COMPLEX INTERACTIONS BETWEEN DIET, EXERCISE, AND THE HPA AXIS ON THE DEVELOPMENT OF OBESITY, INSULIN RESISTANCE, AND TYPE 2 DIABETES

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ABSTRACT

Chronic elevations in glucocorticoids (GCs), resulting from hyperactivation of the hypothalamic-Pituitary-Adrenal (HPA) axis, can induce insulin resistance, hyperglycaemia, and dyslipidemia and consequently has been proposed to result in the development of Type 2 Diabetes Mellitus (T2DM). Exercise, on the other hand, improves insulin resistance and is crucial in the prevention/treatment of T2DM. However, exercise is also a potent stimulator of the HPA axis. Therefore, we investigated the effects of exercise training on both central (HPA axis) and peripheral aspects of GC biology. First, we demonstrated that 2 weeks of regular exercise in healthy Sprague-Dawley rats induces hyperactivation of the HPA axis, but that by 8 weeks of training, HPA activity is restored to control levels. We found that the transient hyperactivity of the HPA axis with short term exercise is mediated through changes in the adrenal gland sensitivity to adrenocorticotropic hormone (ACTH) that are ameliorated with sustained training. Next, to determine the interaction of exercise and GCs in T2DM, we exercise trained Zucker Diabetic Fatty (ZDF) rats, a rodent model of T2DM. We found that sedentary ZDF rats developed elevated HPA activity prior to hyperglycaemia, also due to altered adrenal sensitivity to ACTH. Exercised ZDF rats maintained normal GC levels, remained euglycaemic, and displayed normal adrenal sensitivity. These studies demonstrate that sustained exercise training maintains normal circulating GCs, which may help prevent/delays the development of T2DM. We next explored the adaptive changes in peripheral GC biology caused by exercise. In ZDF rats, exercise training did not change GC exposure in skeletal muscle or liver tissue, however, did increase exposure in adipose

tissue. In fructose-fed hamsters, another rodent model of insulin resistance, exercise training also had modest effects in skeletal muscle and liver tissue, but again increased GC exposure in adipose tissue. The role of GCs in adipose tissue is poorly defined, making it difficult to understand why exercise increases the action of GCs in adipose tissue. Thus, we next determined the effects of GCs on adipose tissue metabolism both *in vitro* and *in vivo*. We found that GCs work on two distinct cell populations in adipose tissue to simultaneously stimulate differentiation in preadipocytes and lipolysis in mature adipocytes. The net balance of these opposing actions appears dependent upon the diet and activity of the animal. Overall, these findings show that sustained exercise training induces positive changes in both central and peripheral aspects of GC biology that help prevent the development of insulin resistance, obesity, and T2DM.

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LIST OF ABBREVIATIONS

11βHSD	-	11 beta hydroxysteroid dehydrogenase
11-DHC	-	11 dehydrocorticosterone
ACTH	-	adrenocorticotropic hormone
AMPK	-	AMP-activated protein kinase
ANOVA	-	anaylsis of variance
AU	-	arbitrary units
AUC	-	area under the curve
AVP	-	arginine-vasopressin
BSA	-	bovine-serum albumin
cAMP	-	cyclic adenosine monophosphate
CBG	-	corticosterone binding globulin
CGI-58	-	comparative gene-identification 58
CNS	-	central nervous system
CORT	-	corticosterone
COX	-	cytochrome c oxidase
CRH	-	corticotrophine releasing hormone
DAG	-	diacylglycerol
DEX	-	dexamethasone
DIO	-	diet-induced obesity
E	-	exercise
ELISA	-	enzyme-linked immunosorbent assay
FAS	-	fatty acid synthase
FBS	-	fetal bovine serum
FFA	-	free fatty acid
FOXO1	-	forkhead box O1
G6P	-	glucose 6-phosphate
G6Pase	-	glucose 6-phosphatase
GAPDH	-	glyceraldehyde 3-phosphate dehydrogenase
GC	-	glucocorticoid
GR	-	glucocorticoid receptor
GRE	-	glucocorticoid response element
HC	-	high-carbohydrate
HF	-	high-fat
HPA	-	hypothalamic-pituitary-adrenal
IBMX	-	3-isobutyl-1-methylxanthine
IHC	-	immunohistochemistry
IPGTT	-	intraperitoneal glucose tolerance test
IRS	-	insulin receptor substrate
MC2R	-	melanocortin 2 receptor
MR	-	mineralocorticoid receptor
NAD	-	nicotinamide adenine dinucleotide
NADP	-	nicotinamide adenine dinucleotide phosphate
OGTT	-	oral glucose tolerance test

PEPCK	-	phosphoenolpyruvate carboxykinase
PFK	-	phosphofructokinase
PI3K	-	phosphoinositide 3-kinase
PKA	-	protein kinase A
POMC	-	proopiomelanocortin
PPAR	-	peroxisome proliferator-activated receptor
PVDF	-	polyvinylidene fluoride
PVN	-	paroventricular nucleus
RIA	-	radioimmunoassay
S	-	sedentary
SAM	-	sympatho/adrenomedullary
SDS	-	sodium dodecyl sulfate
SEM	-	standard error of the mean
SGK1	-	serum and glucocorticoid inducible kinase 1
StAR	-	steroidogenic acute regulatory protein
T1DM	-	type 1 diabetes mellitus
T2DM	-	type 2 diabetes mellitus
TAG	-	triacylglycerol
TNFα	-	tumor necrosis factor alpha
TTBS	-	tween tris buffered saline
V	-	volts
WAT	-	white adipose tissue
ZDF	-	Zucker diabetic fatty

REVIEW OF THE LITERATURE 1

1. Stress and the Activation of the HPA axis

1.1. Overview of Stress

An organism's probability of survival depends upon the maintenance of homeostasis. Stress has been defined as "a state of threatened homeostasis, which is re-established by physiological and behavioural adaptive responses" (1). Typical responses to stressors include increased alertness and focus, appetite suppression/decreased feeding, release and rerouting of nutrients and energy sources to stressed sites, increased cardiovascular and respiratory functions, and inhibition of the immune response (1). The hypothalamic-pituitary-adrenal (HPA) axis is the major system responsible for the physiological response to stress (1). The initiation of this system begins centrally with the corticotrophin-releasing hormone (CRH) and arginine-vasopressin (AVP) neurons of the paraventricular nucleus (PVN) and the locus ceruleus-norepinephrine system of the brainstem (1). This initiates peripheral endocrine responses that are carried out mainly through the HPA axis, however, also involves the participation of the sympatho/adrenomedullary (SAM) system. These two systems have a multitude of tissue-dependent actions that initiate many of the above mentioned processes.

Stressors can be broadly separated according to whether they are: 1) reactive stressors, events that create a genuine homeostatic challenge recognized by somatic, visceral, or circumventricular sensory pathways; or 2) anticipatory stressors, events that elicit a stress response because of conditioning or innate species-specific predispositions (2). Reactive stressors would include physical (e.g., injury) and metabolic (e.g.,

hypoglycaemia) stressor, whereas anticipatory stressors may include psychological (e.g., fear) or social (e.g., dominant/subordinate hierarchies) stressors (3). There tends to be significant overlap among these classifications. For instance, some of the work presented in this dissertation involves the use of restraint stress, where rats are confined to clear, plastic tubes for a duration of time. Although there is no physical threat to the animal, the rat perceives the situation as stressful and reacts with fear and anxiety, exemplified by activation of the HPA axis and SAM system. This psychological stressor can disrupt physiological homeostasis as the animal struggles to free itself, inducing an 'exercise-like' response, including a rise in body temperature, heart rate, respiratory rate, and mild pain. Thus, the need to respond to a stress is essential and generally involves the robust activation of the HPA axis.

1.2. The Hypothalamic-Pituitary-Adrenal Axis

Under normal conditions, the HPA axis displays a diurnal, pulsatile pattern with high activity in the morning and low activity in the evening. Nocturnal animals, such as rodents, have a diurnal pattern opposite of humans, with high activity in the evening and low activity in the morning. In addition to the diurnal activation, the HPA axis is acutely activated by stressors. Although different neural pathways are activated by different stressors, they all converge to activate parvocellular neurons located primarily in the dorsomedial region of the PVN and stimulate the release of CRH into the median eminence (4). Once released, CRH travels through the hypophyseal portal circulation to corticotrophs in the anterior pituitary, where it binds to the CRH1 receptor (CRH1R) (5). CRH1R is a G-protein coupled receptor, which activates adenylate cyclase and

subsequently increases intracellular levels of cAMP (5). This, in turn, activates cAMP-dependent protein kinase A and increases the influx of extracellular calcium (5). These events result in the release of adrenocorticotropic hormone (ACTH) and the synthesis of proopiomelanocortin (POMC), the precursor for ACTH (4). In addition to CRH, parvocellular cells also release AVP in response to stressors (6, 7). AVP potentiates the stimulatory effect of CRH on ACTH synthesis and secretion by binding V1b receptors, which work to also increase intracellular calcium levels (8). However, AVP by itself does not increase the transcription of POMC, and thus has no effect on the synthesis of ACTH (8). The pituitary gland releases ACTH into the systemic circulation, where it eventually binds to melanocortin 2 receptors (MC2R) located in the zona fasciculata of the adrenal cortex (9). This stimulates the production and release of glucocorticoids (GCs), discussed in detail in the following section (Sec 1.3).

GCs are released into the systemic circulation and act upon virtually every tissue type in the body. GC receptors (GRs) are ubiquitously expressed, including neural tissue where negative feedback of the HPA axis takes place (10). Negative feedback occurs when GCs bind to GRs and mineralocorticoid receptors (MRs) at the levels of the hippocampus, hypothalamus (PVN), and pituitary (10). The different binding affinities of these receptors give notion to different types of negative feedback control. In the brain, MRs bind GCs with a 10-fold higher affinity than GR and are virtually saturated at even basal levels of GCs (9). Thus, it has been proposed that MR is responsible for the maintenance of basal HPA activity at the nadir of the diurnal pattern, whereas GR is responsible for terminating HPA activity following stress and during the peak of the diurnal pattern (9). GCs facilitate a negative feedback mechanism by binding GC

receptors in the hypothalamus and pituitary, and causing inhibition the synthesis of CRH and AVP (11) and ACTH synthesis (8), respectively. In the hippocampus, GCs bind to GR which in turn stimulates neurons that project onto the hypothalamus and prevent the synthesis of CRH and AVP (9). Initiation of these events can occur within minutes of GCs becoming elevated in the plasma (12), however, complete restoration of basal GC levels is dependent upon removal of the stress, and the resulting increase in the concentration of GCs, stimulated by the stressor.

1.3. Glucocorticoids

Upon release in the systemic circulation, ACTH binds to G protein-coupled receptors located on the adrenal glands termed melanocortin-2 receptors (MCR2) (112,113). Binding activates adenylate cyclase, consequently increasing the intracellular concentrations of cAMP, and initiating the signalling cascade leading towards GC synthesis and release (114,115). Steroidogenesis is stimulated through an increase in cholesterol delivery to the inner mitochondrial membrane, a process that is regulated by the expression of steroidogenic acute regulatory (StAR) protein (28). GCs are synthesized and secreted from the zona fasiculata region of the adrenal cortex, which contain cells that uniquely express the enzyme 17α-hydroxylase, which is essential in the metabolism of cholesterol to cortisol (the main GC in humans). The zona glomerulosa region of the adrenal cortex does not express 17α-hydroxylase and thus cannot produce GCs and instead is responsible for the production and release of mineralocorticoids such as aldosterone. Interestingly, rodent adrenal glands lack 17α-hydroxylase completely and thus lack the ability to move cholesterol towards the same pathways that are responsible

for cortisol synthesis in humans (13). Therefore, although the main GCs in humans are cortisol and cortisone, in rodents, the main GCs are corticosterone and 11-dehydrocorticosterone (11-DHC). The major rodent GCs are, in fact, precursors towards the mineralocorticoid pathway and are found in humans, however, in much lower quantities (~0.05:1, corticosterone:cortisol under basal conditions) (14). Interestingly, corticosterone does represent approximately 28% of the active GCs in the cerebral spinal fluid of humans (0.387:1, corticosterone:cortisol); however, this does not appear to have significant effects on HPA negative feedback, nor are the functional implications of this known (15).

In addition to releasing active GCs (cortisol and corticosterone), the adrenal glands also release inactive GCs; cortisone and 11-DHC in humans and rodents, respectively. The ratio of active to inactive hormones released by the adrenal gland is controversial, having been reported to be as high as 8:1 (active to inactive) (16) and as low as 3:1 (17). This is likely due to the sample location and the actions of 11β-hydroxysteroid dehydrogenase (11βHSD) enzymes throughout the body. 11βHSD enzymes are responsible for the interconversion of active and inactive GCs, in both humans and rodents. One study demonstrated that the cortisol:cortisone ratio is highest in the venous blood draining the adrenal glands (~8:1), drops significantly in the renal veins (~2:1), and increases again in the hepatic vein (~15:1) (16). This study also reported a ratio of ~5:1 in the systemic circulation, when measured in the femoral vein (16). The drop in active GCs (or the relative increase in inactive GCs) following the kidney is most likely due to the high expression of 11βHSD type 2 (11βHSD2), which converts active GCs to the inactive form. In a similar manner, the increased ratio seen in the hepatic vein

is likely due to the high expression of 11βHSD type 1 (11βHSD1) in the liver, which converts inactive GCs to the active form. However, conversion of inactive to active GCs may also involve visceral adipose depots, which have a high expression of 11βHSD1 and drain into the portal circulation, leading to the liver. To this effect, genetic overexpression of 11βHSD1 in visceral adipose depots increases the concentration of active GCs specifically in the portal circulation, but not the systemic circulation (18). Thus, measurements of systemic GCs may not accurately represent the concentrations of GCs seen by some of the important tissues involved in the pathogenesis of T2DM (liver, skeletal muscle, and adipose tissue), and careful site-specific measurements are required to make proper inferences regarding the role of GCs in the development of this disease. Unfortunately, tissue specific concentrations of inactive and active GC levels are seldom reported in the literature, likely because of technical challenges in measuring these hormones, which may or may not be bound to various chaperone proteins and/or receptors that translocate from the cytosol to the nucleus.

1.4. Peripheral Glucocorticoid Action

1.4.A. Cortisol Binding Globulin

Due to their hydrophobic nature, both cortisol and corticosterone require a protein carrier in the bloodstream. More than 90% of active GCs are bound to a carrier, with the majority of these bound to cortisol-binding globulin (CBG) (19). Because only free GCs are capable of being transported into tissues for biological activity, only a small fraction of active hormone (i.e., unbound) actually enter the cell (20). Conversely, inactive GCs (cortisone and 11-DHC) do not require a carrier protein and consequently, can freely

enter the cell. Still, no studies have reported the relative concentrations of active and inactive GCs within the cell. CBG is synthesized in the liver and circulates at a concentration of 26 mg/dL (21). However, various stimuli are capable of altering this value, including GCs which cause a decrease in circulating CBG concentrations (22). Little work has focused on the effects of physiological stimuli (e.g., exercise) on circulating CBG concentrations or the implications this would have for GC biology and peripheral GC action.

1.4.B. Tissue exposure to GCs and receptor interactions

In addition to activation of the HPA axis and levels of circulating GC, the target tissues also have a level of control of GC action. This control is made possible by the pre-receptor enzyme complex 11βHSD and the amount and isoform expression of the GC receptor (GR).

The pre-receptor enzyme 11βHSD

11βHSDs are intracellular enzymes responsible for pre-receptor metabolism of GCs. 11βHSD2 is a high-affinity, nicotinamide adenine dinucleotide (NAD)-dependent dehydrogenase that converts active GCs to inert forms (23) and is expressed almost exclusively in aldosterone target tissues (e.g., kidney). By inactivating GCs, 11βHSD2 prevents their binding to MR, leaving these receptors free to bind aldosterone. 11βHSD1 is a nicotinamide adenine dinucleotide phosphate (NADPH) dependent enzyme with a much lower affinity for cortisol. Widely distributed throughout the body (24), this enzyme is responsible for the reverse reaction: activating inert GCs which allows them to

bind to GR. Enhancing tissue expression of 11βHSD1 can increase the concentration of active GCs in tissue ~10-15 fold over that found in plasma (18), thus playing a crucial role in determining GC action in tissue. The metabolic significance of altered 11βHSD1 expression in various organs and tissues is highlighted in section 2.7 of this literature review.

The GC receptor (GR)

Tissue GC action is determined by its circulating concentration, the density of tissue receptors, and the expression of enzymes responsible for pre-receptor metabolism of inactive hormone conversion to the active form (24). The latter is of particular importance since the majority of the hormone entering the tissue is inactive and unable to bind to GR. GCs elicit their effect by binding to GR, after which the receptors will form a homodimer and translocate from the cytoplasm to the nucleus to act as a transcription factor (25). Whole body knockout of GR in mice results in death at birth, illustrating the importance for biological function (26). The GR gene is capable of producing multiple splice variants, resulting in the production of either GRα, GRβ, or mineralcorticoid receptor (MR) (27). GCs cause a genomic effect when they bind to GRα, forming homodimers between two GRα receptors. The GRβ variant may act as a dominant negative regulator of GRα, creating inactive heterodimers with GRα once cortisol has bound (28). Therefore, the proportion of GRα to GRβ would be expected to dictate GC action in the various target tissues, although literature supporting this theory is lacking. Recent reports have revealed that the biology of the GR is significantly more complex than initially proposed, reporting that multiple isoforms of GRα can be found in various

tissues as a result of posttranslational splicing of the protein that forms GR (29). As many as seven GRα isoforms have been reported (A, B, C1, C2, D1, D2, D3), with each acting upon a unique set of genes (29). However, the functional relevance of these findings has yet to be elucidated.

1.5. Stress and Food Intake

Food intake and feeding patterns have a strong influence on the HPA axis. In both humans and rodents, GCs are elevated following a meal (30, 31). Furthermore, individuals that have been dieting for a significant period show a more pronounced elevation in GCs following a big meal, whereas non-dieters shows signs of stress with the absence of food (32). GCs have also been shown to change food preference, with both human and rodents having increase consumption of high-sugar and high-fat foods (33). Consumption of these calorically dense foods appears to partially restore the elevation of the HPA axis (34). Interestingly, chronic elevations in GCs appear to decrease total food intake (34, 35), and, if sustained for a significant period of time, this can lead to decreases in body weight (36, 37). The stress-mediated reduction in food intake has been linked to central actions of CRH in neural peptides such as serotonin (38), neuropeptide Y (39), and alpha-melanocyte-stimulating hormone (39). Remarkably, and in contrast to the weight-rebound typically found with diet-induced weight loss, animals experiencing stress-induced weight loss do not appear to regain weight following removal of the stressor (37, 38). Surprisingly, given the profound effect of GCs on food intake and food choices, and the dramatic effect these parameters have on body weight, few studies have investigated the physiological mechanisms for the effects of GCs on body composition.

Based on patients with Cushing's disease, an accumulation of visceral adipose mass and a reduction in skeletal muscle mass and bone mass are the expected results of elevations in systemic GC levels (40).

2. Type 2 diabetes mellitus, insulin resistance, and glucocorticoids

2.1. Introduction and Pathophysiology

Diabetes mellitus is defined as an insufficiency in insulin action (41-44). Type 1 Diabetes Mellitus (T1DM) emerges from an autoimmune disorder that results in a selective targeting and destruction of insulin producing β-cells (42, 43). On the other hand, Type 2 Diabetes Mellitus (T2DM) is characterized by relative insulin deficiency, that is, insulin production does not meet metabolic demands (45-48). With either scenario, a lack of treatment results in chronic hyperglycaemia, which leads to many of the associated disease complications such as cardiovascular disease, retinopathy, nephropathy, and neuropathy (49-52). Control of glycaemia levels through insulin therapy, diet management, and exercise is essential in the prevention of these co-morbidities (43, 49, 53).

Worldwide, the number of individuals with T2DM is estimated to be between 150 and 200 million, and is expected to double by the year 2025 (54). What may be more alarming is that the number of overweight people worldwide is estimated to be 1 billion, with ~300 million being defined as obese (54). A sedentary lifestyle and an obese phenotype are the two strongest predictors for the development of T2DM (55). Furthermore, the predicted increase in the prevalence of T2DM is partially based on the rapid increase in childhood obesity and T2DM (56). It is not surprising, therefore, that

much effort and resources have been dedicated to understanding the link between obesity and the development of T2DM.

Peripheral insulin resistance is widely believed to precede, and consequently contribute to, the development of T2DM (57). Insulin resistance occurs when peripheral tissues begin to lose their responsiveness to the hormone. This, in turn, increases the production of insulin by the β-cells in order to meet demands and maintain euglycaemia. T2DM emerges when β-cell compensation is no longer sufficient and hyperglycaemia develops (57). There is a strong positive correlation between obesity and insulin resistance, suggesting that increased adipose mass is a predictor for the development of T2DM (55).

2.2. Diet-Induced Obesity

Numerous factors play a role in the development of obesity, including genetic and environmental. From an environmental perspective, it is unlikely that a single individual factor is responsible for obesity. Instead, it is likely a synergistic combination of excess food consumption and reduced physical activity (58, 59). In rodent studies, various models of diet-induced obesity (DIO) are used to study obesity and insulin resistance. One of the most commonly used models is a high-fat (HF) diet, given to otherwise healthy animals (e.g. Sprague-Dawley rats (60) or C57BL/6J mice (61)). Many HF diets are similar in caloric density compared to standard rodent chows, but alter the percentage of calories from fat sources to range between 30-60% (62). The source of the fat calories vary, with the majority coming from animal sources (lard, butter, milk) and less so from plant sources (corn oil, safflower oil, coconut oil) (62). Increased adiposity and insulin

resistance are seen in animals fed a HF diet within only 2 weeks, however, these variables become more appreciable with several weeks of treatment (i.e., >4 weeks) (63, 64). The development of T2DM with HF feeding alone is controversial, with the majority of studies reporting a hyperinsulinemic/euglycaemic phenotype (i.e., insulin resistant) and only a few showing overt T2DM, as evidenced either by sustained fasting hyperglycaemia (plasma glucose values >7 mM) or fed hyperglycaemia (plasma glucose values >11 mM) (65, 66).

In addition to obesity and hyperinsulinemia, HF diets also consistently induce elevations in serum triglycerides (TGs), plasma free fatty acids (FFAs), blood pressure, and can initiate the development of atherosclerosis (62, 67). Perhaps because of these consistent effects on developing features of the metabolic syndrome and because much of human cardiometabolic disease is associated with excessive caloric intake (in particular a high intake of saturated fat) (68), HF diets are still commonly used as a model of obesity and insulin resistance.

2.3. The Fructose-Fed Hamster

The epidemic of obesity has not only been attributed to the increased consumption of fat calories, but also to diets high in carbohydrates. Indeed, in recent years the consumption of sugar has increased to comprise 20% of the total caloric intake in a child's diet (69). Animal models given a high-carbohydrate (HC) diet also develop insulin resistance and many of the metabolic complications seen in prediabetes (70-74). Interestingly, HC diets composed of fructose induce insulin resistance; whereas, HC diets composed of glucose do not (70, 71). In rhesus monkeys, the long term consumption of

fructose-sweetened beverages increased body weight and postprandial triacylglycerol concentrations more than an isocaloric beverages containing glucose (75). One theory that explains this difference is that glucose largely escapes first-pass removal by the liver, whereas fructose does not (71). Because of this, a high-fructose diet would first result in hepatic insulin resistance, which in turn increases hepatic glucose output and the production of TGs and lipoproteins (76). With high fructose consumption, the resulting hyperglycaemia and dyslipidemia caused initially by the liver then induces peripheral insulin resistance in other tissues (e.g., skeletal muscle, adipose). Surprisingly, a high-fructose diet does not always appear to induce significant increases in whole body adiposity (72), at least not on the same time line as a HF-diet does. Thus, although both diets induce insulin resistance, they appear to do so through different mechanisms. This may be why many recent studies have been investigating the development of insulin resistance when both a high fat and high fructose diet is combined (77, 78), a so called "Western Diet" induced metabolic syndrome.

The Syrian Golden hamster provides some advantages over other rodent models for the study of glucose and lipid metabolism in the sedentary or physically active state. First, unlike rats, these hamsters tend to develop insulin resistance in response to a high-fructose diet in a manner that is similar to humans (73, 74, 76). A second advantage pertains to studies utilizing a volitional wheel running model. Young rats tend to run voluntarily between 4-8 km/night when given free access to a running wheel (73, 74, 76), whereas young hamsters will voluntarily run upwards of 20 km/night (79, 80). Furthermore, rats introduced to a running wheel later in life tend not to voluntarily exercise an appreciable volume, where as older hamsters will run ~10 km/night (79).

Thus, hamsters may be a better choice to efficiently study the effects of exercise training on the development of diet-induced insulin resistance.

2.4. The Zucker Diabetic Fatty Rat

The Zucker Diabetic Fatty (ZDF) rat was first described in 1990, after selective breeding of Zucker Fatty rats that spontaneously became hyperglycaemic (81). ZDF rats have a mutation affecting the extracellular domain of the leptin receptor, which results in impaired leptin signalling and early-onset obesity attributable to the lack of appetite suppression and reduced thermogenesis (82). ZDF animals display overt obesity, elevated plasma cholesterol and TGs, hyperphagia, polyuria and polydipsia. The animals develop hyperinsulinemia between 4 and 7 weeks, which progresses to hyperglycaemia by weeks 7-12 (83). Thus, the development of T2DM closely follows the disease progression in humans: obesity → insulin resistance → hyperinsulinemia → hyperglycaemia. That being said, as with many rodent models of disease, the ZDF rat is not perfect. Leptin has emerged as an important regulator of many biological actions beyond energy balance (84, 85). This warrants caution when comparing the experimental results in ZDF rats to humans with T2DM that have functioning leptin signalling.

2.5. The HPA Axis in Type 2 Diabetes

The most predominant role of GCs is the regulation of glucose metabolism; specifically, to raise blood glucose levels during hypoglycaemia to restore euglycaemia (86). Because the primary action of GCs is to raise blood sugar, it has long been thought that prolonged elevations of GC secretion would result in insulin resistance,

hyperglycaemia and the development of the metabolic syndrome and T2DM (87). Indeed, it has been well established that GC excess occurs in patients with T2DM and, in fact, is positively correlated with the severity of diabetes (88-90). Interestingly, the strongest correlations are found with respect to elevated basal GC levels (48, 50, 89), specifically in the nadir cycle of the diurnal pattern (91, 92). The mechanism for this increased basal level of GCs is still unknown. However, challenging various levels of the HPA axis in individuals with T2DM also reveals increased sensitivity to CRH and reduced negative feedback (50), although this is not universally found (41). Furthermore, it remains to be seen if the elevated GC levels seen in individuals with T2DM occurs prior to, and thus may contribute to, the development of the disease or if GCs become elevated in response to disease onset.

GC analogs are among the most prescribed drugs for the treatment of many diseases, mostly because of their immunosuppressant functions. Despite the association between GCs and insulin resistance, very few studies have discussed the potential negative side-effects of steroid treatment, including the development of T2DM. Some studies have shown the development of insulin resistance and obesity with exogenous GC treatment (93, 94). Others have suggested that prenatal exposure to elevated GC levels can induce glucose intolerance in adulthood (95, 96). One study showed that exogenous GCs will induce impaired glucose tolerance in people that have impaired beta-cell function prior to treatment (97). Still, there is a lack of conclusive evidence establishing a causal link between elevated GCs, endogenous or exogenous, and the development of insulin resistance and T2DM.

2.6. Glucocorticoids and Glycaemic Hormones

2.6.A. Insulin

GCs induce rapid peripheral insulin resistance in a variety of tissue types (86), The peripheral insulin resistance can lead to a compensatory increase in insulin production and release by the pancreas (98). In healthy animals, this compensatory effect in response to GC treatment is accomplished through increased β-cell mass (99-101). However, GCs also have a direct effect on the pancreas by decreasing glucose-stimulated insulin release (102-104). Genetic mice with selective overexpression of GR in β-cells demonstrate decreased insulin secretion and eventually develop diabetes (105). Multiple mechanisms have been proposed to explain the effects of GCs in β-cells, including: posttranslational degradation of GLUT2 (103); reduced glucokinase (106); reduced insulin biosynthesis (104); increased NPY (107) and α2-adrenergic receptor (105) expression, both of which are inhibitory for insulin secretion; and induction of serum and glucocorticoid inducible kinase 1 (SGK1), which prevents the increase in intracellular calcium and thus the signalling for insulin release (108). Therefore, GCs have a direct effect on β-cells to prevent the synthesis and release of insulin.

2.6.B. Glucagon

In healthy individuals, glucagon is released by the α-cells in the pancreas in response to hypoglycaemia and stimulates hepatic glucose production in order to raise blood sugars. With T2DM, glucagon production is inappropriately elevated, which leads to hyperglycaemia (109). It has been suggested that insulin binds to α-cells and allows cross-talk within the pancreas to limit glucagon production when glycaemic levels are

high (110). In individuals with T2DM, insulin resistance may be present in α-cells, which reduces or prevents this cross-talk (110). Although GCs and glucagon have similar functions with respect to controlling glycaemia, GCs may inhibit glucagon production indirectly through decreased insulin production, as discussed in the previous section. Indeed, administration of GCs prior to the induction of a hypoglycaemia in humans decreases the release of glucagon (111). GCs may also have a direct impact on decreasing glucagon production, as selective overexpression of 11βHSD1 in α-cells prevents the release of glucagon in response to hypoglycaemia (112). The physiological relevance, or consequence, of why GCs appear to decrease glucagon production remains unclear.

2.6.C. Catecholamines

Stressors, in addition to activating the HPA axis, also activate the SAM systems to increase circulating concentrations of catecholamines (norepinephrine and epinephrine). Catecholamines increase hepatic glucose production, stimulate glucagon secretion, and inhibit insulin secretion (113-115). Catecholamines also increase the rate of lipolysis, resulting in the efflux of FFAs and glycerol from adipose depots (116). The action of catecholamines is partially determined by the density and ratio of both α- and β-adrenergic receptors on target cells (117). GCs have been shown to increase the action of catecholamines through a variety of mechanisms; 1) decreased extraneuronal uptake of catecholamines, which increases the plasma concentration (118); 2) increased density of -adrenergic receptors in many tissues (119, 120); and 3) increased synthesis (121, 122) and decreased degradation of catecholamines (123). Thus, GCs indirectly increase blood glucose and circulating lipids through potentiation of catecholamine action.

2.7. Glucocorticoids and Peripheral Insulin Target Tissues

2.7.A. The Insulin Signalling Pathway

Insulin is released by pancreatic β-cells in response to an elevation in circulating nutrients (124). Up to 50% of the insulin released is immediately cleared by the liver before leaving the portal circulation (125). This process involves the hormone binding to the insulin receptor, followed by endocytosis of the hormone-receptor complex, and degradation of this complex by the insulin-degrading enzyme (126). The kidney is responsible for the removal of the majority of the systemic insulin, through filtration and excretion in the urine (125). The remaining insulin is cleared by other tissues, in a mechanism similar to the liver (125). This process gives insulin a half-life of between 3 and 6 minutes in the systemic circulation (125).

In peripheral tissue, insulin binds to a transmembrane, tyrosine kinase receptor called the insulin receptor. This initiates autophosphorylation of the receptor and, in turn, the phosphorylation of various insulin-receptor substrates (IRSs), causing conformational changes in these proteins (127). This allows association of IRSs with the regulatory subunit of phosphoinositide 3-kinase (PI3K). PI3K activates 3-phosphoinositide-dependent protein kinase 1 (PDK1), which in turn, activates AKT, a serine kinase important in glucose and lipid metabolism (127).

2.7.B. Skeletal Muscle

Skeletal muscle tissue is responsible for the majority of glucose disposal (128). Glucose uptake is stimulated by insulin, but can also occur in the absence of insulin,

highlighted by exercise-stimulated glucose uptake (129). In either case, glucose uptake requires the translocation of GLUT4 to the cell membrane through an AKT-mediated pathway (129). Similar to the liver, glucose is trapped in the muscle cell through phosphorylation by hexokinase to create glucose-6-phosphate (G6P). However, unlike the events in hepatocytes, this is not substrate-driven and is dependent more on insulin-regulated intracellular events that control the translocation of GLUT4 (130). Once in the muscle cell, the majority of glucose is either oxidized or stored as glycogen (131). Glucose that is being metabolized for energy first undergoes glycolysis to generate pyruvate, a process where PFK is the rate-limiting enzyme (131). For complete oxidation, pyruvate enters the Kreb's cycle and the electron transport chain, of which both citrate synthase and cytochrome c oxidase are considered the rate-limiting enzymes, respectively (132, 133). Glucose that is diverted for storage is also first phosphorylated by hexokinase to form G6P. The formation of glycogen from G6P is regulated by glycogen synthase (131). This process is regulated by insulin, however, the exact mechanisms are still under debate (134).

Skeletal muscle accounts for the largest portion of glucose uptake. Exogenous treatment with GCs decreases glucose uptake in skeletal muscle (135). GCs do not affect insulin binding (135-137) and, surprisingly, increase total GLUT4 content (138-140), indicating the mechanism behind decreased glucose uptake is due to the transduction of the intracellular signalling pathway. Accordingly, GCs decrease the docking of PI3K to IRS proteins (135, 136, 141), decrease the activity of PI3K (135, 142), decrease the phosphorylation of AKT (135), and decrease the translocation of GLUT4 (143, 144). Treating healthy myocytes with GCs decreases the expression of both GR and 11βHSD1,

likely to elicit a protective mechanism that decreases GC action (145). A failure to downregulate the expression of these proteins may play a role in the development of the metabolic syndrome (146).

Positive correlations exist between the expression of either GR or 11βHSD1 and markers of prediabetes; insulin resistance, body mass index, percent body fat, and blood pressure (146). Blocking GC action with RU486, a GR antagonist, causes these relationships to disappear (146). Increased GC action in skeletal muscle has also been reported in individuals with T2DM (147). Inhibition of 11βHSD1 in diabetic myotubes improves insulin sensitivity and glucose uptake (147). Reduction of GC action in skeletal muscle, potentially through targeted reduction of either GR or 11βHSD1, may have therapeutic effects on the treatment/prevention of T2DM.

2.7.C Liver

Maintenance of euglycaemia levels is one of the major functions of the liver, and is accomplished mainly through two methods; 1) increased uptake of glucose and storage into glycogen, and 2) decreased gluconeogenesis and hepatic glucose output. Post-prandial, when glucose levels are high, the liver removes approximately 1/3 of the glucose from the blood. The uptake of glucose into the liver occurs through a GLUT2 transporter and is directly related to the concentration of both glucose and insulin in the blood (148). The liver is unique compared to muscle and adipose tissue, in that control of glucose metabolism is more tightly regulated by substrate availability (148). The levels of both insulin and glucose regulate the expression and activity of both hexokinase and glucose-6-phosphatase (G6Pase), key enzymes that control glucose metabolism in the

liver (149). Hexokinase activity is increased, causing glucose to be phosphorylated and consequently trapped within the hepatocyte. G6Pase is responsible for dephosphorylating glucose, to allow its exit from the cell; thus, G6Pase is inhibited by high levels of insulin and glucose. Finally, the activity of both phosphofructokinase (PFK) and glycogen synthase are increased by insulin, which stimulate the conversion of glucose to glycogen for storage (149). At the same time, insulin signaling causes the phosphorylation of FOXO1, a transcription factor involved in the regulation of phosphoenolpyruvate carboxykinase (PEPCK) and G6Pase expression, the two rate-limiting enzymes in gluconeogenesis (150). Insulin stimulation of the AKT pathway leads to phosphorylation of FOXO1 and causes it to be exported out of the nucleus, thus decreasing the expression of PEPCK and G6Pase and subsequently decreasing gluconeogenesis (149).

Decreasing hepatic glucose output reduces hyperglycaemia and is therefore a popular target of pharmacological intervention (151, 152). Insulin inhibits gluconeogenesis by decreasing the expression and activity of PEPCK and G6Pase, the two key regulatory enzymes involved in gluconeogenesis (153). Glucocorticoids oppose the action of insulin by increasing the expression of these enzymes (154-156). Rodent models of T2DM display elevated levels of both GR and 11βHSD1, both under basal conditions and in response to elevated glucose concentrations (157). Cultured hepatocytes from these mice demonstrate a robust increase in PEPCK when treated with corticosterone, which is blocked when co-incubated with RU486 (157). Antagonism of hepatic GC action has been shown to improve insulin sensitivity in various models of insulin resistance (158-160). Although insulin treatment decreases the expression of 11βHSD1 in healthy rats, this does not occur in rodent models of T2DM, likely due to

hepatic insulin resistance (161). Furthermore, 11βHSD1 knockout mice have decreased expression of both PEPCK and G6Pase, and subsequently have an attenuated glucose response to either stress or high-fat feeding (162). Increasing GC action specifically in liver reveals a non-obese, insulin resistant phenotype (163). Finally, the use of selective and non-selective 11βHSD1 inhibitors improves hepatic insulin sensitivity and reduces glycaemia (45, 164, 165). Thus, similar to skeletal muscle, a reduction in GC activity in the liver appears to improve insulin sensitivity and glucose tolerance.

2.7.D. Adipose Tissue

Insulin regulates two major pathways in white adipose tissue (WAT); 1) stimulation of glucose uptake for the formation of triacylglycerol (TAG), and 2) inhibition of lipolysis.

The formation of TAG involves the esterification of fatty acids to glycerol-3-phosphate (G3P). Most of the fatty acids utilized come from circulating plasma pools and reuptake of released FFAs from lipolysis. Another source of FFAs comes from the breakdown of circulating TAGs stored in lipoproteins. Lipoproteins are broken down to release FFAs by lipoprotein lipase (LPL), a process which is enhanced by the presence of insulin (166). G3P is sourced from either glycolysis or glyceroneogenesis, as adipocytes have very low glycerokinase activity, the enzyme required for the reuptake and utilization of released glycerol from lipolysis (167). Insulin also activates the PI3K-AKT pathway in adipocytes (168) and increases the uptake of glucose through either GLUT1 or GLUT4 transporters (169). This glucose enters glycolysis to eventually become G3P, which then is esterified to FFAs, a precursor for the formation of TAG. Glyceroneogenesis is an

abbreviated version of gluconeogenesis that provides G3P from substrates such as lactate and pyruvate (167) and is controlled by the expression of PEPCK (170). Insulin, PPARs, and glucocorticoids are some of many circulating factors that regulate the expression and activity of PEPCK (170). Insulin not only stimulates adipogenesis through the formation and storage of TAGs, but also through the inhibition of lipolysis. The rate of lipolysis is governed largely by the activity and expression of hormone sensitive lipase (HSL) and adipose triglyceride lipase (ATGL), the major lipolytic hormones (171). Insulin decreases the activity of both of these enzymes, however, the mechanism that regulates HSL is more defined than that which regulates ATGL (171). There is some evidence suggesting that high concentrations of insulin can increase lipolysis through an AMPK mediated mechanism (172, 173).

