van Remmen H, and Davies KJ**. Modulation of Lon protease activity and aconitase turnover during aging and oxidative stress. *FEBS Lett* 532: 103-106, 2002.
44. **Boucher J, Masri B, Daviaud D, Gesta S, Guigne C, Mazzucotelli A, Castan-Laurell I, Tack I, Knibiehler B, Carpene C, Audigier Y, Saulnier-Blache JS, and Valet P**. Apelin, a newly identified adipokine up-regulated by insulin and obesity. *Endocrinology* 146: 1764-1771, 2005.
45. **Bouloumie A, Sengenes C, Portolan G, Galitzky J, and Lafontan M**. Adipocyte produces matrix metalloproteinases 2 and 9: involvement in adipose differentiation. *Diabetes* 50: 2080-2086, 2001.
46. **Boveris A, and Navarro A**. Systemic and mitochondrial adaptive responses to moderate exercise in rodents. *Free Radic Biol Med* 44: 224-229, 2008.
47. **Bruun JM, Lihn AS, Pedersen SB, and Richelsen B**. Monocyte chemoattractant protein-1 release is higher in visceral than subcutaneous human adipose tissue (AT): implication of macrophages resident in the AT. *J Clin Endocrinol Metab* 90: 2282-2289, 2005.
48. **Cancello R, Henegar C, Viguerie N, Taleb S, Poitou C, Rouault C, Coupaye M, Pelloux V, Hugol D, Bouillot JL, Bouloumie A, Barbatelli G, Cinti S, Svensson PA, Barsh GS, Zucker JD, Basdevant A, Langin D, and Clement K**. Reduction of macrophage infiltration and chemoattractant gene expression changes in white adipose tissue of morbidly obese subjects after surgery-induced weight loss. *Diabetes* 54: 2277-2286, 2005.
49. **Cao Y**. Angiogenesis modulates adipogenesis and obesity. *J Clin Invest* 117: 2362-2368, 2007.
50. **Cartwright A**. *Determination of adipose tissue cellularity*. New YorkL Nostrand Reinhold, 1987.
51. **Carvalho E, Kotani K, Peroni OD, and Kahn BB**. Adipose-specific overexpression of GLUT4 reverses insulin resistance and diabetes in mice lacking GLUT4 selectively in muscle. *Am J Physiol Endocrinol Metab* 289: E551-561, 2005.
52. **Casillas MA, Jr., Lopatina N, Andrews LG, and Tollefsbol TO**. Transcriptional control of the DNA methyltransferases is altered in aging and neoplastically-transformed human fibroblasts. *Mol Cell Biochem* 252: 33-43, 2003.
53. **Catenacci VA, and Wyatt HR**. The role of physical activity in producing and maintaining weight loss. *Nat Clin Pract Endocrinol Metab* 3: 518-529, 2007.

54. **Cettour-Rose P, Samec S, Russell AP, Summermatter S, Mainieri D, Carrillo-Theander C, Montani JP, Seydoux J, Rohner-Jeanrenaud F, and Dulloo AG**. Redistribution of glucose from skeletal muscle to adipose tissue during catch-up fat: a link between catch-up growth and later metabolic syndrome. *Diabetes* 54: 751-756, 2005.
55. **Chakravarthy MV, and Booth FW**. Eating, exercise, and "thrifty" genotypes: connecting the dots toward an evolutionary understanding of modern chronic diseases. *J Appl Physiol* 96: 3-10, 2004.
56. **Chan JL, and Mantzoros CS**. Role of leptin in energy-deprivation states: normal human physiology and clinical implications for hypothalamic amenorrhoea and anorexia nervosa. *Lancet* 366: 74-85, 2005.
57. **Chan MY, Zhao Y, and Heng CK**. Sequential responses to high-fat and high-calorie feeding in an obese mouse model. *Obesity (Silver Spring)* 16: 972-978, 2008.
58. **Chatterjee TK, Stoll LL, Denning GM, Harrelson A, Blomkalns AL, Idelman G, Rothenberg FG, Neltner B, Romig-Martin SA, Dickson EW, Rudich S, and Weintraub NL**. Proinflammatory phenotype of perivascular adipocytes: influence of high-fat feeding. *Circ Res* 104: 541-549, 2009.
59. **Chatzipanteli K, Rudolph S, and Axelrod L**. Coordinate control of lipolysis by prostaglandin E2 and prostacyclin in rat adipose tissue. *Diabetes* 41: 927-935, 1992.
60. **Choo HJ, Kim JH, Kwon OB, Lee CS, Mun JY, Han SS, Yoon YS, Yoon G, Choi KM, and Ko YG**. Mitochondria are impaired in the adipocytes of type 2 diabetic mice. *Diabetologia* 49: 784-791, 2006.
61. **Chun TH, Hotary KB, Sabeh F, Saltiel AR, Allen ED, and Weiss SJ**. A pericellular collagenase directs the 3-dimensional development of white adipose tissue. *Cell* 125: 577-591, 2006.
62. **Churchman AT, and Siow RC**. Isolation, culture and characterisation of vascular smooth muscle cells. *Methods Mol Biol* 467: 127-138, 2009.
63. **Clarkson RW, Chen CM, Harrison S, Wells C, Muscat GE, and Waters MJ**. Early responses of trans-activating factors to growth hormone in preadipocytes: differential regulation of CCAAT enhancer-binding protein-beta (C/EBP beta) and C/EBP delta. *Mol Endocrinol* 9: 108-120, 1995.
64. **Collins F**. In: *House Subcommittees on Labor-HHS Education Appropiations*. Washington, DC: 2006.
65. **Cordain L, Gotshall RW, Eaton SB, and Eaton SB, 3rd**. Physical activity, energy expenditure and fitness: an evolutionary perspective. *Int J Sports Med* 19: 328-335, 1998.
66. **Coulon V, L'Honore A, Ouimette JF, Dumontier E, van den Munckhof P, and Drouin J**. A muscle-specific promoter directs Pitx3 gene expression in skeletal muscle cells. *J Biol Chem* 282: 33192-33200, 2007.
67. **Coyle EF, Martin WH, 3rd, Sinacore DR, Joyner MJ, Hagberg JM, and Holloszy JO**. Time course of loss of adaptations after stopping prolonged intense endurance training. *J Appl Physiol* 57: 1857-1864, 1984.
68. **Craig BW, Garthwaite SM, and Holloszy JO**. Adipocyte insulin resistance: effects of aging, obesity, exercise, and food restriction. *J Appl Physiol* 62: 95-100, 1987.
69. **Craig BW, Thompson K, and Holloszy JO**. Effects of stopping training on size and response to insulin of fat cells in female rats. *J Appl Physiol* 54: 571-575, 1983.
70. **Cui L, Hofer T, Rani A, Leeuwenburgh C, and Foster TC**. Comparison of lifelong and late life exercise on oxidative stress in the cerebellum. *Neurobiol Aging* 2007.
71. **Dahlman I, Forsgren M, Sjogren A, Nordstrom EA, Kaaman M, Naslund E, Attersand A, and Arner P**. Downregulation of electron transport chain genes in visceral adipose tissue in type 2 diabetes independent of obesity and possibly involving tumor necrosis factor-alpha. *Diabetes* 55: 1792-1799, 2006.

72. **De Bono JP, Adlam D, Paterson DJ, and Channon KM**. Novel quantitative phenotypes of exercise training in mouse models. *Am J Physiol Regul Integr Comp Physiol* 290: R926-934, 2006.
73. **Despres JP**. Is visceral obesity the cause of the metabolic syndrome? *Ann Med* 38: 52-63, 2006.
74. **Detmer SA, and Chan DC**. Functions and dysfunctions of mitochondrial dynamics. *Nat Rev Mol Cell Biol* 8: 870-879, 2007.
75. **Dietrich D, Lesche R, Tetzner R, Krispin M, Dietrich J, Haedicke W, Schuster M, and Kristiansen G**. Analysis of DNA Methylation of Multiple Genes in Microdissected Cells From Formalin-fixed and Paraffin-embedded Tissues. *J Histochem Cytochem* 2009.
76. **Dohm GL, Barakat HA, Tapscott EB, and Beecher GR**. Changes in body fat and lipogenic enzyme activities in rats after termination of exercise training. *Proc Soc Exp Biol Med* 155: 157-159, 1977.
77. **Dohm GL, Huston RL, Askew EW, and Weiser PC**. Effects of exercise on activity of heart and muscle mitochondria. *Am J Physiol* 223: 783-787, 1972.
78. **Dresner A, Laurent D, Marcucci M, Griffin ME, Dufour S, Cline GW, Slezak LA, Andersen DK, Hundal RS, Rothman DL, Petersen KF, and Shulman GI**. Effects of free fatty acids on glucose transport and IRS-1-associated phosphatidylinositol 3-kinase activity. *J Clin Invest* 103: 253-259, 1999.
79. **Dube JJ, Amati F, Stefanovic-Racic M, Toledo FG, Sauers SE, and Goodpaster BH**. Exercise-induced alterations in intramyocellular lipids and insulin resistance: the athlete's paradox revisited. *Am J Physiol Endocrinol Metab* 294: E882-888, 2008.
80. **Dubois SG, Heilbronn LK, Smith SR, Albu JB, Kelley DE, and Ravussin E**. Decreased expression of adipogenic genes in obese subjects with type 2 diabetes. *Obesity (Silver Spring)* 14: 1543-1552, 2006.
81. **Dulloo AG**. Regulation of fat storage via suppressed thermogenesis: a thrifty phenotype that predisposes individuals with catch-up growth to insulin resistance and obesity. *Horm Res* 65 Suppl 3: 90-97, 2006.
82. **Eaton RP, Berman M, and Steinberg D**. Kinetic studies of plasma free fatty acid and triglyceride metabolism in man. *J Clin Invest* 48: 1560-1579, 1969.
83. **Fain JN, Buehrer B, Bahouth SW, Tichansky DS, and Madan AK**. Comparison of messenger RNA distribution for 60 proteins in fat cells vs the nonfat cells of human omental adipose tissue. *Metabolism* 57: 1005-1015, 2008.
84. **Fazzari MJ, and Greally JM**. Epigenomics: beyond CpG islands. *Nat Rev Genet* 5: 446-455, 2004.
85. **Fitts RH, Booth FW, Winder WW, and Holloszy JO**. Skeletal muscle respiratory capacity, endurance, and glycogen utilization. *Am J Physiol* 228: 1029-1033, 1975.
86. **Fontana L, Eagon JC, Trujillo ME, Scherer PE, and Klein S**. Visceral fat adipokine secretion is associated with systemic inflammation in obese humans. *Diabetes* 56: 1010-1013, 2007.
87. **Foster SA, Wong DJ, Barrett MT, and Galloway DA**. Inactivation of p16 in human mammary epithelial cells by CpG island methylation. *Mol Cell Biol* 18: 1793-1801, 1998.
88. **Fraga MF, Ballestar E, Paz MF, Ropero S, Setien F, Ballestar ML, Heine-Suner D, Cigudosa JC, Urioste M, Benitez J, Boix-Chornet M, Sanchez-Aguilera A, Ling C, Carlsson E, Poulsen P, Vaag A, Stephan Z, Spector TD, Wu YZ, Plass C, and Esteller M**. Epigenetic differences arise during the lifetime of monozygotic twins. *Proc Natl Acad Sci U S A* 102: 10604-10609, 2005.
89. **Frank S, Gaume B, Bergmann-Leitner ES, Leitner WW, Robert EG, Catez F, Smith CL, and Youle RJ**. The role of dynamin-related protein 1, a mediator of mitochondrial fission, in apoptosis. *Dev Cell* 1: 515-525, 2001.