It is well established that excess fat accumulation is a strong predictor of both insulin resistance and the development of T2DM (46, 174). However, it remains unknown if GC activity in adipose tissue is increased prior to, and thus may contribute to, the onset of obesity, or if GC activity is increased because of obesity. Increased adipose mass alone does not predict abnormal GC activity in adipose tissue (175, 176), in fact, obese individuals that undergo diet-induced weight loss show decreased 11βHSD1 activity (177). However, transgenic mice with increased GC action selectively in adipose tissue become obese and develop the metabolic syndrome (18). Decreasing GC action in adipose tissue also confers protection against diet-induced obesity (178, 179). Similar to other insulin target tissues, GCs stimulate insulin resistance in adipose tissue (180). This may be, in part, mediated through direct and indirect effects on circulating adipokine concentrations (181, 182). Increasing GCs in adipose tissue stimulates the release of

TNFα and decreases the release of adiponectin (18), whereas decreased GC action is associated with reduction in both resistin and TNFα (179). Thus, it appears that similar to skeletal muscle and liver tissue, reducing GC action in adipose tissue may be protective against obesity and insulin resistance. However, exercising hamsters demonstrated an increase in 11βHSD1 activity in adipose tissue, despite a decrease in adipose mass (80). Furthermore, decreased obesity through caloric restriction is also associated with increased 11βHSD1 expression and activity (177). Therefore, GCs may play a role in reducing adipose tissue mass, which would be expected to be protective against insulin resistance. Thus, the role GCs play in adipose tissue metabolism appears to be controversial, and will be discussed in following sections as it is the major focus of a portion of this dissertation.

2.8. Adrenalectomy, GR Antagonists and 11βHSD1 Inhibitors

The close association between chronically elevated GCs and the development of insulin resistance and T2DM has led to a number of strategies for reducing GC action. Early studies in rodents used adrenalectomies to completely remove endogenous production of GCs and have studied the effects on glucose tolerance (158, 183-185). Adrenalectomy in ob/ob mice, a leptin-deficient mouse with a similar phenotype to ZDF rats, reduces body weight, food intake, and glucose levels to values comparable with lean mice (186). Adrenalectomy confers similar positive benefits in mice that have been chemically induced to have a T2DM phenotype (187). Specifically, adrenalectomy improves insulin sensitivity in both skeletal muscle and liver tissue (188), and the removal of a single adrenal gland improves whole body glucose tolerance in humans

(189). However, it is understandable that adrenalectomy is not a viable solution to counteract the epidemic of obesity and T2DM, given that the gland controls the secretion of several hormones that control metabolism, water balance, and the stress response. Consequently, less invasive methods focusing on reducing GC action at the level of the tissue have been proposed. RU486, also termed mifepristone, is a steroid that competitively inhibits GCs binding to GR. Administration of RU486 ameliorates the diabetic phenotype in ob/ob mice (190), db/db mice (mice with mutated leptin receptors) (157), models of pharmacologically induced diabetes (191), Cushing's syndrome (192), and in diet-induce obesity (193). However, RU486 is not specific to GR and also antagonizes the action of MR, resulting in significant disruption of water balance. In response to this, GR specific antagonists are being developed for the treatment of diabetes (194).

11βHSD1 inhibitors represent another method for reducing GC action in tissue. Early studies using non-selective 11βHSD inhibitors demonstrated the potential for a glucose lowering effect in humans with T2DM (45). Since then, the use of various selective 11βHSD1 inhibitors have been successfully used to prevent and reverse the development of T2DM in a variety of rodent models of insulin obesity and resistance (164, 195-198). Although recent reports have shown that selective 11βHSD1 inhibitors improve insulin resistance in human cell cultures (199), no studies have reported the use of these drugs in humans subjects.

3. Glucocorticoids and Exercise

An acute exercise bout has long been known to cause activation of the HPA axis and elevate circulating GCs levels, perhaps to help mobilize fuel sources for the "fight or flight" response (200). Given the above discussion that GCs facilitate the insulin resistant/diabetic phenotype, it is intriguing that regular exercise is of critical importance in the prevention or treatment of insulin resistance and T2DM (201). Accordingly, many studies have utilized various exercise training protocols in rodents to study the physiological and cellular processes related to HPA axis activation and adaptation that occurs with regular physical activity (202). However, before discussing experimental interventions involving exercise training, it is important to note the validity and representative quality of the control group. The control group typically consists of animals housed in standard rodent cages and fed corn- or soy-based diets, *ad libitum*. Physical movement is limited and food intake may be in excess, and thus, such animal can be considered sedentary and overfed (67). Sadly, this is reflective of the majority of the population in areas where T2DM has increased significantly (43). Consequently, it has been proposed that inactivity reflects a diseased state (203, 204), questioning whether the control groups used in exercise studies, and the human subjects they are representative of, can be considered 'healthy controls'. Since a majority of this thesis is related to the adaptive effects of exercise on the HPA axis, a further discussion of the modes of exercise training is provided below.

3.1. Forced vs. Volitional Exercise

The majority of forced exercise protocols can be categorized into 3 variations: 1) rodents running on a treadmill, encouraged by foot shocks at the base of the treadmill (205, 206); 2) forced swimming, often with weighted attachments (207, 208); and 3) more creative approaches such as ladder climbing, again with electrical shock as the motivator (209, 210). Forced exercise provides the advantage of precisely controlling the amount, intensity, and duration of exercise being performed. However, forced exercise invokes fear to motivate the animal to exercise (e.g., electrical shock or drowning). Furthermore, these studies are typically carried out during the daytime, when the animals would normally be sleeping and their diurnal GC levels would typically be at a nadir (211). As a result, forced exercise induces many changes that are also found with chronic stress; adrenal hypertrophy, decreased serum corticosteroid-binding protein, suppressed lymphocyte proliferation, suppressed immunoglobulin M, and increased plasma corticosterone (212, 213). This makes it difficult to delineate the effects of exercise from those due to stress when studying aspects of the HPA axis and GC biology.

Volitional wheel running enables researchers to study the effects of exercise on the HPA axis without causing psychological/circadian stress to animals. This involves attaching a running wheel to a standard rodent cage and allowing the animal to exercise voluntarily, which typically occurs at the onset of the dark cycle (214). The volume of exercise is quantified, but not controlled, by counting the number of wheel revolutions. Mice, rats, squirrels, hamsters, and even rabbits will all voluntarily run in a wheel apparatus (215-217). However, it is difficult to regulate the quantity of exercise, and impossible to regulate the intensity of the exercise amongst individual animals. The

quantity of exercise appears to depend on the species of animal (79, 214, 218), as well as the age of the animal, with younger animals running more than older (79, 219). When placed in a running wheel environment, hamsters reach maximal activity almost immediately (usually >10 km/day) while rats increase their running activity with time and need about 4 weeks to reach an average of about 7 km/day (220). Interestingly, rodents will bar press for wheel access, which suggests that they are motivated to engage in this behavior (221). Thus, contrary to forced exercise, volitional wheel running appears to be a rewarding 'stress' for animals.

3.2. Acute Exercise and Glucocorticoid Biology

The effects of a single bout of exercise on HPA activity are related to the intensity and duration of the exercise. Light to moderate exercise does not induce a noticeable release of GCs, whereas intense exercise stimulates the release of GCs in an intensity related manner (222). Interestingly, when exercise intensity becomes exhaustive, GC levels tend to decrease, perhaps in a protective mechanism to prevent depletion of energy stores (222). It terms of duration, GC levels remain elevated for up to two hours following the cessation of exercise (223). For example, marathon runners exercising for ~3 hours are exposed to elevated GC levels for 5+ hours (223).

The primary function of GCs during exercise is to increase the mobilization of fuel sources, in order to provide energy to skeletal muscle and prevent hypoglycaemia. Adrenalectomized rats undergoing four hours of swimming have less of a glycogen drop in liver and oxidative muscle compared to normal rats, however, they also display severe hypoglycaemia (224). Supply of exogenous GCs to the adrenalectomized rats during

exercise corrects for both of these events (224). The direct effect of GCs on the regulation of fat metabolism during an acute bout of exercise appears to be less important, as adrenalectomized animals do not have impaired lipolytic rates during exercise, measured by the release of FFAs from adipose tissue (225). However, it is unknown if the duration and intensity of exercise affects this relationship, as the adrenalectomized animals in that study were not exercised under conditions that would elicit maximal utilization of fat substrates. Furthermore, activation of the HPA axis during acute exercise may increase lipolysis through elevations in systemic ACTH concentrations, which act on adenylate cyclase to stimulate lipolytic signalling in adipocytes (226). The effect of acute exercise on peripheral tissue exposure to GCs is currently unknown. One study suggested that an acute bout of swimming reduced the expression of GR in renal and myocardial cytosol, however, no reports on the functional relevance of these adaptations were made (227). In isolated blood monocytes from endurance trained men, acute exercise has been shown to cause decreased sensitivity to GCs, perhaps to shut off the resulting muscle inflammatory reaction and cytokine synthesis (228). No studies have reported the effects of a single bout of exercise on the central components of the HPA axis or on GC action in peripheral insulin target tissues.

3.3. Chronic Exercise and Glucocorticoid Biology

Since GCs are steroid hormones that elicit the majority of their effects through genomic actions, it is not surprising that many of the adaptations induced by exercise require a long duration and/or sustained stimulation, as seen with training. One example of the interaction between sustained exercise training and GCs can be seen in the liver.

Exercise training increases the expression of PEPCK, a key regulator of hepatic glucose output, and appears to be dependent upon GC release. Although adrenalectomy alone does not change the expression of PEPCK, adrenalectomized animals undergoing exercise training demonstrate a severely blunted increase in PEPCK expression compared to healthy animals (229). Furthermore, providing exogenous GCs during exercise training rescued this and resulted in comparable PEPCK levels (229). Exercise training also increases the expression of G6Pase, another key regulator of hepatic glucose output, and also appears to be dependent upon GC action (230). Studies have suggested that an exercise-mediated increase in GCs affect the liver by increasing the potential for hepatic glucose output, likely to increase the availability of glucose during exercise. However, sustained (or chronic) hyperactivity of the HPA axis which can result in uncontrolled hepatic glucose output can lead to hyperglycaemia and the development of T2DM. In addition to this, increased activity of the HPA axis has been associated with many other pathologies common to T2DM; insulin resistance (146), increased adiposity (18), immune suppression (231), depression (232), osteoporosis (232), muscle wasting (233) and cardiovascular disease (234, 235). Because exercise elevates GCs both during exercise and for hours following cessation of exercise (223, 236), regular training would result in prolonged phases of endogenous hyperglucocorticoidemia. However, exercise certainly does not induce any of the above mentioned phenotypes and, with the exception of over-training syndrome, leads to positive adaptations and protects against insulin resistance. This has led to the hypothesis that a number of adaptive mechanisms occur in response to sustained exercise training, that decrease both the peripheral sensitivity to GCs and the activity of the HPA axis to protect against hyperglucocorticoidemia (237).

Much of the literature has focused on the effects of exercise on the regulation of the HPA activity. Early studies utilized a forced exercise protocol (swimming or treadmill running) to show varying results within the HPA axis. Some studies show reduction in HPA activity with sustained exercise training (238), others show increased activity (239), while still others report both depending on the stressor activating the axis (240-242). As discussed earlier, using forced exercise protocols makes it difficult to interpret the results, and this is likely the reason for these inconsistent findings. Interestingly, various studies utilizing a volitional wheel running model have reported no changes on diurnal GC patterns after 4-6 weeks of sustained training (214, 243-246). However, volitional wheel running over a shorter time frame appears to increase HPA axis activity (214). Therefore, it appears that voluntary exercise initially disrupts normal HPA function, but that with sustained training this is ameliorated. The mechanism behind these events still remains vague and largely unknown.

Recent studies have focused on the individual components of the HPA axis to better understand the effects of exercise on GC release. In the hypothalamus, 4 weeks of voluntary wheel running (~ 4 km/day) in mice was shown to decrease the mRNA and protein levels of CRH (243, 245), suggesting that exercise training reduces HPA activity at the level of the hypothalamus. Interestingly, the same group showed that 4 weeks of wheel running in rats (~4-7 km/day) does not change the level of CRH mRNA found in the hypothalamus, and also reported that that GR mRNA levels were unchanged with exercise (244). It is unclear if this is due to species differences, or because the rats ran a slightly greater distance per night in the later study thus facilitating a greater drive of the central components of the HPA axis. Taken together, volitional exercise appears to either

decrease or have no effect on the hypothalamic contribution to HPA activity. Contrary to this, forced exercise may increase hypothalamic activity, at least transiently. Swim training (45 min/day) in rats caused a decrease in hypothalamic GR mRNA from after 2 weeks, which continued to remain below control animals up to 6 weeks (247). This decrease in GR was accompanied by a transient increase in CRH content during the same period of time (247). The levels of GR and CRH were not different between trained and control animals after 6 weeks. From this, it appears that volitional exercise training either has no impact, or decreases, hypothalamic activation of the HPA axis, whereas forced exercise temporarily increases this action.

The effects of exercise at the level of the pituitary also appear to be dependent on the type of training. Treadmill training in rats results in an increased release of both basal and stimulated ACTH (241, 248), suggesting an increase in pituitary contribution. Swim training causes a transient decrease in pituitary GR mRNA, suggesting that this increase in pituitary contribution may be due to impaired negative feedback (247). In a human study, elite endurance trained men who consistently ran 50-70 km/week were resistant to GC suppression during the combined dexamethasone-CRH test, suggesting a decrease in pituitary sensitivity to negative feedback signals (249). A similar group of athletes had elevated morning ACTH levels compared to untrained men (250), also indicating increased pituitary action. From this, it appears that strenuous exercise training (i.e., forced exercise protocol or very high intensity) decreases negative feedback at the level of the pituitary and results in increased systemic levels of ACTH and subsequent increases in GC levels. Contrary to this, volitional training in rodents preserves the negative feedback mechanism at the pituitary, maintaining normal ACTH and GC levels.

4 weeks of wheel running in rats does not alter pituitary levels of GR mRNA (244) or protein (214), indicating that negative feedback is preserved in the pituitary. In turn, trained rats do not differ from sedentary rats with respect to basal ACTH or GC levels (214, 244). Interestingly, wheel running in rats increases the transcription of POMC, the ACTH/melanocortin precursor, but this increase does not translate to an increase in ACTH levels (251), thus further supporting that volitional exercise may increase HPA negative feedback functioning at the level of the pituitary.

In addition to the apparent controversy about the effects of regular exercise on the central components of the HPA axis (i.e., brain and pituitary gland), the effects of training on the adrenal glands are also unclear. Six weeks of treadmill training decreases adrenal sensitivity to ACTH in rats (241), possibly as a compensatory mechanism to counteract the increased hypothalamic-pituitary drive seen in these animals (241). In humans, highly trained men who run 50-70 km/week show no differences in adrenal sensitivity to ACTH compared to sedentary men (236). However, another study showed that competitive athletes demonstrate a more robust release of cortisol compared to sedentary controls in response to a single bout of strenuous exercise, despite similar ACTH levels (252). This finding suggests that strenuous training can increase adrenal sensitivity to ACTH (252). Volitional wheel running in rodents also seems to increase adrenal sensitivity to ACTH at least during the first few weeks of training. After 4 weeks of wheel running, compared to sedentary mice, trained mice have similar basal ACTH levels, but a 2-fold higher increase in CORT concentrations (245). Furthermore, trained mice respond to a cross-stressor (i.e., restraint stress, cage-switch) with a higher CORT:ACTH ratio compared to untrained mice (243, 245, 253). Similar findings have

been reported in rats trained with wheel running in the short term (214, 244). The proposed mechanism behind the increase in adrenal sensitivity to ACTH with chronic voluntary exercise is an increase in steriogenic acute regulatory (StAR) protein expression within the adrenal glands (254). StAR is responsible for cholesterol shuttling to the mitochondria and is the rate-limiting step in steroidogenesis (255). Although this was only shown with 2 weeks of training, where CORT levels in the trained animals were substantially elevated compared to sedentary animals. This mechanism still needs to be tested in conditions of prolonged training (> 4 weeks), when CORT levels do not differ between groups (214, 243-245).

Although the effects of exercise training on the various components of the HPA axis is still unclear, even less attention has been given to the effects of training on peripheral GC action. As mentioned in the above section (2.7), peripheral GC action is largely determined by the tissue expression of both GR and 11βHSD1 (24). As mentioned above, compared to sedentary men, endurance trained men have decreased sensitivity to exogenous GCs in their blood monocytes immediately following exercise (228), and the expression of GR in renal and myocardial tissue is decreased following chronic training in rats (227). Yet, in order to better understand the relationship between GCs, exercise, and T2DM, it is important to determine the effects of exercise on GC action in insulin target tissues; liver, skeletal muscle, and adipose tissue. One study reported that 5-7 weeks of treadmill training in rats decreases the expression of GR in liver tissue (256). Coutinho et al., found that 4 weeks of wheel running in hamsters had no significant effect on the expression of GR in liver, but did decrease hepatic expression of 11βHSD1 (80). In another study, eight weeks of treadmill training prevented

hyperinsulinemia, muscular glycogen loss, and muscle atrophy induced by dexamethasone treatment in rats, suggesting that GC action in skeletal muscle had been reduced (257). Treadmill exercise training had a protective effect against the cardiometabolic alterations induced by the chronic use of glucocorticoids, indicating reduced GC action in cardiac muscle (258).

Limited studies have been conducted on the effects of exercise on skeletal muscle GC exposure. In one study, Coutinho et al., (80) reported that 4 weeks of wheel running in hamsters decreases both GR and 11βHSD1 in skeletal muscle. These findings suggest that exercise training has the potential to decrease GC exposure in skeletal muscle and liver; two major tissues involved in glucose metabolism. However, no studies have identified the significance these potential adaptations have in protecting against the development of insulin resistance and/or T2DM. Furthermore, intense physical exercise increases systemic 11βHSD1 activity in humans (259), indicating the protective effect of exercise may be limited to specific exercise intensities. However, the increase in whole body 11βHSD1 activity, as measured by urinary GC metabolites in a later study by Dovio et al., (259) may not be reflective of what is occurring in each of the GC target tissues. One tissue that requires further investigation is adipose tissue, where 11βHSD1 activity increases GCs regeneration to the systemic circulation (260). Indeed, we have previously reported that high volume exercise in hamsters increases 11βHSD1 activity specifically in adipose tissue (80). It is odd that adipose tissue would respond to exercise training opposite to what is seen in skeletal muscle and liver tissue, as GC overexposure in adipose tissue also results in detrimental metabolic actions (18). No studies have elaborated on the functional relevance of increased GC action in adipose tissue in

response to exercise training. In fact, the effects of GCs in adipose tissue are controversial and lack clear definition.

4. Glucocorticoids and Adipose Tissue Metabolism

4.1. Lessons from the Cushing's Phenotype

Cushing's syndrome results from a chronic elevation of GCs, usually due to the presence of a tumour somewhere along the HPA axis (234). Individuals with Cushing's syndrome present many similar pathologies found in the metabolic syndrome and T2DM: hypertension, dyslipidemia, insulin resistance, and profound adiposity (234). Interestingly, Cushing's syndrome demonstrates a phenotype of central adiposity, particularly in the visceral regions and face (234). It is the remarkable similarities between the phenotype of Cushing's syndrome and T2DM that has led to the hypothesis that GCs play a detrimental role in the development of insulin resistance and diabetes. However, individuals with T2DM do not consistently present elevations in circulating GCs. Consequently, the metabolic syndrome and T2DM has been coined tissue-specific Cushing's syndrome (261), where increased expression of GR and 11βHSD1 causes intracellular concentrations of GCs to reach pathological levels. Although increasing GC concentrations, specifically in skeletal muscle or liver tissues, does cause glucose intolerance, other features of the metabolic syndrome/Cushing's syndrome are not present, including obesity (145, 146, 157). On the other hand, increasing GC concentrations specifically within adipose tissue creates a phenotype that is remarkably similar to the metabolic syndrome/Cushing's syndrome (18). Mice overexpressing 11βHSD1 specifically in adipose tissue develop central obesity, hypertension,

dyslipidemia, and severe insulin resistance (18). Therefore, it appears that adipose tissue may be among the most critical tissues responsible for the development of a Cushing's syndrome phenotype. Still, the role of GCs in the adipose tissue of Cushing's patients is unclear. The severe obesity indicates an anabolic role for GCs, yet the rate of lipolysis in Cushing's patients is elevated (262), indicating that a catabolic role is also present. Also, no reports clarify why individuals with Cushing's syndrome display a drastic increase specifically within visceral adipose depots, rather than subcutaneous depots. Thus, although Cushing's syndrome highlights the importance of GCs in adipose tissue biology, there is still much to be resolved.

4.2. Visceral Adiposity and Insulin Resistance

The phenotypes of both Cushing's syndrome and T2DM suggest that visceral adiposity is more detrimental to health than subcutaneous adiposity. Studies have shown that visceral adipose accumulation is a major risk factor for the development of insulin resistance and the metabolic syndrome (47). Currently, there are two proposed mechanisms to explain the detrimental effects of increased visceral adiposity; 1) visceral adipocytes secrete more negative factors into the circulation and 2) visceral adipose depots drain into the portal circulation ("portal hypothesis") (263). The first hypothesis is based on the idea that visceral adipocytes have a higher rate of basal and stimulated lipolysis compared to subcutaneous adipocytes (264, 265). Increased visceral adiposity would also increase the circulating concentrations of 'bad' adipokines, such as TNF-α, IL-6, and resistin, which are released in greater proportion by visceral adipose depots (266-268). Finally, visceral adipocytes have the highest activity of 11βHSD1 (269) and

have been shown to generate and release active GCs, increasing the concentrations in the systemic circulation (260). Therefore, increased visceral adiposity may lead to increased circulating FFAs, harmful adipokines, and GCs, all of which have been shown to be associated with the development of insulin resistance and T2DM.

The second mechanism builds upon the first and stems from the anatomical position of visceral adipose tissue, such that the venous drainage goes directly into the liver. Therefore, the portal hypothesis suggests that increased visceral adiposity substantially increases the concentration of negative factors (FFAs, adipokines, GCs) in the portal circulation, thereby causing insulin resistance in the liver. This may explain why some studies fail to find increases in FFAs, adipokines, and GCs in the systemic circulation of obese/insulin resistant individuals (270). The liver may be taking up these factors released by visceral adipocytes, thereby preventing their accumulation in the systemic circulation. Indeed, blood sampled directly from the portal circulation shows higher concentrations of FFAs and adipokines (263) and GCs (18) compared to systemic concentrations. Therefore, under conditions of high visceral adiposity, one would expect that hepatic insulin resistance would precede the development of whole body insulin resistance, and ultimately contribute to the progression towards a T2DM phenotype. This has yet to be conclusively shown.

4.3. Glucocorticoids and Lipolysis

Complete lipolysis is the hydrolysis of a triacylglycerol (TAG) into 3 FFAs and glycerol. Each of the FFAs released during this process can be either released into the vasculature or re-esterified back into the original adipose tissue. Re-esterification into adipose tissue, classified as primary re-esterification, creates a futile cycle that is constantly taking place, even during basal, unstimulated states. Under fasting conditions, approximately 40% of hydrolyzed FFAs undergo primary re-esterification into adipose fat (271). The remaining 60% that escape into the plasma are either oxidized for energy or undergo secondary re-esterification in other tissues (liver, muscle, other adipose depots). Hormone sensitive lipase (HSL) was discovered almost 50 years ago and was appropriately named due to the enzymes role in hormone driven lipolysis (272). The role of HSL in lipolysis has been extensively studied since its discovery, and the signaling pathway has been well defined (116, 273, 274). Consequently, HSL had previously been considered the rate-limiting enzyme in TAG lipolysis. However, recent generation of HSL knockout mice illustrated a non-obese phenotype with blunted catecholamine-induced lipolysis but normal unstimulated (or basal) lipolysis (275, 276). These mice have an accumulation of diacylglycerol (DAG) in the adipose tissue, suggesting a different enzyme is present and capable of hydrolyzing TAG. It has now been proposed that HSL is the rate-limiting enzyme for DAG lipolysis and is only necessary for efficient lipolysis (171). Adipose triglyceride lipase (ATGL) is predominately expressed in adipose tissue and has been proposed to be the rate-limiting enzyme in TAG hydrolysis (277, 278). ATGL is a cytosolic protein localized to the intracellular lipid droplets (277, 278). Stimulation with isoproterenol, a beta-adrenergic agonist with similar effects as

epinephrine, does not change the location of ATGL. This suggests that ATGL is always associated with the lipid droplet regardless of basal or stimulated states, opposite of HSL which translocates towards the lipid droplet following stimulation. Because ATGL is always proximal to the lipid droplet, it may be more involved than HSL in basal lipolysis. Overexpression of ATGL in 3T3-L1 adipocytes increased basal lipolysis, while silencing gene expression with siRNA or antisense-RNA significantly decreased basal lipolysis (277, 279). In contrast to this, silencing HSL in 3T3-L1 adipocytes had little to no effect on basal lipolysis (279).

The role of GCs in adipose tissue lipolysis is controversial and represents somewhat of a dichotomy. Most of the *in vitro* studies investigating the direct effects of GCs on adipocytes state that the hormone is lipolytic (280-282). Others claim that cortisol is antilipolytic in human adipose tissue (283). The mechanism behind GC action in adipose tissue has not been resolved. Some evidence has been provided that GCs increase the expression of HSL in rodent primary adipocytes (280). Others have shown that the combination of growth hormone and dexamethasone slightly increase adenylate cyclase and protein kinase A (PKA) activity (281). GCs have also been shown to increase the expression of ATGL in a dose-response manner; however the impact of this on lipolysis was not measured (278). Therefore, more conclusive evidence is required to determine the role of GCs in adipose tissue lipolysis.

4.4. Glucocorticoids and Adipogenesis

During times when energy intake is in excess of energy expenditure, adipocytes undergo fatty acid and triglyceride synthesis in order to store surplus energy. The energy

is ultimately stored in lipid droplets within adipocytes in the form of triglycerides. The fatty acids that undergo esterification with glycerol to generate the triglyceride come either directly from the circulation, or are created within the adipocyte. FFAs circulate in the plasma mostly in the form of TAGs packed into lipoproteins and non-esterified fatty acids (NEFAs) bound to albumin. Lipoproteins are unable to directly enter adipose tissue. Instead, they must be first hydrolyzed within the capillary by lipoprotein lipase (LPL). LPL is a lipase enzyme expressed by the adipocyte and located such that the catalytic portion of the enzyme is located in the lumen of the capillary. FFAs enter the adipocyte where they are readily esterified into TAGs. Adipocytes also have the ability to synthesize new fatty acids, which can then be esterified into TAGs. The essential substrate required for *de novo* fatty acid synthesis is acetyl-coenzyme A (CoA) which comes from the metabolism of glucose. The rate-limiting enzyme for *de novo* fatty acid synthesis is fatty acid synthase (FAS). Intracellular fatty acids are quickly esterified into glycerol-3-phosphate (G3P) to begin the process of creating a TAG. Adipocytes do not have glycerol kinase and must obtain G3P from glucose. Therefore, the uptake of glucose into adipocytes is essential for lipogenesis. In addition to lipogenesis, which results in hypertrophy of adipocytes, adipogenesis can also occur when preadipocytes differentiate into mature adipocytes, and thus increase the number of cells (hyperplasia). Much of the insight into preadipocyte differentiation came from experiments conducted in 3T3-L1 fibroblasts. Maximal differentiation of preadipocytes occurs with the combination of insulin, a glucocorticoid, an agent that elevates intracellular cAMP levels, and fetal bovine serum (284). This cocktail increases the expression of c-fos, c-jun, junB, c-myc, and CCAAT/enhancer binding proteins (C/EBP) β and δ, all essential for the

differentiation process (285). The activity of C/EBP β and δ is thought to mediate the expression of peroxisome proliferator-activated receptor γ (PPARγ) (286, 287). PPARγ activation is essential for adipocyte maturation and treating preadipocytes with rosiglitazone alone (a synthetic PPARγ agonist) can drive differentiation (288, 289). Overall, an increase in adipose tissue mass can occur through either mechanism, lipogenesis and/or preadipocyte differentiation.

Although GCs are essential in standard differentiation of preadipocytes and GC excess results in adipose tissue accumulation (18), there are very few studies examining the effects of GCs on adipogenesis. Several studies have described the role of GCs in 3T3-L1 differentiation (290-293), but no studies have attempted to replicate these results *in vivo*. Some believe the effects of GCs on adipogenesis are indirect; occurring secondary to the increase in glucose and FFAs in the circulation caused by GCs (294). Several studies are content to make the observation that increases in circulating GCs are associated with the development of obesity and the metabolic syndrome (18, 178, 180, 295). Yet, to date, there are no studies that look at the direct effects of GCs on adipocytes or preadipocytes in attempt to understand the mechanism for how GCs stimulate adipogenesis and increase obesity.

MANUSCRIPT #1 – EXERCISE AND THE HPA-AXIS IN THE SPRAGUE- DAWLEY RAT

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Rationale for manuscript #1:

Much of the work focusing on the effects of exercise training on the HPA axis has shown that the major adaptations occur at the level of the adrenal glands. Other mechanisms that control the HPA axis have been reported to also change with training; however, the influence of the exercise on these processes is relatively moderate in comparison to the effects on the adrenal glands. Studies have shown that many types of exercise training modalities alter the ACTH:CORT ratio in rodents and humans, suggesting a change in adrenal sensitivity to ACTH. However, only one study has attempted to elucidate the mechanism controlling this adaptation (254). Furthermore, no studies have directly tested adrenal sensitivity to ACTH following exercise training using a standardized ACTH challenge (a gold standard for measuring adrenal sensitivity to ACTH in a clinical setting). Finally, the effects of exercise training also appear to be time-dependent, as short-duration training (< 4 weeks) causes hyperactivation of the HPA axis, whereas long duration training (>4 weeks) does not. Therefore, the goal of this study was to determine the effect of voluntary wheel running on the HPA axis at the level of the adrenal glands, through direct measurement of adrenal sensitivity to ACTH, with both short- and long-duration training protocols.

Author Contributions:

Conceived and designed the experiments: JEC (33%), NR (33%), MCR (33%). Trained the animals: NR (66%), JEC (33%). Performed the experiments: JEC (60%), NR (30%), SF (10%). Analyzed the data: JEC (60%), NR (15%), SB (15%), MCR (10%). Wrote the paper: JEC (60%), NR (10%), MCR (30%).

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Voluntary Wheel Running Initially Increases Adrenal Sensitivity to Adrenocorticotrophic Hormone, which is Attenuated with Long-term Training

Running Title: Exercise Transiently Increases Adrenal Sensitivity to ACTH

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Abstract

Although exercise is a common and potent activator of the hypothalamic-pituitary adrenal (HPA) axis, the effects on the acute stress response are not well understood. Here, we investigated the effects of short (2wk)- and long-term (8wk) voluntary wheel running on adrenal sensitivity to ACTH stimulation and the acute stress response to restraint in male rats. Diurnal glucocorticoid (GC) patterns were measured on days 7 (all groups) and 35 (8 wk groups). Rats were subjected to a 20 minute restraint stress protocol on either week one of treatment or on week seven of treatment to assess HPA activation. One week later, exogenous ACTH (75 ng/kg) was administered to assess adrenal sensitivity to ACTH. Following this, adrenal glands were collected and analyzed for key proteins involved in corticosterone synthesis. By the end of week one, exercising (E) animals had a 2-fold higher peak diurnal corticosterone (CORT) levels compared with sedentary (S) animals ($p<0.01$). CORT values were not different between groups at week 8. In response to restraint stress at week 2, CORT values in E were ~3-fold greater than in S ($p<0.05$). No difference was found between E and S animals in the response to, or recovery from, restraint stress at week 8. During the ACTH challenge at week 2, E demonstrated ~2.5 fold increase in adrenal sensitivity compared to S, while no difference was found between E and S at week 8. The expression of steriogenic acute regulatory (StAR) protein was found to be ~50% higher in the adrenal glands in E compared with S at week 2 ($P<0.05$), but no difference existed between groups at week 8. These results show that volitional wheel running initially causes hyperactivation of the HPA-axis, due to enhanced adrenal sensitivity to ACTH, but that these alterations in HPA activity are completely restored with 8 weeks of training.

Introduction:

The hypothalamic-pituitary-adrenal (HPA) axis is a vital component of the body's stress response system. This pathway is activated by a variety of stressors that increase the pituitary release of adrenocorticotropic hormone (ACTH) into the circulation. ACTH stimulates the adrenal release of glucocorticoids (GCs), which in turn mobilize energy sources for the increased metabolic demands placed on the body. Acutely, this recruitment of energy sources is essential for events such as physical exercise, however, restoration of the axis is imperative to prevent overexposure to GCs. It has been well established that chronically elevated levels of plasma GCs can lead to the development of a number of harmful pathologies, including impaired insulin signaling, central adiposity, skeletal muscle atrophy, immune suppression and type II diabetes mellitus (240, 250, 296, 297).

Exercise is a potent stressor, activating the HPA-axis in animals (214, 238, 243-245, 298) and in humans (299-302). The effects of regular exercise on diurnal GC levels and adrenal sensitivity to ACTH secretion are unclear. Previously, a number of rodent studies investigating the effects of training on the HPA axis have employed forced exercise paradigms for their method of training (241, 254, 298, 303). Such studies have produced findings that are similar to those of investigations employing chronic stress (e.g. repeated restraint stress) as an activator of the axis (302, 304), making it difficult to delineate the effects of perceived stress from exercise. Forced exercise studies have shown an enlargement of the adrenal glands, elevations in diurnal corticosterone (CORT) levels, decreased central (i.e. brain and pituitary) glucocorticoid receptor (GR) density and a reduction in the circulating ACTH to GC ratio (suggestive of a reduced adrenal

sensitivity to ACTH). In contrast, using a voluntary running wheel design, Dishman et al (246) found that 6-weeks of endurance training in rats attenuated the ACTH response to foot shock stress, but not the GC response, suggesting that an increase in adrenal sensitivity to ACTH occurs with training. This was one of the first studies to illustrate that volitional exercise may differ from forced exercise in the adaptive responses of the HPA axis. Employing a similar voluntary exercise model, Droste and colleagues (245) recently found that rats, subjected to 4 weeks of voluntary wheel running, had similar ACTH levels but higher GC levels during forced swim stress when compared to previously sedentary rats. Thus, unlike with forced exercise, voluntary exercise is thought to increase adrenal sensitivity to ACTH. In line with this hypothesis, we have previously shown that long-term wheel running (4 weeks) in rats yields a normal GC response to restraint stress despite attenuated ACTH levels (214). However, it is still unclear 1) if voluntary wheel running increases adrenal sensitivity to exogenous ACTH administration, 2) if the increased adrenal sensitivity to ACTH is sustained with prolonged training, and 3) how these apparent changes in adrenal sensitivity occur.

We hypothesized that voluntary exercise training transiently increases adrenal sensitivity to ACTH by acting through molecular changes in the adrenal glands. We also hypothesized that these adrenal adaptations are due to the novel stress of exercise and that long-term training would result in the return to pre-exercise conditions and better control of homeostasis. Therefore, the aim of this study was to: 1) reveal the effects of short- and long-term voluntary exercise training on the acute stress response to, and recovery from, a novel cross stressor, 2) directly measure adrenal sensitivity through administration of

exogenous ACTH and, 3) determine the influence of voluntary wheel running on key regulatory proteins involved in the secretion of GCs from the adrenal glands.

Methods:

Animals

Thirty-two, male, Sprague-Dawley rats (Charles River Laboratories Inc. Quebec, Canada), with initial weights of 175-200g, were equally divided into four groups consisting of two sets of sedentary (S) and two sets of exercise (E) trained animals (n=8 per group). In addition, a fifth set of animals (n=6) were sacrificed at day 0, to establish a baseline group for protein analysis. All animals had a seven-day habituation period to a 12-h light, 12-h dark cycle (lights on at 0800h and lights off at 2000h) in a temperature (22-23 °C) and humidity (50-60%) controlled room.

Design

Exercising rats were singly housed in standard rodent cages (height:36.4 cm, width 26.8 cm, depth: 50 cm) for either 2 weeks (E2, n=8) or 8 weeks (E8, n=8), and were given 24-hour access to a standard running wheel (Harvard Apparatus). Wheel revolutions were counted daily and multiplied by the wheel circumference (106 cm) to obtain daily running distances. The sedentary groups were singly housed in similar cages for either 2 weeks (S2, n=8) or for 8 weeks (S8, n=8), but without a running wheel. All animals were given food (Ralston Purina Co.) and water *ad libitum*. All experiments were approved by the York University Animal Care Committee, Toronto, Canada.

Cannulation Surgery:

Cannulation surgeries were performed on either the day prior to the introduction to the running wheels (S2 and E2 rats) or after six weeks of treatment (S8 and E8 rats) as

previously described (214). Briefly, all animals had a microrenathane tube (MRE O40, 0.040" O.D. x 0.025" I.D., Braintree Scientific, Inc., MA, USA) inserted retrograde into the left carotid artery and secured with silk sutures to nearby fascia. In order to maintain integrity, the cannula was primed with mixture of heparin, saline, glycerol in a ratio of 1:2:2, respectively. Following surgery, the animals recovered in their home cages and received oral antibiotic treatment (amoxicillin) for seven days to help prevent infection.

Diurnal Corticosterone Sampling:

On the seventh day (all animals) and thirty-fifth day (8 wk groups), whole blood was sampled at 2100h, 0300h and 1000h via a tail nick bleed to obtain diurnal plasma corticosterone concentrations, as described previously (214). During nighttime sampling (2100h, 0300h), the lights were kept off and the only source of light was a battery-operated headlamp (white light). All sampling was performed in less than 60 seconds to minimize stress to the animal and to avoid rapid elevations in GCs. During each sampling, ~200 µl of whole blood was collected in heparinized capillary tubes (Sarstedt, Inc., Montreal, QC, Canada), centrifuged for 90s, and the plasma was removed and stored at –20 °C for later analysis.

Restraint Stress Protocol:

One week following cannulation surgery, rats underwent a standardized 20 minute restraint stress protocol at 0800h, as previously described (214). During the restraint protocol, an initial blood sample was taken to establish baseline corticosterone and ACTH concentrations (time 0), after which the animal was placed in a clear, plastic, aerated restraint stress tube (Harvard Apparatus, Inc., Holliston, MA, USA) for twenty minutes. Following restraint stress the animal was removed from the restraint tube and

allowed to recover in their respective cage for 95 minutes with no access to food or water. Throughout the experiment, blood samples were taken via the carotid artery at 0, 5, 10, 20, 30, 40, 55, 70, 85, 100, 115 minutes for plasma corticosterone levels and at 0, 20, 85, 115 minutes for plasma ACTH levels. The volume of blood obtained for analysis of corticosterone and ACTH was ~200 µl and ~500 µl, respectively. The total blood volume taken from the animals throughout the procedure was approximately 3.5 ml/animal (1.82 ml/hr), which is below the suggested maximum volume of 2.0 ml/hr (305).

ACTH Challenge:

Seven days after the restraint stress experiment, all animals underwent a standardized exogenous ACTH challenge to gauge adrenal sensitivity to ACTH at 0800h. Following an initial baseline blood sample, a low dose of human $ACTH_{1-24}$ (75 ng/kg; Peninsula Laboratories, Inc., San Carlos, CA, USA) was administered through the carotid artery cannula, after which five blood samples were taken via the carotid artery at 5, 10, 15, 30, 60, and 120 minutes for corticosterone concentrations. Approximately 200 µl of blood was collected at each time point and all blood samples were frozen at -20°C until used.

Upon completion of this experiment, the rats were anaesthetized under inhaled isoflurane and sacrificed by decapitation. Various tissues were collected, weighed, and stored at –80°C until subsequent analysis.

Hormone concentrations:

Plasma corticosterone and ACTH concentrations were measured using commercially available radioimmunoassay kits (MP Biomedicals, Costa Messa, CA). The

detection limit of corticosterone was 0.4 ng/ml and the inter- and intra-assay coefficients of variance were 7% and 4%, respectively. The detection limit of ACTH was 2 pg/ml and the inter- and intra-assay coefficients of variance were 7% and 5%, respectively.

Immunoblotting

Western blotting was performed as previously described (79), with the following modifications. Seventy-five micrograms of total protein obtained from the right adrenal glands was electrophoretically resolved on a 12% SDS-polyacrylamide gel. Three gels were run with two standards on each gel in addition to the samples to account for any variability found between gels. Intergel variability was less than 4% in all experiments. The protein was then transferred overnight at 20V to *PVDF* paper. Blots were blocked for 2 hours using 5% BSA in TTBS and then incubated overnight with either steroidogenic acute regulatory (StAR) antibody or melanocortin receptor 2 (MC2R) antibody at 4°C (StAR: AbCam, Cat#:ab3343, 1:1000; MC2R: Chemicon, Cat#:AB5128, 1:1000). Blots were then washed 5x10 min in TTBS and incubated with secondary antibody for 1 hour at room temperature (anti-rabbit conjugated with horseradish peroxidase; 1:5,000). Hybridization signals were visualized using the Western Lightning Chemiluminescence Reagent Plus kit (Millipore, Cat#WBKLS0500) after exposure to Kodak X-Omat Blue x-ray film (Rochester, NY). The densitometry of each band was determined and presented relative to the basal group. GAPDH was used as a loading control.

Data analysis

For all experiments, a t-test, one-way, or two-way ANOVA was performed, as appropriate, to identify significant differences in between treatment groups using Statistica 6.0 software, with $p<0.05$ as the criterion. When a significant difference was

observed with an ANOVA, post-hoc analysis was performed using a Tukey-Kramer test to determine specific differences. Data are presented as mean ± SEM.

Results

Anthropometry

All groups gained body weight with time, however, S rats gained weight at a faster rate than E rats ($p<0.001$). As a result, sedentary animals weighed significantly more than their matching exercise group by day 8 (+13.6g and +17.2g in the 2- and 8-week groups respectively, $p<0.05$). All animals demonstrated a decrease in body weight in response to the cannulation surgery (day 7 and day 38, 2 week and 8 week groups respectively, $p<0.05$). However, following surgery all groups continued to gain weight as expected. Exercising animals also demonstrated decreased epididymal and perirenal fat mass in comparison with their sedentary counterpart (data not shown). No difference was found in adrenal, soleus, or plantaris weight (data not shown).

Running Distance

Both E2 and E8 had similar running distances during the first 2 weeks (Table 1). E8 continued to progressively increase their average weekly distance ran to a maximum of 8875±2731 m/day during week 4, where it remained until cannulation surgery during week 6. Following cannulation surgery, the average weekly running distance dropped to 3445±1029 m/day in the E8 group but then rebounded in these animals to that observed pre-surgery, averaging 5973±1176m in week 8.

Diurnal Corticosterone Results:

After one week of treatment, E demonstrated higher corticosterone concentrations at 0300h (table 2, $p<0.01$) than S animals, indicating hyperactivation of the HPA-axis in

the former group following lights out. No differences were found at 1000h or 2100h between E and S at week one. At day 49, no differences were found in diurnal GC levels between S and E rats, despite the high running distances observed in the later group.

Restraint Stress Experiments:

To assess the effects of both short- and long-term training to a novel stressor, restraint stress protocols were carried out eight days following surgery in all groups. The restraints stress experiments show significant differences between S and E animals at day 8, but not at day 50. E and S rats had similar basal CORT values prior to the restraint on day 8, suggesting that the animals had recovered from the cannulation surgery similarly (figure 2c), but E responded to the novel stress with a greater increase in CORT concentrations than S for the first 10 minutes of the restraint ($p<0.01$). Peak CORT levels at 20 minutes were also higher in E than S, but only a trend was noted for statistical significance ($p=0.09$). S and E had similar CORT values throughout the recovery period and values returned to baseline by ~90 minutes following the end of restraint in both groups. Differences in ACTH concentrations were also noted during the restraint protocol in the short-term treated rats. In particular, E responded to restraint with lower ACTH concentrations compared to S at 20 minutes ($p<0.01$, figure 2a). No differences in ACTH were observed between the two groups during the recovery period, however. At day 50, the CORT response to and recovery from restraint was similar between S and E animals (figure 2d). Furthermore, no differences were seen in ACTH at any point during the restraint stress protocol (figure 2b).

ACTH Challenge:

To assess adrenal sensitivity to ACTH, a standardized ACTH challenge was given 1 week following the restraint stress experiment. Similar to the restraint experiments that had occurred one week previously, differences between E and S animals were seen at short-term but not at long-term treatment. In particular, on day 14, E demonstrated increased adrenal sensitivity to ACTH compared to S (figure 3a) with CORT concentrations higher in E than in S at 5, 10, and 15 minutes ($p<0.05$). E and S had similar values after 30 minutes and thereafter. In contrast, no difference in ACTH sensitivity was found in the 8 week animals at any time points (figure 3b).

MC2R and StAR

To confirm the molecular mechanisms behind increased adrenal sensitivity to ACTH in short-term training, we determined the expression of two key adrenal proteins involved in the synthesis and release of CORT in short and long-term treated S and E animals and in animals prior to the training intervention (basal rats). MC2R binds ACTH in the adrenal glands, initiating the signaling process to release CORT. A trend was noted for increased expression of MC2R in E compared to S at 2 weeks and basal rats ($p=0.08$, figure 4b). No difference was found between S and E groups in MC2R expression at 8 weeks of treatment. StAR protein is involved in the import of cholesterol into the adrenocortical cells and is proposed to be the rate-limiting step in GC biosynthesis (255). StAR expression was significantly increased in E compared to S at 2 weeks and Basal rats ($p<0.05$, figure 4a). No difference was found in StAR between S and E rats at 8 weeks.

Discussion

It is well known that acute exercise is a potent stressor, activating the HPA axis in humans and in rodents (214, 243-245, 299, 300). This study reveals that with regular exercise, in the form of voluntary wheel running, healthy rodents initially have hyperactivation of the HPA axis, as evidenced by elevations in diurnal CORT levels, an enhanced CORT response to restraint stress and an increased adrenal response to ACTH challenge. Furthermore, we confirm that even with increased wheel running, long-term endurance trained rats have normal diurnal HPA activity and normal response to restraint stress and ACTH challenge. We propose the mechanism behind these adaptive effects to be at the level of the adrenal gland, specifically through transient changes in ACTH sensitivity via alterations in the receptor (MC2R) and the rate limiting steroidogenic protein called StAR. These novel findings suggest that short-term endurance training in untrained individuals may result in a temporary increase in GC levels (i.e. from days to weeks depending on their adaptive capacity) and increased adrenal sensitivity to ACTH, however sustained training is associated with normal diurnal GC activity, a restored adrenal sensitivity to ACTH and a normal HPA responsiveness to a non-exercise stressor.

The effect of endurance training on basal (i.e. unstimulated) GC levels is somewhat controversial. Marathon runners have normal resting GC concentrations despite higher concentrations of ACTH, suggesting that training may decrease adrenal sensitivity to ACTH (236, 250). In rodents, the effect of exercise on diurnal HPA activity and adrenal sensitivity to ACTH is also unclear. For example, compared with sedentary animals, trained animals have increased resting stress hormones (240, 306), while others show similar resting concentrations (214, 247) or even decreased resting concentrations

(79, 208, 245, 246). It is likely that the variety of training protocols and the varying training durations used, as well as the timing of blood sampling in these rodent studies, contributes to the discrepancies in findings. With respect to HPA activation, voluntary exercise models are better than forced exercise models, as the former allows animals to exercise during their normal wake cycle (i.e. evening with lights off) and without the negative reinforcement usually required to initiate and maintain exercise intensities (i.e. foot shocks). Our voluntary wheel running model was undertaken to remove such confounding variables and allow the measurement of exercise interventions directly on the HPA axis.

In this study, and previously (214), we show that short-term voluntary wheel running (i.e. 1-2 weeks of training) elicits higher diurnal CORT patterns compared to sedentary animals and that this elevation in HPA axis activity becomes attenuated with increased training time, despite an increase in exercise volume (tables 1 and 2). This alteration in diurnal CORT pattern with short-term wheel running is similar to what has been observed recently by others using a similar training paradigm in mice (244). However, we also show that continuous training (lasting >5 weeks in duration) is associated with similar diurnal CORT patterns as those observed in sedentary animals (table 2). In agreement with this, long-term wheel running with or without the antidepressant Tianeptine in mice is associated with slightly lower basal GC levels compared with sedentary mice (245). Thus, it is clear that the HPA axis adapts with training likely to protect the exercising animals from the negative consequences of elevated GC concentrations. Indeed, chronic elevations in HPA activity, to levels observed during the initial 2 weeks of training, would likely counter the beneficial effects

of exercise training itself by contributing to insulin resistance, immune suppression, muscle proteolysis and mitochondrial loss (301). These findings in rodents coincide well with the observations that endurance-trained men have normal AM plasma ACTH and cortisol levels and normal 24 hour urinary free cortisol levels on a sedentary day (249).

It is possible that the increased HPA activity during the first 2 weeks of training is due to impaired central negative feedback. Reductions in central GR and MR levels have been previously been illustrated to cause prolonged elevations in CORT following a chronic variable-stressor paradigm that included forced vigorous exercise (306). However, the same group also found no significant changes in hippocampus GR with milder exercise intensities (304). Indeed, neither long-term forced swim training (254) nor voluntary wheel running (214, 244) lowered central corticoreceptors. In fact, Droste et al., found that exercise can increase GR mRNA expression in distinct hippocampal cell layers (244), suggesting increased negative feedback sensitivity exists with four weeks of wheel running in mice. In this study and previously (214), we found no difference in the recovery of exercise and sedentary animals following the restraint stress in either short-term or long-term trained animals (figure 2). This suggests that impaired negative feedback sensitivity is likely not the cause of HPA hyperactivity during the early stages of exercise training. Rather, we and others (244), believe that significant adaptations in the adrenal glands occur with prolonged training, such that adrenal sensitivity to ACTH is enhanced. Indeed, we clearly confirm this hypothesis in this study by using a standardized ACTH challenge to show that exercising animals have increased adrenal sensitivity at two weeks (figure 3). In further support of this, after short term training, E rats responded to novel restraint stress with elevated CORT concentrations but with

attenuated ACTH values when compared to S rats (figure 2). Protein analysis of the adrenal glands showed that this transient increase in adrenal sensitivity to ACTH with short-term training is due to increased expression of StAR and a tendency for an increased expression of the ACTH receptor MC2R (figure 4). These two proteins are thought to be the key determinants of adrenal sensitivity to ACTH (255). We believe that these short-term adrenal adaptations may be driven by the initial increase in HPA activity resulting from the exercise stress. Indeed, ACTH upregulates the expression of its own receptors on adrenocortical cells in such a feed forward manner (298, 307). In accordance with this, 2 weeks of voluntary wheel running has recently been shown to increase mRNA levels of StAR (254). Alternatively, a recent study has also suggested that enhanced sympathoadrenomedullary activity, found to be associated with exercise training, may positively modulate adrenal sensitivity to ACTH (244). Considering adrenaline has previously been demonstrated to stimulate adrenocortical steroidogensis and secretion of GCs (244), our study proposes possible target genes on which this increased sympathoadrenomedullary input may be operating.

The return to basal or sedentary values with prolonged exercise training points towards the potential for desensitization of the adrenal glands, as seen with the return in StAR protein levels by week 8 in the E rats. In this regard, others have shown that exercise initially causes hyperactivity of the HPA axis (measured by morning cortisol) but that prolonged training results in restoration of normal values, suggesting that a desensitization of the HPA axis exists with long-term training (308). We are the first to show that the mechanism occurs, at least partially, at the level of the adrenals through restoration in StAR protein to sedentary levels. Additionally, we confirm this by directly

measuring adrenal sensitivity to ACTH challenge and show that the transient increase seen at 2 weeks is restored by 8 weeks. The mechanism that drives this desensitization (i.e. the restoration of StAR protein) clearly merits future investigations. It is also worth mentioning that compared with 2 week sedentary rats, 8 week sedentary rats also had a apparent decrease in adrenal sensitivity to exogenous ACTH (figure 3). Elevations in stress reactivity in prepubertal female rats compared with postpubertal female rats have been observed by Romeo et al. (309), and these maturational changes have also been attributed to changes in adrenal sensitivity to ACTH.

Our study has some important limitations that warrant discussion. First, because of the nature of our study design, we can not equivocally say that the upregulation in StAR protein was the cause of increase adrenal sensitivity early on in training. Rather, we report an association between these two variables, as others have done recently (310). Second, it is unclear why diurnal GC levels changed from 7 days to 42 days in the sedentary rodents even though enrichment and cage conditions remained constant. It may be that these rats had a reduction in basal glucocorticoid levels as a result of sexual maturation (309) or perhaps because of an accommodation to their cage conditions. It is also important to note that as an alternative, sedentary rats could have been exposed to the identical cage condition as the exercising rats, with the addition of a locked wheel, as has recently been used to study the metabolic effects of exercise cessation in rodents (203). Finally, it is important to note that the "short-term" training measurements, that included diurnal GC profiling, restraint stress and ACTH challenge, were made over a span of six days to allow for a recovery from the restraint (see figure 1). It is possible, therefore, because of the apparent dynamic changes in the HPA axis at the start of

training that the transient increase in MC2R and StAR expression and elevated adrenal sensitivity to ACTH might have already been returning toward baseline. The dynamic changes in HPA axis activity that occurs during the initial period of training thus require careful profiling in future studies.

In conclusion, we demonstrate that exercise initially causes upregulation of HPA axis activity resulting in increased circulating concentrations of CORT in rats, particularly during the lights off cycle. Importantly, long-term training ameliorates this difference, resulting in a similar diurnal CORT pattern, as well as a similar stress response in exercising and sedentary animals. We propose that these adaptations are found at the level of the adrenal gland, specifically with transient differences in StAR protein expression and concomitant increases in adrenal sensitivity to ACTH. Overall, the data presented here indicates that, although exercise initially causes perturbations in the HPA axis, long-term training can be performed without concern for chronic elevations in CORT concentrations, and associated health detriments.

Table 1: ***Average Running Distances***

	2 Week Treatment Group		8 Week Treatment Group	
Week	**Mean Distance (m/day)**	**SEM**	**Mean Distance (m/day)**	**SEM**
1	2099	503	2490	573
2	4162	1791	5601	950
3			6841	1271
4			8875	2731
5			8332	1905
6			3445*	1029
7			5601	1262
8			5973	1176

Average weekly running distances for E2 and E8 animals, expressed as meters/day. Both groups increased running distance with time ($P<0.001$). There was a transient decrease in running distance immediately following cannulation surgery in E8 rats (week 6), * - $p<0.01$.

Table 2: ***Average Diurnal Corticosterone***

	2 Week Treatment Group Corticosterone (ng/ml)		8 Week Treatment Group Corticosterone (ng/ml)	
Time of Day	**Sedentary**	**Exercise**	**Sedentary**	**Exercise**
0300h	16.59 ± 3.37	35.81 ± 9.29 *	19.85 ± 10.33	28.6 ± 4.48
1000h	16.19 ± 4.49	23.57 ± 8.11	8.20 ± 4.37	7.64 ± 2.77
2100h	44.75 ± 9.95	46.87 ± 11.50	77.94 ± 20.54	84.71 ± 15.48

Diurnal corticosterone values for sedentary and exercise animals at 1 week and 7 weeks. Blood was sampled at lights out (2100h), in the middle of the night (0300h), and at lights on (1000h) to assess daily diurnal corticosterone patterns. E2 animals had higher corticosterone values at 0300h. Corticosterone concentrations (ng/ml) are expressed as mean ± SEM. *= $p<0.05$ vs. S2.

Figure Legends

Figure 1: ***Schematic of the experimental design.***

All exercise animals were introduced to the running wheel at day 0. The 2 week animals were cannulated on day 0, had daily diurnal CORT measurements taken at day 7, underwent the stress restraint experiment on day 8, and underwent the ACTH challenge on day 14.The 8 week animals were cannulated on day 42, had their diurnal CORT measurements taken at day 49, underwent the stress restraint experiment on day 50, and underwent the ACTH challenge on day 56. All animals were euthanized immediately after the ACTH challenge (day 14 and 56, 2 week and 8 week groups, respectively).

Figure 2: ***The response and recovery profile of ACTH and CORT in response to, and recovery from, 20 minutes restraint in the 2 week and 8 week groups.***

E2 were found to have a higher ACTH response to restraint stress compared with S2 at the 20 minute time point (a, *-$p<0.01$). No difference in ACTH levels between S8 and E8 were found at any time point (b). E2 were found to have a higher CORT response to restraint stress compared with S2 at the 5 and 10 minute time points (b,*-$p<0.01$). No difference in CORT levels between S8 and E8 were found at any time point (d).

Figure 3: ***The CORT response to exogenous ACTH challenge (75ng/kg) in the 2 week animals and 8 week animals.***

E2 had a significantly higher CORT response to the ACTH injection during the 5, 10, and 15 minute time points ($p<0.01$). There was no difference in CORT levels between S8 and E8 at any time point during the ACTH challenge.

Figure 4: ***Expression of StAR and MC2R protein in the adrenal glands.***

StAR protein is involved in the import of cholesterol into the adrenocortical cells, which is the rate-limiting step in steroid biogenesis. MC2R is the ATCH receptor in the adrenal glands. E2 had significantly higher StAR protein compared with S2 and B groups (*-$p<0.05$). No difference was found in the 8 week animals. There was a tendency for increased expression of MC2R in E2 compared to Basal groups ($p=0.08$), no other differences were found. ($n=6$ for Basal, $n=8$ for all other groups)

Figure 1

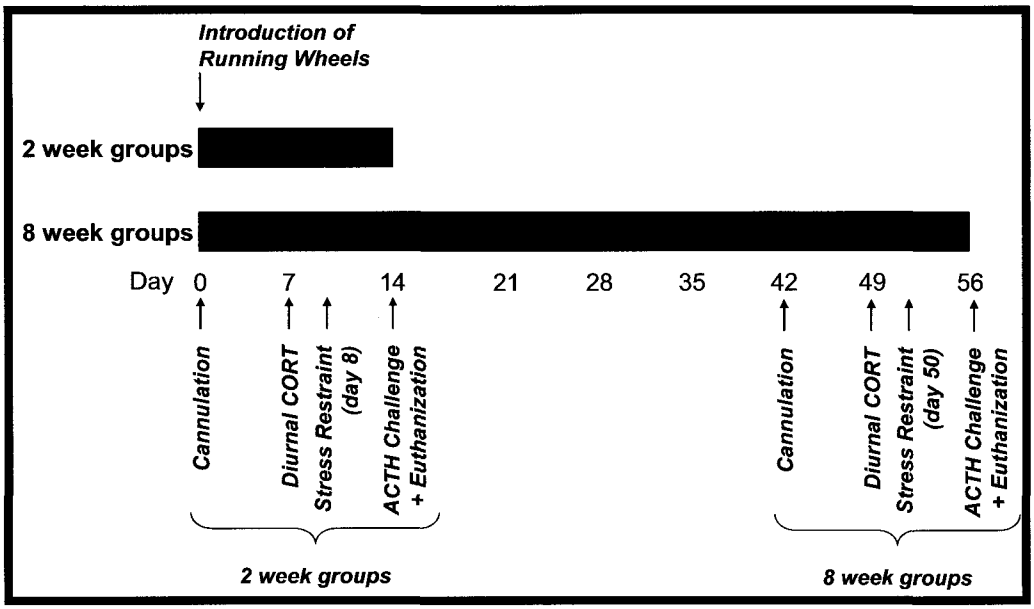


Figure 2

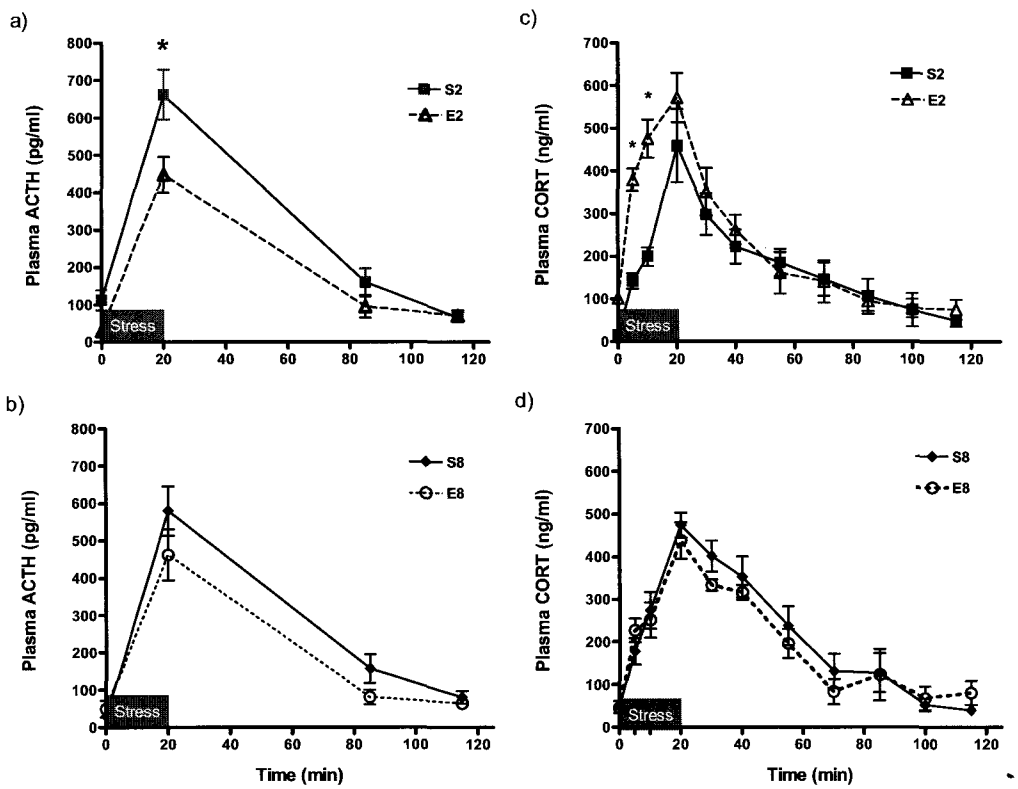


Figure 3

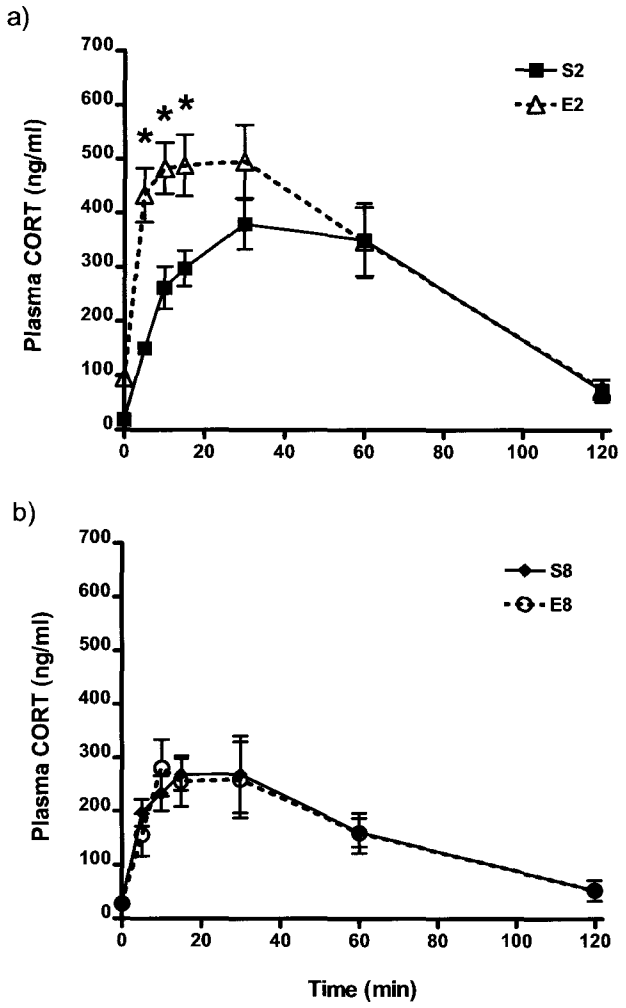
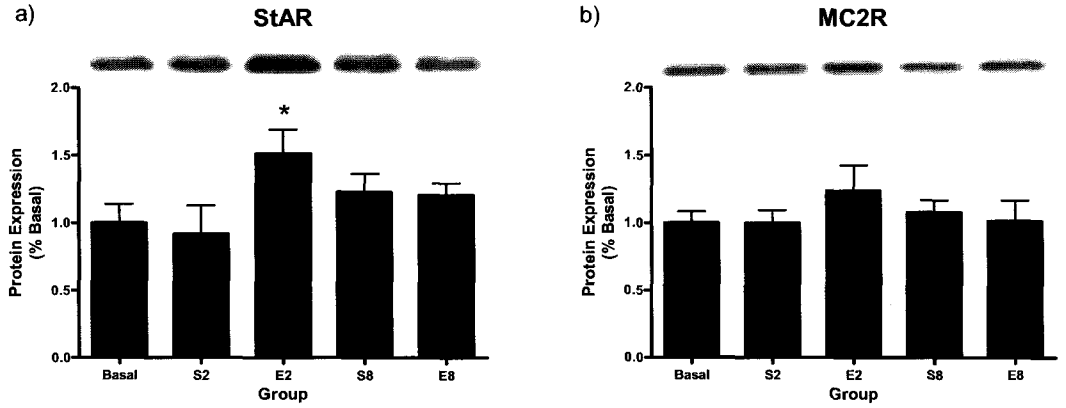


Figure 4



MANUSCRIPT #2 – EXERCISE AND THE HPA-AXIS IN THE ZUCKER-DIABETIC-FATTY RAT

3

Rationale for manuscript #2:

Manuscript #1 demonstrated that voluntary exercise initially increases adrenal sensitivity to ACTH, which is attenuated with sustained training. This, along with our previous publications (214, 247), demonstrates that sustained training does not cause hyperactivation of the HPA axis in either basal or stimulated conditions. Hyperactivity of the HPA axis may contribute to the development of insulin resistance, obesity, and eventually T2DM. Prevention of hyperglucocorticoidemia through adrenalectomy has been shown to prevent the progression towards T2DM in ZDF rats. We hypothesized that volitional exercise training in ZDF rats would prevent hyperglucocorticoidemia and subsequently attenuate the development of T2DM. Furthermore, we hypothesized that training would prevent hyperglucocorticoidemia at the level of the adrenal glands, through similar mechanisms reported in manuscript #1. This was the first study to report the time-course of hyperglycaemia concurrently with the activity of the HPA axis in both sedentary and exercise trained ZDF rats.

Author Contributions:

Conceived and designed the experiments: JEC (30%), MAK (30%), MV (10%), MCR (30%). Trained the animals: MAK (100%). Performed the experiments: JEC (50%), MAK (20%), AMD (10%), DJA (20%). Analyzed the data: JEC (75%), MCR (25%). Wrote the paper: JEC (60%), MCR (40%).

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Regular exercise prevents the development of hyperglucocorticoidemia via adaptations in the brain and adrenal glands in male Zucker Diabetic Fatty rats

Running Title: Exercise prevents hyperglucocorticoidemia in ZDF rats

Key Words: glucocorticoid, stress, hippocampus, type 2 diabetes, wheel running

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Abstract

We determined the effects of voluntary wheel running on the hypothalamic-pituitary-adrenal (HPA) axis, and the peripheral determinants of glucocorticoids (GC) action, in male Zucker diabetic fatty (ZDF) rats. Six-week-old euglycemic ZDF rats were divided into baseline (B), sedentary (S), and exercise (E) groups (n= 8-9 per group). B animals were immediately sacrificed, whereas S and E were monitored for 10 weeks. Basal (i.e. AM) GC levels increased 2.3-fold by week 3 in S rats where they remained elevated for the duration of the study. After an initial elevation in basal GC levels at week 1, E rats maintained low GC levels from week 3 through week 10. Hyperglycemia was evident in sedentary animals by week 7, whereas exercising animals maintained euglycemia throughout. At the time of sacrifice, S had ~40% lower GC receptor (GR) content in the hippocampus, compared to B and E ($P<0.05$), suggesting that the former group had impaired negative feedback regulation of the HPA axis. Both S and E groups had elevated ACTH compared to B rats indicating that central drive of the axis was similar between groups. However, S, but not E, animals had elevated adrenal ACTH receptor and steroidogenic acute regulatory (StAR) protein content compared with B, suggesting that regular exercise protects against elevations in GCs by a downregulation of adrenal sensitivity to ACTH. GR and 11β-hydroxysteroid dehydrogenase type 1 content in skeletal muscle and liver were similar between groups, however, GR content in adipose tissue was elevated in S compared with B and E ($P<0.05$). Thus, the gradual elevations in GC levels associated with the development of insulin resistance in male ZDF rats can be prevented with regular exercise, likely because of adaptations that occur primarily in the adrenal glands.

Introduction

Glucocorticoid (GC) excess is characterized by increased central adiposity, insulin resistance, hyperlipidemia and elevated glucose production (86), while in the pancreas, sustained elevations in GCs adversely affect β cells and directly attenuate insulin release (44) – all features that make these hormones potent diabetogenic agents (311). Moreover, GCs are vasoactive and their elevation is an independent risk factor for cardiovascular disease and other diabetes related complications (88). Animal models of both type 1 (312, 313) and type 2 (314-316) diabetes show elevations in circulating GCs, supporting the hypothesis that hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis is tightly coupled to the pathophysiology of both forms of the disease. Evidence is also mounting that elevations in GCs, either by increased central drive of the HPA axis or by peripheral activation of glucocorticoids through 11beta-hydroxysteroid dehydrogenase-1 activity (11βHSD1), plays a pathological role in the development of the metabolic syndrome (180).

We have shown previously that hyperglycemia resulting from streptozotocin-induced diabetes increases the activity of the HPA axis, and that normalizing glucose with either insulin or phloridzin corrects this hyperactivity (317, 318). Based on these findings, one might assume that the hypercortisolemia that is also observed in animal models of type 2 diabetes (50, 319, 320) is a result of hyperglycemia and relative hypoinsulinemia. Surprisingly, however, no known studies report on the levels of GCs during the progression from prediabetes to T2DM in animal models of this disease.

While several studies have measured GC levels in models of T2DM (50, 145, 319, 320), few have examined the central (brain) and peripheral (adrenal gland and target

tissues) components of GC release and action. Although much of the data point towards increased HPA activity with hyperglycemia (50, 316), the results are not consistent (2) and the mechanism(s) behind the observed increase in HPA activity in these rodent models are also unclear. These studies suggest that increases in corticotrophin releasing hormone (CRH) sensitivity (50) or adrenocorticotropic hormone (ACTH) sensitivity (314) may account for these changes, while others have shown decreased negative feedback following a dexamethasone suppression test (50, 52) suggesting abnormalities at the level of the hippocampus or hypothalamus. To our knowledge, no study has profiled the time course change in basal (resting AM) GC levels in male ZDF rats to determine if the hyperglucocorticoidemia precedes the development of hyperglycemia, or results after the alteration in glucose homeostasis.

Exercise acutely activates the HPA axis and raises basal GC levels (321). Regular exercise, however, is well known to prevent the development of insulin resistance and to delay the progression towards type 2 diabetes in both humans with prediabetes (322) and in rodent models of the disease (207, 208, 323-325). We have recently shown that adaptations exist in healthy rodents that normalize a transiently elevated activity of the HPA axis within days to weeks after the start of training (214, 247, 326). We attribute this restoration in HPA axis activity in healthy rats to adaptations in the hypothalamus and adrenal gland that promote a lower CRH production and reduced adrenal sensitivity to ACTH, respectively (214, 247, 326). Whether these adaptations also exist in the Zucker Diabetic Fatty (ZDF) rat (a rodent model of T2DM that exhibits elevated HPA axis activity) is unknown.

In this study, we set out to determine: 1) if elevations in HPA axis activity precede, or responds to, the development of hyperglycemia in the ZDF rat and 2) if regular exercise, which is known to prevent hyperglycemia in this animal model, prevents the central and peripheral hyperactivity of the HPA axis that is associated with disease development. We show that sedentary ZDF rats have elevations in HPA axis activity that precedes the development of hyperglycemia, suggesting that GCs have a causative role in this model of T2DM. Furthermore, we show that regular exercise induces positive adaptations, primarily in the adrenal gland, that lowers hypercortisolemia.. These novel findings are important as they illustrate new mechanisms for how regular exercise can prevent, or at least delay, the development of hypercortisolemia and its associated metabolic disturbances.

Methods

Animals

Male ZDF rats were obtained from Charles River Laboratories (Saint-Constant, Quebec, Canada) at 5 weeks of age with initial body weights of 150-175g, and were individually housed in clear cages and kept in a temperature (23-25˚C) and humidity (40-50%) controlled room for a 7-day habituation period. The animals were given standard rodent chow (Purina 5001, 4.3kcals/g metabolizable energy) and water ad libitum throughout the study duration. Following the habituation period, rats were randomly assigned to one of three groups: basal (n=8), sedentary (n=8), or volitional exercising ("exercise", n=9). Exercise animals were individually housed in specialized activity wheel cages (height: 36.4 cm, width: 26.8 cm, depth 50 cm) with unrestricted, 24-hour access to their wheels (circumference: 108 cm, width: 9 cm). Basal and sedentary animals were housed in

similarly sized cages, but without activity wheels. Wheel revolutions, body weight, and food intake were recorded daily. Running distance was calculated as the circumference times recorded revolutions. Basal animals were euthanized following the habituation period at 6 weeks of age. Sedentary and exercise animals were euthanized 10 weeks later at 16 weeks of age. All experiments were approved by the Animal Care Committee of the Faculty of Medicine at the University of Toronto in accordance with regulations set forth by the Canadian Council for Animal Care.

Blood sampling

The glycemic profile and glucose tolerance of these animals have been published elsewhere (327). Once per week (Thursdays), the rats were fasted overnight, for 15-18h, after which blood samples were taken via a venous 'tail nick' for glucose and insulin concentrations. The first drop of blood was used to measure glucose concentrations using a blood glucose monitor (ASCENSIA ELITE™ XL Blood Glucose Meter, Bayer, Toronto, Canada). Approximately 100 µl of whole blood was collected into heparinized microvettes (Sarstedt, Montreal, Canada) and the plasma was separated by centrifugation at 400 x g for 1 min and stored at -20°C. Fasting insulin concentrations were analyzed using a rat insulin ELISA assay kit (Crystal Chem Inc, Illinois, USA). Additional blood samples were taken via 'tail nick' once per week (Mondays) at 0800h under normal (i.e. non-fasted) conditions for the determination of fed glucose (whole blood), insulin (plasma), and corticosterone (plasma) concentrations. Corticosterone concentrations were analyzed with a commercially available radioimmunoassay (RIA) kit (Medicorp Inc., Montreal Canada).

Intraperitoneal glucose tolerance test

All groups were subjected to an intraperitoneal glucose tolerance test (IPGTT) 3 days prior to euthanasia. Rats were fasted overnight for 15-18 hours and were then administered an intra-peritoneal injection of 50% dextrose (Abbott Laboratories Limited, Montreal, Canada) at a dose of 2g/kg body weight between 0900h and 1200h. Blood for glucose and insulin levels was collected via tail nick at 30 minute intervals for 2 hours. Blood was immediately centrifuged as previously described and frozen at -20C for subsequent analysis.