90. **Fried SK, Bunkin DA, and Greenberg AS**. Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: depot difference and regulation by glucocorticoid. *J Clin Endocrinol Metab* 83: 847-850, 1998.
91. **Fukuhara A, Matsuda M, Nishizawa M, Segawa K, Tanaka M, Kishimoto K, Matsuki Y, Murakami M, Ichisaka T, Murakami H, Watanabe E, Takagi T, Akiyoshi M, Ohtsubo T, Kihara S, Yamashita S, Makishima M, Funahashi T, Yamanaka S, Hiramatsu R, Matsuzawa Y, and Shimomura I**. Visfatin: a protein secreted by visceral fat that mimics the effects of insulin. *Science* 307: 426-430, 2005.
92. **Galitzky J, Sengenes C, Thalamas C, Marques MA, Senard JM, Lafontan M, and Berlan M**. The lipid-mobilizing effect of atrial natriuretic peptide is unrelated to sympathetic nervous system activation or obesity in young men. *J Lipid Res* 42: 536-544, 2001.
93. **Garaulet M, Hernandez-Morante JJ, Lujan J, Tebar FJ, and Zamora S**. Relationship between fat cell size and number and fatty acid composition in adipose tissue from different fat depots in overweight/obese humans. *Int J Obes (Lond)* 30: 899-905, 2006.
94. **Gardiner-Garden M, and Frommer M**. CpG islands in vertebrate genomes. *J Mol Biol* 196: 261-282, 1987.
95. **Garrick D, Fiering S, Martin DI, and Whitelaw E**. Repeat-induced gene silencing in mammals. *Nat Genet* 18: 56-59, 1998.
96. **Gasic S, Tian B, and Green A**. Tumor necrosis factor alpha stimulates lipolysis in adipocytes by decreasing Gi protein concentrations. *J Biol Chem* 274: 6770-6775, 1999.
97. **Gaudet F, Hodgson JG, Eden A, Jackson-Grusby L, Dausman J, Gray JW, Leonhardt H, and Jaenisch R**. Induction of tumors in mice by genomic hypomethylation. *Science* 300: 489-492, 2003.
98. **Gauthier MS, Couturier K, Charbonneau A, and Lavoie JM**. Effects of introducing physical training in the course of a 16-week high-fat diet regimen on hepatic steatosis, adipose tissue fat accumulation, and plasma lipid profile. *Int J Obes Relat Metab Disord* 28: 1064-1071, 2004.
99. **Giada F, Vigna GB, Vitale E, Baldo-Enzi G, Bertaglia M, Crecca R, and Fellin R**. Effect of age on the response of blood lipids, body composition, and aerobic power to physical conditioning and deconditioning. *Metabolism* 44: 161-165, 1995.
100. **Girard J, Perdereau D, Foufelle F, Prip-Buus C, and Ferre P**. Regulation of lipogenic enzyme gene expression by nutrients and hormones. *FASEB J* 8: 36-42, 1994.
101. **Goodpaster BH, Thaete FL, and Kelley DE**. Thigh adipose tissue distribution is associated with insulin resistance in obesity and in type 2 diabetes mellitus. *Am J Clin Nutr* 71: 885-892, 2000.
102. **Gordon-Larsen P, Hou N, Sidney S, Sternfeld B, Lewis CE, Jacobs DR, Jr., and Popkin BM**. Fifteen-year longitudinal trends in walking patterns and their impact on weight change. *Am J Clin Nutr* 89: 19-26, 2009.
103. **Graff JR, Herman JG, Myohanen S, Baylin SB, and Vertino PM**. Mapping patterns of CpG island methylation in normal and neoplastic cells implicates both upstream and downstream regions in de novo methylation. *J Biol Chem* 272: 22322-22329, 1997.
104. **Graham TE, Yang Q, Bluher M, Hammarstedt A, Ciaraldi TP, Henry RR, Wason CJ, Oberbach A, Jansson PA, Smith U, and Kahn BB**. Retinol-binding protein 4 and insulin resistance in lean, obese, and diabetic subjects. *N Engl J Med* 354: 2552-2563, 2006.
105. **Gravholt CH, Schmitz O, Simonsen L, Bulow J, Christiansen JS, and Moller N**. Effects of a physiological GH pulse on interstitial glycerol in abdominal and femoral adipose tissue. *Am J Physiol* 277: E848-854, 1999.
106. **Greenberg AS, Egan JJ, Wek SA, Garty NB, Blanchette-Mackie EJ, and Londos C**. Perilipin, a major hormonally regulated adipocyte-specific phosphoprotein associated with the periphery of lipid storage droplets. *J Biol Chem* 266: 11341-11346, 1991.

107. **Gregg EW, Cadwell BL, Cheng YJ, Cowie CC, Williams DE, Geiss L, Engelgau MM, and Vinicor F**. Trends in the prevalence and ratio of diagnosed to undiagnosed diabetes according to obesity levels in the U.S. *Diabetes Care* 27: 2806-2812, 2004.
108. **Gregoire FM**. Adipocyte differentiation: from fibroblast to endocrine cell. *Exp Biol Med (Maywood)* 226: 997-1002, 2001.
109. **Gregor MF, Yang L, Fabbrini E, Mohammed BS, Eagon JC, Hotamisligil GS, and Klein S**. Endoplasmic reticulum stress is reduced in tissues of obese subjects after weight loss. *Diabetes* 58: 693-700, 2009.
110. **Halperin ML, and Robinson BH**. The role of the cytoplasmic redox potential in the control of fatty acid synthesis from glucose, pyruvate and lactate in white adipose tissue. *Biochem J* 116: 235-240, 1970.
111. **Hamm JK, el Jack AK, Pilch PF, and Farmer SR**. Role of PPAR gamma in regulating adipocyte differentiation and insulin-responsive glucose uptake. *Ann N Y Acad Sci* 892: 134-145, 1999.
112. **Hamm JK, Park BH, and Farmer SR**. A role for C/EBPbeta in regulating peroxisome proliferator-activated receptor gamma activity during adipogenesis in 3T3-L1 preadipocytes. *J Biol Chem* 276: 18464-18471, 2001.
113. **Hancock CR, Han DH, Chen M, Terada S, Yasuda T, Wright DC, and Holloszy JO**. High-fat diets cause insulin resistance despite an increase in muscle mitochondria. *Proceedings of the National Academy of Sciences* 105: 7815-7820, 2008.
114. **Hark AT, Schoenherr CJ, Katz DJ, Ingram RS, Levorse JM, and Tilghman SM**. CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2 locus. *Nature* 405: 486-489, 2000.
115. **Harman-Boehm I, Bluher M, Redel H, Sion-Vardy N, Ovadia S, Avinoach E, Shai I, Kloting N, Stumvoll M, Bashan N, and Rudich A**. Macrophage infiltration into omental versus subcutaneous fat across different populations: effect of regional adiposity and the comorbidities of obesity. *J Clin Endocrinol Metab* 92: 2240-2247, 2007.
116. **Hartman WM, Stroud M, Sweet DM, and Saxton J**. Long-term maintenance of weight loss following supplemented fasting. *Int J Eat Disord* 14: 87-93, 1993.
117. **Haskell WL, Lee IM, Pate RR, Powell KE, Blair SN, Franklin BA, Macera CA, Heath GW, Thompson PD, and Bauman A**. Physical activity and public health: updated recommendation for adults from the American College of Sports Medicine and the American Heart Association. *Circulation* 116: 1081-1093, 2007.
118. **Hattori N, Abe T, Suzuki M, Matsuyama T, Yoshida S, Li E, and Shiota K**. Preference of DNA methyltransferases for CpG islands in mouse embryonic stem cells. *Genome Res* 14: 1733-1740, 2004.
119. **Hausman DB, Park HJ, and Hausman GJ**. Isolation and culture of preadipocytes from rodent white adipose tissue. *Methods Mol Biol* 456: 201-219, 2008.
120. **He J, Watkins S, and Kelley DE**. Skeletal muscle lipid content and oxidative enzyme activity in relation to muscle fiber type in type 2 diabetes and obesity. *Diabetes* 50: 817-823, 2001.
121. **Hickner RC, Privette J, McIver K, and Barakat H**. Fatty acid oxidation in African-American and Caucasian women during physical activity. *J Appl Physiol* 90: 2319-2324, 2001.
122. **Higashida K, Higuchi M, and Terada S**. Potential role of lipin-1 in exercise-induced mitochondrial biogenesis. *Biochem Biophys Res Commun* 374: 587-591, 2008.
123. **Hirosumi J, Tuncman G, Chang L, Gorgun CZ, Uysal KT, Maeda K, Karin M, and Hotamisligil GS**. A central role for JNK in obesity and insulin resistance. *Nature* 420: 333-336, 2002.
124. **Holliday R**. Epigenetics: a historical overview. *Epigenetics* 1: 76-80, 2006.
125. **Holloszy JO**. Skeletal muscle "mitochondrial deficiency" does not mediate insulin resistance. *Am J Clin Nutr* 89: 463S-466S, 2009.