Euthanization

As previously mentioned, basal animals were euthanized at 6 weeks of age, whereas sedentary and exercise animals were euthanized at 16 weeks of age. Euthanasia occurred in the morning (0800h to 1000h) to obtain basal plasma hormone levels, within five hours of the last bout of exercise. Trunk blood was collected in EDTA and trasylol coated tubes, immediately centrifuged, and stored at -20C for subsequent hormone analysis. Post-prandial blood glucose was measured with a blood glucose meter. Plasma free fatty acids (FFA) and triglycerides (TG) were determined by an enzymatic colorimetric method (ACS-ACOD; Wako Chemicals, Richmond, VA). Plasma ACTH concentrations were determined by a commercially available RIA (Medicorp Inc., Montreal Canada).

Immunoblotting

This method for protein preparation and quantification has been previously described (328) with some modifications. Briefly, tissue samples were homogenized to obtain total protein, centrifuged at 1650 x g for 30 min, and the supernatants were

collected. Protein concentrations were assessed by Bradford method. Seventy-five micrograms of total protein was electrophoretically resolved on either an 8% SDS-polyacrylamide gel (GR), or a 12% SDS-polyacrylamide gel (11βHSD1) and transferred overnight at 20V to PVDF paper. Blots were blocked with 5% BSA in TTBS and then incubated overnight in primary antibody at 4°C (GR: Affinity BioReagents, Cat#:PA1-511A, 1:5000; 11βHSD1:Alpha Diagnostic, Cat#:BHSD11-S). Blots were incubated with the appropriate secondary antibody (Abcam) for 1 hour at room temperature and hybridization signals were visualized using the Western Lightning Chemiluminescence Reagent Plus kit (PerkinElmer, Wellesley, MA) after exposure to Kodak X-Omat Blue x-ray film (Rochester, NY). β-actin and α-tubulin were used as a loading controls (Abcam).

Tissue cryosectioning

Brains were mounted on annular discs using tissue freezing medium (Triangle Biomedical Sciences), sectioned to 10 microns in a refrigerated microtome (ThermoShandon Cryotome) at -10°C, and mounted on SuperFrost Plus Gold slides (Thermo Fisher Scientific). Correct brain orientation was confirmed by hematoxylin staining and architectural examination under a light microscope.

Immunohistochemical (IHC) staining

Slide-mounted tissue sections were air-dried, fixed in 4% paraformaldehyde (Sigma-Aldrich), and permeabilized using either 0.1% (GR, MR) or 0.3% (CRH) Triton X-100 (Sigma). Tissues were then blocked in either 10% normal goat serum (GR, CRH; Vector Laboratories) with 1.5% bovine serum albumin (BioShop) or 10% normal horse serum (MR; Vector Laboratories) with 1.5% bovine serum albumin, and all tissues were incubated in a streptavidin/biotin blocking kit (Vector Laboratories; SP-2002). Sections

were incubated in a humidified chamber at 4°C for 18 hours with their respective primary antibodies (GR: 1:250, Santa Cruz M-20; MR: 1:200, Santa Cruz N-17; CRH: 1:1000, Peninsula Laboratories T-4037) in 1.5% of their respective blocking sera. Signals were detected using either anti-rabbit (GR, CRH) or anti-goat (MR) biotinylated secondary antibodies (Vector Laboratories; BA-1000, BA-9500 respectively) and a TxRed-conjugated streptavidin tertiary antibody (Vector Laboratories; SA-5006). Autofluorescence was reduced by sequential incubation in solutions of 0.3% Sudan Black (BioShop) in 70% ethyl alcohol, and $CuSO_4$ / NH_4Ac (Acros Organics and BioShop, respectively). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; Sigma) before sections were cover-slipped with Fluoromount (Sigma) to preserve fluorescence. For all sections, a negative control was incubated with PBS instead of primary antibody to determine the degree of non-specific secondary antibody binding. Slides were visualized using a fluorescence-equipped microscope (Nikon Eclipse 90i) and image overlays were performed using Adobe Photoshop CS software. Images were converted to black and white and then inverted. Signal intensities were recorded in Adobe Photoshop™, subtracted from background values and then made absolute. Signal intensities were then expressed as arbitrary units (AU). Some samples were significantly damaged by the freezing process, leading to freeze fractures and making analysis not possible. Subsequently, IHC results are based upon 5 animals from each group.

Data analysis

For all experiments, the appropriate t-test, one-way, or two-way ANOVA was performed to identify significant differences between treatment groups using Statistica 6.0 software, with $p<0.05$ as the criterion. When a significant difference was observed

with an ANOVA, a post-hoc analysis using contrasts with a Bonferroni correction factor were performed to determine specific differences. Data are presented as mean ± S.E.M.

Results

Food intake, body weights, and running distance

Food intake did not differ between groups at any time point. Sedentary and exercise animals gradually increased food intake from week 1 (23.5 ± 0.7 g and 22.3 ± 0.6 g, respectively) to week 10 (34.5 ± 0.7g and 35.2 ± 0.4 g, respectively). Average daily food intake for the sedentary and exercise groups, over the entire study, was 29.2 ± 0.5 g and 30.0 ± 0.7 g (not significantly different; Table 1). Sedentary animals gained slightly more weight compared to exercise animals throughout the 10 weeks, and were statistically heavier at the end of the study ($p<0.05$; Table 1). Average daily running distances for the exercising animals began at 3.4 ± 0.2 km/day during week 1, peaked at 6.5 ± 0.5 km/day during week 6, and then slowly declined to 4.5 ± 0.7 km/day during week 10.

Weekly corticosterone and glycemia

As noted earlier, weekly fed and fasted blood glucose values in these animals have been previously reported (327). Exercise initially caused elevations in HPA activity, with GC concentrations being higher than sedentary animals during week 1 ($p<0.05$; Fig. 1A). However, the GC concentrations gradually decreased in the exercise group from week 1 to week 3, and then remained relatively constant until week 10. Contrary to this, GC concentrations gradually increased in the sedentary group from week 1 to week 3, and remained elevated compared to exercise animals throughout the study ($p<0.05$; Fig. 1A). Both groups had fasting euglycemic levels until week 7, whereupon the sedentary

developed hyperglycemia while the exercise animals remained euglycemic for the remainder of the experimental period ($p<0.05$; data reported previously (327)).

End point glucose tolerance, tissue weights, and blood chemistry analysis

Exercise prevented the impaired glucose tolerance seen in the sedentary animals. During the IPGTT, the sedentary group had a higher area under the curve (AUC) compared to the basal group for both glucose and insulin ($p<0.01$, Table 1). The sedentary group also had elevated fed glucose levels compared to basal group at euthanasia ($p<0.01$; Table 1). Exercise animals had smaller glucose AUC values and fed glucose levels compared to sedentary animals, however, did have the highest AUC for insulin during the IPGTTs ($p<0.05$; Table 1). Sedentary animals also presented elevated plasma concentrations of TGs and FFAs compared to basal animals at euthanasia ($p<0.01$; Table 1). Exercise animals did not differ from the basal group in regards to FFAs and had lower levels of TGs compared to the sedentary group, although still higher than the basal ($p<0.05$; Table 1). Both sedentary and exercise groups had higher plasma ACTH concentrations compared to the basal group ($p<0.01$; Fig. 1B). Exercise animals had less adiposity and more skeletal muscle mass compared to sedentary animals, as shown by lower epididymal and higher plantaris weights ($p<0.01$; Table 1).

Regulation of hypothalamic-pituitary-adrenal axis activity

Exercise prevented the decrease in hippocampal GR receptor protein content seen in sedentary animals. IHC analysis for GR protein in the hippocampus showe a sedentary animals to have a lower signal intensity compared to basal and exercise groups ($p<0.05$; Fig. 2A-C). Western blot analysis confirmed that GR protein was lower in sedentary animals compared to basal and exercise groups ($p<0.05$; Fig. 2D). IHC and western blot

analysis in the hippocampus for mineralocorticoid receptor showed no differences between groups (CA1 region IHC: B, 4.76±0385 AU; S, 5.05±0.58 AU; E, 5.41±0.77 AU; P=0.85 Western: B, 100.0±12.1%; S, 92.5±8.6%; E, 93.7±13.2%; P=0.69). Interestingly, IHC analysis of the hypothalamus showed CRH protein to be highest in the exercise group ($p<0.05$; Fig. 3). Western blot analysis for GR protein in the pituitary gland revealed no group differences (Fig. 4A). Western blot analysis for regulatory proteins for the production of GCs, namely adrenal MC2R and StAR, showed a higher expression for both proteins in the sedentary group compared to the exercise group ($p<0.05$; Fig. 4B and 4C). In addition, sedentary animals had higher MC2R protein compared to basal animals ($p<0.05$; Fig. 4B), but only a trend was found for elevations in StAR between these two groups ($p=0.08$; Fig. 4C).

Glucocorticoid action in peripheral tissues, expression of GR and 11βHSD1

To determine the effects of insulin resistance and exercise on peripheral tissue exposure to circulating GCs, skeletal muscle (mixed quadriceps), liver, and adipose tissues were probed for GR and 11βHSD1 protein content. No group differences were found for GR and 11βHSD1 levels in the skeletal muscle or liver tissues (Fig. 5A and 5B). Sedentary animals had higher GR content in epididymal adipose tissue compared to basal animals ($p<0.05$), though no difference was found between exercise and basal groups (Fig. 5C, left panel). Furthermore, a trend was found for elevated 11βHSD1 content in the adipose tissue of exercise animals compared with basal animals ($p=0.07$; Fig. 5C right panel).

Discussion

This study shows that there is a gradual increase in basal HPA axis activity in sedentary male ZDF rats that precedes their development of hyperglycemia. We also show that the elevations in HPA activity in this rodent model of T2DM coincide with increased adrenal cortical proteins, which, in turn, increase adrenal sensitivity to ACTH. In contrast to sedentary behavior, we show that regular exercise prevents hyperglucocorticoidemia for at least 10 weeks duration – all the while maintaining euglycemia, likely through reduced adrenal sensitivity to ACTH. These novel findings indicate a new potential mechanism for the prevention of hyperglycemia in this rodent model of T2DM.

Researchers and clinicians have long postulated that stress hormones may be involved in the development of type 2 diabetes and that exercise may be beneficial for stress reduction. Indeed, the close phenotypic parallels between the metabolic syndrome and cortisol excess (e.g., Cushing's syndrome) indicate a common underlying role for GC action in these disease processes (180, 329). GC induced metabolic complications are ameliorated by adrenalectomy in rodents and reinstated by exogenous GCs (183). Mechanistically, GCs promote visceral adiposity (18), increase free fatty acid release (163), elevate liver glucose production (330), and exacerbate muscle insulin resistance (143). Despite these previous research findings, to our knowledge, no study has previously shown that increased activity of the HPA axis proceeds, and thus potentially contributes to, the onset of hyperglycemia and insulin resistance in ZDF rats. Our study clearly demonstrates that elevations in GCs occur prior to the development of glucose intolerance and may facilitate the subsequent onset of hyperglycemia in the ZDF rat. In

sedentary rats, hypercortisolemia was evident by week 3, whereas hyperglycemia emerged on week 7, likely a result of diminished insulin production in the face of elevated insulin resistance (207).

We show the mechanisms associated with elevations in HPA activity in sedentary ZDF rats appear to be a combination of several factors, including: 1) reduced negative feedback regulation of the axis as a result of diminished hippocampal GR content, 2) increased central drive of the axis, as evidenced by increased ACTH levels, and 3) increased adrenal sensitivity to ACTH demonstrated by decreased StAR and MC2R protein content. It has been previously shown that obese Zucker rats have reduced MR content, which would also potentially contribute to the dysregulation of the HPA axis (331). In our study, both IHC and western analysis showed no difference between groups. We also show that although exercise maintains normal hippocampal GR protein (indicating the continuance of adequate negative feedback compared to sedentary animals), exercising animals also have elevations in both hypothalamic CRH protein content and circulating ACTH levels. Therefore, we conclude that regular exercise prevents elevations in circulating GCs in this rodent model of T2DM mainly through reductions in adrenal sensitivity to ACTH via downregulation of both MC2R and StAR proteins.

Our previous work with ZDF rats has shown that both swim training and intermittent restraint stress help maintain β cell mass and prevents (or at least delays) the onset of type 2 diabetes (207, 208, 314, 315). Furthermore, we have previously demonstrated that wheel running exercise in healthy non-diabetic rodents causes initial hyperactivity of the HPA axis that is followed by a complete restoration to basal states

(214, 328). Importantly, we found that the initial increase in HPA activity following the onset of exercise is the result of a transient increase in adrenal sensitivity to ACTH (328). We extend our findings in this study by showing that regular exercise elicits a similar mechanism for reduced GC production in ZDF rats. Indeed, the elevations in GCs seen in the exercising group during the first 1-2 weeks in this study may be due, at least in part, to transient increases in adrenal gland sensitivity to ACTH, as we seen previously in healthy rats undergoing training (328). Importantly, however, despite the initial increase in HPA axis activity, sustained exercise for greater than 2 weeks is associated with low basal HPA axis activity in ZDF rats, with similar adaptations occurring in the brain and adrenal gland as observed in non-diabetic exercise-trained rodents (214, 328).

Local amplification of GC action, through increased tissue expression of GR and 11βHSD1, can lead to the development of metabolic complications in the absence of high circulating GCs (18, 199, 332). Therefore, we probed insulin target tissues for GR and 11βHSD1 in basal, sedentary and exercise trained rats. We found no differences in skeletal muscle or liver tissues, though a higher expression of GR in the epididymal adipose tissue of the sedentary ZDF rats was found compared with basal rats (Fig. 5C). Increased GC action in visceral adipose tissue induces the metabolic syndrome through the development of central obesity and dyslipidemia (18). Thus, exercise training may also be protecting against the development of insulin resistance by lowering GC exposure in adipose tissue and subsequently preventing dyslipidemia, as was observed in the exercise group (Table 1). A reduction in GR content with training may also be important in lowering adipose tissue exposure to reactivated GCs since elevations in the prereceptor

enzyme 11βHSD1 is known to increase with both dietary weight loss (177) and with exercise (80, 328).

It is important to note some of the limitations of our study. First, exercise training reduced adipose tissue mass, which in turn, leads to improvements in insulin sensitivity (324). The exercising ZDF rats in our study also experienced an attenuated gain in fat mass compared to the sedentary animals (Table 1), making it difficult to delineate the contributions of the HPA axis from the effects of decreased adiposity on the prevention of hyperglycemia. Although a calorically restricted group of sedentary ZDF animals could have been added to the experimental design to help tease out the effects of decreased fat mass on diabetes development caused by exercise, we have previously shown that modest caloric restriction also activates the HPA axis in sedentary ZDF rats (315), which would make it difficult to compare this group to the exercising group. Another important limitation to our study is that we propose that exercise training lowers adrenal sensitivity to ACTH, thus resulting in lower GC levels, although we did not directly measure adrenal sensitivity *per se*. However, we have previously shown that the protein levels of StAR and MC2R are directly associated to the sensitivity of the adrenals to ACTH in trained and untrained rats by using exogenous ACTH challenge (326).

Perspectives and Significance:

This study is the first to show that hyperactivity of the HPA axis and elevated plasma glucocorticoids precedes the development of, and thus may contribute to, hyperglycemia in ZDF rats. Furthermore, we demonstrate that exercise training is capable of attenuating these alterations in the HPA axis, allowing for maintenance of normal plasma glucocorticoids in this model of T2DM development. These data reveal novel

neuroendocrine mechanisms for the beneficial effects of exercise on the management of the stress axis, which may aid in the prevention of type 2 diabetes.

Table 1 – *Animal Characteristics, blood hormone concentrations, and IPGTT results*

	Basal	Sedentary	Exercise
Final Body Weight (g)	166.6 ± 3.5	454.0 ± 10.5*	426.4 ± 6.4*
Average Daily Food Intake (g)	-	29.2 ± 0.5	30.0 ± 0.7
Epididymal Adipose Weight (g)	0.87 ± 0.06	5.45 ± 0.2*	4.10 ± 0.2*†
Plantaris Weight (mg)	-	108.5 ± 2	141.3 ± 7†
Fasted FFA (uEq/l)	313.4 ± 15.1	816.8 ± 78.4*	324.9 ± 21.7†
Fasted TG (mM)	1.51 ± 0.09	5.61 ± 0.38*	3.74 ± 0.29*†
Fed Glucose (mM)	6.5 ± 0.2	16.0 ± 2.7*	5.1 ± 0.3†
IPGTT AUC Glucose (AU)	1104 ± 56	2141 ± 144*	1376 ± 125†
IPGTT AUC Insulin (AU)	326 ± 63	752 ± 77*	1020 ± 125*†

* - denotes significantly different from Basal group
† - denotes significantly different from Sedentary group

Figure Legends

Figure 1 – *Weekly measurements of corticosterone and plasma ACTH concentrations at euthanasia.*
(A) Corticosterone concentrations in sedentary animals were elevated by week 3, whereas corticosterone in exercising animals decreased from week 1 to week 3, and remained low thereafter. • – sedentary, ○ – exercise. (B) ACTH concentrations at euthanasia were elevated in both sedentary and exercising animals compared to the basal group. * - $p<0.05$; † - $p<0.05$ vs. basal group. Values are means ± SEM, n=8 for basal and sedentary, n=9 for exercise.

Figure 2 – *Hippocampal GR protein*
(A) Representative staining of the entire hippocampus regions were for GR and DAPI to confirm the highest GR:nuclei ratio to be in the CA1 regions. (B) Representative images showing hippocampal GR protein staining for basal, sedentary, and exercise groups. (C) Quantification of the relative signal intensity for each group showing a decrease in GR in the sedentary group compared to both basal and exercise (n=5/group). AU = arbitrary units. (D) Western blotting of the hippocampus showed that sedentary animals had decreased hippocampal GR protein compared to basal and exercise groups. * - $p<0.05$ vs. basal and exercise groups. Values are means ± SEM, n=8 for basal and sedentary, n=9 for exercise.

Figure 3 – *Hypothalamic CRH protein*
(A) Representative DAPI staining to confirm the location of the hypothalamus. (B) Representative images showing CRH (red) and nuclei (blue) for basal, sedentary, and exercise groups. (C) Quantification of the relative signal intensity for each group showing no difference in CRH protein between the basal and sedentary groups, and an elevation in CRH in the exercise group. AU = arbitrary units. * - $p<0.05$ vs. basal and sedentary. Values are means ± SEM, n=5 for all groups.

Figure 4 – *Pituitary GR protein, adrenal MC2R and StAR proteins*
(A) Western blotting for GR protein in the pituitary showed no group differences. (B) Western blotting for MC2R in the adrenal glands showed increased protein in the sedentary group compared to the basal and exercise groups. (C) Western blotting for StAR in the adrenal glands showed the sedentary animals to have higher expression compared to the exercise animals. The exercise group did not differ from the basal group. * - $p<0.05$ vs basal and exercise; † - $p<0.05$ vs sedentary. Values are means ± SEM, n=8 for basal and sedentary, n=9 for exercise.

Figure 5 – *GR and 11βHSD1 protein in liver, skeletal muscle, and adipose tissue*
Western blotting showed no differences between groups for either GR or 11βHSD1 in the (A) liver and (B) skeletal muscle. (C) The sedentary animals had higher expression of GR protein compared to the basal group in epididymal adipose tissue, whereas the exercise animals did not differ from the basal. The exercise animals showed a trend for higher expression of 11βHSD1 compared to the basal animals ($p=0.07$), but no differences were

found between sedentary and basal groups. * - $p<0.05$ vs. basal. Values are means ± SEM, n=8 for basal and sedentary, n=9 for exercise.

Figure 6 – *Summary of the effects of type 2 diabetes and exercise on the HPA axis in ZDF rats.*

Sedentary ZDF rats have increased circulating GCs prior to the development of insulin resistance, likely through increased adrenal sensitivity to ACTH. In contrast, exercising ZDF rats maintain low circulating GCs through the maintenance of normal adrenal sensitivity to ACTH. ↓, ↔ and ↑ symbols represent decreases, no change and increases respectively, compared to basal ZDF rats.

Figure 1

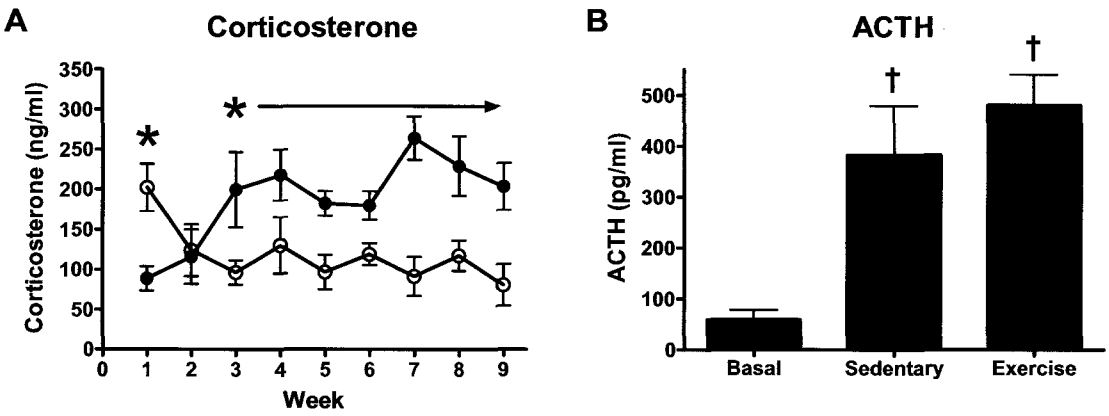

A
Corticosterone
Corticosterone (ng/ml)
350
300
250
200
150
100
50
0
0 1 2 3 4 5 6 7 8 9
Week
*
*
B
ACTH
ACTH (pg/ml)
500
400
300
200
100
0
†
†
Basal
Sedentary
Exercise

Figure 2

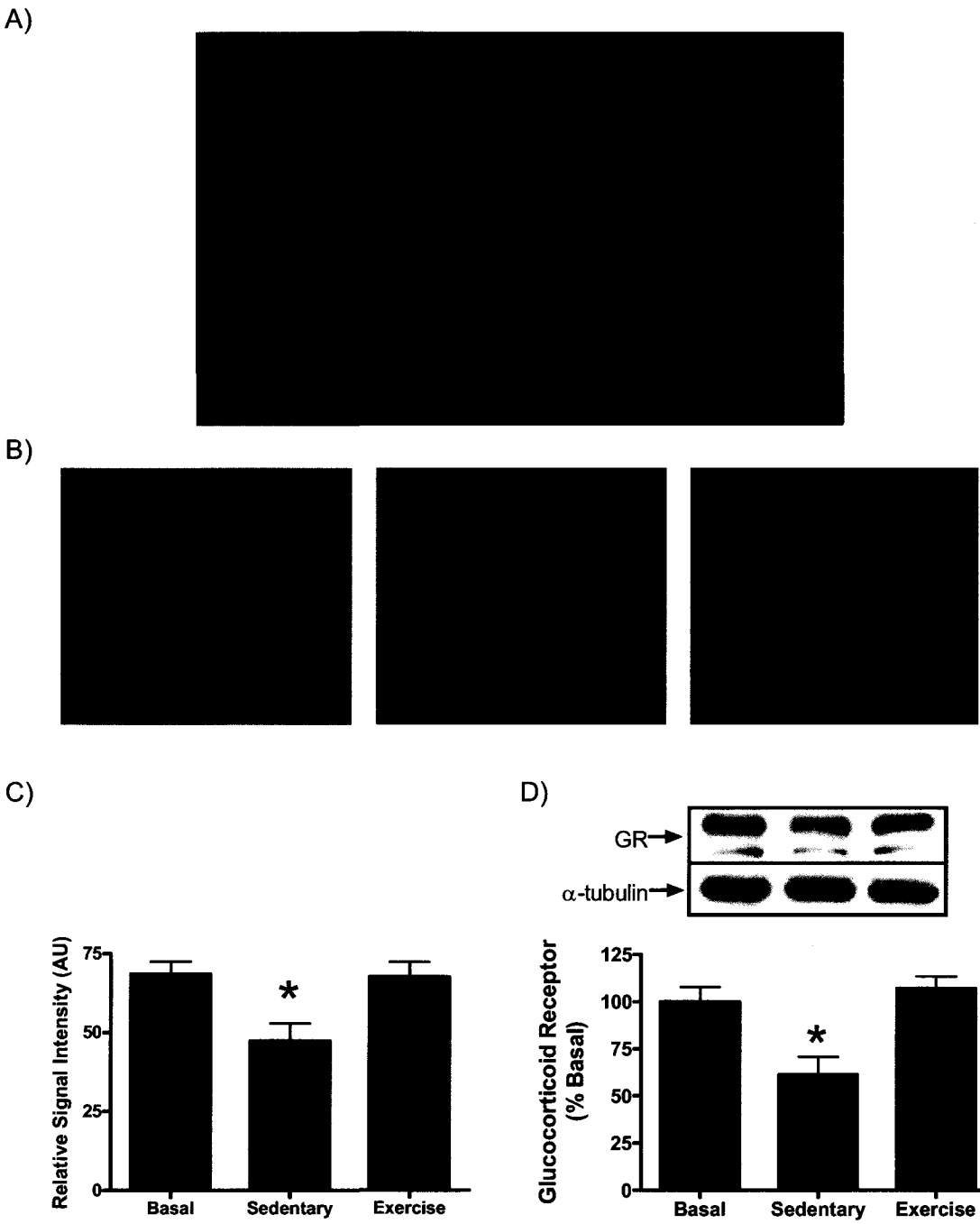
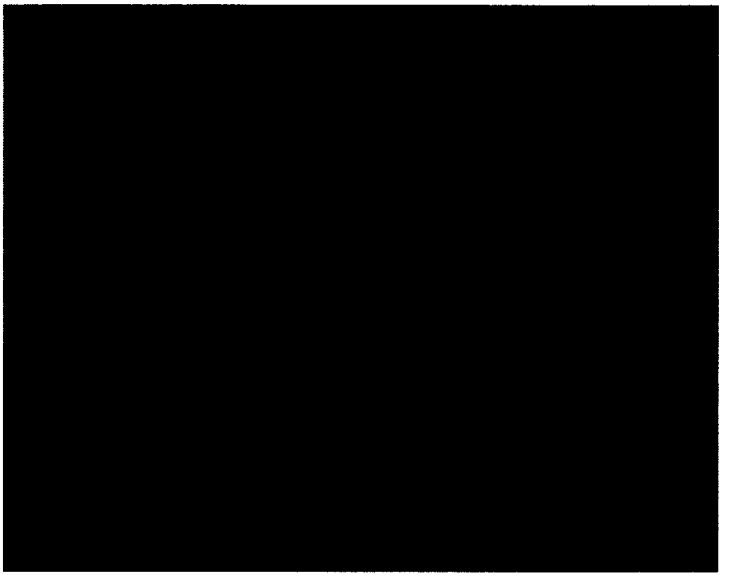

A)
B)
C)
D)
GR
α-tubulin
Relative Signal Intensity (AU)
75
50
25
0
*
Basal
Sedentary
Exercise
Glucocorticoid Receptor (% Basal)
125
100
75
50
25
0
*
Basal
Sedentary
Exercise

Figure 3

A)



B)

C)

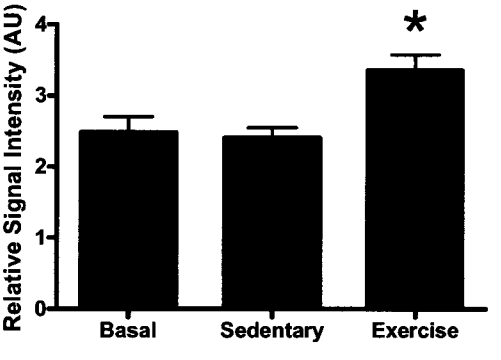

Relative Signal Intensity (AU)
0
1
2
3
4
*
Basal
Sedentary
Exercise

Figure 4

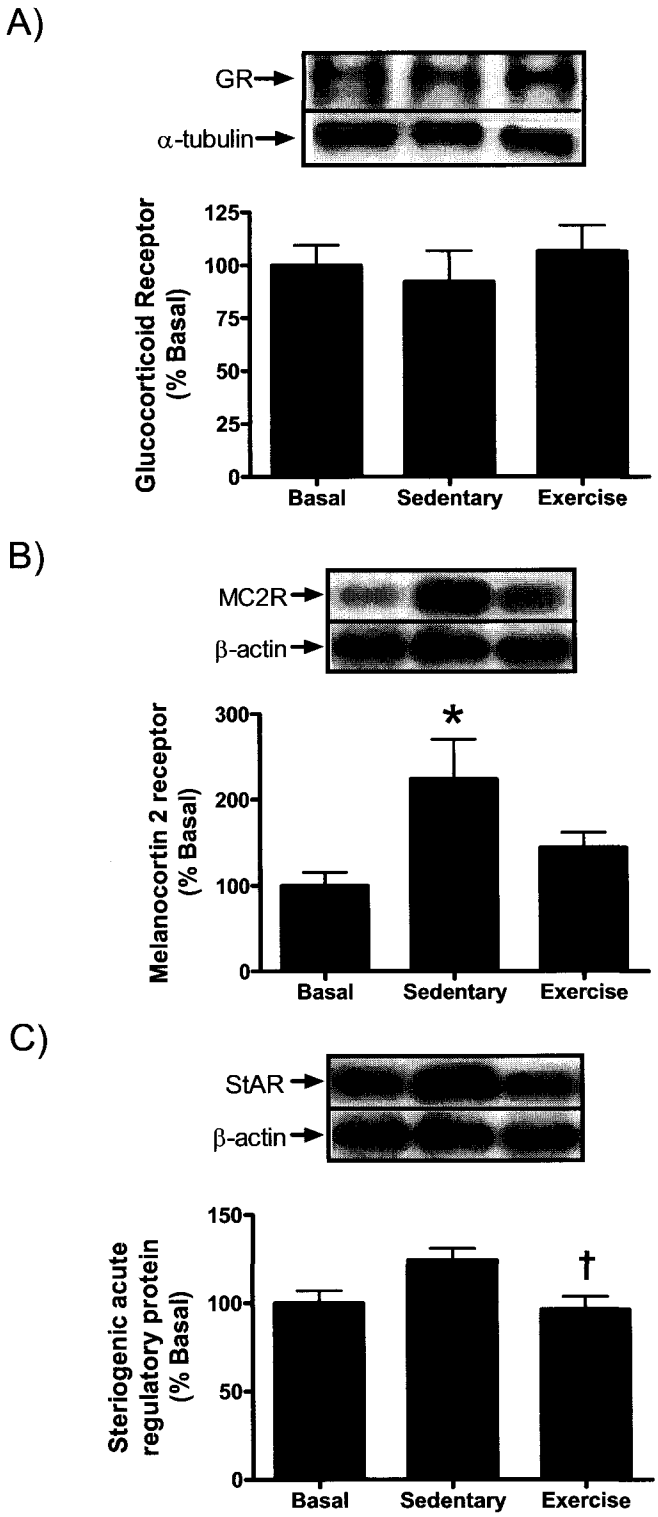

A)
GR
α-tubulin
Glucocorticoid Receptor (% Basal)
125
100
75
50
25
0
Basal
Sedentary
Exercise
B)
MC2R
β-actin
Melanocortin 2 receptor (% Basal)
300
200
100
0
*
Basal
Sedentary
Exercise
C)
StAR
β-actin
Steriogenic acute regulatory protein (% Basal)
150
100
50
0
†
Basal
Sedentary
Exercise

Figure 5

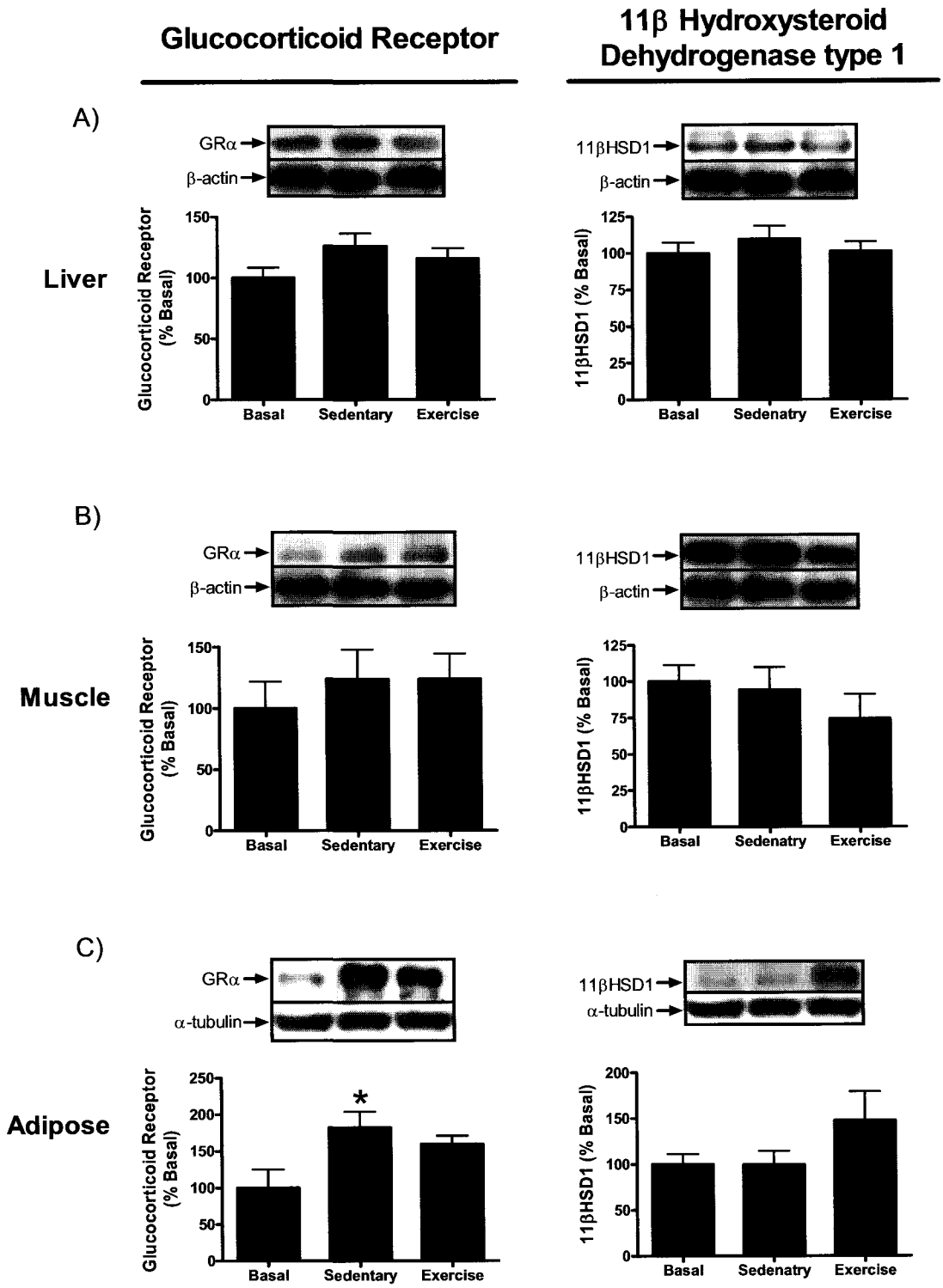
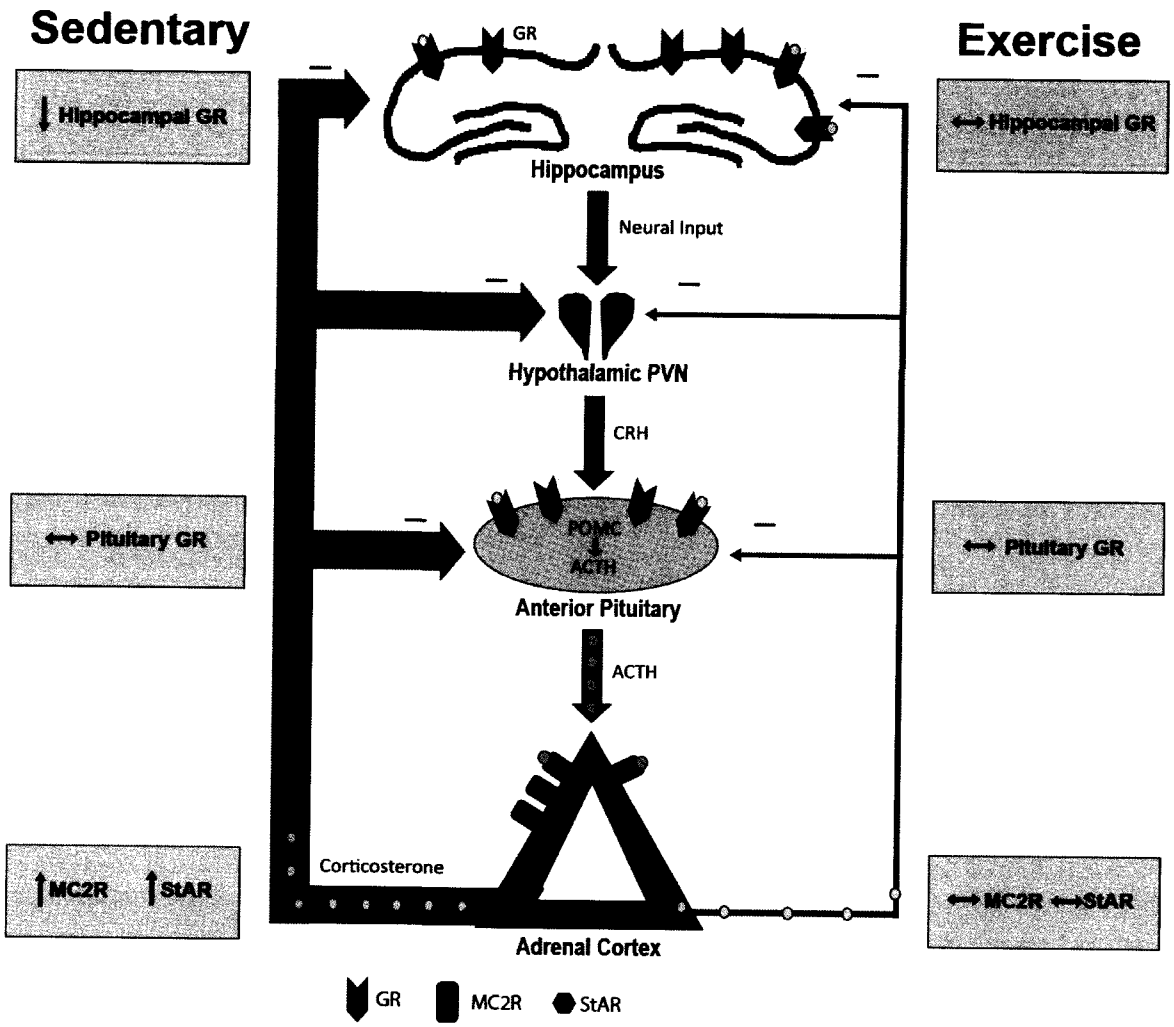

Glucocorticoid Receptor
11β Hydroxysteroid Dehydrogenase type 1
A)
GRα
β-actin
11βHSD1
β-actin
Liver
Glucocorticoid Receptor (% Basal)
11βHSD1 (% Basal)
Basal
Sedentary
Sedenatry
Exercise
B)
GRα
β-actin
11βHSD1
β-actin
Muscle
Glucocorticoid Receptor (% Basal)
11βHSD1 (% Basal)
Basal
Sedentary
Sedenatry
Exercise
C)
GRα
α-tubulin
11βHSD1
α-tubulin
Adipose
*
Glucocorticoid Receptor (% Basal)
11βHSD1 (% Basal)
Basal
Sedentary
Sedenatry
Exercise

Figure 6


Sedentary
Exercise
GR
Hippocampus
↓ Hippocampal GR
↔ Hippocampal GR
Neural Input
Hypothalamic PVN
CRH
↔ Pituitary GR
POMC
ACTH
Anterior Pituitary
↔ Pituitary GR
ACTH
Corticosterone
↑MC2R ↑StAR
↔MC2R ↔StAR
Adrenal Cortex
GR
MC2R
StAR

MANUSCRIPT #3 – EXERCISE INCREASES GLUCOCORTICOID ACTION IN ADIPOSE TISSUE

4

Rationale for manuscript #3:

Manuscript #1 and manuscript #2 focused on the effects of exercise training on the central components of the HPA axis. Very little literature is available investigating the effects of exercise training on the components of peripheral GC action. In manuscript #2, we reported that exercise training had modest effects on GR and 11βHSD1 in peripheral insulin target tissues, however there was a tendency for an increase in GC action in adipose tissue. A previous publication from our laboratory reported that exercise training in hamsters increases 11βHSD1 enzyme activity in adipose tissue (80). These animals voluntarily exercise at a much higher volume (15-20 km/night) compared to the ZDF rats (3-7 km/night). From this, we hypothesized that exercise training, at an adequate volume, would increase GC action in adipose tissue. However, increased GC exposure in adipose tissue has been associated with the development of insulin resistance and T2DM (18). Therefore, our goals in these experiments were to determine if exercise training in various models of insulin resistance (diet-induced obesity and aging) would increase adipose tissue exposure to GCs and what implications this would have on insulin resistance. This, along with our preceding publication (80), were the first studies to investigate the effects of exercise on peripheral GC action in insulin target tissues.

Author Contributions:

Conceived and designed the experiments: JEC (50%), MCR (50%). Trained the animals: JEC (100%). Performed the experiments: JEC (80%), SF (20%). Analyzed the data: JEC (50%), TJH (20%), MCR (30%). Wrote the paper: JEC (50%), TJH (20%), MCR (30%).

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Endurance exercise training increases adipose tissue glucocorticoid exposure: Adaptations that facilitate lipolysis

Running Title: Exercise Increases Adipose Tissue GR and 11βHSD1

Key Words: visceral fat, subcutaneous fat, voluntary wheel running, adipose triglyceride lipase, hormone sensitive lipase

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Abstract

Glucocorticoids (GC) have long been thought to be lipolytic in nature. Recently, however, increased exposure to glucocorticoids (GCs) in insulin-sensitive tissues has been associated with lipid accumulation and metabolic complications, regardless of plasma concentrations. Intracellular GC action is determined by both 11-beta hydroxysteroid dehydrogenase type 1 (11βHSD1) and the GC receptor (GR). We hypothesized that exercise training would increase 11βHSD1 and GR protein in adipose tissue, resulting in increased lipolysis. To test the effects of exercise on adipose tissue GR and 11βHSD1 protein, two sets of hamsters were trained for 6 weeks; young, diet-induced obese animals and older, overweight animals. Young (6 week) hamsters, fructose-fed to induce an obese phenotype, and older (6 month) hamsters were randomly divided into exercising and sedentary groups. Exercise training decreased adipose tissue mass in both fructose-fed and older hamsters. Additionally, exercise training increased 11βHSD1 (31.5±15% and 20.0±7%, fructose-fed and older, respectively) and GR (45.6±14% and 61.1±27%, fructose-fed and older, respectively) protein expression in the perirenal adipose depot and increased 11βHSD1 (16.7±7%, $p=0.09$) and GR (47.4±19%, $p<0.05$) in the subcutaneous adipose depot of the older hamsters. To determine the metabolic effect of increased GC exposure in adipocytes, 3T3-L1 adipocytes were treated with corticosterone for 24h and measures of lipolytic rates were conducted. Low concentrations of GCs (0.01-0.1 μM) increased GR (44.1±18%, $p<0.05$) and 11βHSD1 (95.3±24%) protein expression, as well as increasing lipolytic rates (34.6±6%) as measured by glycerol release. The increased lipolysis was blocked by RU486, a GR antagonist, suggesting the elevated lipolysis was a direct result of GC action. These

results suggest that exercise training amplifies the activity of GCs in adipose tissue of overweight animals, through alterations in 11βHSD1 and GR, despite differences in age and amounts of adiposity. *In vitro*, GCs are capable of increasing lipolysis, but depend upon the presence of GR. We purpose GCs play a significant role in changing the phenotype of adipose tissue during exercise training, resulting in decreased fat mass.

Introduction

Glucocorticoids (GCs) are steroid hormones released from the adrenal glands in response to a variety of physiological and psychological stressors. These stress hormones act by binding to cytosolic glucocorticoid receptors (GR), resulting in a positive or negative genomic effect (86). Two major glucocorticoids are released from the adrenal glands in humans, cortisol and cortisone, with the former considered active and the latter biologically inactive (86). In rodents, the equivalent glucocorticoids are corticosterone (active) and 11-dehydrocortisone (inactive). 11-beta hydroxysteroid dehydrogenase type 1 (11βHSD1) is a pre-receptor enzyme that is capable of converting inactive GCs into their active form (i.e. cortisone/11-DHC into cortisol/corticosterone) (333). Previous research has shown that tissue specific overexpression of 11βHSD1 results in a dramatic increase in intracellular concentrations of active GCs, despite normal plasma concentrations (18). Therefore, physiological activity of GCs is determined not only by circulating concentrations of these hormones but also by the protein levels of both GR and 11βHSD1 (145).

GCs have many important physiological roles, including increasing the amount of energy substrates in the blood during energy deprivation (i.e. hypoglycaemia) (86). While the effects in other tissues such as liver or skeletal muscle are more clearly defined, the effect of GCs in adipose tissue is controversial. GCs promote pre-adipocyte differentiation and adipogenesis, while inhibition of 11βHSD1 prevents cortisone-induced pre-adipocyte differentiation (334). In humans, elevated 11βHSD1 expression in visceral adipose tissue is associated with central obesity and increased fat cell size (335, 336). Mice overexpressing 11βHSD1 under the adipocyte-specific aP2 promoter have

higher tissue GC levels and develop central obesity as a result of increased adipocyte size (18). On the other hand, weight loss via dietary restriction increases 11βHSD1 expression in the adipose tissue of obese persons (177). If this increase in 11βHSD1 with diet is a compensatory response to decreased fat mass or if it helps to facilitate increased adipose tissue lipolysis is unclear.

In human (337, 338) and rat adipocytes (280), GCs have been shown to increase lipolysis, possibly through increased expression of hormone sensitive lipase (HSL) (280). Yet in human adipocytes, GCs are thought to inhibit lipid mobilization in the presence of insulin, thus leading to triglyceride accumulation and retention (339). Since the density of GC receptors is higher in intra-abdominal (visceral) fat than in other fat depots (336), the increased activity of GCs is thought to be accentuated in visceral adipose tissue (340). Therefore, it may be that the greater GC action observed in central fat depots, in the presence of elevated insulin, results in the accumulation of fat. This increase in abdominal obesity is consistent with some pathological conditions such as Cushing's' Syndrome and the metabolic syndrome. Indeed, reducing GC exposure in adipose tissue protects from diet-induced obesity in rodents (178, 179). Taken together, these findings suggest that GCs may play a paradoxical role in adipose tissue, causing lipolysis in some situations while facilitating fat accumulation and, thus, obesity in others.

Previous work from our laboratory demonstrates that exercise training in hamsters reduces GR and 11βHSD1 in skeletal muscle and liver tissue, which would decrease GC activity, however, exercise paradoxically increases 11βHSD1 activity in central fat stores (80). We hypothesize that this increase in 11βHSD1 activity results from increased protein levels and would elevate the action of GCs. This study shows that exercise

training results in elevated levels of both 11βHSD1 and GR protein. Importantly, we show that this effect of exercise takes place in both our animal models of excessive adiposity and that both exercising groups have decreased fat mass. Furthermore, we use *in vitro* experiments to show that GCs are capable of increasing lipolysis and that this is dependent upon the presence of GR. This data shows the important role that GCs have during exercise-mediated weight loss.

Methods

In vivo studies

Animals and Diets

Study 1: To assess the effects of exercise on adipose tissue in a model of diet-induced obesity, 16 young, male Syrian golden hamsters (*Mesocricetus auratus,* Charles River, Montreal, QC, Canada), at an initial age of 6 weeks and an initial weight of 87.7 ± 0.84g (mean ± SEM), were individually housed in clear cages and kept in a temperature (23-25°C) and humidity (40-50%) controlled room for a 7 day habituation period. During habituation, the animals were fed standard rodent chow (Purina 5001, 4.3kcals/g metabolizable energy). Following this period, the hamsters were randomly assigned to either sedentary or exercise groups. Animals were given a high fructose diet *ad libitum* (*Dyets*, 60% fructose, 4.1 kcals/g metabolizable energy), to induce visceral obesity and whole body insulin resistance (73, 74). Exercising animals were individually housed in specialized activity wheel cages (height: 36.4 cm, width: 26.8 cm, depth 50 cm) with unrestricted, 24-hour access to their wheels (circumference: 108 cm, width: 9 cm). Sedentary animals were housed in similar sized cages, but without activity wheels. Wheel

revolutions, body weight, and food intake were recorded daily. Running distance was calculated daily as the circumference times the revolutions.

Study 2: To assess the influence of exercise on adipose tissue of an older sedentary, over-weight model, 16 male Syrian golden hamsters, at an initial age of 6 months and an initial weight of 151.2 ± 3.12g, were individually housed in a similar environment as previously described for the fructose-fed hamsters for a 7 day habituation period and fed a standard rodent chow. Following habituation, the hamsters were randomly assigned to either a sedentary or exercise group. Exercising and sedentary animals were housed in running wheel cages and standard cages, respectively, as previously described for the fructose-fed hamsters. Wheel revolutions, body weight, and food intake was recorded daily. Daily body weight and food intake for these animals have been previously published (79).

All animal experiments were approved by the Animal Care Committee at York University and were conducted in accordance with guidelines set forth by the Canadian Council for Animal Care.

Blood sampling

Non-fasted blood samples were taken via saphenous vein puncture on the first day of every week at approximately 1300h for determination of whole blood glucose and plasma insulin concentrations. This method of blood sampling causes minimal stress to the animal and has been described previously (341). Approximately 90µl of whole blood was collected into heparin coated microvettes (SARSTEDT Inc., Montreal, QC, Canada), centrifuged at 4°C and 2000 RPM for 5 minutes, and the plasma was frozen and stored at -20°C until further analysis.

Intraperitoneal glucose tolerance test

At the end of the 38 days, intraperitoneal glucose tolerance tests (IPGTT) were performed on all hamsters after an 18h overnight fast. To minimize the effect of stress due to repeated blood sampling, the hamsters were lightly anaesthetized with sodium pentobarbital (0.05 g/kg). Previous studies using sodium pentobarbital during glucose tolerance tests report no effect of the anesthesia on glucose dynamics (305). Prior to the IPGTT, a fasted blood sample was taken at time 0 min (0830h), 30 minutes following the administration of the sedative. Glucose (2 g/kg) was then administered intraperitoneally, dissolved in saline, via a 23-gauge needle. Subsequent blood samples were taken at 30, 60, 90, and 120 min. All blood samples were collected via saphenous vein puncture in the same method as describe above, with the first drop of blood (~15µl) used for whole blood glucose measurements and the next 40µl used for determination of insulin concentration.

Euthanization

Following the IPGTT, animals were euthanized by decapitation and adipose tissue (perirenal in young hamsters; perirenal and subcutaneous in old hamsters) and various skeletal muscles were removed, weighed and snap frozen on dry ice.

Metabolic hormone concentrations

Fed plasma insulin, leptin, and glucagon hormone concentrations were determined from the final week saphenous vein blood samples using the Luminex^{TM} 100 instrument and the LINCOplex^{TM} Well Plate Assay rat/mouse endocrine panel (LINCO Research, INC, St. Charles, MO, United States). Corticosterone concentrations in the plasma collected during the final week were determined with a commercially available RIA kit (MP Biomedicals, Cat# 07-120102).

Plasma insulin collected during the IPGTT was determined using a commercially available ELISA kit (Crystal Chem Inc., Cat# INSKR020).

Skeletal muscle cytochrome c oxidase activity

Cytochrome C Oxidase activity in the plantaris muscle was determined as previously described (342). Enzyme activity was determined as the maximal rate of oxidation of fully reduced cytochrome C, measured by the change in absorbance at 550 nm in a Microplate Reader (ELx800 Universal, Bio-tek instruments).

In vitro studies

To assess the role of GCs on adipose tissue 11βHSD1 and GR expression and on rates of adipose tissue lipolysis *in vitro*, 3T3-L1 cells were grown for 2 days post-confluence in 10% FBS/DMEM at 37°C and 5% CO_2. Differentiation was induced with 10% FBS/DMEM containing 500 μM IBMX, 0.25 μM DEX and 200 pM insulin for 4 days. The cells were then incubated in 10% FBS/DMEM containing 200 pM insulin for 4 days. Following this, the cells were incubated in 10% FBS/DMEM until greater than 95% of cells contained lipid droplets (~2-4 days). The medium was changed every other day throughout the entire differentiating period.

Glucocorticoid Dose Response

Following differentiation, 3T3-L1 cells were incubated in a 3% FBS/DMEM containing various concentrations of corticosterone (Sigma, Cat#C2505) for 24h at 37°C and 5% CO_2. Following this, the cells were immediately placed on ice and lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% Triton-x-100, 10% glycerol, pH 7.4) was added. The cells were scraped into separated chilled tubes and

centrifuged at 5000 rpm for 30 minutes. The supernatant was removed and stored at -80 until used. For lipolysis experiments, 3T3-L1 cells were incubated in 3% FBS/DMEM without phenol red, containing various concentrations of corticosterone for 24h at 37°C and 5% CO_2. In addition to the corticosterone, cells were incubated either with DMSO or 10 μM RU486 (Sigma, Cat#M8046), the GR antagonist. A sample of the media was removed at 0h and 24h to determine glucocorticoid stimulated lipolysis. Lipolysis was determined as the rate of glycerol appearance and expressed as a percent of control cells. Glycerol concentrations were determined with a commercially available kit (Sigma, Cat#FG0100). All *in vitro* data are an average of three separate experiments, with two replicates per condition in each experiment.

Immunoblotting

This method for protein preparation and quantification has been previously described (80). Briefly, tissue samples were homogenized to obtain total protein, centrifuged at 5000 rpm for 30 min, and the supernatants were collected. Protein concentrations were assessed by Bradford method. Seventy-five micrograms of total protein was electrophoretically resolved on either an 8% SDS-polyacrylamide gel (GR), 10% (HSL and ATGL) or 12% SDS-polyacrylamide gel (11βHSD1) and transferred overnight at 20V to *PVDF* paper. Blots were blocked with 5% milk in TTBS and then incubated overnight in primary antibody at 4°C (GR: Santa Cruz, Cat#:sc-8992, 1:1000; 11βHSD1:Alpha Diagnostic, Cat#:BHSD11-S, 1:1500, HSL: Cell Signaling, Cat#4107, 1:1000, ATGL: Cell Signaling, Cat#2138, 1:1000). Blots were incubated with secondary antibody for 1 hour at room temperature and hybridization signals were visualized using the Western Lightning Chemiluminescence Reagent Plus kit (PerkinElmer, Wellesley,

MA) after exposure to Kodak X-Omat Blue x-ray film (Rochester, NY). β-actin and GAPDH were used as a loading controls in tissue and 3T3-L1 cells, respectively.

Data analysis

For all experiments, the appropriate t-test, one-way, or two-way ANOVA was performed to identify significant differences between treatment groups using Statistica 6.0 software, with $p<0.05$ as the criterion. When a significant difference was observed with an ANOVA, post-hoc analysis using contrasts with a Bonferroni correction factor were performed to determine specific differences. Data are presented as mean ± S.E.M.

Results

In vivo studies

Wheel running causes a transient reduction in body mass gain and an increase in food intake in both fructose-fed and older hamsters.

Body weights for the fructose-fed and older hamsters are shown in figure 1A. Both exercise groups demonstrated a transient decrease in body weight following the introduction of the running wheel. This occurred from day 1 to day 5 in the fructose-fed hamsters (left panel, $p<0.05$) and from day 3 to day 10 in the older chow-fed animals (right panel, $p<0.05$). A significant main effect for time was found in the fructose-fed hamsters, but not in the older hamsters. In both groups of hamsters, body mass was similar between exercising and sedentary groups by the end of the experimental period.

In both groups, food intake remained similar between the exercising and sedentary hamsters at the beginning of the study (figure 1B). It is important to note that the fructose and standard chow diets were isocaloric. The exercising hamsters began to

consume more food than the respective sedentary group after the introduction of the running wheel, and this became statistically different in both groups by day 8 ($p<0.05$).

Wheel running causes an increase in skeletal muscle mass and increases mitochondrial oxidative capacity.

Both the fructose-fed and older hamsters ran similar distances through out the protocol, with the average distance for both groups being 11.35±0.13km/day over the entire protocol.

Exercise training resulted in heavier plantaris weights in the fructose-fed hamsters, but not the older hamsters (Table 1). However, soleus weight was significantly higher in both exercising groups compared with their respective sedentary group ($p<0.05$). Cytochrome c oxidase activity was ~35% higher in exercising hamsters compared to sedentary hamsters ($p<0.05$; Table 1).

Wheel running causes a reduction in fed insulin and leptin levels in both fructose-fed and older hamsters.

Table 1 shows non-fasted hormone concentrations in the plasma taken during the final week of training. Both exercise groups had lower fed plasma insulin concentrations in comparison with their respective sedentary controls, however, only in the fructose-fed animals did this difference reach statistical significance ($p<0.05$). No differences were found with glucagon concentrations in any of the groups (data not shown). Exercising hamsters had significantly lower plasma leptin than sedentary hamsters in both groups ($p<0.05$). Exercise had no effect on either basal corticosterone or fed blood glucose concentrations.

Wheel running improved glucose tolerance in fructose-fed young, but not older over-weight hamsters.

Fructose-fed exercising hamsters had lower blood glucose and insulin concentrations compared to the sedentary hamsters throughout the IPGTT ($p<0.05$; Table 2.). In the older hamsters, the exercise group demonstrated smaller differences in blood glucose and insulin concentrations, with only the baseline and 120 minute insulin time points achieving statistical significance ($p<0.05$). An insulin sensitivity index (ISI = $2/[(AUC_{glucose} \times AUC_{insulin}) +1]$)) was calculated based on the area under the curve for both glucose and insulin, during the IPGTT, as has previously been reported (22) (Table 2). Using this formula, both exercise groups had improved insulin sensitivity compared to their sedentary controls ($p<0.01$).

Wheel running increases the intracellular determinants of glucocorticoid action in adipose tissue and lowers adipose tissue mass.

To investigate the effects of exercise training on adipose tissue exposure to GCs, western blotting for both 11βHSD1 and GR was completed in the adipose tissue of the fructose-fed and older hamsters. Figure 2 shows the perirenal adipose tissue mass and the protein expression for both 11βHSD1 and GR in the fructose-fed hamsters. The exercise group had lower fat mass compared with the sedentary group ($p<0.01$) and a 46% increase in adipose GR protein expression ($p<0.05$) and a 32% increase in 11βHSD1 protein expression ($p=0.08$).

Similar to the fructose-fed hamsters, the older exercise group had significantly decreased perirenal adipose mass and increased 11βHSD1 and GR protein expression compared to sedentary group (20% and 61%, respectively; $p<0.05$). The older exercise

group also demonstrated a 47% increase in GR ($p<0.05$) and a trend for a 16% increase in 11βHSD1 ($p=0.09$) in the subcutaneous adipose tissue (Figure 3).

Wheel running increases perirenal and subcutaneous adipose tissue HSL and ATGL protein expression.

Figure 4 shows the expression of HSL and ATGL in the perirenal and subcutaneous adipose depots in the older hamsters. The exercise group had increased HSL and ATGL protein expression in both the perirenal and subcutaneous adipose depots, compared to sedentary group (all $p<0.01$). This data suggests that the adipose tissue of the exercising hamsters has an increased potential for lipolytic activity. This may be one potential mechanism for the exercise-induced weight loss seen in these hamsters, which may be mediated through increased GC exposure.

<u>In vitro studies</u>

Glucocorticoids alter adipocyte lipolytic rates and the protein expression of 11βHSD1 and GR in a concentration-dependent fashion.

To determine a potential mechanism for the increased GR and 11βHSD1 protein expression in adipose tissue, 3T3-L1 adipocytes were incubated in various concentrations of corticosterone for 24 hours (figure 5). Lower concentrations (0.01-0.1 μM) of GCs caused an increase in both 11βHSD1 and GR protein levels compared with control cells, whereas higher concentrations (1-100 μM) had no effect on 11βHSD1 but caused a decrease in GR protein expression ($p<0.05$). This data shows that low concentrations of GCs are able to increase 11βHSD1 and GR protein to magnitude seen *in vivo* in the exercising hamsters.

To assess the role of GCs on adipose tissue lipolysis, 3T3-L1 adipocytes were incubated in corticosterone for 24 hours with or without RU486, a GR antagonist (figure 6). A concentration-dependent increase in lipolysis was found with increasing corticosterone concentrations up to a maximum of 10 μM (35%, $p<0.05$). Higher concentrations of corticosterone did not increase lipolytic rate. Co-incubation with RU486 prevented the GC induced increases in lipolysis at all concentrations over the 24 hour period. This indicates that the effects of GCs on lipolysis are mediated through GR and the greatest effect observed at low-to moderate concentrations.

Discussion

The activity of GCs within cells is dependent largely on the conversion of inactive hormone to biologically-active hormone by 11βHSD1 and the expression levels of the hormone receptor, GR. While elevated GCs have largely been associated with negative effects on human health, including central adiposity and insulin resistance, it is interesting to note that exercise results in decreased adiposity and increased insulin sensitivity despite elevations in GCs. To address this apparent disparity in GC action, two distinct populations of hamsters with elevated central adiposity which would mimic the human over-weight conditions (diet-induced young hamsters and sedentary older hamsters) were exercise trained for 6 weeks.

We demonstrate that exercise training increases the potential for GC action in adipose tissue by increasing the expression of 11βHSD1 and GR. Furthermore, exercise training resulted in an increased expression of HSL and ATGL, two key lipolytic enzymes. In order to determine the functional consequences of GC action in adipocytes,

we undertook *in vitro* experiments on 3T3 adipocytes. These experiments demonstrate that GCs are capable of increasing lipolysis in a concentration dependent manner and this effect is dependent on the presence of GR as antagonism of this receptor abolishes any change in lipolytic rate. From this, we believe that in the situation of exercise training, GC exposure is enhanced in various adipose depots and this upregulation facilitates an increase in lipolysis.

Our previous research indicated that exercise training increases the activity of 11βHSD1 in the adipose tissue of young, healthy hamsters (79). Here, we strengthen these findings by showing that exercise increases both 11βHSD1 and GR in two distinct populations of hamsters with elevated central adiposity. Both groups had more adiposity, relative to their body weight, compared with the young, healthy hamsters from our previous study (79). The fructose-fed, young hamsters had more intra-abdominal fat compared with the over-weight, older hamsters (figures 2 and 3), which likely led to the elevated levels of leptin (table 1) and decreased insulin sensitivity (table 2) observed in the sedentary fructose-fed animals compared with the older sedentary animals. Interestingly, both the fructose-fed and older hamsters exercised at approximately the same volume and consequently had similar amounts of perirenal adipose mass and similar results during the IPGTT. Both exercise groups displayed similar increases in 11βHSD1 and GR protein content, which shows that exercise training has the ability to increase GC action in adipose tissue regardless of diet or age. The functional consequence of this event is still not completely understood, however, it is tempting to believe that GCs are driving the observed increases in HSL and ATGL found in the exercising hamsters. GCs have been purposed to be lipolytic in their activity (280, 321,

338). Previous research has shown that exercise is capable of increasing HSL in adipose tissue (343), but no data exists on the relationship between exercise and ATGL. GCs are capable of increasing HSL mRNA in adipocytes (280), and others have shown that GCs can increase ATGL mRNA in non-adipocytes (278). Our own unpublished data shows that incubating 3T3-L1 adipocytes in GCs increases ATGL protein in a concentration dependent manner (data not shown). Still, more evidence is needed before a direct link between the elevated concentrations of GCs during exercise and the increased protein levels of HSL and ATGL in the exercise trained animals reported here.

A number of studies suggest that GCs are adipogenic, particularly when circulating insulin levels are also high (18, 24, 178, 260). Thus, it interesting that the trained animals have significantly less adipose mass, suggesting an elevated state of lipolysis rather than lipogenesis, despite elevations in GCs. Our observation that fat mass reduction is associated with increased 11βHSD1 and GR expression is in line with the findings by Tomlinson et al, (177) which show that dietary induced weight loss increases the expression of 11βHSD1 in subcutaneous adipose tissue. One discrepancy between our findings, and those that show GCs to be adipogenic, may be the metabolic state, which influences the levels of several circulating hormones and substrates, of the subjects. The adipogenic effects of GCs are often seen in animals with elevated levels of insulin (18, 24, 178). In a situation of low insulin, such as dieting, elevated GC action is associated with decreased adiposity (177). The exercise-trained hamsters in our study were very glucose-tolerant (table 2), and thus would have low circulating concentrations of insulin. In this situation, GCs may be permitted to stimulate lipolysis and result in the reduced

adiposity observed. The relationship between insulin and GCs is interesting and certainly merits further attention.

To identify the direct effects of GCs in adipocytes, we incubated 3T3-L1 cells in increasing concentrations of corticosterone similar to, and above, values observed in active animals (208, 214, 247). Lower concentrations of corticosterone, similar to the values typically found in exercising animals, increased lipolysis over a 24h period. Importantly, we show that the lipolytic action of GCs is dependent upon the availability of GR. Co-incubation with a GR antagonist prevented any increase in lipolysis. We also show that lower concentrations of GCs increase the protein content of 11βHSD1 and GR in adipocytes. It is interesting to note that lipolysis is still increased at moderate to higher concentrations of GCs, despite no differences in 11βHSD1 protein and a progressive decline in GR protein. All of these measurements were taken after 24h incubation, making it difficult to determine the timing of these events. One possibility is that the GC-mediated events that increase lipolysis (i.e. increased ATGL) occur at an earlier time point and remain active through to 24h. However, down regulation of 11βHSD1 and/or GR with sustained exposure to the moderate-high concentrations may take place as a secondary action, in a negative-feedback manner. In this situation, it is possible to have increased lipolysis despite the observed changes in 11βHSD1 and GR. Indeed, the highest concentration (100 μM) may activate the negative-feedback (i.e. reduced GR) before the lipolytic actions can take place. This would explain why lipolysis did not continue to increase from the 10 μM to 100 μM concentrations, where GR protein was the lowest. This situation is likely representative of a pathological state such as Cushing's syndrome, where an organism is chronically exposed to elevated levels of GCs, and

subsequently develops severe obesity. Data from our laboratory has shown that chronically elevating GCs through exogenous administration in rodents increases adiposity, even if the animal undergoes exercise training (Mejia K and Riddell MC, unpublished observations). It is unlikely that the animals in the current study are chronically exposure to GCs because of exercise, but rather experience intermittent increases during activity. Still, further experiments aimed at delineating a time course for these events would help to clarify these inconsistencies.

In summary, we show that two different animal models of excessive adiposity, undergoing exercise training, display increased protein levels of 11βHSD1 and GR in adipose tissue. This adaptation likely results in increased GC action within the adipocyte, which is accompanied by decreases in adipose mass. Our *in vitro* data shows that GCs are capable of increasing lipolysis and that this depends on GR. We purpose that exercise-induced increases in GC action, through elevations in GR and 11βHSD1, increase the lipolytic action in adipose tissue and facilitate a decrease in adiposity.

Table 1: ***Skeletal Muscle Characteristics and Post-prandial Hormone Concentrations***

	Fructose-fed Sedentary	Fructose-fed Exercise	Older Sedentary	Older Exercise
Skeletal Muscle Properties				
Plantaris (mg)	28.8 ± 0.9	38.1 ± 1.4*	39.5 ± 1.8	39.1 ± 2.2
Soleus (mg)	14.1 ± 0.8	26.2 ± 1.9*	25.7 ± 0.9	38.8 ± 1.8*
Cytochrome C Oxidase Activity (U/g)	8.05 ± 0.3	10.76 ± 0.9*	6.74 ± 0.3	9.24 ± 0.3*
Post-prandial Hormones				
Glucose (mM)	4.43 ± 0.3	4.28 ± 0.2	4.66 ± 0.3	4.44 ± 0.2
Insulin (pM)	697.2 ± 59	417.4 ± 65*	210.6 ± 53	176.9 ± 37
Leptin (pM)	124.4 ± 17	59.1 ± 12*	34.0 ± 7	21.0 ± 7*
Corticosterone (ng/ml)	45.8 ± 1.9	45.0 ± 1.9	47.8 ± 2.1	49.2 ± 1.3

* - $p<0.05$ vs. respective sedentary group

Table 2: ***Results of the IPGTT; Plasma Glucose, Insulin, and the Insulin Sensitivity Index***

	Fructose-fed Sedentary	**Fructose-fed Exercise**	**Older Sedentary**	**Older Exercise**
Glucose (mM)				
Baseline	6.6 ± 0.4	6.1 ± 0.3	5.9 ± 0.3	5.6 ± 0.5
30 min	14.4 ± 1.0	11.6 ± 1.4*	12.5 ± 1.6	10.5 ± 0.8
60 min	12.5 ± 0.6	10.2 ± 1.5*	9.9 ± 1.6	9.3 ± 1.5
90 min	9.1 ± 0.9	8.2 ± 1.0*	7.9 ± 0.8	7.4 ± 1.0
120 min	8.0 ± 1.0	6.6 ± 0.5	6.2 ± 0.5	6.0 ± 0.7
Insulin (ng/ml)				
Baseline	3.36 ± 0.4	2.84 ± 0.5	3.33 ± 0.2	2.17 ± 0.2*
30 min	6.49 ± 0.6	4.05 ± 0.6*	3.77 ± 0.4	3.38 ± 0.4
60 min	6.52 ± 0.7	3.89 ± 0.4*	1.97 ± 0.6	1.74 ± 0.3
120 min	4.11 ± 0.2	2.76 ± 0.4*	2.64 ± 0.5	1.39 ± 0.2*
Insulin Sensitivity Index (AU, %Sedentary Control)	1.0 ± 0.05	1.49 ± 0.10*	1.0 ± 0.1	1.29 ± 0.06*

* - $p<0.05$ vs. respective sedentary group

Figure Legends

Figure 1: ***Daily changes in body weight and food intake throughout the training protocol in fructose-fed and older hamsters.***
A) The fructose-fed hamsters gained weight throughout the protocol, whereas the older hamsters had stable weights. Both exercise groups experienced a transient decrease in body weight immediately following the introduction of the running wheel. However, exercising hamsters had similar body weight compared to their sedentary group by the end of the 6 weeks B) Both exercise groups consumed more food compared to their sedentary group after the introduction of the running wheel. This became statistically different at day 8 in both groups. * denotes $p<0.05$ vs. respective sedentary group.

Figure 2: ***Perirenal adipose tissue characteristics in the fructose-fed hamsters.***
The left panel shows that the exercise group had significantly less perirenal adipose mass compared with the sedentary group. The middle and right panels show representative blots and the average densitometry for 11βHSD1 and GR, respectively. The expression of both proteins was increased in the exercise group, suggesting the potential for increased GC action in the perirenal adipose of these animals. A trend was found for 11βHSD1 ($p=0.08$), * denotes $p<0.05$ vs. the sedentary group. Average densitometry is expressed as a percent of the sedentary group.

Figure 3: ***Perirenal and subcutaneous adipose tissue in the older hamsters.***
The left panel shows the adipose mass for the perirenal depot, the middle and right panels show the representative blots and average densitometry for 11βHSD1 and GR, respectively. The exercise group had significantly less adipose mass compared to the sedentary group, which was accompanied by increased protein expression for both 11βHSD1 and GR. Furthermore, the exercise group had increased GR, and a trend for increase 11βHSD1 ($p=0.09$) in the subcutaneous adipose. Similar to the perirenal adipose in fructose-fed hamsters, the adipose depots in older hamsters demonstrated increased potential for elevated GC action.). * denotes $p<0.05$ vs. sedentary group. Average densitometry is expressed as a percent of the sedentary group.

Figure 4: ***The expression of HSL and ATGL, the two major lipolytic enzymes in adipose tissue, in the perirenal and subcutaneous adipose tissue of the older hamsters.***
Exercise training was associated with increased ATGL and HSL expression in both the perirenal (A) and subcutaneous (B) adipose depots. This potential increase in lipolytic activity provides a potential mechanism for the exercise-mediated decrease in adiposity.* denotes $p<0.01$ vs. the sedentary group.

Figure 5: ***The effects of GCs on 11βHSD1 and GR protein expression in 3T3-L1 adipocytes.***
The representative blots (A) and the average densitometry (B) for 11βHSD1 and GR show a dose-response change in protein expression. 11βHSD1 protein was increased a low concentrations of GCs, but unchanged at high concentrations. GR protein was increased at low concentrations of GCs, but decreased at high concentrations. This shows

that low concentrations of GCs are able to increase 11βHSD1 and GR protein to levels seen in our *in vivo* studies. Concentrations of corticosterone are presented as μM, with ng/ml in parenthesis below. Values are an average of three separate experiments. * denotes $p<0.05$ vs. control conditions.

Figure 6: ***GCs increase lipolysis in a dose-dependent manner, which can be blocked through co-incubation with the GR antagonist RU486.***
Corticosterone increased lipolysis in the dose range of 0.1-10 μM. Co-incubation with RU486 blocked the lipolytic effects of corticosterone at all concentrations. Lipolysis is an average of three separate experiments, expressed as a percent of control conditions. Concentrations of corticosterone are presented as μM, with ng/ml in parenthesis below. * denotes $p<0.05$ vs. control conditions (DMSO).