126. **Houmard JA**. Intramuscular lipid oxidation and obesity. *AJP - Regulatory, Integrative and Comparative Physiology* 294: R1111-R1116, 2008.
127. **Hu FB, Manson JE, Stampfer MJ, Colditz G, Liu S, Solomon CG, and Willett WC**. Diet, lifestyle, and the risk of type 2 diabetes mellitus in women. *N Engl J Med* 345: 790-797, 2001.
128. **Hu G, Lakka TA, Kilpelainen TO, and Tuomilehto J**. Epidemiological studies of exercise in diabetes prevention. *Appl Physiol Nutr Metab* 32: 583-595, 2007.
129. **Hulver MW, Berggren JR, Carper MJ, Miyazaki M, Ntambi JM, Hoffman EP, Thyfault JP, Stevens R, Dohm GL, Houmard JA, and Muoio DM**. Elevated stearoyl-CoA desaturase-1 expression in skeletal muscle contributes to abnormal fatty acid partitioning in obese humans. *Cell Metab* 2: 251-261, 2005.
130. **Hulver MW, Berggren JR, Cortright RN, Dudek RW, Thompson RP, Pories WJ, MacDonald KG, Cline GW, Shulman GI, Dohm GL, and Houmard JA**. Skeletal muscle lipid metabolism with obesity. *Am J Physiol Endocrinol Metab* 284: E741-E747, 2003.
131. **Ibdah JA, Perlegas P, Zhao Y, Angdisen J, Borgerink H, Shadoan MK, Wagner JD, Matern D, Rinaldo P, and Cline JM**. Mice Heterozygous for a Defect in Mitochondrial Trifunctional Protein Develop Hepatic Steatosis and Insulin Resistance. *Gastroenterology* 128: 1381-1390, 2005.
132. **Ieronimakis N, Balasundaram G, and Reyes M**. Direct isolation, culture and transplant of mouse skeletal muscle derived endothelial cells with angiogenic potential. *PLoS ONE* 3: e0001753, 2008.
133. **International Commission on Radiological Protection. Task Group on Reference Man.** *Report of the Task Group on Reference Man : a report*. Oxford ; New York: Pergamon Press, 1975, p. xix, 480 p.
134. **Issa JP, Ottaviano YL, Celano P, Hamilton SR, Davidson NE, and Baylin SB**. Methylation of the oestrogen receptor CpG island links ageing and neoplasia in human colon. *Nat Genet* 7: 536-540, 1994.
135. **Jackman MR, Steig A, Higgins JA, Johnson GC, Fleming-Elder BK, Bessesen DH, and MacLean PS**. Weight regain after sustained weight reduction is accompanied by suppressed oxidation of dietary fat and adipocyte hyperplasia. *Am J Physiol Regul Integr Comp Physiol* 294: R1117-1129, 2008.
136. **Ji H, and Friedman MI**. Reduced hepatocyte fatty acid oxidation in outbred rats prescreened for susceptibility to diet-induced obesity. *Int J Obes (Lond)* 2008.
137. **Jones PA, and Baylin SB**. The epigenomics of cancer. *Cell* 128: 683-692, 2007.
138. **Jurgens HS, Schurmann A, Kluge R, Ortmann S, Klaus S, Joost HG, and Tschop MH**. Hyperphagia, lower body temperature, and reduced running wheel activity precede development of morbid obesity in New Zealand obese mice. *Physiol Genomics* 25: 234-241, 2006.
139. **Kaaman M, Sparks LM, van Harmelen V, Smith SR, Sjolin E, Dahlman I, and Arner P**. Strong association between mitochondrial DNA copy number and lipogenesis in human white adipose tissue. *Diabetologia* 50: 2526-2533, 2007.
140. **Kadi F, Charifi N, Denis C, Lexell J, Andersen JL, Schjerling P, Olsen S, and Kjaer M**. The behaviour of satellite cells in response to exercise: what have we learned from human studies? *Pflugers Arch* 451: 319-327, 2005.
141. **Kalderon B, Mayorek N, Berry E, Zevit N, and Bar-Tana J**. Fatty acid cycling in the fasting rat. *Am J Physiol Endocrinol Metab* 279: E221-227, 2000.
142. **Karpe F, and Frayn KN**. The nicotinic acid receptor--a new mechanism for an old drug. *Lancet* 363: 1892-1894, 2004.
143. **Katic M, Kennedy AR, Leykin I, Norris A, McGettrick A, Gesta S, Russell SJ, Bluher M, Maratos-Flier E, and Kahn CR**. Mitochondrial gene expression and increased

oxidative metabolism: role in increased lifespan of fat-specific insulin receptor knock-out mice. *Aging Cell* 6: 827-839, 2007.
144. **Kawano K, Hirashima T, Mori S, Saitoh Y, Kurosumi M, and Natori T**. Spontaneous long-term hyperglycemic rat with diabetic complications. Otsuka Long-Evans Tokushima Fatty (OLETF) strain. *Diabetes* 41: 1422-1428, 1992.
145. **Kayman S, Bruvold W, and Stern JS**. Maintenance and relapse after weight loss in women: behavioral aspects. *Am J Clin Nutr* 52: 800-807, 1990.
146. **Kelley DE, and Simoneau JA**. Impaired free fatty acid utilization by skeletal muscle in non-insulin-dependent diabetes mellitus. *J Clin Invest* 94: 2349-2356, 1994.
147. **Kim J, Inoue K, Ishii J, Vanti WB, Voronov SV, Murchison E, Hannon G, and Abeliovich A**. A MicroRNA feedback circuit in midbrain dopamine neurons. *Science* 317: 1220-1224, 2007.
148. **Kim J, Kim JY, Song KS, Lee YH, Seo JS, Jelinek J, Goldschmidt-Clermont PJ, and Issa JP**. Epigenetic changes in estrogen receptor beta gene in atherosclerotic cardiovascular tissues and in-vitro vascular senescence. *Biochim Biophys Acta* 1772: 72-80, 2007.
149. **Kim JY, Hickner RC, Cortright RL, Dohm GL, and Houmard JA**. Lipid oxidation is reduced in obese human skeletal muscle. *AJP - Endocrinology and Metabolism* 279: E1039-E1044, 2000.
150. **Kim JY, van de Wall E, Laplante M, Azzara A, Trujillo ME, Hofmann SM, Schraw T, Durand JL, Li H, Li G, Jelicks LA, Mehler MF, Hui DY, Deshaies Y, Shulman GI, Schwartz GJ, and Scherer PE**. Obesity-associated improvements in metabolic profile through expansion of adipose tissue. *J Clin Invest* 117: 2621-2637, 2007.
151. **Kim KH, Song MJ, Chung J, Park H, and Kim JB**. Hypoxia inhibits adipocyte differentiation in a HDAC-independent manner. *Biochem Biophys Res Commun* 333: 1178-1184, 2005.
152. **Kivela R, Silvennoinen M, Lehti M, Jalava S, Vihko V, and Kainulainen H**. Exercise-induced expression of angiogenic growth factors in skeletal muscle and in capillaries of healthy and diabetic mice. *Cardiovasc Diabetol* 7: 13, 2008.
153. **Klem ML, Wing RR, McGuire MT, Seagle HM, and Hill JO**. A descriptive study of individuals successful at long-term maintenance of substantial weight loss. *Am J Clin Nutr* 66: 239-246, 1997.
154. **Kloting N, Graham TE, Berndt J, Kralisch S, Kovacs P, Wason CJ, Fasshauer M, Schon MR, Stumvoll M, Bluher M, and Kahn BB**. Serum retinol-binding protein is more highly expressed in visceral than in subcutaneous adipose tissue and is a marker of intra-abdominal fat mass. *Cell Metab* 6: 79-87, 2007.
155. **Knott AB, Perkins G, Schwarzenbacher R, and Bossy-Wetzel E**. Mitochondrial fragmentation in neurodegeneration. *Nat Rev Neurosci* 9: 505-518, 2008.
156. **Knowler WC, Barrett-Connor E, Fowler SE, Hamman RF, Lachin JM, Walker EA, and Nathan DM**. Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. *N Engl J Med* 346: 393-403, 2002.
157. **Koh EH, Park JY, Park HS, Jeon MJ, Ryu JW, Kim M, Kim SY, Kim MS, Kim SW, Park IS, Youn JH, and Lee KU**. Essential role of mitochondrial function in adiponectin synthesis in adipocytes. *Diabetes* 56: 2973-2981, 2007.
158. **Kouzarides T**. Chromatin modifications and their function. *Cell* 128: 693-705, 2007.
159. **Koves TR, Li P, An J, Akimoto T, Slentz D, Ilkayeva O, Dohm GL, Yan Z, Newgard CB, and Muoio DM**. Peroxisome proliferator-activated receptor-gamma co-activator 1alpha-mediated metabolic remodeling of skeletal myocytes mimics exercise training and reverses lipid-induced mitochondrial inefficiency. *J Biol Chem* 280: 33588-33598, 2005.
160. **Koves TR, Noland RC, Bates AL, Henes ST, Muoio DM, and Cortright RN**. Subsarcolemmal and intermyofibrillar mitochondria play distinct roles in regulating skeletal muscle fatty acid metabolism. *Am J Physiol Cell Physiol* 288: C1074-C1082, 2005.