Figure 1

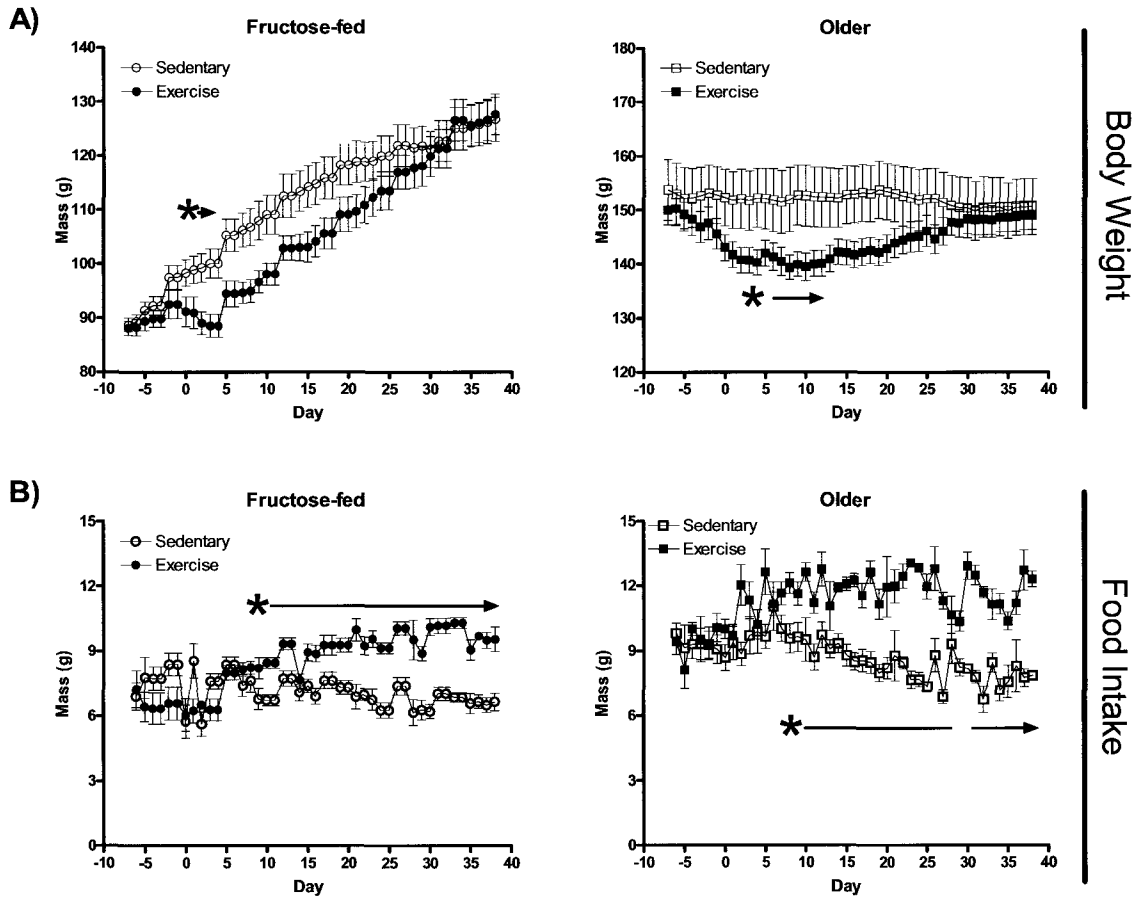


Figure 2

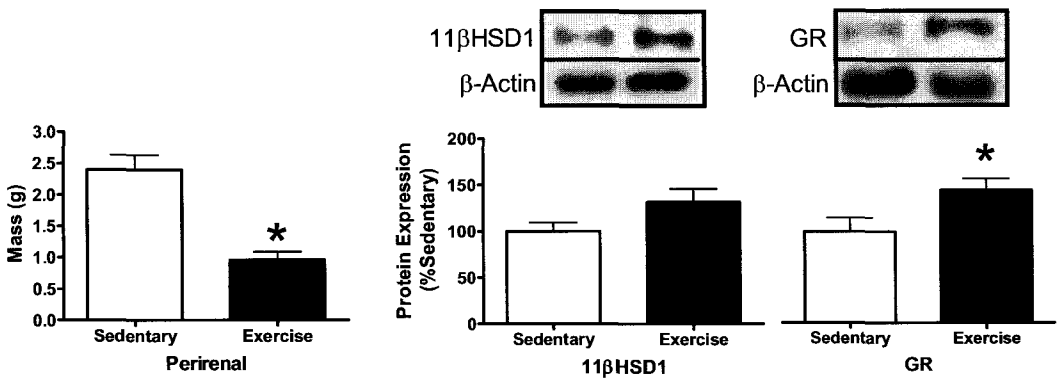


Figure 3

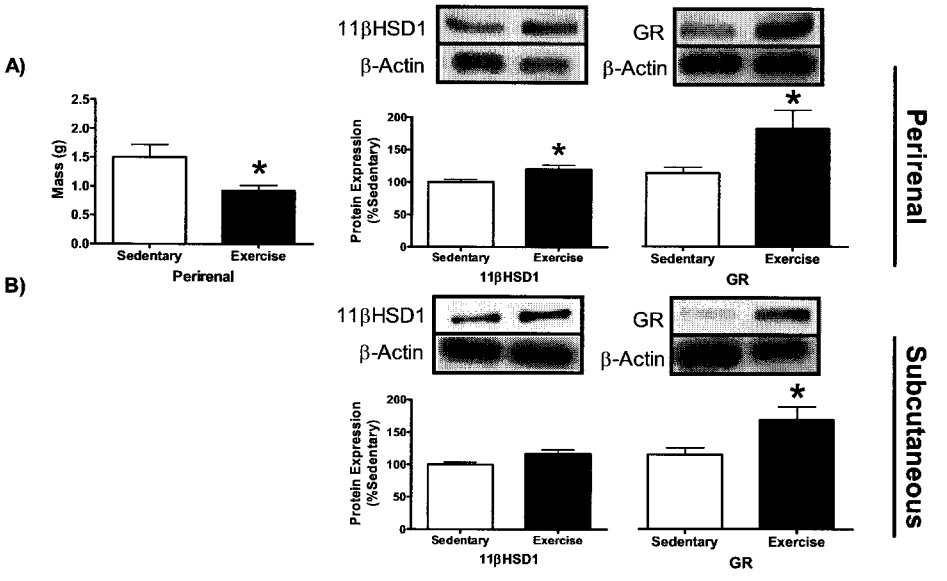


Figure 4

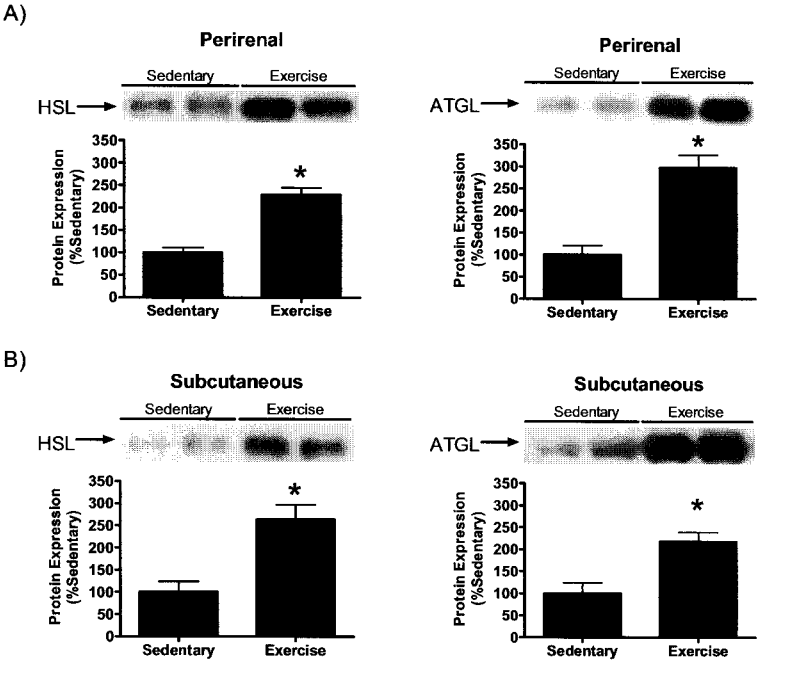


Figure 5

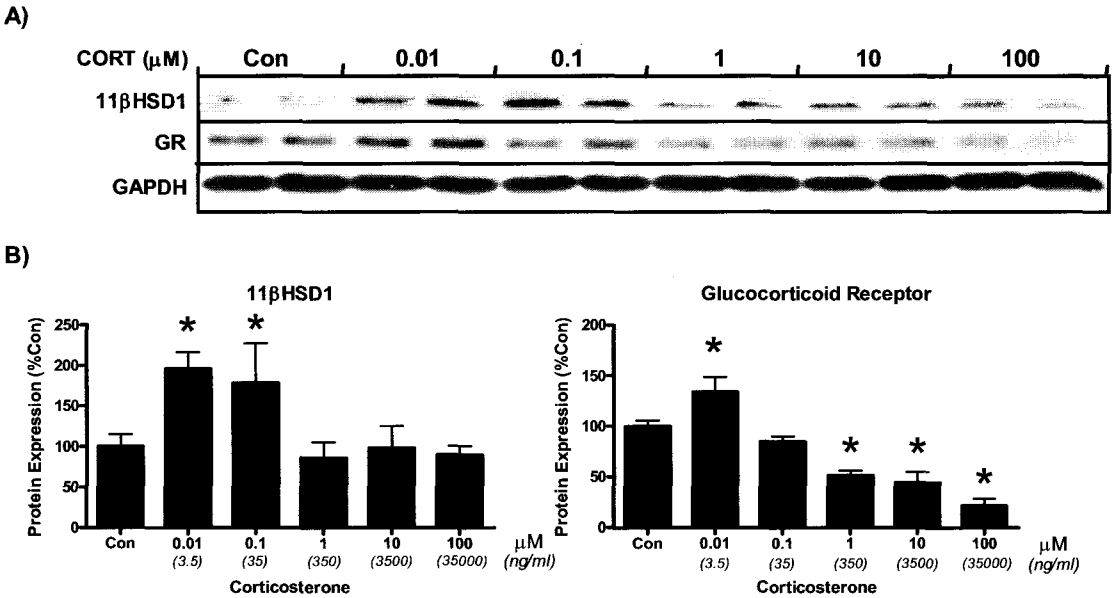
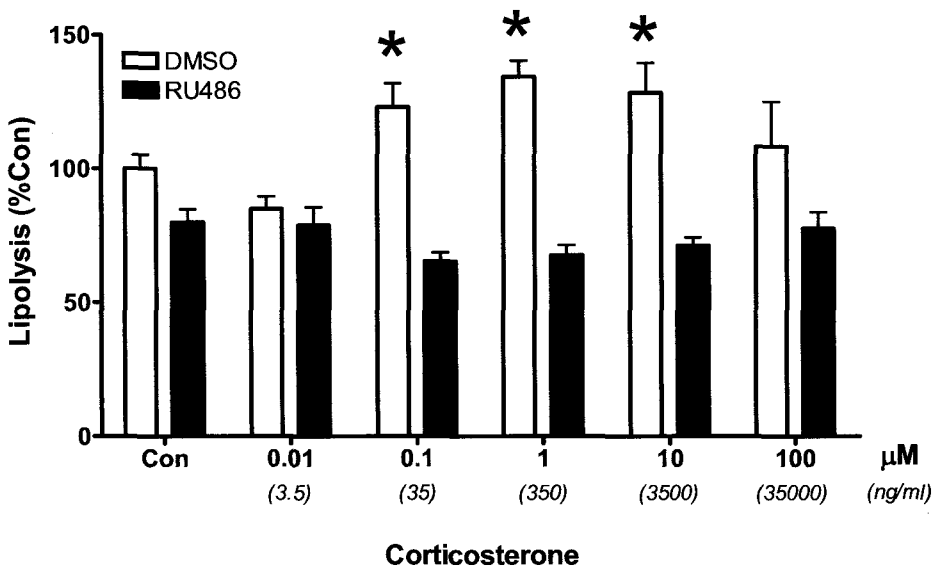


Figure 6



MANUSCRIPT #4 – ADIPOGENIC AND LIPOLYTIC EFFECTS OF CHRONIC GLUCOCORTICOIDS IN ADIPOSE TISSUE

5

Rationale for manuscript #4:

Manuscript #3 demonstrated that exercise training increases GC action in adipose tissue and proposed that this occurs in order to increase lipolysis and the provision of lipolytic products as substrates for energy. However, the question still remained as to what role GCs have in adipose tissue. Manuscript #3 is in agreement with a body of literature that suggests that GCs have a catabolic role and increase lipolysis. However, another body of literature suggests that GCs are anabolic and increase adipose tissue mass through either anti-lipolytic pathways or through increased adipogenesis. This characteristic of GCs in adipose tissue might have implications on adiposity rebound when individuals transisition from a state of neutral or negative energy balance (e.g., regular exercise, dieting) and enter a positive energy balance. Therefore, the goal of manuscript #4 was to elucidate the role of GCs in adipose tissue in both an *in vitro* and *in vivo* model. This is the first study to investigate both the catabolic and anabolic actions of GCs concurrently, with respect to both concentration and duration.

Author Contributions:

Conceived and designed the experiments: JEC (60%), TJH (10%), MCR (30%). Performed the experiments: JEC (100%). Oil Red O data: AP (50%), AMD (50%). Analyzed the data: JEC (75%), MCR (25%). Wrote the paper: JEC (60%), TJH (10%), MCR (20%).

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Adipogenic and Lipolytic Effects of Chronic Glucocorticoid Exposure

Running Title: Glucocorticoids are both adipogenic and lipolytic

Key Words: Visceral Adipose, Subcutaneous Adipose, 3T3-L1 Adipocytes, Corticosterone

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Abstract

Glucocorticoids (GCs) have been proposed to be both adipogenic and lipolytic in action within adipose tissue, although it is currently unknown if these actions can occur simultaneously. We determined the effects of corticosterone (CORT) treatment on adipose tissue lipid metabolism both *in vitro* and *in vivo*. *In vitro*, we treated 3T3-L1 preadipocytes and mature adipocytes with CORT at different concentrations (0-250μM) and measured indices of lipolysis and adipogenesis. We found that GCs are essential for preadipocyte differentiation, however do not increase lipogenesis in adipocytes. Exposing adipocytes to CORT increased lipolysis in a concentration-dependent manner, with the maximum effect at 1-10 μM (~170-185% above control, $P<0.05$). Surprisingly, removal of CORT caused a further increase in lipolytic rates (10 μM pre-treatment = ~320% above control, $P<0.05$), indicating a residual effect on basal lipolytic rates. Our *in vitro* findings allowed us to conclude that the adipogenic effects of CORT are mediated through preadipocytes, increasing differentiation and resulting in a greater number of new adipocytes. However, the actions of CORT in mature adipocytes increase lipolysis, but not lipogenesis. We next set out to replicate these findings *in vivo* by subcutaneously implanting 300 mg CORT pellets in Sprague-Dawley rats. Animals were monitored for 10 days, after which, adipose depots were removed and cultured *ex vivo*. Lipolytic rates were elevated in both fed and fasting conditions, evident by circulating concentrations of free-fatty acids and glycerol ($P<0.05$). Visceral, but not subcutaneous, adipocyte cultures from CORT-treated rats had a 1.5-fold increase in lipolytic rates compared to sham-treated rats ($P<0.05$). CORT-treated rats had significantly more visceral adiposity than sham-treated rats (10.2 vs. 6.9 g/kg, $P<0.05$). Furthermore, the visceral adipose depots in

the CORT-treated animals consisted of smaller, more numerous adipocytes, suggesting the adipogenesis occurred through preadipocyte differentiation rather than adipocyte hypertrophy. Taken together, our findings demonstrate that chronic GC exposure stimulates both lipolysis and adipogenesis in adipose tissue, but favours adipogenesis mediated through preadipocyte differentiation under normal circumstances.

Introduction

Glucocorticoids (GCs) are steroid hormones released from the adrenal cortex upon activation of the hypothalamic-pituitary-adrenal (HPA) axis in response to stress (41). One of the major actions of GCs is to stimulate metabolic processes that collectively serve to increase fuel availability in times of physical, neurogenic or metabolic stress. While the actions of GCs on carbohydrate and protein metabolism are more well-defined (41, 344), the role of these stress hormones in lipid metabolism are much more controversial. GCs are widely cited as being both lipolytic and adipogenic in action, depending on the dose, duration and type of GC investigated, as well as the experimental model utilized (18, 41, 279, 345).

The synthetic GC, dexamethasone (DEX), has been shown to increase adipose tissue lipolysis, as assessed by free fatty acid (FFA) release, either directly (280, 345) or possibly through increases in the lipolytic potential of growth hormone (346, 347). In humans, six hours of hydrocortisone infusion, at rates that cause physiological elevations in cortisol, increased circulating FFAs (337). A number of mechanisms for GCs apparent lipolytic activity have been proposed including: (i) increased gene transcription of lipolytic enzymes such as hormone sensitive lipase (HSL) (280) and adipose triglyceride lipase (ATGL) (278, 328) (ii) altered characteristics of inhibitory G-proteins (281) and (iii) downregulation of phosphodiesterase 3B and upregulation of intracellular concentrations of cAMP (345).

Though the aforementioned studies demonstrate that GCs are capable of stimulating lipolysis, at least in the short term, prolonged exposure to GCs promotes adiposity, particularly in the central adipose regions. This "obesogenic" nature of GCs is

best observed in patients with Cushing's syndrome or in those receiving treatment with synthetic glucocorticoids for various inflammatory conditions such as rheumatoid arthritis and asthma (235). Moreover, enhanced GC activity in adipose tissue through increased adipose expression of the GC-activating enzyme, 11β-hydroxysteroid dehydrogenase type 1 (11βHSD1), results in marked central obesity (18). In contrast, we recently demonstrated that increased GC activity specifically in the adipose tissue of exercise-trained rodents is associated with elevations in lipolysis and markedly diminished adiposity (326, 328). This discrepancy between GC levels and adiposity makes drawing generalized conclusions regarding the significance of GC's lipolytic actions difficult.

Using both *in vitro* and *in vivo* methodologies, the present study aids in clarifying the effects of GC action within adipose tissue. We demonstrate that in the presence of GCs, 3T3-L1 adipocytes displayed a concentration-dependent increase in lipolysis and upon GC removal, the basal lipolysis rates were further elevated. GC exposure resulted in a concentration-dependent increase in preadipocyte differentiation into mature adipocytes, suggesting that GC-stimulated adipogenesis is the result of new adipocyte formation. To investigate this further, rats were implanted with CORT pellets to increase circulating CORT levels for 10 days. Within 2 days, lipolytic rates were increased above Shams, as demonstrated by increased plasma FFA and glycerol levels. Consistent with increased lipolysis, expression of the lipolytic enzymes, ATGL and HSL, were increased above Shams in the adipose tissue of 10 day CORT-treated rats. Primary visceral adipocyte cultures from CORT-treated rats displayed a 1.5-fold increase in lipolytic rates compared to Shams, however, CORT-treated rats also displayed an increase in visceral

adipose tissue with a greater number of small adipocytes. The net effect is an increase of visceral adipose tissue mass despite the concomitant stimulation of lipolysis.

Methods

In vitro studies

Cell Culture

3T3-L1 fibroblasts were grown for 2 days post-confluence in 10% Fetal Bovine Serum/Dulbecco's Modified Eagle Medium (FBS/DMEM) at 37°C and 5% CO_2. Differentiation was induced with 500 μM isobutylmethylxanthine (IBMX), 500 nM DEX and 200 pM insulin for 4 days. The cells were then maintained in 10% FBS/DMEM containing 200 pM insulin for 4 days and then in 10% FBS/DMEM until greater than 95% of cells contained lipid droplets (~2-4 days). The medium was changed every other day throughout the differentiating period.

3T3-L1 Lipolysis

Fully differentiated cells (termed 3T3-L1 adipocytes) were treated with corticosterone (CORT; Sigma, Cat#C2505) for 24 or 48 hrs as previously described (328). To measure lipolytic rates in the presence of CORT, media was sampled at 0 and 48 hrs and assayed for glycerol concentrations using a commercially available kit (Sigma, Cat#FG0100). Cells were then washed 3x with PBS, scraped on ice in 100 μl of lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% Triton-x-100, 10% glycerol, pH 7.4) and assayed for protein concentrations with the Bradford method. Lipolytic rates are expressed as μM glycerol released per μg protein per hour. Separate experiments normalizing lipolytic rates with Oil Red O staining triglyceride content yielded similar results (data not shown). For measurement of basal lipolysis, adipocytes were first treated

with corticosterone for 48 hours in the manner described, washed 3x with warm phosphate buffered saline (PBS) and resuspended in Krebs-Ringer-Phosphate-Hepes (KRPH) buffer containing 3.5% (w/v) bovine serum albumin (BSA) (125 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 2.5 mM CaCl2, 1 mM KH2PO4, 25 mM Hepes, 5 mM D-glucose). Media was sampled at hour 48 and 49 for basal glycerol release, the cells were harvested for protein, and lipolysis was expressed as described above. For epinephrine stimulated lipolysis, cells were treated to identical conditions described for basal lipolysis, with the addition of 10 μM epinephrine (Sigma, Cat# E4250). For experiments using RU486, a non-specific glucocorticoid receptor antagonist, cells were co-incubated with the specified corticosterone concentration and 10 μM RU486 (Sigma, Cat#M8046).

Cell Viability

To ensure that incubation of 3T3-L1 adipocytes with CORT was not inducing cell death, 3T3-L1 cells were treated with increasing concentrations of CORT (0.01-250 μM) for 48 hours and cell viability was assessed with a trypan blue exclusion assay. No evidence of increased cell death was seen at any concentrations of CORT.

Glucose Incorporation into Lipids

Glucose uptake was measured as previously described (348). Briefly, 3T3-L1 adipocytes were treated with 0, 1 and 100 μM corticosterone for 48 hours under the same conditions described for the lipolysis experiments. Afterwards, the cells were starved for 2 hours in serum-free media and then resuspended in KRPH buffer with 4% (w/v) BSA containing 0.2 μCi/ml D- [U-^{14}C] glucose (GE healthcare, Cat#CFB2), with or without 100 nM insulin for 1 hour. The media was immediately removed, the cells were washed 3x with

PBS and then treated with Dole's reagent (isopropyl alcohol/n-heptane/H_2SO_4, 4:1:0.25, v/v/v) to isolate the total lipid fraction and counted for radioactivity.

Oil Red O Analysis

Corticosterone treated adipocytes in a 6-well plate were stained with Oil Red O to determine triglyceride content. First, a stock solution was prepared by mixing 0.7g of Oil Red O (Sigma, Cat# O-0625) with 200 ml of isopropanol and filtering through 0.2 μm paper. A working solution (3:2, stock solution:ddH_2O, v/v) was filtered through 0.2 μm paper, incubated at room temperature for 20 minutes and then immediately used. The majority of media in the 6-well plates was discarded and the cells were fixed with 10% formalin for 5 min at RT. The formalin was removed and the cells were washed with 60% isopropanol and allowed to fully dry. The cells were then incubated in 1 ml of the Oil Red O working solution for 10 minutes and immediately washed with ddH_2O four times. After completely drying, 3.5 ml of 100% isopropanol was added and the plate was agitated to fully elute the Oil Red O. The eluted solution was read at 500 nm in a spectrophotometer with 100% isopropanol used as the blank. The values were made relative to control cells (no CORT).

Cell Microscopy

To visually determine the impact of corticosterone treatment on adipocytes, 3T3-L1 adipocytes were treated with 1 μM CORT for 48 hours and visualized with an inverted microscope equipped with a motorized stage system (Nikon Eclipse TE2000-U; Prior Scientific ProScan II Series). Coordinates for three random fields per well were ascertained for initial and subsequent imaging. Adipocyte cross-sectional area and lipid droplet cross-sectional area were calculated utilizing the area measuring tool of the

Adobe Photoshop CS software suite. Approximately 25 distinct adipocytes were measured per field and measurements were normalized to control cells at time 0.

In vivo studies

Animals

Male Sprague-Dawley rats, with an initial weight of ~250 g, were divided into 3 groups; Sham, Corticosterone (CORT), and Pair-fed (n=5 animals/group). Animals were housed individually in standard rodent cages and in a 12 hour light/dark cycle for 4 days prior to experimental conditions. On day 0, both Sham and pair-fed animals had two 150 mg wax pellets subcutaneously implanted between their scapulae, whereas CORT animals had two 150 mg CORT (Sigma, Cat#C2505) pellets subcutaneously implanted. Sham and CORT animals were fed *ad libitum,* with food intake measure daily for 10 days. Pair-fed animals were given the average amount of food consumed by the CORT animals the prior day. Tail vein blood was collected two days following treatment at 0100, 0800, and 2000 to determine the diurnal CORT concentrations. Blood was also collected on days 2 and 10 at 0800 for measurements of FFAs and glycerol concentrations and day 6 for insulin concentrations. Blood was immediately centrifuged and the plasma was collected and frozen at -20C until further use. Commercially available kits were used to measure plasma glycerol (Sigma, Cat#FG0100), FFAs (Wako, Cat#994-75409), insulin (Crystal Chem, Cat#INSKR020) and CORT (MP Biomedicals, Cat#07-120102). On day nine, one day prior to sacrifice, all animals underwent an overnight fast. On day ten, the adrenal glands and various skeletal muscles were removed, weighed, and frozen. Epididymal, retroperitoneal, and the inguinal subcutaneous adipose depots were removed and weighed. A small portion of each adipose tissue depot (100 mg) was separated and frozen

for protein analysis. The remaining adipose tissue was used for primary cell culture. Both epididymal and retroperitoneal depots were used for representation of visceral adipose. These depots did not differ, and the data presented as visceral adipose is from epididymal depots. The inguinal adipose was used as a representative depot for subcutaneous adipose.

Primary Cell Culture

Adipocytes from visceral and subcutaneous adipose depots were isolated as previously described (349). Briefly, ~ 1g from individual depots were finely minced and then shaken (~120 rpm) for 20-30 minutes at 37°C in Hank's solution containing 1 mg/ml type II collagenase (Sigma, Cat#C6885). Isolated adipocytes were obtained by filtering the solution through a nylon mesh. Cells were washed 3x using KRPH buffer and then allowed to rest in 10ml of KRPH buffer with 4% BSA for 1 hour before being resuspended in a final volume to make a 10% lipocrit solution. Media was sampled at 0 and 90 minutes for glycerol release. Following this, adipocytes were lysed and protein concentrations were measured using the Bradford method. Lipolysis was expressed as μM glycerol release per mg protein per hour.

Determination of adipocyte size and number

A 10 μl aliquot of isolated adipocytes were removed and analyzed during the rest period to determine adipocyte volume and number as previously described (350). Briefly, mean adipocyte diameter was determined microscopically by counting 150 individual adipocytes. Adipocyte volume was calculated as $V=4\pi(\text{diameter}/2)^3/3$. Cell density (volume of cells/volume of cell suspension), or lipocrit, was estimated from the packed cell volume obtained after centrifugation of a capillary tube. Adipocyte number was

calculated by dividing the cell density by the average adipocyte volume and expressed per gram of adipose tissue.

mRNA analysis

Total RNA was harvested from 3T3-L1 adipocytes using TRIzol® Reagent according to the manufacture's instructions. Briefly, 1ml of TRIzol reagent was added per well and the cells were scraped off the plate. The cells were collected and homogenized for 15 seconds in 2 ml tubes. 200 ul of chloroform was added and the tubes were vortex for 15 seconds. Following a 3 minute incubation time, the tubes were centrifuged at 12000xg for 15 minutes at 4°C. The upper phase (containing mRNA) was removed and transferred to a fresh tube. mRNA was analyzed using primers for ATGL (AY894805: GAGCCCCGGGGTGGAACAAG) and HSL (NM_010719: GCCGGTGACGCTGAAAGTGG) (Sigma).

Immunoblotting

Protein quantification from both 3T3-L1 and primary adipocytes was done as previously described (328). For 3T3-L1 adipocytes, cells in 6-well plates were washed with PBS, placed on ice and 100 µl of lysis buffer was added to each well. Cells were scraped, collected into 0.5ml tubes, and underwent sonication for 5 seconds. For primary adipocytes, ~100mg of adipose tissue was homogenized with 300 µl of lysis buffer. In both situations, tubes were centrifuged for 20 minutes at 13000 rpm at 4°C and the supernatant was collected and assayed for protein concentration with the Bradford method. Total protein (50 µg) was electrophoretically resolved on a 10% SDS-polyacrylamide gel and transferred overnight at 20V to PVDF paper. Blots were blocked with 5% BSA then incubated overnight in primary antibody at 4°C (HSL: Cell Signaling,

Cat#4107, 1:5000; pHSL^{Ser563}: Cell Signaling Cat#4139, 1:1000; pHSL^{Ser565}: Cell Signaling Cat#4137, 1:5000; pHSL^{Ser660}, Cat#4126, 1:5000; ATGL: Cell Signaling, Cat#2138, 1:5000). Blots were incubated with the appropriate secondary antibody for 1 hour at room temperature and hybridization signals were visualized using the Western Lightning Chemiluminescence Reagent Plus kit (PerkinElmer, Wellesley, MA) after exposure to Kodak X-Omat Blue x-ray film (Rochester, NY). GAPDH (Abcam Cat#ab9484) and α-tubulin (Abcam Cat#ab7291) were used as loading controls in tissue and 3T3-L1 cells, respectively.

Skeletal Muscle and Liver Tissue IMTG content and Respiratory Cages

Intramuscular and intrahepatic triglyceride content was determined by histological staining, as previously described (351). Briefly, muscle and liver sections (10 μm thick) were sliced in a cryostat maintained at a temperature of -20°C. Sections were stained for lipid content using an Oil Red O solution composed of 0.5g of Oil Red O powdered dye (Sigma Aldrich, St. Louis MO) and 100 ml of 60% triethyl phosphate (Sigma Aldrich). Slides were left to air dry for 10 minutes followed by fixation in 3.7% formaldehyde (Sigma Aldrich) for 1 hour at room temperature. After 3 rinses with distilled water, slides were then placed in a double filtered ORO solution for 30 minutes at room temperature. Immediately following Oil Red O staining, liver sections were counterstained for 1 minute with Harrison's Hematoxlyin solution (Sigma Aldrich). All slides were then allowed to air dry for 10 minutes and sealed with Crystal Mount (Sigma Aldrich). Tissue sections were viewed using light microscopy (Nikon Eclipse 90i microscope, Nikon Canada) and digital images were taken at a magnification of 10X (liver) or 20X (muscle). On day 7, 3 animals from Sham and CORT groups were placed in a metabolic cage for

24 hours (Windows Oxymax Code #0246-002M Columbus Instruments Ohio, USA) to measure the respiratory exchange ratio.

Data Analysis

For all experiments, the appropriate t-test, one-way, or two-way ANOVA was performed to identify significant differences between treatment groups using Statistica 6.0 software, with $P<0.05$ as the criterion. When a significant difference was observed with an ANOVA, post-hoc analysis using contrasts with a Bonferroni correction factor were performed to determine specific differences. Data are presented as mean ± S.E.M.

Results

Glucocorticoid treatment does not increase lipogenesis, but is required for preadipocyte differentiation

3T3-L1 fibroblasts were induced to differentiate with 500 mM IBMX, 200 pM insulin and various amounts of dexamethasone. The number of lipid-containing mature adipocytes was quantified with Oil Red O staining, and a concentration-dependent increase in the differentiation of preadipocytes was seen with increasing GCs ($P<0.05$, Fig 1A and 1B). To determine the impact of CORT treatment on lipid content *in vitro*, 3T3-L1 adipocytes were treated for 48 hrs with 1 μM CORT and analyzed for evidence of lipogenesis. CORT treatment did not change the basal or insulin-stimulated glucose incorporation into lipids (Fig 1C). Furthermore, CORT treatment did cause a small, but consistent, decrease in the amount of triglyceride content measured with Oil Red O staining ($P<0.05$, Fig 1D). This coincided with the results of analyzing the cells with microscopy (represented in Fig 1E), where CORT treatment did not affect the cross-

sectional area of adipocytes (Fig 1F), but did prevent the increase in lipid droplet cross-sectional area seen in the control cells ($P<0.05$, Fig 1G).

Corticosterone alters lipolysis in a concentration-dependent manner

Adipocytes treated with increasing concentrations of CORT demonstrated increased lipolytic rates, with maximal effects occurring between 1 and 10 μM concentrations ($P<0.05$, Fig 2A). Further increases in CORT resulted in a progressive decrease in lipolysis with the highest concentration of CORT (250 μM) resulting in decreased lipolytic rates to below control values ($P<0.05$, Fig 2A). To demonstrate the effects of CORT are mediated through the glucocorticoid receptor (GR), cells were co-incubated with 10 μM RU486, a GR antagonist. RU486 prevented the CORT-mediated increase in lipolysis (Fig 2B).

Corticosterone increases lipolysis through elevations in basal lipolytic rates, not epinephrine stimulated rates

3T3-L1 adipocytes treated with CORT for 48 hrs were washed and resuspended in KRPH buffer containing only 3.5% BSA (basal) or KRPH buffer + 3.5% BSA + 10 μM epinephrine stimulated lipolysis for 1 hr. Basal lipolytic rates following CORT treatment were increased in a concentration-dependent manner, with 250 μM CORT pre-treatment causing residual basal lipolysis to increase the most (~425% vs. Con, $P<0.05$, Fig 2C). These effects were abolished with co-incubation of RU486 at all concentrations expect 100 μM CORT, likely due to the 10-fold higher concentration of CORT compared to RU486 (Fig 2D). Neither CORT nor RU486 had an effect on epinephrine stimulated lipolysis (Fig 2E).

Corticosterone treatment increases the protein expression of ATGL and the phosphorylation state of HSL

In 3T3-L1 adipocytes. CORT increased the protein expression of ATGL ($P<0.05$, Fig 3A). mRNA analysis confirmed that the increased ATGL protein was due to an increase in transcription ($P<0.05$, Fig 3B). Interestingly, HSL mRNA was increased at 48 hrs ($P<0.05$, Fig 3C), but not protein levels (Fig 3A). Analysis of adipocytes treated for longer than 48 hrs revealed a small increase in HSL protein levels that did not achieve statistical significance (data not shown). Probing for the phosphorylated states of HSL following 48 hrs of CORT treatment revealed an increase in the basal phosphorylation of HSL at Ser563 and Ser660 with 1 μM concentrations ($P<0.05$, Fig 3D and 3E, respectively). However, high concentrations of CORT (100 μM) did not increase the phosphorylation of HSL above control levels. Furthermore, 1 μM CORT did not change the phosphorylation status of HSL following epinephrine stimulation, whereas 100 μM CORT decreased HSL phosphorylation at both Ser563 and Ser660 ($P<0.05$, Fig 3D and 3E, respectively).

Subcutaneous corticosterone pellets increased nadir plasma corticosterone and decreased body weight and food intake.

Rats implanted with CORT pellets had significantly elevated levels of CORT only at 0800, in the nadir of the diurnal pattern ($P<0.01$, Fig 4A). CORT animals displayed decreased rates of body growth immediately after the receiving the pellets and weighed significantly less than Sham and Pair-fed animals by day 2 ($P<0.01$, Fig 4B). CORT animals continued to grow at a slower rate and weighed ~10% less than Sham animals on day 9 ($P<0.01$, Fig 4B). Although all animals lost weight during the fast from day 9 to 10,

the CORT group lost significantly more mass than either Sham or Pair-fed animals ($P<0.01$, Fig 4B). CORT treatment also decreased total food intake compared to the Sham animals (main effect of food: $P<0.05$, Fig 4C).

Corticosterone pellets increased the concentration of FFAs and glycerol, but not insulin, in both fed and fasting conditions.

Plasma FFAs, glycerol and insulin concentrations were measured on day 2 at 0800 (fed) and on day 10 at 0800 (fasted). CORT treatment increased FFA levels under both fed and fasting conditions compared to either Sham or Pair-fed groups ($P<0.05$, Table 1). CORT treatment also increased fed glycerol concentrations compared to Sham and Pair-fed groups ($p<0.05$, Table 1). Fasting glycerol concentrations were also elevated in the CORT group compared to the Sham group ($P<0.05$, Table 1). No differences were found in either fed or fasting insulin concentrations between groups (Table 1).

CORT treatment increased visceral, but not subcutaneous, adipose mass and visceral adipose depots were comprised of a greater number of small adipocytes.

Despite smaller body masses, CORT animals had greater relative amounts of visceral adipose mass ($P<0.05$, Table 2), but not subcutaneous adipose mass (Table 2), compared to Sham and Pair-fed rats. The groups did not differ with respect to relative skeletal muscle mass, including plantaris (Table 2), soleus and tibialis anterior (data not shown), but CORT animals did have significantly smaller adrenal gland masses compared to Sham and Pair-fed animals ($P<0.01$, Table 2). The adipose depots from all animals were removed and the adipocytes were isolated and analyzed for volume and number as previously described (350). CORT animals had smaller adipocytes in visceral adipose tissue compared with either Sham or Pair-fed animals ($P<0.05$, Table 2). CORT animals

also had a greater number of adipocytes per mg of adipose tissue in visceral adipose depots when compared to the other two groups ($P<0.05$, Table 2), suggesting that the increase in visceral adipose depot induced by CORT treatment is the result of adipocyte hyperplasia.

Isolated visceral adipocytes from CORT animals have an increased basal lipolytic rate and increased expression of lipolytic enzymes

Adipocytes from both visceral and subcutaneous depots were isolated and observed for basal lipolytic rates for 90 minutes. CORT animals demonstrated an increased basal lipolytic rates in visceral adipocytes compared to Sham and Pair-fed animals ($P<0.05$, Fig 5). Though no difference was seen between groups in the lipolytic rates of adipocytes isolated from subcutaneous depots (Fig 5), protein analyses revealed that CORT animals had increased expression of ATGL and HSL in both visceral and subcutaneous depots ($P<0.05$, Fig 5)

Corticosterone treatment increases ectopic adipose deposition

To determine the metabolic fate of the increased lipolytic products, we measured the respiratory quotient (oxidation) and the triglyceride content of skeletal muscle and liver (storage). CORT treatment did not significantly alter the fat oxidation compared to sham animals, shown by the respiratory exchange ratio over a 24-hour period (0.928 ± 0.003 vs. 0.920 ± 0.004, Sham vs. CORT, $n=3$). However, CORT treatment did increase the lipid content in both skeletal muscle and liver tissue, measured by intracellular triglyceride content (Fig 6).

Discussion

The contribution of GCs to adipose tissue metabolism is controversial. One body of research classifies GCs as catabolic, promoting energy substrate release. Consistent with this, studies have demonstrated that GCs are lipolytic in action, resulting in the release of free fatty acids (280, 345, 352). However, GCs may also act in an anabolic fashion within adipose tissue by increasing adipose mass, particularly in visceral regions (18, 353, 354), by either promoting adipogenesis or by reducing lipolysis (283). This anabolic action is consistent with the well known centrally-obese phenotype associated with populations with elevated GC levels, e.g. Cushing's syndrome patients (261). Given these studies, it remained unknown as to whether this hormone actually elicits these disparate actions simultaneously within adipose tissue. Here we show *in vitro* and *in vivo* evidence that elevations in corticosterone stimulate adipogenesis by acting on preadipocytes, while concomitantly increasing lipolysis in fully mature adipocytes. Furthermore, we demonstrate in rats that the adipogenic/lipolytic balance favours a net increase in adipose tissue mass. Importantly, we demonstrate that these effects are most notably seen in visceral adipose tissue, which has important metabolic implications in promoting whole body insulin resistance. Taken together, our results show that CORT treatment causes visceral obesity and increases circulating lipids, which, if sustained, would likely contribute to the development of metabolic complications such as type 2 diabetes.

Adipogenesis can be accomplished through either increased preadipocyte differentiation resulting in a greater number of mature adipocytes (hyperplasia) or through hypertrophy of existing adipocytes (355). Here we demonstrate that GCs are

essential for the differentiation of preadipocytes, showing one potential mechanism for increased adipogenesis. Importantly, when we treated mature adipocytes with CORT, we did not find any evidence that GC stimulated adipogenesis through adipocyte hypertrophy. This finding strengthens the hypothesis that GCs increase adipose tissue mass by acting on preadipocytes, not mature adipocytes, to increase differentiation.

To determine the action of GCs in mature adipocytes, we treated fully differentiated 3T3-L1 cells with increasing concentrations of CORT. Our finding that low to moderate concentrations of CORT increase lipolysis, but higher doses have a diminished or suppressive effect, illustrates an interesting relationship between the concentration of GCs and lipolytic rates. Early studies have also shown that CORT increases lipolysis in isolate adipocytes until a plateau somewhere between 1-10 μM (346). We continued past these concentrations and found that further increases in CORT exposure clearly caused a progressive decline in lipolytic rates to below control values. This suggests that the adipogenic effects of CORT may be mediated, in part, through anti-lipolytic actions that are clearly evident at higher doses.