161. **Koves TR, Ussher JR, Noland RC, Slentz D, Mosedale M, Ilkayeva O, Bain J, Stevens R, Dyck JR, Newgard CB, Lopaschuk GD, and Muoio DM**. Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. *Cell Metab* 7: 45-56, 2008.
162. **Kump DS, and Booth FW**. Alterations in insulin receptor signalling in the rat epitrochlearis muscle upon cessation of voluntary exercise. *J Physiol* 562: 829-838, 2005.
163. **Kump DS, and Booth FW**. Sustained rise in triacylglycerol synthesis and increased epididymal fat mass when rats cease voluntary wheel running. *J Physiol* 565: 911-925, 2005.
164. **Kump DS, Laye MJ, and Booth FW**. Increased mitochondrial glycerol-3-phosphate acyltransferase protein and enzyme activity in rat epididymal fat upon cessation of wheel running. *Am J Physiol Endocrinol Metab* 290: E480-489, 2006.
165. **Kwabi-Addo B, Chung W, Shen L, Ittmann M, Wheeler T, Jelinek J, and Issa JP**. Age-related DNA methylation changes in normal human prostate tissues. *Clin Cancer Res* 13: 3796-3802, 2007.
166. **L'Honore A, Coulon V, Marcil A, Lebel M, Lafrance-Vanasse J, Gage P, Camper S, and Drouin J**. Sequential expression and redundancy of Pitx2 and Pitx3 genes during muscle development. *Dev Biol* 307: 421-433, 2007.
167. **Laaksonen DE, Lindstrom J, Lakka TA, Eriksson JG, Niskanen L, Wikstrom K, Aunola S, Keinanen-Kiukaanniemi S, Laakso M, Valle TT, Ilanne-Parikka P, Louheranta A, Hamalainen H, Rastas M, Salminen V, Cepaitis Z, Hakumaki M, Kaikkonen H, Harkonen P, Sundvall J, Tuomilehto J, and Uusitupa M**. Physical activity in the prevention of type 2 diabetes: the Finnish diabetes prevention study. *Diabetes* 54: 158-165, 2005.
168. **Labarca C, and Paigen K**. A simple, rapid, and sensitive DNA assay procedure. *AnalBiochem* 102: 344-352, 1980.
169. **Lacasa D, Taleb S, Keophiphath M, Miranville A, and Clement K**. Macrophage-secreted factors impair human adipogenesis: involvement of proinflammatory state in preadipocytes. *Endocrinology* 148: 868-877, 2007.
170. **Lambert EV, Wooding G, Lambert MI, Koeslag JH, and Noakes TD**. Enhanced adipose tissue lipoprotein lipase activity in detrained rats: independent of changes in food intake. *J Appl Physiol* 77: 2564-2571, 1994.
171. **Laplante M, Festuccia WT, Soucy G, Gelinas Y, Lalonde J, Berger JP, and Deshaies Y**. Mechanisms of the depot specificity of peroxisome proliferator-activated receptor gamma action on adipose tissue metabolism. *Diabetes* 55: 2771-2778, 2006.
172. **Laye MJ, Rector RS, Borengasser SJ, Naples SP, Uptergrove GM, Ibdah JA, Booth FW, and Thyfault JP**. Cessation of daily wheel running differentially alters fat oxidation capacity in liver, muscle, and adipose tissue. *J Appl Physiol* 106: 161-168, 2009.
173. **Laye MJ, Thyfault JP, Stump CS, and Booth FW**. Inactivity induces increases in abdominal fat. *J Appl Physiol* 102: 1341-1347, 2007.
174. **Lee S, Jeong SY, Lim WC, Kim S, Park YY, Sun X, Youle RJ, and Cho H**. Mitochondrial fission and fusion mediators, hFis1 and OPA1, modulate cellular senescence. *J Biol Chem* 282: 22977-22983, 2007.
175. **Levin BE, and Dunn-Meynell AA**. Chronic exercise lowers the defended body weight gain and adiposity in diet-induced obese rats. *Am J Physiol Regul Integr Comp Physiol* 286: R771-778, 2004.
176. **Levine JA, Jensen MD, Eberhardt NL, and O'Brien T**. Adipocyte macrophage colony-stimulating factor is a mediator of adipose tissue growth. *J Clin Invest* 101: 1557-1564, 1998.
177. **Li E, Bestor TH, and Jaenisch R**. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* 69: 915-926, 1992.
178. **Li J, Yu X, Pan W, and Unger RH**. Gene expression profile of rat adipose tissue at the onset of high-fat-diet obesity. *Am J Physiol Endocrinol Metab* 282: E1334-1341, 2002.

179. **Ljubicic V, Adhihetty PJ, and Hood DA**. Application of animal models: chronic electrical stimulation-induced contractile activity. *Can J Appl Physiol* 30: 625-643, 2005.
180. **Louet JF, Hayhurst G, Gonzalez FJ, Girard J, and Decaux JF**. The Coactivator PGC-1 Is Involved in the Regulation of the Liver Carnitine Palmitoyltransferase I Gene Expression by cAMP in Combination with HNF4alpha and cAMP-response Element-binding Protein (CREB). *JBiolChem* 277: 37991-38000, 2002.
181. **Mallon PW, Sedwell R, Rogers G, Nolan D, Unemori P, Hoy J, Samaras K, Kelleher A, Emery S, Cooper DA, and Carr A**. Effect of rosiglitazone on peroxisome proliferator-activated receptor gamma gene expression in human adipose tissue is limited by antiretroviral drug-induced mitochondrial dysfunction. *J Infect Dis* 198: 1794-1803, 2008.
182. **Marcinek DJ, Schenkman KA, Ciesielski WA, Lee D, and Conley KE**. Reduced mitochondrial coupling in vivo alters cellular energetics in aged mouse skeletal muscle. *J Physiol* 569: 467-473, 2005.
183. **Marques BG, Hausman DB, and Martin RJ**. Association of fat cell size and paracrine growth factors in development of hyperplastic obesity. *Am J Physiol* 275: R1898-1908, 1998.
184. **McGarry JD, and Brown NF**. The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. *EurJ Biochem* 244: 1-14, 1997.
185. **McGee SL, van Denderen BJ, Howlett KF, Mollica J, Schertzer JD, Kemp BE, and Hargreaves M**. AMP-activated protein kinase regulates GLUT4 transcription by phosphorylating histone deacetylase 5. *Diabetes* 57: 860-867, 2008.
186. **Menshikova EV, Ritov VB, Fairfull L, Ferrell RE, Kelley DE, and Goodpaster BH**. Effects of exercise on mitochondrial content and function in aging human skeletal muscle. *J Gerontol A Biol Sci Med Sci* 61: 534-540, 2006.
187. **Miller WC, Koceja DM, and Hamilton EJ**. A meta-analysis of the past 25 years of weight loss research using diet, exercise or diet plus exercise intervention. *Int J Obes Relat Metab Disord* 21: 941-947, 1997.
188. **Miyasaka K, Ichikawa M, Kawanami T, Kanai S, Ohta M, Sato N, Ebisawa H, and Funakoshi A**. Physical activity prevented age-related decline in energy metabolism in genetically obese and diabetic rats, but not in control rats. *Mech Ageing Dev* 124: 183-190, 2003.
189. **Mokdad AH, Marks JS, Stroup DF, and Gerberding JL**. Actual causes of death in the United States, 2000. *JAMA* 291: 1238-1245, 2004.
190. **Mokdad AH, Marks JS, Stroup DF, and Gerberding JL**. Correction: actual causes of death in the United States, 2000. *JAMA* 293: 293-294, 2005.
191. **Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, Sihag S, Lehar J, Puigserver P, Carlsson E, Ridderstrale M, Laurila E, Houstis N, Daly MJ, Patterson N, Mesirov JP, Golub TR, Tamayo P, Spiegelman B, Lander ES, Hirschhorn JN, Altshuler D, and Groop LC**. PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet* 34: 267-273, 2003.
192. **Moran TH, and Bi S**. Hyperphagia and obesity of OLETF rats lacking CCK1 receptors: developmental aspects. *Dev Psychobiol* 48: 360-367, 2006.
193. **Mori Y, Murakawa Y, Okada K, Horikoshi H, Yokoyama J, Tajima N, and Ikeda Y**. Effect of troglitazone on body fat distribution in type 2 diabetic patients. *Diabetes Care* 22: 908-912, 1999.
194. **Moro C, Crampes F, Sengenes C, De Glisezinski I, Galitzky J, Thalamas C, Lafontan M, and Berlan M**. Atrial natriuretic peptide contributes to physiological control of lipid mobilization in humans. *FASEB J* 18: 908-910, 2004.
195. **Morris RT, Laye MJ, Lees SJ, Rector RS, Thyfault JP, and Booth FW**. Exercise-induced attenuation of obesity, hyperinsulinemia, and skeletal muscle lipid peroxidation in the OLETF rat. *J Appl Physiol* 104: 708-715, 2008.
196. **Motoshima H, Wu X, Sinha MK, Hardy VE, Rosato EL, Barbot DJ, Rosato FE, and Goldstein BJ**. Differential regulation of adiponectin secretion from cultured human omental and

subcutaneous adipocytes: effects of insulin and rosiglitazone. *J Clin Endocrinol Metab* 87: 5662-5667, 2002.
197. **Nedachi T, Fujita H, and Kanzaki M**. Contractile C2C12 myotube model for studying exercise-inducible responses in skeletal muscle. *Am J Physiol Endocrinol Metab* 295: E1191-1204, 2008.
198. **Neel JV**. Diabetes mellitus: a "thrifty" genotype rendered detrimental by "progress"? *Am J Hum Genet* 14: 353-362, 1962.
199. **Nile CJ, Read RC, Akil M, Duff GW, and Wilson AG**. Methylation status of a single CpG site in the IL6 promoter is related to IL6 messenger RNA levels and rheumatoid arthritis. *Arthritis Rheum* 58: 2686-2693, 2008.
200. **Nishimura S, Manabe I, Nagasaki M, Hosoya Y, Yamashita H, Fujita H, Ohsugi M, Tobe K, Kadowaki T, Nagai R, and Sugiura S**. Adipogenesis in Obesity Requires Close Interplay Between Differentiating Adipocytes, Stromal Cells, and Blood Vessels. *Diabetes* 56: 1517-1526, 2007.
201. **Nogalska A, Pankiewicz A, Goyke E, and Swierczynski J**. The age-related inverse relationship between ob and lipogenic enzymes genes expression in rat white adipose tissue. *Exp Gerontol* 38: 415-422, 2003.
202. **Nogalska A, and Swierczynski J**. Potential role of high serum leptin concentration in age-related decrease of fatty acid synthase gene expression in rat white adipose tissue. *Exp Gerontol* 39: 147-150, 2004.
203. **Nunes I, Tovmasian LT, Silva RM, Burke RE, and Goff SP**. Pitx3 is required for development of substantia nigra dopaminergic neurons. *Proc Natl Acad Sci U S A* 100: 4245-4250, 2003.
204. **Ogden CL, Carroll MD, Curtin LR, McDowell MA, Tabak CJ, and Flegal KM**. Prevalence of overweight and obesity in the United States, 1999-2004. *JAMA* 295: 1549-1555, 2006.
205. **Ojuka EO, Jones TE, Han DH, Chen M, and Holloszy JO**. Raising Ca2+ in L6 myotubes mimics effects of exercise on mitochondrial biogenesis in muscle. *FASEB J* 17: 675-681, 2003.
206. **Oka M, Rodic N, Graddy J, Chang LJ, and Terada N**. CpG sites preferentially methylated by Dnmt3a in vivo. *J Biol Chem* 281: 9901-9908, 2006.
207. **Okada T, Kawano Y, Sakakibara T, Hazeki O, and Ui M**. Essential role of phosphatidylinositol 3-kinase in insulin-induced glucose transport and antilipolysis in rat adipocytes. Studies with a selective inhibitor wortmannin. *J Biol Chem* 269: 3568-3573, 1994.
208. **Okano M, Bell DW, Haber DA, and Li E**. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* 99: 247-257, 1999.
209. **Olsen RH, Krogh-Madsen R, Thomsen C, Booth FW, and Pedersen BK**. Metabolic responses to reduced daily steps in healthy nonexercising men. *JAMA* 299: 1261-1263, 2008.
210. **Ong KK**. Size at birth, postnatal growth and risk of obesity. *Horm Res* 65 Suppl 3: 65-69, 2006.
211. **Oscai LB, Spirakis CN, Wolff CA, and Beck RJ**. Effects of exercise and of food restriction on adipose tissue cellularity. *J Lipid Res* 13: 588-592, 1972.
212. **Ozanne SE, and Constancia M**. Mechanisms of disease: the developmental origins of disease and the role of the epigenotype. *Nat Clin Pract Endocrinol Metab* 3: 539-546, 2007.
213. **Park SY, Choi GH, Choi HI, Ryu J, Jung CY, and Lee W**. Calorie restriction improves whole-body glucose disposal and insulin resistance in association with the increased adipocyte-specific GLUT4 expression in Otsuka Long-Evans Tokushima fatty rats. *Arch Biochem Biophys* 436: 276-284, 2005.

214. **Patel MS, Jomain-Baum M, Ballard FJ, and Hanson RW**. Pathway of carbon flow during fatty acid synthesis from lactate and pyruvate in rat adipose tissue. *J Lipid Res* 12: 179-191, 1971.
215. **Patterson CM, Dunn-Meynell AA, and Levin BE**. Three weeks of early-onset exercise prolongs obesity resistance in DIO rats after exercise cessation. *Am J Physiol Regul Integr Comp Physiol* 294: R290-301, 2008.
216. **Patti ME, Butte AJ, Crunkhorn S, Cusi K, Berria R, Kashyap S, Miyazaki Y, Kohane I, Costello M, Saccone R, Landaker EJ, Goldfine AB, Mun E, DeFronzo R, Finlayson J, Kahn CR, and Mandarino LJ**. Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of PGC1 and NRF1. *ProcNatlAcad Sci USA* 100: 8466-8471, 2003.
217. **Perez-Carreras M, Del Hoyo P, Martin MA, Rubio JC, Martin A, Castellano G, Colina F, Arenas J, and Solis-Herruzo JA**. Defective hepatic mitochondrial respiratory chain in patients with nonalcoholic steatohepatitis. *Hepatology* 38: 999-1007, 2003.
218. **Perri MG, McAdoo WG, McAllister DA, Lauer JB, and Yancey DZ**. Enhancing the efficacy of behavior therapy for obesity: effects of aerobic exercise and a multicomponent maintenance program. *J Consult Clin Psychol* 54: 670-675, 1986.
219. **Perseghin G, Burska A, Lattuada G, Alberti G, Costantino F, Ragogna F, Oggionni S, Scollo A, Terruzzi I, and Luzi L**. Increased serum resistin in elite endurance athletes with high insulin sensitivity. *Diabetologia* 49: 1893-1900, 2006.
220. **Petridou A, Tsalouhidou S, Tsalis G, Schulz T, Michna H, and Mougios V**. Long-term exercise increases the DNA binding activity of peroxisome proliferator-activated receptor gamma in rat adipose tissue. *Metabolism* 56: 1029-1036, 2007.
221. **Pette D**. Historical Perspectives: plasticity of mammalian skeletal muscle. *J Appl Physiol* 90: 1119-1124, 2001.
222. **Piestrzeniewicz K, Luczak K, Komorowski J, Maciejewski M, Jankiewicz Wika J, and Goch JH**. Resistin increases with obesity and atherosclerotic risk factors in patients with myocardial infarction. *Metabolism* 57: 488-493, 2008.
223. **Pilegaard H, Saltin B, and Neufer PD**. Exercise induces transient transcriptional activation of the PGC-1alpha gene in human skeletal muscle. *J Physiol* 546: 851-858, 2003.
224. **Poehlman ET, Tremblay A, Despres JP, Fontaine E, Perusse L, Theriault G, and Bouchard C**. Genotype-controlled changes in body composition and fat morphology following overfeeding in twins. *Am J Clin Nutr* 43: 723-731, 1986.
225. **Post WS, Goldschmidt-Clermont PJ, Wilhide CC, Heldman AW, Sussman MS, Ouyang P, Milliken EE, and Issa JP**. Methylation of the estrogen receptor gene is associated with aging and atherosclerosis in the cardiovascular system. *Cardiovasc Res* 43: 985-991, 1999.
226. **Poussin C, Hall D, Minehira K, Galzin AM, Tarussio D, and Thorens B**. Different transcriptional control of metabolism and extracellular matrix in visceral and subcutaneous fat of obese and rimonabant treated mice. *PLoS ONE* 3: e3385, 2008.
227. **Powelka AM, Seth A, Virbasius JV, Kiskinis E, Nicoloro SM, Guilherme A, Tang X, Straubhaar J, Cherniack AD, Parker MG, and Czech MP**. Suppression of oxidative metabolism and mitochondrial biogenesis by the transcriptional corepressor RIP140 in mouse adipocytes. *J Clin Invest* 116: 125-136, 2006.
228. **Pradhan S, Bacolla A, Wells RD, and Roberts RJ**. Recombinant human DNA (cytosine-5) methyltransferase. I. Expression, purification, and comparison of de novo and maintenance methylation. *J Biol Chem* 274: 33002-33010, 1999.
229. **Puri V, Konda S, Ranjit S, Aouadi M, Chawla A, Chouinard M, Chakladar A, and Czech MP**. Fat-specific protein 27, a novel lipid droplet protein that enhances triglyceride storage. *J Biol Chem* 282: 34213-34218, 2007.