The high concentrations of CORT used in our study may be reflective of intracellular concentrations, particularly in disease states or if patients are given exogenous GCs. It should be noted that intracellular physiological CORT concentrations are difficult to determine *in vivo* and largely depend on the location of interest. Moreover, considerable evidence exists to suggest that intracellular GC levels may be much higher than that observed in blood because of the cellular activation of inactive GCs by 11βHSD1, which freely crosses the cell plasma membrane in high abundance (86). This intracellular enzyme is capable of increasing the concentration of active CORT

substantially over that found in the plasma depending on the tissue type. In adipose tissue, elevating 11βHSD1 activity increases the concentration of CORT up to 15-fold over the levels found in the circulation (18). In our study, 1-3 μM (~330-1000 ng/ml) represents concentrations typically found in the blood, including what we report in our *in vivo* model. Therefore, concentrations between 10 and 50 μM, or higher, may be more representative of the intracellular concentrations found in adipocytes. Still, efforts to accurately measure intracellular concentrations of CORT are required.

We propose that GCs influence lipolysis in adipocytes mainly through increases in basal lipolysis. We found that CORT concentrations between 1 and 10 μM increased lipolytic rates ~175% above control cells, whereas the lipolytic rates at these concentrations were ~300% above control once CORT was removed and the cells underwent basal lipolysis. Furthermore, incubation of adipocytes with 250 μM CORT decreased lipolysis below control cells, but increased the subsequent basal lipolysis ~425% above control. Clearly, CORT has a greater effect on the basal lipolysis following treatment rather than on lipolytic rates that occur while the steroid is present. It has been recently proposed that ATGL plays a more significant role than HSL in the regulation of basal lipolysis, given that ATGL is constantly proximal to the lipid droplet (279). Our data shows the effects of CORT on basal lipolysis are accompanied by a similar concentration-dependent increase in ATGL. We propose, therefore, that GCs increase lipolytic rates through elevations of ATGL protein content and the subsequent increase in basal lipolysis. Furthermore, we suggest that this effect is blunted while GCs are present at high concentrations, through a yet to be determined anti-lipolytic action. Whether the anti-lipolytic effects of high CORT concentrations are inhibiting the lipolytic action of

ATGL, as well as the impact this may have on the adipogenic effects of GCs, requires further study.

Contrary to the work of others (356), we found little evidence that increases in GCs suppress lipolysis *in vivo*. Instead, we found lipolytic rates to be elevated in both fed (day 2) and fasting (day 10) conditions in animals treated with CORT. In addition, we found that isolated adipocytes from CORT animals on day 10 demonstrated elevated lipolytic rates in culture, along with increases in lipolytic enzymes. Interestingly, we demonstrate that CORT treatment *in vivo* also increases visceral adiposity, despite a lower food intake. Furthermore, the visceral adipose tissue displayed a phenotype consisting of a greater number of small adipocytes. Although we did not directly measure differentiation *in vivo*, this phenotype strongly supports the hypothesis that CORT increases adipogenesis primarily through increasing the number of adipocytes, rather than through adipocyte hypertrophy. This is in agreement with the findings by Aria *et al.*, showing that significant food restriction in rats for 14 days elevates plasma CORT concentrations to similar levels caused by our pellet implantation, leading to a greater number of small adipocytes rather than adipocyte hypertrophy (357). Thus, our *in vivo* findings are in agreement with our *in vitro* findings, showing that CORT treatment acts on mature adipocytes to increase lipolysis through an ATGL mediated mechanism, and suggesting a concurrent action on preadipocytes to increase differentiation and adipose tissue mass.

Finally, it is important to note that our findings show CORT to have a more pronounced effect on visceral adipose tissue compared to subcutaneous adipose tissue. Ten days of CORT treatment significantly increased visceral adipose tissue mass, but not

subcutaneous mass, consistent with the central adiposity phenotype seen in Cushing's syndrome (261). Furthermore, isolated visceral adipocytes from CORT-treated rats displayed elevated lipolytic rates compared to Sham and Pair-fed controls, whereas no difference in the lipolytic rates of subcutaneous adipocytes was found amongst groups. Our findings are in agreement with observations in humans, where GCs preferentially increased lipolysis in abdominal versus peripheral adipocytes (282). GCs have also been shown to decrease glucose uptake preferentially in visceral, as opposed to subcutaneous, adipose tissue (358). Thus, our results are consistent with the current paradigm that visceral adipose depots have increased GR expression and therefore are more sensitive to changes in GCs (336, 340, 355).

In summary, this study is the first to demonstrate that corticosterone simultaneously played dual role in adipose tissue biology, acting on two distinct cell populations within the tissue. On one hand, we show that the lipolytic effects are targeted towards mature adipocytes; increasing basal lipolysis through an ATGL mediated mechanism. On the other hand, GCs concurrently act on preadipocytes to induce differentiation and increase adipose mass through hyperplasia. Finally, we show that under normal conditions, the actions of corticosterone favour adipogenesis, despite an increase in lipolysis. Thus, the net effect of GC treatment is increased adipose tissue mass, increased circulating FFAs, and ectopic storage of lipids in liver and skeletal muscle. Additional studies are warranted to determine if the balance of lipolysis and adipogenesis changes under conditions of significant positive energy balance (i.e. high-fat diet) or negative energy balance (i.e. exercise). Understanding these mechanisms will

lead to the development of future interventions aimed at decreasing adipose tissue to help prevent obesity and type 2 diabetes.

Table 1: ***Plasma fed and fasting FFAs, glycerol, and insulin concentrations***

	Fed Concentrations (day 2)			*Fasting Concentrations (day 10)*		
	Sham	**CORT**	**Pair-fed**	**Sham**	**CORT**	**Pair-fed**
FFAs (mM)	247.8 ± 13.3	441.3 ± 43.0 *	171.4 ± 21.6	219.4 ± 10.5	298.5 ± 32.4 *	190.3 ± 17.6
Glycerol (mM)	140.1 ± 7.1	235.3 ± 10.6 *	159.3 ± 21.9	115.0 ± 14.1	156.0 ± 5.8 †	133.7 ± 0.6
Insulin (pg/ml)	3446 ± 560	3907 ± 480	2916 ± 305	305 ± 38	185 ± 62	539 ± 271

* - P<0.05 CORT vs. Sham and Pair-fed
† - P<0.05 CORT vs. Sham

Table 2: ***Anthropometric characteristics on day 10 and epididymal adipocytes characteristics***

	Sham	**CORT**	**Pair-fed**
Epididymal Adipose Mass ($g/kg_{B.W.}$)	6.80 ± 0.47	10.27 ± 0.63 §	6.54 ± 0.54
Subcutaneous Adipose Mass ($g/kg_{B.W.}$)	10.02 ± 1.64	12.89 ± 0.54	10.83 ± 1.13
Plantaris Mass ($mg/kg_{B.W.}$)	1.03 ± 0.02	0.95 ± 0.03	1.01 ± 0.04
Adrenal Mass (mg) *Left*	34.1 ± 2	19.6 ± 2 §	33.2 ± 2
Right	31.1 ± 2	15.2 ± 3 §	32.6 ± 1
Pellet Mass (mg)	300.0 ± 9	269.5 ± 6 *	309.9 ± 9
Adipocyte Size ($L \times 10^{-9}$)	16.02 ± 2.3	6.44 ± 0.7 §	13.96 ± 1.3
Adipocyte Number (per mg tissue)	33.37 ± 4.7	83.81 ± 7.8 §	48.90 ± 4.1

* - P<0.05 CORT vs. Sham and Pair-fed
§ - P<0.01 CORT vs. Sham and Pair-fed

Figure 1: ***Corticosterone increases adipogenesis through preadipocyte differentiation rather than lipogenesis.***
(A) A concentration-dependent increase in preadipocyte differentiation was found with increasing concentrations of glucocorticoids. (B) Representative plate with cells induced to differentiate with increasing concentrations of glucocorticoids and stained with Oil Red O. (C) Treatment of 3T3-L1 adipocytes with corticosterone (CORT) for 48 hours had no effect on basal or insulin stimulated glucose uptake. Insulin stimulated glucose uptake was significantly higher than basal in all conditions ($P<0.001$) (D) Fully differentiated 3T3-L1 adipocytes incubated in 1 μM CORT for 48 hours display a slight decrease in total lipid content. (E) Fully differentiated cells treated with 1 μM CORT were monitored with microscopy for 48 hours. CORT treatment had no affect on adipocyte cross-sectional area, but did prevent the increase in lipid content seen in the control cells. (F) CORT treatment had no effect on the average cross-sectional area for both adipocytes. (G) CORT treatment attenuated the increase in average cross-sectional area of lipid droplets seen in the control cells. Values are means ± SEM, n=3-6. * - $P<0.05$ vs. CON.

Figure 2: ***The lipolytic effects of corticosterone in 3T3-L1 adipocytes are concentration-dependent and are facilitated through basal lipolysis.***
(A) Lipolysis was increased in a parabolic manner in response to increasing concentrations of CORT, with maximal lipolysis occurring between 1-10 μM CORT, and then progressively decreasing. (B) RU486, a glucocorticoid receptor antagonist, prevented the increases in lipolysis during the 48 hour incubation. (C) Basal lipolysis following CORT incubation was increased in a concentration-dependent manner. (D) RU486 prevented the subsequent increase in basal lipolysis following CORT treatment (E). 48 hour treatment with CORT had no effect on the response to epinephrine. Values are means ± SEM, n=5-6. * - $P<0.05$ vs. CON (A and C) or CON DMSO (B, D, and E).

Figure 3: ***Basal lipolysis is due to an increase in ATGL protein expression***
3T3-L1 adipocytes were incubated in increasing concentrations of corticosterone (CORT) with or without RU486 for 48 hours and then immediately harvested for protein and mRNA analysis. (A) CORT induced a concentration-dependent increase in ATGL ($P<0.01$, Con vs. 100 μM), but not HSL (B) mRNA analysis confirmed that the changes in ATGL protein levels are due to increased protein expression. (C) HSL mRNA levels were increased with CORT treatment, but this did not translate to elevated protein levels at 48 hrs. In separate experiments, adipocytes were treated similarly with CORT, washed 3x with PBS and then incubated in Krebs-Ringer buffer with or without 10 μM epinephrine. 1 μM CORT treatment increased the basal phosphorylated state of HSL at (D) Ser563 and (E) Ser660, but did not increase the ability of epinephrine to phosphorylate HSL. 100 μM CORT did not change in basal phosphorylated state of HSL, but did decrease the epinephrine-stimulated phosphorylated state. Values are means ± SEM, n=4. * - $P<0.05$ vs. CON (B) or CON without epinephrine (C), Φ – $P<0.05$ vs. CON + epinephrine (C).

Figure 4: ***Animals implanted with corticosterone pellets had sustained elevations in corticosterone, decreased body weight and food intake***

(A) CORT animals had significantly elevated levels of the hormone in the nadir (0800h) of the circadian rhythm on day 2, which lasted throughout the 10 day protocol (data not shown). (B) Corticosterone (CORT) animals weighed significantly less than Sham and Pair-fed animals from day 2 onwards. (C) CORT animals ate less food than Sham animals on most days (group effect – $P<0.05$). Values are means ± SEM, n=5. * - $P<0.01$ CORT vs. Sham and Pair-fed

Figure 5: ***Corticosterone treatment increases the basal lipolytic rate in isolated adipocytes from visceral, but not subcutaneous, adipose tissue.***
Visceral and subcutaneous adipocytes were isolated and incubated in Krebs-Ringer buffer for 1.5 hrs. Visceral adipocytes isolated from animals treated with corticosterone (CORT) pellets had a higher rate of basal lipolysis compared with visceral adipocytes from either sham or pair-fed animals. No difference was found between groups in the basal lipolytic rate of subcutaneous adipocytes. Animals receiving CORT treatment had higher levels of ATGL and HSL in both visceral and subcutaneous adipose tissue compared to either sham of pair-fed animals. Values are means ± SEM, n=5. * - $P<0.05$ vs. Sham and Pair-fed.

Figure 6: ***Corticosterone treatment increases ectopic accumulation of lipid storage in skeletal muscle and liver tissue.***
To determine the fate of lipolytic products, samples of liver and skeletal muscle were sectioned and stained with Oil Red O for triglyceride content. (A) Liver sections were first stained with Oil Red O and then counterstained with hematoxylin and eosin. CORT animals demonstrated significant increases in intrahepatic triglycerides compared to Sham animals. (B) Tibialis anterior muscle sections were stained with Oil Red O. CORT animals demonstrated significantly increased intramuscular triglycerides compared to Sham animals.

Figure 1:

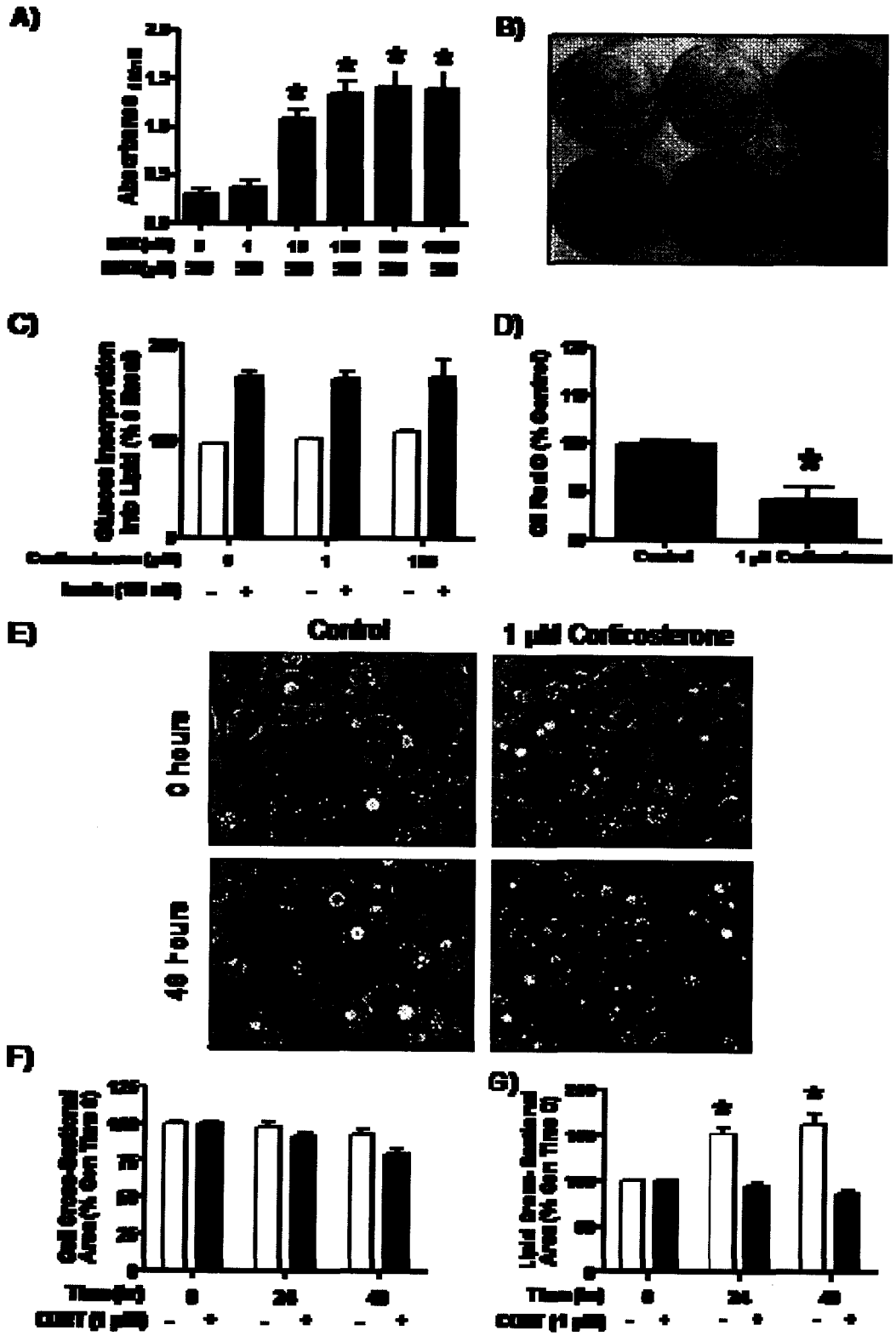


Figure 2:

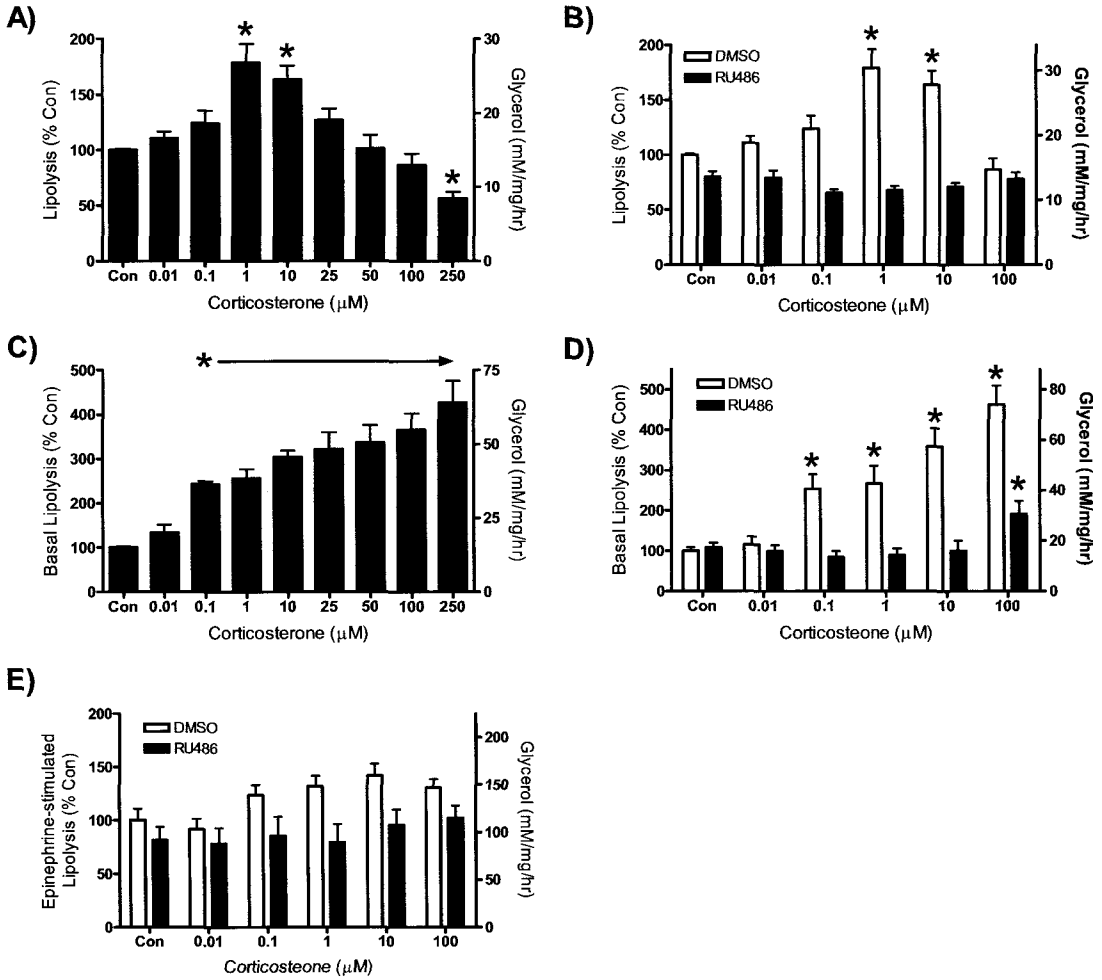

A)
Lipolysis (% Con)
Glycerol (mM/mg/hr)
Con 0.01 0.1 1 10 25 50 100 250
Corticosterone (μM)
B)
DMSO
RU486
Lipolysis (% Con)
Glycerol (mM/mg/hr)
Con 0.01 0.1 1 10 100
Corticosteone (μM)
C)
Basal Lipolysis (% Con)
Glycerol (mM/mg/hr)
Con 0.01 0.1 1 10 25 50 100 250
Corticosterone (μM)
D)
DMSO
RU486
Basal Lipolysis (% Con)
Glycerol (mM/mg/hr)
Con 0.01 0.1 1 10 100
Corticosteone (μM)
E)
DMSO
RU486
Epinephrine-stimulated Lipolysis (% Con)
Glycerol (mM/mg/hr)
Con 0.01 0.1 1 10 100
Corticosteone (μM)

Figure 3:

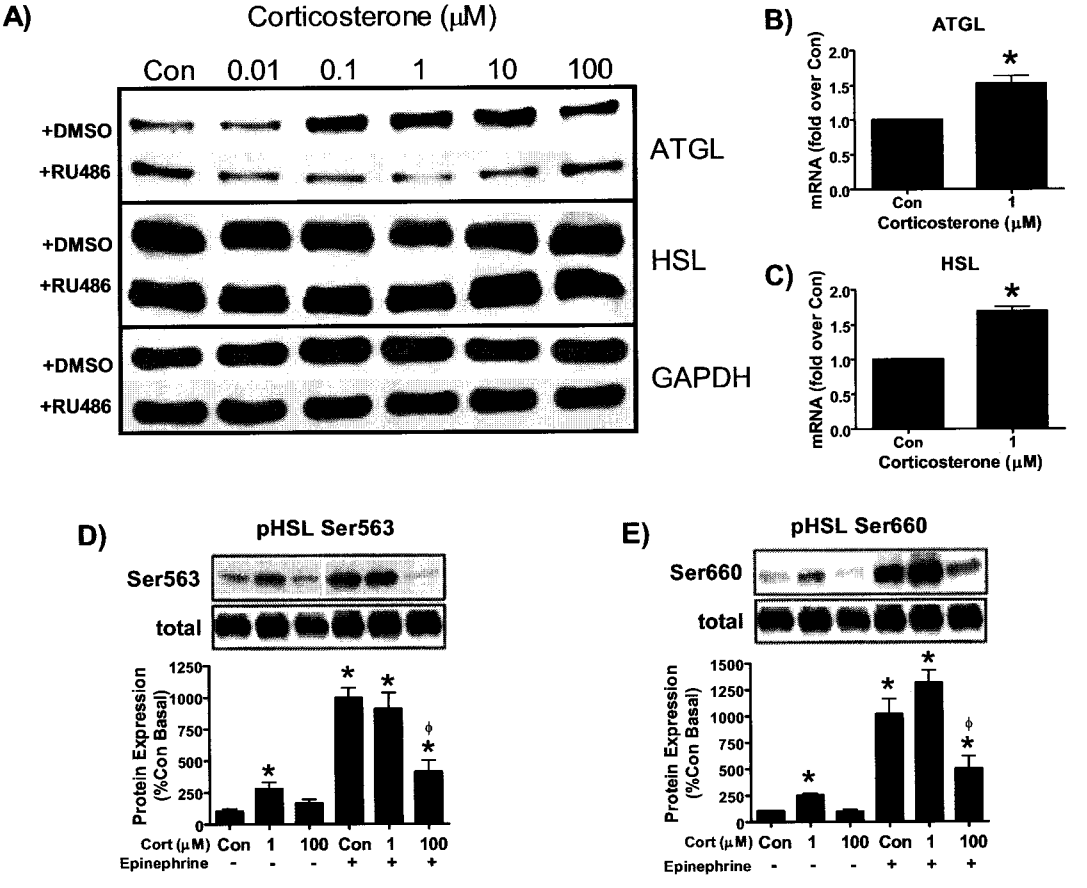

A)
Corticosterone (μM)
Con 0.01 0.1 1 10 100
+DMSO
+RU486
ATGL
HSL
GAPDH
B)
ATGL
mRNA (fold over Con)
Con
1
Corticosterone (μM)
C)
HSL
D)
pHSL Ser563
Ser563
total
Protein Expression (%Con Basal)
Cort (μM) Con 1 100 Con 1 100
Epinephrine - - - + + +
E)
pHSL Ser660
Ser660
total

Figure 4:

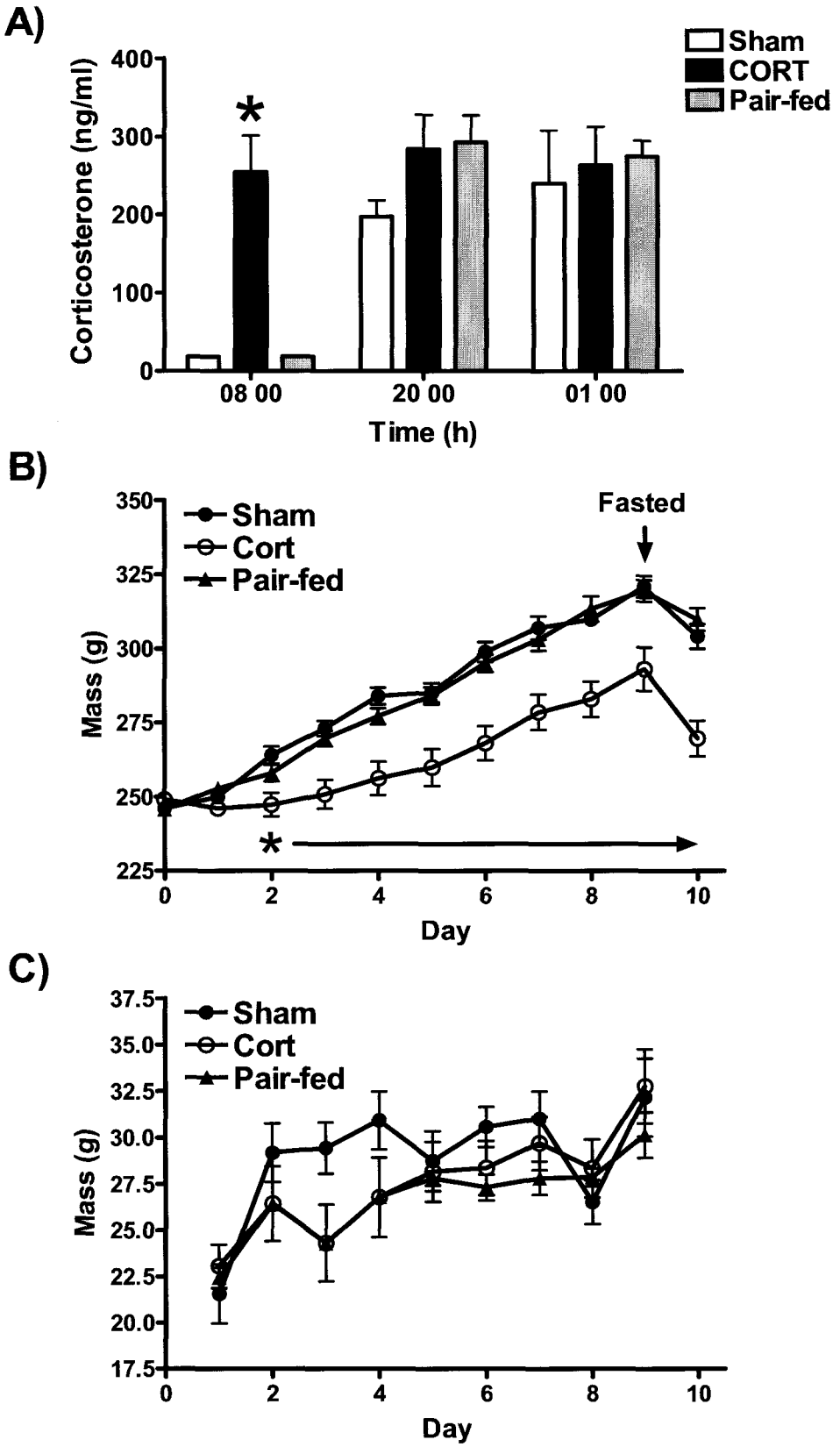

A)
Sham
CORT
Pair-fed
Corticosterone (ng/ml)
400
300
200
100
0
*
08 00
20 00
01 00
Time (h)
B)
Sham
Cort
Pair-fed
Fasted
Mass (g)
350
325
300
275
250
225
*
0
2
4
6
8
10
Day
C)
Sham
Cort
Pair-fed
Mass (g)
37.5
35.0
32.5
30.0
27.5
25.0
22.5
20.0
17.5
0
2
4
6
8
10
Day

Figure 5:

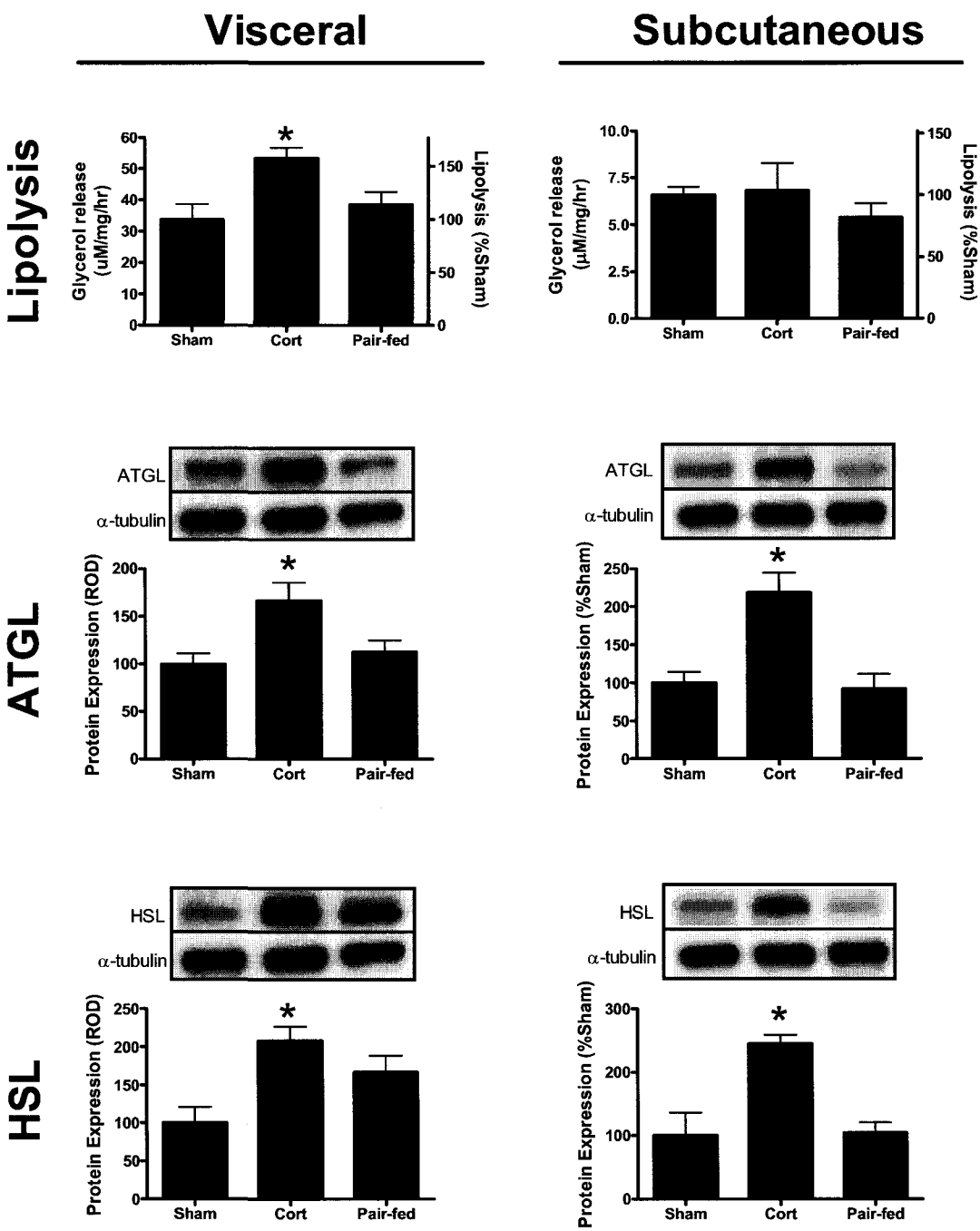
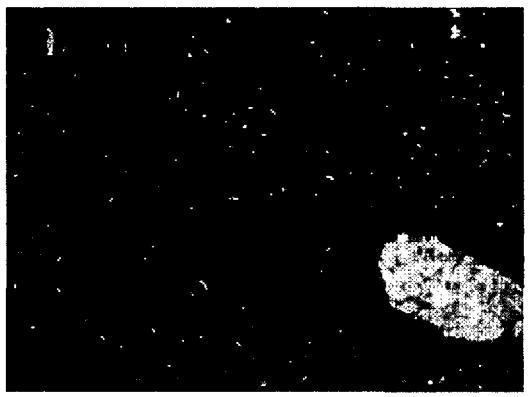

Visceral
Subcutaneous
Lipolysis
Glycerol release (uM/mg/hr)
Lipolysis (%Sham)
Glycerol release (μM/mg/hr)
Sham
Cort
Pair-fed
ATGL
α-tubulin
Protein Expression (ROD)
Protein Expression (%Sham)
HSL

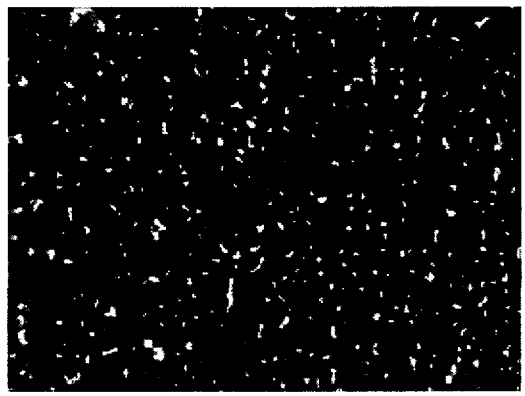
Figure 6:

A)

Liver – Wax

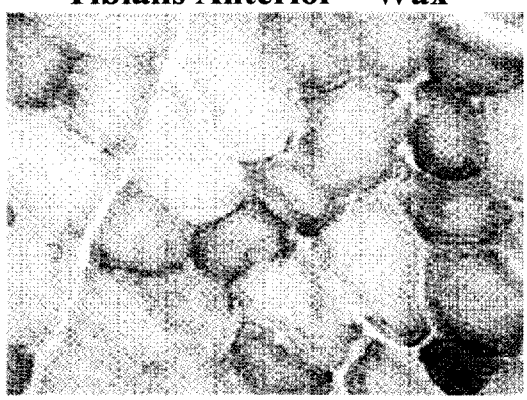


Liver - CORT



C)

Tibialis Anterior – Wax



D)

Tibialis Anterior – CORT

DISSERTATION SUMMARY 6

The hypothalamic-pituitary-adrenal axis is activated in response to a variety of stressors, including perceived, physical, and physiological (1). Upon activation, the hypothalamus releases CRH into the hypophyseal portal circulation, where it subsequently binds to receptors on the anterior pituitary. This initiates the synthesis and release of ACTH into the systemic circulation, where it ultimately binds to receptors on the adrenal glands. Binding of ACTH in the adrenal glands begins a signalling cascade that leads to the synthesis and release of GCs into the systemic circulation. In rodents, corticosterone and 11-DHC are the major GCs released, with the majority (>90%) of corticosterone being bound to a carrier protein (CBG or albumin) (19). The remaining corticosterone, along with 11-DHC, are considered 'free-hormones', and are capable of entering a cell, however, only free corticosterone is able to bind to the glucocorticoid receptor. 11-DHC requires the conversion to corticosterone by the intracellular enzyme 11βHSD1, which is expressed in varied quantities in a tissue-specific manner. Consequently, the action of GCs in tissue is largely determined by the expression and activity of GR and 11βHSD1.

One of the major roles of GCs is to increase the concentrations of energy substrates in the circulation. GCs were first defined by their ability to increase the concentration of glucose in the blood, in response to hypoglycaemia (200). This is largely accomplished by increasing hepatic glucose output and decreasing glucose uptake into skeletal muscle and adipose tissue (86). A less defined role for GCs is their ability to

increase lipolysis and release FFAs and glycerol into the circulation (280, 283, 346, 347). These actions are beneficial when energy sources are low (e.g., fasting) or when energy demand is high (e.g., exercise). Accordingly, exercise is a potent activator of the HPA axis, increasing blood GC concentrations both during exercise and for hours following cessation of exercise (223). However, chronic activation of the HPA axis, resulting in a sustained elevation of GCs, can have serious negative consequences. Cushing's syndrome, a disease characterized by chronic elevations of GCs, presents many metabolic complications seen in T2DM, including insulin resistance, hypertension, dyslipidemia, and increased adiposity (52, 359). Yet, exercise training, which robustly increases GCs for sustained periods of time, also protects against each of these harmful symptoms. One would hypothesize that exercise training elicits a compensatory mechanism that protects an organism from being chronically exposed to GCs. Thus, much effort has been put forth towards understanding the mechanisms behind the effects of exercise on the HPA axis and peripheral GC action.

The HPA axis is activated by both physical (e.g., exercise) and psychological (e.g., perceived danger) stressors. Exercise training models that utilize forced exercise training, such as treadmill or swimming, make it difficult to disassociate the physical stress from the psychological stress. Therefore, a number of studies investigating the HPA axis have utilized a volitional wheel running model that allows the animal to exercise voluntarily, at times when the animal would normally be active. This model provided evidence that exercise training lasting greater than 4 weeks results in animals having comparable basal HPA activity compared to sedentary controls training (214, 243-246), as well as, a similar response to a novel cross-stressor (214). Interestingly, trained

animals also showed the potential for increased adrenal sensitivity to ACTH, identified by an increased corticosterone:ACTH ratio (243, 245, 252, 253). However, no studies had directly measured adrenal sensitivity to ACTH to definitively state the effects of exercise training. Furthermore, the effects of exercise training on HPA activity had only been demonstrated with 4-6 weeks of training (243, 245, 253). Thus, it remained to be seen if exercise initially perturbed the HPA axis, followed by a compensatory adaptation resulting to a similar profile to sedentary controls by week 4. Therefore, the objectives of manuscript #1 were to address the questions that remain: 1) what are the effects of short-term (2 weeks) exercise training on the HPA axis, and 2) what effect does exercise training have on adrenal sensitivity to ACTH. Using short-term (2 weeks) and long-term (8 weeks) groups, as well as a basal group, we were able to demonstrate that exercise training does initially cause hyperactivation of the HPA axis that is ameliorated with sustained training. This was demonstrated by both basal GC diurnal patterns, as well as, HPA axis reactivity to a novel cross-stressor (restraint stress). Furthermore, we demonstrated that the early hyperactivity of the HPA axis in response to exercise training was due to increased adrenal sensitivity to ACTH, and that sustained training restored this to basal conditions. We also showed that the mechanism behind the changes in adrenal sensitivity was due to the protein levels of both the ACTH receptor (MC2R) and StAR, the rate-limiting protein in steroidogenesis (255). Manuscript #1 confirmed that exercise training has a dramatic effect on the HPA axis, acting mainly at the level of the adrenal glands. Furthermore, this manuscript highlighted the importance of regular exercise training and confirms that, if sustained, the activity of the HPA axis is restored to normal conditions.