230. **Reaven GM, Hollenbeck C, Jeng CY, Wu MS, and Chen YD**. Measurement of plasma glucose, free fatty acid, lactate, and insulin for 24 h in patients with NIDDM. *Diabetes* 37: 1020-1024, 1988.
231. **Rector RS, Thyfault JP, Laye MJ, Morris RT, Borengasser SJ, Uptergrove GM, Chakravarthy MV, Booth FW, and Ibdah JA**. Cessation of daily exercise dramatically alters precursors of hepatic steatosis in Otsuka Long-Evans Tokushima Fatty (OLETF) rats. *J Physiol* 2008.
232. **Rector RS, Thyfault JP, Morris RT, Laye MJ, Borengasser SJ, Booth FW, and Ibdah JA**. Daily exercise increases hepatic fatty acid oxidation and prevents steatosis in Otsuka Long-Evans Tokushima Fatty rats. *Am J Physiol Gastrointest Liver Physiol* 294: G619-626, 2008.
233. **Reeve AK, Krishnan KJ, and Turnbull D**. Mitochondrial DNA mutations in disease, aging, and neurodegeneration. *Ann N Y Acad Sci* 1147: 21-29, 2008.
234. **Rhee I, Jair KW, Yen RW, Lengauer C, Herman JG, Kinzler KW, Vogelstein B, Baylin SB, and Schuebel KE**. CpG methylation is maintained in human cancer cells lacking DNMT1. *Nature* 404: 1003-1007, 2000.
235. **Robert JJ, Rakotoambinina B, Cochet I, Foussier V, Magre J, Darmaun D, Chevenne D, and Capeau J**. The development of hyperglycaemia in patients with insulin-resistant generalized lipoatrophic syndromes. *Diabetologia* 36: 1288-1292, 1993.
236. **Robinson SW, Dinulescu DM, and Cone RD**. Genetic models of obesity and energy balance in the mouse. *Annu Rev Genet* 34: 687-745, 2000.
237. **Rodbell M**. Metabolism of Isolated Fat Cells. I. Effects of Hormones on Glucose Metabolism and Lipolysis. *J Biol Chem* 239: 375-380, 1964.
238. **Rodnick KJ, Reaven GM, Haskell WL, Sims CR, and Mondon CE**. Variations in running activity and enzymatic adaptations in voluntary running rats. *J Appl Physiol* 66: 1250-1257, 1989.
239. **Rong JX, Qiu Y, Hansen MK, Zhu L, Zhang V, Xie M, Okamoto Y, Mattie MD, Higashiyama H, Asano S, Strum JC, and Ryan TE**. Adipose mitochondrial biogenesis is suppressed in db/db and high-fat diet-fed mice and improved by rosiglitazone. *Diabetes* 56: 1751-1760, 2007.
240. **Rooyackers OE, Adey DB, Ades PA, and Nair KS**. Effect of age on in vivo rates of mitochondrial protein synthesis in human skeletal muscle. *Proc Natl Acad Sci U S A* 93: 15364-15369, 1996.
241. **Rosenbaum M, Goldsmith R, Bloomfield D, Magnano A, Weimer L, Heymsfield S, Gallagher D, Mayer L, Murphy E, and Leibel RL**. Low-dose leptin reverses skeletal muscle, autonomic, and neuroendocrine adaptations to maintenance of reduced weight. *J Clin Invest* 115: 3579-3586, 2005.
242. **Ryan DH**. Diet and exercise in the prevention of diabetes. *Int J Clin PractSuppl* 28-35, 2003.
243. **Saggerson ED**. Lipogenesis in rat and guinea-pig isolated epididymal fat-cells. *Biochem J* 140: 211-224, 1974.
244. **Saint-Marc P, Kozak LP, Ailhaud G, Darimont C, and Negrel R**. Angiotensin II as a trophic factor of white adipose tissue: stimulation of adipose cell formation. *Endocrinology* 142: 487-492, 2001.
245. **Sandri M, Carraro U, Podhorska-Okolov M, Rizzi C, Arslan P, Monti D, and Franceschi C**. Apoptosis, DNA damage and ubiquitin expression in normal and mdx muscle fibers after exercise. *FEBS Lett* 373: 291-295, 1995.
246. **Santomauro AT, Boden G, Silva ME, Rocha DM, Santos RF, Ursich MJ, Strassmann PG, and Wajchenberg BL**. Overnight lowering of free fatty acids with Acipimox improves insulin resistance and glucose tolerance in obese diabetic and nondiabetic subjects. *Diabetes* 48: 1836-1841, 1999.

247. **Saxonov S, Berg P, and Brutlag DL**. A genome-wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters. *Proc Natl Acad Sci U S A* 103: 1412-1417, 2006.
248. **Scarpace PJ, and Zhang Y**. Leptin resistance: a prediposing factor for diet-induced obesity. *Am J Physiol Regul Integr Comp Physiol* 296: R493-500, 2009.
249. **Schaffer JE**. Lipotoxicity: when tissues overeat. *Curr Opin Lipidol* 14: 281-287, 2003.
250. **Scherer PE, Williams S, Fogliano M, Baldini G, and Lodish HF**. A novel serum protein similar to C1q, produced exclusively in adipocytes. *J Biol Chem* 270: 26746-26749, 1995.
251. **Schmitz KH, Jacobs DR, Jr., Leon AS, Schreiner PJ, and Sternfeld B**. Physical activity and body weight: associations over ten years in the CARDIA study. Coronary Artery Risk Development in Young Adults. *Int J Obes Relat Metab Disord* 24: 1475-1487, 2000.
252. **Schoeller DA, Shay K, and Kushner RF**. How much physical activity is needed to minimize weight gain in previously obese women? *Am J Clin Nutr* 66: 551-556, 1997.
253. **Seale P, Bjork B, Yang W, Kajimura S, Chin S, Kuang S, Scime A, Devarakonda S, Conroe HM, Erdjument-Bromage H, Tempst P, Rudnicki MA, Beier DR, and Spiegelman BM**. PRDM16 controls a brown fat/skeletal muscle switch. *Nature* 454: 961-967, 2008.
254. **Selvarajan S, Lund LR, Takeuchi T, Craik CS, and Werb Z**. A plasma kallikrein-dependent plasminogen cascade required for adipocyte differentiation. *Nat Cell Biol* 3: 267-275, 2001.
255. **Sene-Fiorese M, Duarte FO, Scarmagnani FR, Cheik NC, Manzoni MS, Nonaka KO, Rossi EA, de Oliveira Duarte AC, and Damaso AR**. Efficiency of intermittent exercise on adiposity and fatty liver in rats fed with high-fat diet. *Obesity (Silver Spring)* 16: 2217-2222, 2008.
256. **Sengenes C, Berlan M, De Glisezinski I, Lafontan M, and Galitzky J**. Natriuretic peptides: a new lipolytic pathway in human adipocytes. *FASEB J* 14: 1345-1351, 2000.
257. **Seppala-Lindroos A, Vehkavaara S, Hakkinen AM, Goto T, Westerbacka J, Sovijarvi A, Halavaara J, and Yki-Jarvinen H**. Fat accumulation in the liver is associated with defects in insulin suppression of glucose production and serum free fatty acids independent of obesity in normal men. *J Clin Endocrinol Metab* 87: 3023-3028, 2002.
258. **Serradas P, Giroix MH, Saulnier C, Gangnerau MN, Borg LA, Welsh M, Portha B, and Welsh N**. Mitochondrial deoxyribonucleic acid content is specifically decreased in adult, but not fetal, pancreatic islets of the Goto-Kakizaki rat, a genetic model of noninsulin-dependent diabetes. *Endocrinology* 136: 5623-5631, 1995.
259. **Shepherd PR, Gnudi L, Tozzo E, Yang H, Leach F, and Kahn BB**. Adipose cell hyperplasia and enhanced glucose disposal in transgenic mice overexpressing GLUT4 selectively in adipose tissue. *J Biol Chem* 268: 22243-22246, 1993.
260. **Shi X, Burkart A, Nicoloro SM, Czech MP, Straubhaar J, and Corvera S**. Paradoxical effect of mitochondrial respiratory chain impairment on insulin signaling and glucose transport in adipose cells. *J Biol Chem* 283: 30658-30667, 2008.
261. **Shima K, Shi K, Sano T, Iwami T, Mizuno A, and Noma Y**. Is exercise training effective in preventing diabetes mellitus in the Otsuka-Long-Evans-Tokushima fatty rat, a model of spontaneous non-insulin-dependent diabetes mellitus? *Metabolism* 42: 971-977, 1993.
262. **Shimabukuro M, Koyama K, Chen G, Wang MY, Trieu F, Lee Y, Newgard CB, and Unger RH**. Direct antidiabetic effect of leptin through triglyceride depletion of tissues. *Proc Natl Acad Sci U S A* 94: 4637-4641, 1997.
263. **Shimabukuro M, Zhou YT, Levi M, and Unger RH**. Fatty acid-induced beta cell apoptosis: a link between obesity and diabetes. *Proc Natl Acad Sci U S A* 95: 2498-2502, 1998.
264. **Simoneau JA, Veerkamp JH, Turcotte LP, and Kelley DE**. Markers of capacity to utilize fatty acids in human skeletal muscle: relation to insulin resistance and obesity and effects of weight loss. *FASEB J* 13: 2051-2060, 1999.

265. **Skurk T, Alberti-Huber C, Herder C, and Hauner H**. Relationship between adipocyte size and adipokine expression and secretion. *J Clin Endocrinol Metab* 92: 1023-1033, 2007.
266. **Slentz CA, Aiken LB, Houmard JA, Bales CW, Johnson JL, Tanner CJ, Duscha BD, and Kraus WE**. Inactivity, exercise, and visceral fat. STRRIDE: a randomized, controlled study of exercise intensity and amount. *J Appl Physiol* 99: 1613-1618, 2005.
267. **Smith AJ, Thompson BR, Sanders MA, and Bernlohr DA**. Interaction of the adipocyte fatty acid-binding protein with the hormone-sensitive lipase: regulation by fatty acids and phosphorylation. *J Biol Chem* 282: 32424-32432, 2007.
268. **Smith FM, Garfield AS, and Ward A**. Regulation of growth and metabolism by imprinted genes. *Cytogenet Genome Res* 113: 279-291, 2006.
269. **Smith JA, Kohn TA, Chetty AK, and Ojuka EO**. CaMK activation during exercise is required for histone hyperacetylation and MEF2A binding at the MEF2 site on the Glut4 gene. *Am J Physiol Endocrinol Metab* 295: E698-704, 2008.
270. **Smith SJ, and Saggerson ED**. Regulation of pyruvate dehydrogenase activity in white adipocyte mitochondria by palmitoyl carnitine and citrate. *Int J Biochem* 10: 785-790, 1979.
271. **Smyth S, and Heron A**. Diabetes and obesity: the twin epidemics. *Nat Med* 12: 75-80, 2006.
272. **Spalding KL, Arner E, Westermark PO, Bernard S, Buchholz BA, Bergmann O, Blomqvist L, Hoffstedt J, Naslund E, Britton T, Concha H, Hassan M, Ryden M, Frisen J, and Arner P**. Dynamics of fat cell turnover in humans. *Nature* 453: 783-787, 2008.
273. **Spiegelman BM, and Ginty CA**. Fibronectin modulation of cell shape and lipogenic gene expression in 3T3-adipocytes. *Cell* 35: 657-666, 1983.
274. **Srere PA**. Citrate synthase. *Meth Enzymol* 13: 3-5, 1969.
275. **Stallknecht B, Vinten J, Ploug T, and Galbo H**. Increased activities of mitochondrial enzymes in white adipose tissue in trained rats. *Am J Physiol* 261: E410-414, 1991.
276. **Steinberg GR, and Dyck DJ**. Development of leptin resistance in rat soleus muscle in response to high-fat diets. *Am J Physiol Endocrinol Metab* 279: E1374-1382, 2000.
277. **Stenvinkel P, Karimi M, Johansson S, Axelsson J, Suliman M, Lindholm B, Heimburger O, Barany P, Alvestrand A, Nordfors L, Qureshi AR, Ekstrom TJ, and Schalling M**. Impact of inflammation on epigenetic DNA methylation - a novel risk factor for cardiovascular disease? *J Intern Med* 261: 488-499, 2007.
278. **Steppan CM, Bailey ST, Bhat S, Brown EJ, Banerjee RR, Wright CM, Patel HR, Ahima RS, and Lazar MA**. The hormone resistin links obesity to diabetes. *Nature* 409: 307-312, 2001.
279. **Stern JS, and Johnson PR**. Spontaneous activity and adipose cellularity in the genetically obese Zucker rat (fafa). *Metabolism* 26: 371-380, 1977.
280. **Strissel KJ, Stancheva Z, Miyoshi H, Perfield JW, 2nd, DeFuria J, Jick Z, Greenberg AS, and Obin MS**. Adipocyte death, adipose tissue remodeling, and obesity complications. *Diabetes* 56: 2910-2918, 2007.
281. **Stump CS, Short KR, Bigelow ML, Schimke JM, and Nair KS**. Effect of insulin on human skeletal muscle mitochondrial ATP production, protein synthesis, and mRNA transcripts. *Proc Natl Acad Sci U S A* 100: 7996-8001, 2003.
282. **Surwit RS, Kuhn CM, Cochrane C, McCubbin JA, and Feinglos MN**. Diet-induced type II diabetes in C57BL/6J mice. *Diabetes* 37: 1163-1167, 1988.
283. **Sutherland LN, Bomhof MR, Capozzi LC, Basaraba SA, and Wright DC**. Exercise and epinephrine increase PGC-1{alpha}mRNA expression in rat adipose tissue. *J Physiol* 2009.
284. **Sutherland LN, Capozzi LC, Turchinsky NJ, Bell RC, and Wright DC**. Time course of high-fat diet-induced reductions in adipose tissue mitochondrial proteins: potential mechanisms and the relationship to glucose intolerance. *Am J Physiol Endocrinol Metab* 295: E1076-1083, 2008.
285. **Sweeney G**. Leptin signalling. *Cell Signal* 14: 655-663, 2002.

286. **Takahashi K, Mizuarai S, Araki H, Mashiko S, Ishihara A, Kanatani A, Itadani H, and Kotani H**. Adiposity elevates plasma MCP-1 levels leading to the increased CD11b-positive monocytes in mice. *J Biol Chem* 278: 46654-46660, 2003.
287. **Tamura Y, Sugimoto M, Murayama T, Ueda Y, Kanamori H, Ono K, Ariyasu H, Akamizu T, Kita T, Yokode M, and Arai H**. Inhibition of CCR2 ameliorates insulin resistance and hepatic steatosis in db/db mice. *Arterioscler Thromb Vasc Biol* 28: 2195-2201, 2008.
288. **Thomason DB, and Booth FW**. Atrophy of the soleus muscle by hindlimb unweighting. *J Appl Physiol* 68: 1-12, 1990.
289. **Thule PM, Campbell AG, Kleinhenz DJ, Olson DE, Boutwell JJ, Sutliff RL, and Hart CM**. Hepatic insulin gene therapy prevents deterioration of vascular function and improves adipocytokine profile in STZ-diabetic rats. *Am J Physiol Endocrinol Metab* 290: E114-E122, 2006.
290. **Thyfault JP, Kraus RM, Hickner RC, Howell AW, Wolfe RR, and Dohm GL**. Impaired plasma fatty acid oxidation in extremely obese women. *Am J Physiol Endocrinol Metab* 287: E1076-E1081, 2004.
291. **Timmons JA, Norrbom J, Scheele C, Thonberg H, Wahlestedt C, and Tesch P**. Expression profiling following local muscle inactivity in humans provides new perspective on diabetes-related genes. *Genomics* 87: 165-172, 2006.
292. **Timmons JA, Wennmalm K, Larsson O, Walden TB, Lassmann T, Petrovic N, Hamilton DL, Gimeno RE, Wahlestedt C, Baar K, Nedergaard J, and Cannon B**. Myogenic gene expression signature establishes that brown and white adipocytes originate from distinct cell lineages. *Proc Natl Acad Sci U S A* 104: 4401-4406, 2007.
293. **Tost J, and Gut IG**. Analysis of gene-specific DNA methylation patterns by pyrosequencing technology. *Methods Mol Biol* 373: 89-102, 2007.
294. **Tsalouhidou S, Petridou A, and Mougios V**. Effect of chronic exercise on DNA fragmentation and on lipid profiles in rat skeletal muscle. *Exp Physiol* 94: 362-370, 2009.
295. **Turek-Plewa J, and Jagodzinski PP**. The role of mammalian DNA methyltransferases in the regulation of gene expression. *Cell Mol Biol Lett* 10: 631-647, 2005.
296. **Unger RH**. Lipotoxic diseases. *Annu Rev Med* 53: 319-336, 2002.
297. **Ungvari Z, Labinskyy N, Gupte S, Chander PN, Edwards JG, and Csiszar A**. Dysregulation of mitochondrial biogenesis in vascular endothelial and smooth muscle cells of aged rats. *Am J Physiol Heart Circ Physiol* 294: H2121-2128, 2008.
298. **Ushijima T, and Okochi-Takada E**. Aberrant methylations in cancer cells: where do they come from? *Cancer Sci* 96: 206-211, 2005.
299. **Vague J**. The degree of masculine differentiation of obesities: a factor determining predisposition to diabetes, atherosclerosis, gout, and uric calculous disease. *Am J Clin Nutr* 4: 20-34, 1956.
300. **Valerio A, Cardile A, Cozzi V, Bracale R, Tedesco L, Pisconti A, Palomba L, Cantoni O, Clementi E, Moncada S, Carruba MO, and Nisoli E**. TNF-alpha downregulates eNOS expression and mitochondrial biogenesis in fat and muscle of obese rodents. *J Clin Invest* 116: 2791-2798, 2006.
301. **Valet P, Berlan M, Beauville M, Crampes F, Montastruc JL, and Lafontan M**. Neuropeptide Y and peptide YY inhibit lipolysis in human and dog fat cells through a pertussis toxin-sensitive G protein. *J Clin Invest* 85: 291-295, 1990.
302. **van Dale D, Saris WH, and ten Hoor F**. Weight maintenance and resting metabolic rate 18-40 months after a diet/exercise treatment. *Int J Obes* 14: 347-359, 1990.
303. **Van Harmelen V, Reynisdottir S, Eriksson P, Thorne A, Hoffstedt J, Lonnqvist F, and Arner P**. Leptin secretion from subcutaneous and visceral adipose tissue in women. *Diabetes* 47: 913-917, 1998.

304. **Van Harmelen V, Rohrig K, and Hauner H**. Comparison of proliferation and differentiation capacity of human adipocyte precursor cells from the omental and subcutaneous adipose tissue depot of obese subjects. *Metabolism* 53: 632-637, 2004.
305. **van Harmelen V, Skurk T, Rohrig K, Lee YM, Halbleib M, Aprath-Husmann I, and Hauner H**. Effect of BMI and age on adipose tissue cellularity and differentiation capacity in women. *Int J Obes Relat Metab Disord* 27: 889-895, 2003.
306. **Vazquez-Vela ME, Torres N, and Tovar AR**. White adipose tissue as endocrine organ and its role in obesity. *Arch Med Res* 39: 715-728, 2008.
307. **Vega GL, Adams-Huet B, Peshock R, Willett D, Shah B, and Grundy SM**. Influence of body fat content and distribution on variation in metabolic risk. *J Clin Endocrinol Metab* 91: 4459-4466, 2006.
308. **Virtanen KA, Hallsten K, Parkkola R, Janatuinen T, Lonnqvist F, Viljanen T, Ronnemaa T, Knuuti J, Huupponen R, Lonnroth P, and Nuutila P**. Differential effects of rosiglitazone and metformin on adipose tissue distribution and glucose uptake in type 2 diabetic subjects. *Diabetes* 52: 283-290, 2003.
309. **Wakil SJ, Titchener EB, and Gibson DM**. Evidence for the participation of biotin in the enzymic synthesis of fatty acids. *Biochim Biophys Acta* 29: 225-226, 1958.
310. **Walker GE, Verti B, Marzullo P, Savia G, Mencarelli M, Zurleni F, Liuzzi A, and Di Blasio AM**. Deep subcutaneous adipose tissue: a distinct abdominal adipose depot. *Obesity (Silver Spring)* 15: 1933-1943, 2007.
311. **Wang MY, Orci L, Ravazzola M, and Unger RH**. Fat storage in adipocytes requires inactivation of leptin's paracrine activity: implications for treatment of human obesity. *Proc Natl Acad Sci U S A* 102: 18011-18016, 2005.
312. **Wang T, Zang Y, Ling W, Corkey BE, and Guo W**. Metabolic partitioning of endogenous fatty acid in adipocytes. *Obes Res* 11: 880-887, 2003.
313. **Wang YA, Kamarova Y, Shen KC, Jiang Z, Hahn MJ, Wang Y, and Brooks SC**. DNA methyltransferase-3a interacts with p53 and represses p53-mediated gene expression. *Cancer Biol Ther* 4: 1138-1143, 2005.
314. **Wang ZM, Pierson RN, Jr., and Heymsfield SB**. The five-level model: a new approach to organizing body-composition research. *Am J Clin Nutr* 56: 19-28, 1992.
315. **Waterland RA, and Jirtle RL**. Early nutrition, epigenetic changes at transposons and imprinted genes, and enhanced susceptibility to adult chronic diseases. *Nutrition* 20: 63-68, 2004.
316. **Waterland RA, Lin JR, Smith CA, and Jirtle RL**. Post-weaning diet affects genomic imprinting at the insulin-like growth factor 2 (Igf2) locus. *Hum Mol Genet* 15: 705-716, 2006.
317. **Waterland RA, and Michels KB**. Epigenetic epidemiology of the developmental origins hypothesis. *Annu Rev Nutr* 27: 363-388, 2007.
318. **Weaver IC, Cervoni N, Champagne FA, D'Alessio AC, Sharma S, Seckl JR, Dymov S, Szyf M, and Meaney MJ**. Epigenetic programming by maternal behavior. *Nat Neurosci* 7: 847-854, 2004.
319. **Weber M, Hellmann I, Stadler MB, Ramos L, Paabo S, Rebhan M, and Schubeler D**. Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. *Nat Genet* 39: 457-466, 2007.
320. **Weinsier RL, Hunter GR, Desmond RA, Byrne NM, Zuckerman PA, and Darnell BE**. Free-living activity energy expenditure in women successful and unsuccessful at maintaining a normal body weight. *Am J Clin Nutr* 75: 499-504, 2002.
321. **Weisberg SP, Hunter D, Huber R, Lemieux J, Slaymaker S, Vaddi K, Charo I, Leibel RL, and Ferrante AW, Jr.** CCR2 modulates inflammatory and metabolic effects of high-fat feeding. *J Clin Invest* 116: 115-124, 2006.
322. **Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, and Ferrante AW, Jr.** Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* 112: 1796-1808, 2003.

323. **Weiss A, Keshet I, Razin A, and Cedar H**. DNA demethylation in vitro: involvement of RNA. *Cell* 86: 709-718, 1996.
324. **Wells JC**. The evolution of human fatness and susceptibility to obesity: an ethological approach. *Biol Rev Camb Philos Soc* 81: 183-205, 2006.
325. **Wende AR, Huss JM, Schaeffer PJ, Giguere V, and Kelly DP**. PGC-1{alpha} Coactivates PDK4 Gene Expression via the Orphan Nuclear Receptor ERR{alpha}: a Mechanism for Transcriptional Control of Muscle Glucose Metabolism. *MolCellBiol* 25: 10684-10694, 2005.
326. **Wickings EJ, and Dixson AF**. Testicular function, secondary sexual development, and social status in male mandrills (Mandrillus sphinx). *Physiol Behav* 52: 909-916, 1992.
327. **Wigler M, Levy D, and Perucho M**. The somatic replication of DNA methylation. *Cell* 24: 33-40, 1981.
328. **William WN, Jr., Ceddia RB, and Curi R**. Leptin controls the fate of fatty acids in isolated rat white adipocytes. *J Endocrinol* 175: 735-744, 2002.
329. **Williams PT, and Thompson PD**. Dose-dependent effects of training and detraining on weight in 6406 runners during 7.4 years. *Obesity (Silver Spring)* 14: 1975-1984, 2006.
330. **Williamson DF, Madans J, Anda RF, Kleinman JC, Kahn HS, and Byers T**. Recreational physical activity and ten-year weight change in a US national cohort. *Int J Obes Relat Metab Disord* 17: 279-286, 1993.
331. **Wilson-Fritch L, Burkart A, Bell G, Mendelson K, Leszyk J, Nicoloro S, Czech M, and Corvera S**. Mitochondrial biogenesis and remodeling during adipogenesis and in response to the insulin sensitizer rosiglitazone. *Mol Cell Biol* 23: 1085-1094, 2003.
332. **Wing RR, Venditti E, Jakicic JM, Polley BA, and Lang W**. Lifestyle intervention in overweight individuals with a family history of diabetes. *Diabetes Care* 21: 350-359, 1998.
333. **Wolff GL, Kodell RL, Moore SR, and Cooney CA**. Maternal epigenetics and methyl supplements affect agouti gene expression in Avy/a mice. *FASEB J* 12: 949-957, 1998.
334. **Wong TS, and Booth FW**. Protein metabolism in rat tibialis anterior muscle after stimulated chronic eccentric exercise. *J Appl Physiol* 69: 1718-1724, 1990.
335. **Wozniak AC, and Anderson JE**. Single-fiber isolation and maintenance of satellite cell quiescence. *Biochem Cell Biol* 83: 674-676, 2005.
336. **Wu Z, Bucher NL, and Farmer SR**. Induction of peroxisome proliferator-activated receptor gamma during the conversion of 3T3 fibroblasts into adipocytes is mediated by C/EBPbeta, C/EBPdelta, and glucocorticoids. *Mol Cell Biol* 16: 4128-4136, 1996.
337. **Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA, and Chen H**. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest* 112: 1821-1830, 2003.
338. **Yablonka-Reuveni Z, and Nameroff M**. Skeletal muscle cell populations. Separation and partial characterization of fibroblast-like cells from embryonic tissue using density centrifugation. *Histochemistry* 87: 27-38, 1987.
339. **Yamagata Y, Asada H, Tamura I, Lee L, Maekawa R, Taniguchi K, Taketani T, Matsuoka A, Tamura H, and Sugino N**. DNA methyltransferase expression in the human endometrium: down-regulation by progesterone and estrogen. *Hum Reprod* 2009.
340. **Yang Q, Graham TE, Mody N, Preitner F, Peroni OD, Zabolotny JM, Kotani K, Quadro L, and Kahn BB**. Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes. *Nature* 436: 356-362, 2005.
341. **Yanovski JA, Yanovski SZ, Sovik KN, Nguyen TT, O'Neil PM, and Sebring NG**. A prospective study of holiday weight gain. *N Engl J Med* 342: 861-867, 2000.
342. **Yoon JC, Puigserver P, Chen G, Donovan J, Wu Z, Rhee J, Adelmant G, Stafford J, Kahn CR, Granner DK, Newgard CB, and Spiegelman BM**. Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1. *Nature* 413: 131-138, 2001.

343. **Yoon Y, Krueger EW, Oswald BJ, and McNiven MA**. The mitochondrial protein hFis1 regulates mitochondrial fission in mammalian cells through an interaction with the dynamin-like protein DLP1. *Mol Cell Biol* 23: 5409-5420, 2003.
344. **Zabolotny JM, Bence-Hanulec KK, Stricker-Krongrad A, Haj F, Wang Y, Minokoshi Y, Kim YB, Elmquist JK, Tartaglia LA, Kahn BB, and Neel BG**. PTP1B regulates leptin signal transduction in vivo. *Dev Cell* 2: 489-495, 2002.
345. **Zhang K, and Kaufman RJ**. From endoplasmic-reticulum stress to the inflammatory response. *Nature* 454: 455-462, 2008.
346. **Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, and Friedman JM**. Positional cloning of the mouse obese gene and its human homologue. *Nature* 372: 425-432, 1994.
347. **Zhao Q, Rank G, Tan YT, Li H, Moritz RL, Simpson RJ, Cerruti L, Curtis DJ, Patel DJ, Allis CD, Cunningham JM, and Jane SM**. PRMT5-mediated methylation of histone H4R3 recruits DNMT3A, coupling histone and DNA methylation in gene silencing. *Nat Struct Mol Biol* 16: 304-311, 2009.
348. **Zhong JC, Yu XY, Huang Y, Yung LM, Lau CW, and Lin SG**. Apelin modulates aortic vascular tone via endothelial nitric oxide synthase phosphorylation pathway in diabetic mice. *Cardiovasc Res* 74: 388-395, 2007.
349. **Zhu M, Lee GD, Ding L, Hu J, Qiu G, de Cabo R, Bernier M, Ingram DK, and Zou S**. Adipogenic signaling in rat white adipose tissue: modulation by aging and calorie restriction. *Exp Gerontol* 42: 733-744, 2007.
350. **Zhu XH, He QL, and Lin ZH**. [Effects of catecholamines on human preadipocyte proliferation and differentiation]. *Zhonghua Zheng Xing Wai Ke Za Zhi* 19: 282-284, 2003.

VITA

Matthew James Laye was born on March 21st, 1981 in San Jose, CA to Ann and Richard Laye. His family and he moved to Livermore, CA in 1982 and where he spent the remainder of childhood graduating from Granada High School in 1999. In the fall of 1999 he enrolled at University of California – Davis. While at Davis he ran 3 years of intercollegiate cross-country and track and field, during which he was captain of the cross country team in 2004. His love for running helped spark his academic interest and he earned a Bachelor of Science with honors in Exercise Biology in the Fall of 2004. Matthew enrolled in graduate school at University of Missouri in the Fall of 2005. While at Missouri he has served as Vice-President of the department student association and the department liason to the graduate professional council. Still involved in running, Matthew has coached a youth cross-country team for the past 3 years, and actively competes in national championship events. Matthew has accepted a post-doctoral position in the laboratory of Dr. Bente Pedersen at the Center for Inflammation and Metabolism in Copenhagen, Denmark.