GCs have been reported to contribute, at least in part, to the development of a T2DM phenotype in the ZDF rat. This comes from the fact that adrenalectomy of rodent models of insulin resistance prevents the onset of diabetes (158, 183-186). However, it still remained to be seen if elevation of HPA activity proceeds, and thus can contribute to, the development of insulin resistance and eventually T2DM. It is possible that the elevated GCs seen in ZDF rats (327) comes after the development of hyperglycemia and circumvents any compensatory mechanism from occurring to prevent overt diabetes. Manuscript #1 demonstrated that sustained exercise training attenuates an initially hyperactive HPA axis. Therefore, the goal of manuscript #2 was to determine if exercise training can prevent, or correct, the hyperactivity of the HPA axis and what effect this would have on the development of insulin resistance and T2DM. In manuscript #2, we measured glycaemia and GCs levels on a weekly basis and showed in sedentary ZDF animals that hyperactivity of the HPA axis proceeds, and thus potentially contributes to the development of both hyperinsulinemia and hyperglycemia. Furthermore, we demonstrated that volitional exercise training prevents the hyperactivity of the HPA axis and that exercise trained animals do not display any evidence of insulin resistance. We then set out to investigate the mechanism behind the effects of exercise on the HPA axis. We did find that exercise training maintained normal GR density in the hippocampus, whereas sedentary ZDF animals had decreased GR protein. Although this would indicate that negative feedback was decreased in sedentary animals, yet maintained in exercise trained animals, the levels of hypothalamic CRH and blood ACTH did not support this conclusion. CRH protein was actually increased in the exercise trained animals compared to sedentary animals, but both groups had comparable ACTH concentrations in the blood.

Instead, we found that exercise training attenuated (or potentially corrected) the increase of both MC2R and StAR proteins found in the sedentary group. Thus, similar to our findings in manuscript #1, sustained exercise training in ZDF rats resulted in normal adrenal sensitivity to ACTH, whereas this was increased in sedentary animals. This indicates that adaptations in the HPA axis, specifically at the level of the adrenal glands, can be included among the mechanism by which exercise training prevents the onset of T2DM.

In addition to determining the impact of exercise training on central components of the HPA axis, manuscript #2 also reported the effects of exercise training on peripheral aspects of GC biology. More specifically, we measured the protein expression of GR and 11βHSD1 (the intracellular determinants of GC action) in skeletal muscle, liver, and adipose tissue. We found trends for modest effects of both T2DM and exercise training in skeletal muscle and liver tissues. It was in adipose tissue that we saw the most significance. We found that sedentary ZDF animals with overt T2DM displayed increased protein expression of GR in visceral adipose tissue. Furthermore, exercise training only partially corrected this and actually increased ($p=0.07$) 11βHSD1 protein levels. This data indicated that exercise training may increase GC action in visceral adipose tissue, which supports one of our previous publications that showed exercise training in hamsters increased 11βHSD1 enzyme activity in adipose tissue (80). To date, no other studies have investigated the impact of exercise training on GC action in peripheral insulin target tissues.

Previous studies have shown that increased GC action in adipose tissue leads to the development of obesity and insulin resistance (18), and that reducing GC action in

adipose tissue protects against diet-induced obesity (179). However, we have shown, in both healthy hamsters and ZDF rats, that exercise training increases GC action in adipose tissue, but at the same time, confers protection against obesity and insulin resistance. Therefore, in manuscript #3, we attempted to address these discrepancies and to determine what result exercise training would have in adipose tissue in two models of insulin resistance; fructose-fed hamsters and older hamsters. In both models, we found that exercise training increased the protein expression of both GR and 11βHSD1 in adipose tissue, indicating an increase in GC action in this tissue. Using 3T3-L1 adipocytes, we showed the potential for a feed-forward mechanism, where concentrations of GCs that are typically seen with exercise training can induce the increase of both GR and 11βHSD1 protein levels. To give a functional implication for increased GC action in adipose tissue, we demonstrated that GCs increase lipolysis in a concentration-dependent manner. Based on the findings in manuscript #3, we concluded that exercise training increases GC action in adipose tissue, while still providing protection against the development of insulin resistance. We also proposed that this increase in GC action allows for greater rates of lipolysis and the subsequent release of lipid sources to be used as energy substrates to sustain high-volume exercise.

Our work with exercise training in various models of insulin resistance points towards an increase in GC action in adipose tissue in response to exercise. Based on these results, it would appear that GCs initiate catabolic events in adipose tissue, predominantly increasing lipolytic rates. However, the Cushing's syndrome phenotype suggests that GCs are, in fact, anabolic and stimulate an increase in adiposity (52, 180, 329, 359). Thus, the objective of manuscript #4 was to clarify the role of GCs in adipose tissue. We

utilized *in vitro* and *in vivo* techniques to investigate both anabolic and catabolic aspects of adipose tissue metabolism. We found that in fully mature 3T3-L1 adipocytes, GCs do not directly impact lipogenesis. Instead, we found that GCs were essential for preadipocyte differentiation, suggesting that any anabolic effects of GCs in adipose tissue occur because of adipocyte hyperplasia. Within adipocytes themselves, we found GCs to be catabolic, initiating genomic effects that increase lipolytic rates, particularly in the basal state. We concluded that GCs act on two distinct cell populations within adipose tissue. In preadipocytes, they increase differentiation and result in a greater number of adipocytes, contributing to adipogenesis. However, in adipocytes GCs increase lipolysis and attenuate the increase in cellular lipid content. In rats, GC treatment increased adiposity, while at the same time increasing lipolysis. Sedentary rats treated with GCs became obese, demonstrating increased blood lipids and ectopic lipid storage in skeletal muscle and liver. From this, we proposed that increased GC action, without substantial increases in energy expenditure such as that seen with exercise training, initiates a negative phenotype that would likely lead to the eventual development of insulin resistance and T2DM.

Finally, in the last set of experiments performed for this dissertation, we determined the consequences of GC treatment in an environment of energy surplus, by treating rats with exogenous corticosterone and providing a high-fat diet (Appendix B, Figures 8-11). Manuscript #4 demonstrated that exogenous corticosterone with a standard rodent diet causes obesity and dyslipidemia, but not insulin resistance. When we substitute the chow diet with a high-fat diet, the rats develop rapid insulin resistance and a diabetic phenotype within days. This final piece of data strongly supports our previous

conclusions that the overall effect of GCs in adipose tissue largely depends on the energy balance of the animals. In manuscripts #2 and #3, we show that exercising animals that have increased GC exposure in adipose tissue, decreased adiposity and demonstrate protection against insulin resistance. In manuscript #4, we show that sedentary animals with increased GC action in adipose tissue due to exogenous corticosterone treatment, but given a normal chow diet, develop modest obesity without insulin resistance. Finally, we demonstrated that animals treated with corticosterone and given a high-fat diet develop rapid insulin resistance. Thus, the impact of GCs, both in adipose tissue and on whole body glucose tolerance, largely depends upon the feeding and activity patterns of the animals. In other words, proper diet and regular exercise will prevent the deleterious effects of a stressful lifestyle.

In summary, this dissertation investigated the complex interactions between exercise, diet, and the HPA axis on the development of insulin resistance and T2DM. This work demonstrates that although exercise initially disrupts the HPA axis, sustained training restores this to basal conditions. Furthermore, the adaptations brought about by exercise are elicited mainly through changes in the adrenal glands. We also demonstrate that exercise training is capable of inducing significant adaptations in the adrenal glands, can avert hypercortisolemia in ZDF rats, and prevents the development of T2DM. Finally, we demonstrate that exercise training increases peripheral GC action specifically within adipose tissue. Importantly, the daily energy balance of animals with increased GC action in adipose tissue determines the functional consequences of this adaptation. With high energy expenditure, GCs favour lipolysis and the loss of adipose tissue mass. With a standard diet and sedentary lifestyle, GCs slightly favour adipogenesis and a gain in

adiposity. Finally, with the energy surplus induced by a high-fat diet, GCs cause severe obesity and rapid onset of insulin resistance. Thus, this dissertation shows that regular exercise positively affects the HPA axis and encourages a GC-mediated loss of adipose tissue mass, both of which protect against the development of insulin resistance and T2DM.

APPEDICES 7

A. Extended Methods

Cytochrome C Oxidase Assay

Enzyme extraction protocol

Extraction Buffer (pH=7.2)

100 mM Na-K-Phosphate; 2 mM EDTA

A) 500 ml of 0.1M Na_2HPO_4:
   - 8.90 g/500 ml of Na_2HPO_4
   - 0.372 g/500 ml of EDTA

B) 200 ml of 0.1 M KH_2HPO_4
   - 2.72 g/200 ml of KH_2PO_4
   - 0.149 g/200 ml of EDTA

Combine A and B, then check for pH. Always make fresh.

Steps:

i) Add 100 µl of extraction buffer to 1.5 ml eppendorf tubes, on ice
ii) Weight ~20 µg of frozen tissue powder into tubes and record the exact tissue mass
iii) Add volume of extraction buffer required for 20-fold dilution to each sample tube
iv) Stir extracts for 15 minutes using mini-magnetic stir bars
v) Remove magnets, then sonicate extracts four times, 10 seconds each time. Always clean sonicator tip between each sample
vi) Spin samples for 6 minutes in mini-centrifuge at 4C at max speed
vii) Pipette 250-300 µl of supernatant into a new set of tubes, on ice, and record volute save and discard the remainder.
viii) Repeat steps v-vii.
ix) Add the same volume of extraction buffer (250-300 µl) to make a 40-fold dilution.
x) Extracts are snap frozen and stored in liquid N_2 until used, remember to make a small hole by burning though the cap.

Cytochrome c oxidase assay

Reagents:

A) 20 mM KCN (MW=65.12)
   - 13.02 mg/10 ml ddH_2O
B) 100 mM K-Phosphate Buffer
   - make up 0.1 M of KH_2PO_4 (MW=136.09): 13.6 g/1 L (pH~5 at RT)
   - make up 0.1 M of $K_2HPO_4 \cdot 3H_2O$ (MW=174.18): 17.4 g/1 L (pH~8 at RT)
   - mix in equal proportions, then pH to 7.0
C) 10 mM K-Phosphate Buffer
   - dilute 0.1 M KPO_4 buffer prepared above in a 1:10 ratio in ddH_2O
D) Test Solution (Reduced cytochrome c; 2 mg/ml) (10ml)
   - weigh out 20 mg of horse heart cytochrome c (Sigma, C-2506) into a scintillation vial
   - add 1 ml of 10mM KPO_4 buffer and dissolve cytochrome c
   - make up a small volume of 10 mg/ml sodium dithionite-10 mM KPO_4 stock solution (make fresh each experiment and use within 20 minutes.)
   - add 40 µl of the dithionite stock solution to the test solution and observe red-orange color change
   - add 8 ml of ddH_2O
   - add 1 ml of 100mM KPO_4 buffer. Wrap solution bottle in foil
E) Extraction buffer (100mM Na-K-Phosphate, 2mM EDTA, pH=7.2):
   - see 'enzyme extraction protocol for cytox assay

Steps:

1. Pippette 980 µl of test solution into 7 cuvettes and incubate at 30C for 10 minutes to stabilize the temperature and absorbance
2. Place on cuvette in to the spectrophotometer chamber and add 20 µl of tissue extract. Stir the solution and immediately start the kinetics program, recording the change in absorbance for 0 to 5 minutes (starting absorbance should be greater than 2.8 at 550 nm)
3. add 20 µl KCN to the seventh cuvette to measure any absorbance changes in the presence of the CYTOX inhibitor.

Calculation:

CYTOX activity (µmole/min/g tissue)

$$= \frac{\Delta\text{absorbance/min x total volume x 40 (dilution)}}{\text{18.5 (µmole/ml extraction coefficient) x 0.02 ml sample volume}}$$

Bradford Assay for Determining Protein Concentration

Bradford Reagent:

- reagent is from Biorad (Cat# 500-0006)
- reagent is 5x stock and must be dilute to 1x with ddH_2O
- approximately 1 mL of Bradford reagent is required for each sample/standard to be read

Standards

- standards should be made and read prior to each set of samples
- a number of standards can be used, usually 6 is best
- standard concentrations should be between 0 and 16 mg/ml (usually 0,2,4,8,12,16 mg/ml)
- to make x standard, add x µl of 1mg/ml BSA into 1000-x µl of Bradford reagent (example x=4 mg/ml standard → 4 µl of BSA into 996 µl of Bradford)
- vortex and wait 10 minutes
- read standards to make standard curve

Standard Curve

- turn on the spec
- choose option 2 – applications
- choose option 4 – standard curve
- choose standards (bottom right corner)
- make new
- follow instructions and read standards
- the wavelength is 595 nm
- use the 0 standard for the reference too

Samples

- 5 µl of sample is added to 995 µl of bradford
- vortex and wait 10 minutes
- use 0 standard for reference
- read samples
- divide the concentration given by the spec by 5 to obtain actual protein concentration of the sample

Sample Preparation for Western Blot

Lysis Buffer for protein

NaCl – 135 mM (MW = 58.44)
$MgCl_2$ – 1 mM (MW = 203.3)
KCl – 2.7 mM (MW = 74.55)
Tris (pH 8) – 20 mM (121.14)
Sodium vanadate (Na_3VO_4) – 0.5 mM (MW = 183.9)
Sodium fluoride (NaF) – 10 mM (MW = 41.99)
PMSF – 0.2 mM
Leupeptin – 10 ug/ml
Triton – 1%
Glycerol – 10%
*** sodium vanadate, sodium fluoride, PMSF, and leupeptin can be replaced with a protease cocktail and phosphotase cocktail from sigma

For 50 mL of buffer

NaCl → 0.394 g
$MgCl_2$ → 0.010 g
KCl → 0.010 g
Tris (pH 8) → 0.121 g
Sodium vanadate (Na_3VO_4) → 125 µL
Sodium fluoride (NaF) → 0.021 g
PMSF → 100 uL
Leupeptin → 100 µL
Triton → 0.5 mL
Glycerol → 5 mL

Homogenize in lysis buffer and centrifuge @ 12,000 rpm for 10 min (4C). Remove supernantant and transfer to a new tube.
Use an aliquot of the supernantant for protein determination

4x SDS Laemmli Loading Buffer

1M Tris (pH – 6.8)	- 2.5 mL
SDS	- 0.8 g
Glycerol	- 4 mL
Bromophenol Blue (0.5% w/v)	- 200 µL
βME	- 2 mL
Total with ddH_2O	10 mL

Aliquot and store at -20
βME can be added fresh: make the above minus βME (total volume 8mL), aliquot into 0.8ml tubes and add 200 µl prior to use

SDS-polyacrylamide gel (SDS-Page) electrophoresis

Polyacylamide Solution:
30% (w/v) Acrylamide
0.8% (w/v) Bisacrylamide (NN'-Methylenebisacrylamide)
For 400 ml: Using gloves, measure out 120g of acrylamide and 3.2g of bisacrylamide and volume up to 400 ml with ddH_2O. Suction filter using 15 cm Whatman circular filter paper. (Change filter paper every 200 ml)
Store at 4C

Resolving Gel Tris Buffer:
1.5 M Tris
pH – 8.8
Store at 4C

Stacking Gel Tris Buffer:
0.5 M Tris
pH – 6.8
Store at 4C

Ammonium Persulfate (APS):
10% (w/v) in ddH_2O
Make within a week
Store at 4C

Sodium Dodecyl Sulfate:
10% (w/v) in ddH_2O
Store at RT

Butanol Overlay:
Mix 18.75 ml of 1M Tris (pH=8.8) with 0.5ml of 10% SDS, volume up to 150 ml with ddH_2O, mix 1:1 with butanol

10X Electrophoresis/Running Buffer, pH=8.3:

30.34 g	25.0 mM Tris
144 g	192.0 mM Glycine
10 g	0.1%(w/v) SDS

Dissolve contents in 1 L of ddH_2O, store at RT or in 4C Fridge. If precipitation occurs, warm to room temperature before use.

1X Electrophoresis/Running Buffer, pH=8.3
Dilute 10X 1:10.

10X Transfer Buffer (pH=8.3):
30.3 g Tris base
144 g Glycine

Dissolve contents in 1L ddH_2O, store at RT or 4C fridge. If precipitation occurs, warm to room temperature before use.

1X Transfer Buffer (pH=8.3)

100 ml	10X Transfer buffer
200 ml	Methanol
700 ml	ddH_2O

Gel Recipe:

Gel %	ddH2O		Acrylamide		0.5M Tris (pH=6.8)		1.5M Tris (pH = 8.8)		10% SDS		10% APS		TEMED	
	2x	*4x*	*2x*	*4x*	*2x*	*4x*	*2x*	*4x*	*2x*	*4x*	*2x*	*4x*	*2x*	*4x*
4%	6.1	12.2	1.34	2.68	2.5	5	-	-	100	200	25	50	30	60
7.50%	9.7	19.4	5	10	-	-	5	10	200	400	50	100	30	60
8%	11.4	22.8	5.34	10.7	-	-	5	10	200	400	50	100	30	60
10%	8	16	6.66	13.3	-	-	5	10	200	400	50	100	30	60
12%	6.7	13.4	8	16	-	-	5	10	200	400	50	100	30	60

TTBS (Wash Buffer):

10X
350.0 g NaCl
121.1 g Tris-Base
20 ml Tween-20
20 ml NP40

Make to 4L with ddH_2O
pH 7.5

Use at 1X with a 1:10 dilution

Strip Buffer:

25 mM glycine (1.875 g in 1 L)
1% SDS (10 g in 1 L)
pH – 2.0

Strip buffer for 30 minutes
Wash with TBS-T 3x15 minutes
Block and re-probe

Procedures to grow 3T3-L1 adipocytes

Medium used for preadipocyte stage:

High Glucose DMEM (25 mM)
10% FBS
1% Antibiotic solution

NOTE: Do not grow the cells confluent. Split regularly when they are ~70% confluent.

Protocol for Differentiation:

After plating the cells, wait 2 days post 100% confluency. Change the media to differentiation media for 6 days:

High glucose DMEM
10% FBS
200 nM insulin
0.25 μM dexamethasone
500 μM isobutylmethylxanthine

After 6 days, the media is changed to DMEM containing 10% FBS and 200 nM insulin. After an additional 2 days, the cells are placed in DMEM containing 10% FBS without any additives for 2 days. After the 2 days, the cells are used for the desired experiment.

Reagents:

Wisent (1 888 947 3681)
High glucose DMEM (4.5 g/L), cat# 10013CV
Fetal Bovine Serum, cat# 080150
Antibiotic solution, cat# 450201EL
PBS Sterile, cat# 311010CL
Tripsin/EDTA, cat# 325043EL

Sigma
Dexamethasone, cat #D4902, 25mg
3-Isobutyl-1-methylxanthine (IBMX), cat# I7018, 1g
Insulin (Bovine pancreas), cat# I6634, 100mg

Glucose Uptake in 3T3-L1 adipocytes

Materials:

- fully differentiated 3T3-L1 cells
- starve media (DMEM with 1% pen/strep, NO FBS)
- KREBS-RINGER-HEPES Buffer (KRH)
- Labelled ^{3}H 2-Deoxyglucose
- Unlabelled glucose (10 mM stock)
- Insulin media (make 1000x stock solutions based on final concentrations)
- 0.1% SDS lysis buffer (w/v in KRH buffer)

KREBS-RINGER-HEPES (KRH) buffer

Stock solutions (10x):

1. 1.21 M NaCl → 3.5 g in 50 ml
2. 49 mM KCl → 0.183 g in 50 ml
3. 12 mM $MgSO_4$ → 0.072 g in 50 ml
4. 3.3 mM $CaCl_2$ → 0.0183 g in 50 ml
5. 120 mM Hepes → 1.43 g in 50 ml

For 100 ml:

- add 10 ml of each stock solution
- add 45 ml of ddH_2O
- pH to 7.4
- bring final volume to 100 ml

***store at 4 C

For 1 24-well plate

Prior to experiment:

- warm 25 ml of starve media (37 C) in a 15 ml tube (Tube 1)
- warm 25 ml of KRH buffer (37 C) in a 50 ml tube (Tube 2)
- warm 12.5 ml of KRH buffer (37 C) in a 15 ml tube (Tube 3)
- put 37 ml of KRH buffer in a 50 ml tube and store on ice (Tube 4)
- prepare insulin media, warm to 37 C (for basal, use KRH without insulin)
- prepare glucose media
    - add 4 µl of stock unlabelled glucose (10 mM) to 4 ml KRH buffer
    - add 1-4 µl of ^{3}H-glucose, depending on source
    - vortex and warm to 37 C
- prepare Cytochalasin B controls (CB controls); insulin and glucose
    - add 500 µl of insulin media (if using different concentrations, use the max) to an eppendorf tube. Add 1 µl of CB and warm to 37 C.
    - add 300 µl of glucose media to and eppendorf tube. Add 1 µl of CB and warm to 37 C
- put 10 ml of 0.1% SDS lysis buffer in a 15 ml tube, store at RT
- label 1.5ml eppendorf tubes 1 through 24, corresponding to the appropriate well
- label 26 scintillation vials, 1-24 + 2 for total activity

Experiment:

1. Under sterile conditions, wash cells 1x with starve media (Tube 1) and then add 500 µl/well of starve media (Tube 1). Incubate for 2 hours at 37 C.
2. Wash cells 2x with 500 µl of warm wash buffer (Tube 2).
3. Add 250 µl of insulin/control/CB media and incubate for 20 min at 37 C
4. Quickly aspirate insulin media and wash 1x with 500 µl of warm KRH (Tube 3).
5. Immediately aspirate and quickly add 150 µl of glucose media, start timer right before adding the glucose. Make sure to add CB-glucose media to the appropriate wells. Incubate at 37 C.
6. At 4 min, place the plate on ice and quickly aspirate the glucose media.
7. Quickly wash 3x with ice-cold KRH (Tube 4).
8. Add 400 µl of 0.1% SDS lysis buffer and shake the plate for 10 minutes.
9. Transfer the contents of the well to the appropriate eppendorf tube and vortex thoroughly. Centrifuge the tubes for 10 minutes at max speed.
10. While tubes are centrifuging, add 4 ml of scintillation fluid to the labelled vials.
11. Add 200 µl of infranatent to the appropriate scintillation vial.
12. Add 150 µl of remaining glucose media to each of the total scintillation vials (25 and 26)
13. Tightly cap, wipe, and vortex the scintillation vials. Count them immediately and then 3-4 hours later.
14. Measure protein by Bradford by adding 20 µl of remaining contents from the eppendorf tubes to 980 µl of Bradford reagent. Multiply values by 20 to get total protein in each well (µg).

Calculating glucose uptake:

1. Calculate the average CPM for CB and Total.
2. Convert individual CPM to µM glucose uptake using the equation:

$$\text{Glucose uptake } (\mu M) = \frac{(CPM-CB)*10}{Total}$$

3. Divide individual glucose uptake values by 4 to get µM/min.
4. Divide individual glucose uptake values by protein content to get µM/min/µg.
5. Calculate the control basal values.
6. Divide all individual glucose uptake values by the average for control basal to get fold increase/decrease in glucose uptake.

Oil Red O Staining

3T3-L1 adipocytes:

REAGENTS:

Oil Red O stock
FW 408.5, Sigma O-0625
0.7 g Oil Red O
200 ml Isopropanol
Stir O/N, then filter with 0.2 μm and store at +4°C

Oil Red O Working Solution
6 parts Oil Red O stock
4 parts dH2O
Mix and let sit at room temp for 20 min
Filter 0.2 μm

10% Formalin in PBS

Isopropanol 100%
Isopropanol 60%

METHOD

- Remove most of the medium
- Add 10% formalin in incubate 5 min, RT
- Discard formalin and add the same volume of fresh formalin. Incubate at least 1 hour, or longer. Note: Cells can be kept in formalin for a couple of days before staining. Wrap parafilm around the plate to prevent from drying and cover with aluminum foil.
- Remove all the formalin with small transfer pipette
- Wash wells with 60% isopropanol
- Let the wells dry completely
- Add Oil Red O working solution for 10 min (do not touch walls of the wells)
- Remove all Oil Red O and IMMEDIATELY add dH2O, wash with H2O 4 times (you can wash under running tap water)
- Take pictures if desired
- Remove all water and let dry
- Elute Oil Red O by adding 100% isopropanol, incubate about 10 min (can be longer)
- Pipette the isopropanol with Oil Red O up and down several times to be sure that all Oil Red O is in the solution
- Transfer to 1.5 ml tubes if storing, or directly into cuvettes for the spec
- Measure OD at 500 nm, 0.5 sec reading
- As blank use 100% isopropanol. As control use isopropanol from empty well stained as previously described

Tissue Sections:

Tissue harvesting:
Tissue must be flash frozen immediately after dissection. Immerse tissue in a small container of isopentane (2-methylbutane), and place container into liquid nitrogen, being careful not to allow isopentane and liquid nitrogen to mix. Freezing should take place in matter of seconds. Tissue can be stored at –84OC until use.

Sectioning tissue:
Using the cryostat, obtain sections at a thickness of 8μm or less. Set the sample temperature to between –29o and –20o. To ensure that sections are good cross sections, try a few cuts and then look at them under the microscope. You may need to play with the orientation of the blade or the tissue to correct this. Replace blade frequently for best results.

Stock solution:
300mg Oil Red O powder in 100ml isopropanol (2-propanol-2). Store in a cool dark place. This should be done under the fume hood.

Working solution:
Prepare just prior to staining: 12mL Oil Red O stock in 8mL double distilled H2O. Filtered through Whatman #42 filter paper.

Phosphate buffered saline:
137mM NaCl (sodium chloride), 3mM KCl (potassium chloride), 8mM Na2HPO4 (disodium hydrogen phosphate), 3mM KH2PO4 (potassium dihydrogen phosphate), pH 7.4.

Dole's Lipid Extraction

Dole's Reagent:

40 parts isopropanol
10 parts heptane
1 part H_2SO_4 (1N)

Store at 4C

Before beginning:
- label the appropriate number of 15 mL tubes
- label and weight the appropriate number of scintillation vials

For 6 well plates:

Experimental volume should be 1 mL. At the end of the experiment remove 500 µl for analysis, leaving 500 µl in the well. Add 1.5 ml of dole's reagent to the well and shake. Using a cell scraper, removed all cells from the bottom of the plate and mix all the contents of the well. Tip the plate on an angle and allow all the contents of the well to move to the bottom. Using a pipette, wash the top half of the plate with the dole's reagent from the bottom half to ensure all contents are located in the bottom portion of the well. Pipette all the contents into a labeled 15 ml tube. Add 1 mL of heptane and 1 mL of ddH_2O to each of the 15 mL tubes. Vortex for 2.5 minutes at 2.5g. Carefully remove 900 µl from the upper layer (if 900 µl cannot be removed accurately, remove as much as possible and note the volume). Pipette the 900 µl into pre-weight and pre-labeled scintillation vials. Allow the scintillation vials to sit overnight, the heptane will evaporate and leave the lipid content. Weight the vials once the heptane has evaporated.

Lipid content:

Lipid weight = (post-weight – pre-weight) x total heptane volume/volume extracted

If 900 µl was extracted:

Volume extracted = 0.9 mL
Total heptane volume = 1.294 mL (dole's + 1 mL added)
Dole's heptane = (10 parts out of 51) x 1.5 mL = 0.294 mL

Extraction of Primary Adipocytes

Materials required prior to extraction day:

- Autoclaved:
  - surgical tools
  - Pasteur pipettes
  - Sieves (1 for epididymal, 1 for perirenal, 3 for subcutaneous)
  - 5 mL pipette tips, with cut ends
  - p200 and p1000 pipette tips
- Hank's solution
- Weight boats
  - Ethanol and UV
  - Small for epididymal and perirenal
  - Larger for subcutaneous
- Collagenase (sigma)
- DMEM without phenol

1. prepare digestion media
   - weight out collagenase, 5mg per fat type
   - place collagenase under UV light for ~1 min to sterilize
   - dissolve collagenase into Hank's balanced salt solution
   - 15 mL for epididymal and perirenal, 20 mL for subcutaneous
   - place digestive solution into water bath to warm to 37C
2. place DMEM without phenol into 37C to warm up
3. place digestion media into the hood
4. extract fat pad
   - remove a piece of fat and dip into digestion media
   - place fat into respective weight boat
5. after all fat has been extracted from all animals, place digestion media back into the water bath
6. mince adipose tissue, ~5-8 minutes for epididymal and perirenal, ~12-15 minutes for subcutaneous
7. dump minced tissue back into digestion media
8. swirl urine container with digestion media and adipose tissue in the water bath by hand, ~8-10 minutes for epididymal and perirenal, ~10-15 minutes for subcutaneous
9. strain adipose tissue through sieve into another urine container, 1 sieve for epididymal and perirenal, 3 sieves for subcutaneous
10. wash sieves with DMEM (~10 mL)
11. place strained adipose tissue into 50 mL falcon tube and add another 5-10 mL of DMEM (final volume → epi and perirenal = ~25-30mL, subcutaneous = ~30-35 mL)
12. Wash cells 3x:
    - centrifuge the falcon tube to separate the connective tissue from the adipose cells

- using a Pasteur pipette, remove the bottom connective tissue leaving only adipose cells
- resuspend with 15 mL of DMEM
- centrifuge and repeat until last wash has been completed
- subcutaneous tissue may need an additional wash

13. after final wash, resuspend the cells in volume of DMEM require for experiment
14. place in incubator to equilibrate the cells for ~30 minutes and then aliquot appropriately according to experiment

Assessing primary adipocyte number and volume

After isolation of primary adipocytes:
- remove an aliquot for microscopy
- remove an aliquot for determining lipocrit

Use the following equations:

1. Determine the average cell volume by measuring the average diameter of 50-100 individual adipocytes under the microscope.

Average Cell Volume (um^3/cell)

Volume = $(3.14 \times d^3)/6$
***simple volume of sphere using the average diameter counted

Average Cell Volume (pL/cell)

1000 um^3 = 1 pL
***divide average cell volume (um^3/cell) by 1000

2. Centrifuge the aliquot of cells in the microtube to determine the lipocrit fraction

Lipocrit

Volume of cells in microtube (cm^3) = (3.14 x diameter of microtube^2 x height of cells)/4
= $3.14*(0.4^2)$*average height/4

1 cm^3 = 1 mL
***multiply volume of cells in microtube (cm^3) by 1000 to get volume in µL

Lipocrit (%) = volume of cells (µL)/total volume (µL)
***total volume in microtube in usually 300 µL

Cell number

Volume of total cells = total volume (mL) x Lipocrit
Number of cells = volume of cells (µL) x 10^9 (pL/µL) / average volume of cells (pL)

****number is multiplied by 10^6 to express cell number in millions

Free Glycerol Determination Kit

Sigma
FG0100
Storage: 2-8 C

Assay Theory:

The glycerol in the sample is phosphorylated by adenosine-5`-triphophate (ATP) forming glycerol-1-phosphate (G-1-P) and adenosine-5`-diphosphate (ADP). This reaction is catalyzed by glycerol kinase (GK). G-1-P is then oxidized by glycerol phosphate oxidase (GPO) to dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H_2O_2). Peroxidase (POD) catalyzes the coupling of H_2O_2 with 4-aminoantipyrine (4-AAP) and sodium N-ethyl-N-(3-sulfopropyl) m-anisidine (ESPA) to produce a quinoneimine dye that shows an absorbance maximum at 540 nm. The increase in absorbance at 540 nm is directly proportional to the free glycerol concentration in the sample.

Reactions:

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{G-1-P} + \text{ADP}$$

$$\text{G-1-P} + O_2 \xrightarrow{\text{GPO}} \text{DAP} + H_2O_2$$

$$H_2O_2 + \text{4-AAP} + \text{ESPA} \xrightarrow{\text{POD}} \text{Quinoneimine Dye} + H_2O$$

Procedure:

1. Prepare glycerol reagent:
   - reconstitute 1 bottle with 40 mL of ddH_2O
   - gently mix by inverting
   - determine the final volume of reagent required for the current experiment
   - in a new glass container, dilute the reconstituted reagent 1:1 with ddH_2O for a volume that is equal to the final volume
     - example: 16 samples + 4 standards
       - 20 x 0.8 mL = 16 mL
       - add 8.5 mL reagent + 8.5 mL ddH_2O for 17 mL
   - store the remaining reconstituted reagent in the amber bottle at 4C

2. Standard Curve:
   - add 0.8 mL of diluted reagent to 4 cuvettes
   - add nothing to cuvette 1 (0 mg/ml or blank)
   - add 5 µl of glycerol standard to cuvette 2 (1.25 mg/ml triolene)
   - add 10 µl of glycerol standard to cuvette 3 (2.5 mg/ml triolene)
   - add 20 µl of glycerol standard to cuvette 4 (5 mg/ml triolene)
   - place cuvettes in 37C for 5 minutes
   - immediately read at 540 nm

3. Samples:
   - add 0.8 ml to the appropriate number of cuvettes
   - add 100 µl of each sample to the appropriate cuvette
   - place in incubator at 37C for 5 minutes
   - immediately read at 540 nm
   - the concentration given by the spectrophometer is 10x triolene (mg/ml)

4. Determining concentration
   - using the concentration provided by the spectrophometer (conc.):

Glycerol (µM) = spec. conc. x (0.26/2.5) x 1/92 x 1/10 x 1000000

(0.26/2.5) = conversion factor from triolene to glycerol
1/92 = conversion factor from mg/ml to M (MW of glycerol = 92 g/mole)
1/10 = conversion factor from 10x to 1 x
1000000 = conversion factor from M to µM

B. Additional Data

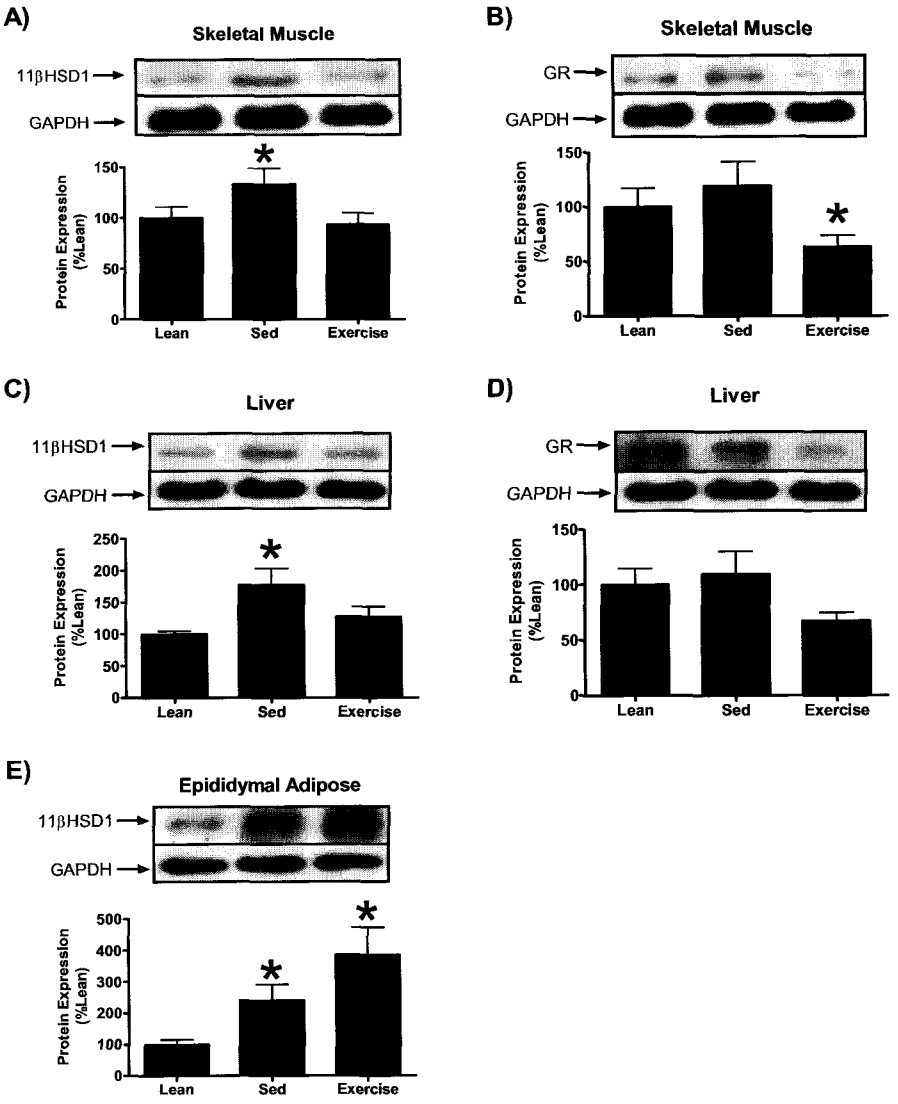


Figure 1: GR and 11βHSD1 protein expression in skeletal muscle, liver, and epididymal adipose tissue after 13 weeks of swim training in male, ZDF and ZF (lean) rats.

In skeletal muscle, sedentary ZDF rats demonstrated an increase in 11βHSD1 protein, which was attenuate by exercise (A). Exercising ZDF animals also had a decrease in GR protein compared to lean and sedentary animals (B). In the liver, sedentary ZDF rats had higher expression of 11βHSD1, which was attenuated with exercise training (C). A trend (P=0.07) was found for decreased expression of GR protein in the exercise ZDF animals, compared to both lean and sedentary animals (D). Finally, in visceral adipose tissue, both sedentary and ZDF rats demonstrated an increase in 11βHSD1 protein compared to lean animals (E). A trend (P=0.06) for higher expression of 11βHSD1 protein was found in the exercised animals compared to the sedentary animals (E). Values are expressed as means ± SEM, n=8, * - $P<0.05$ vs. lean.

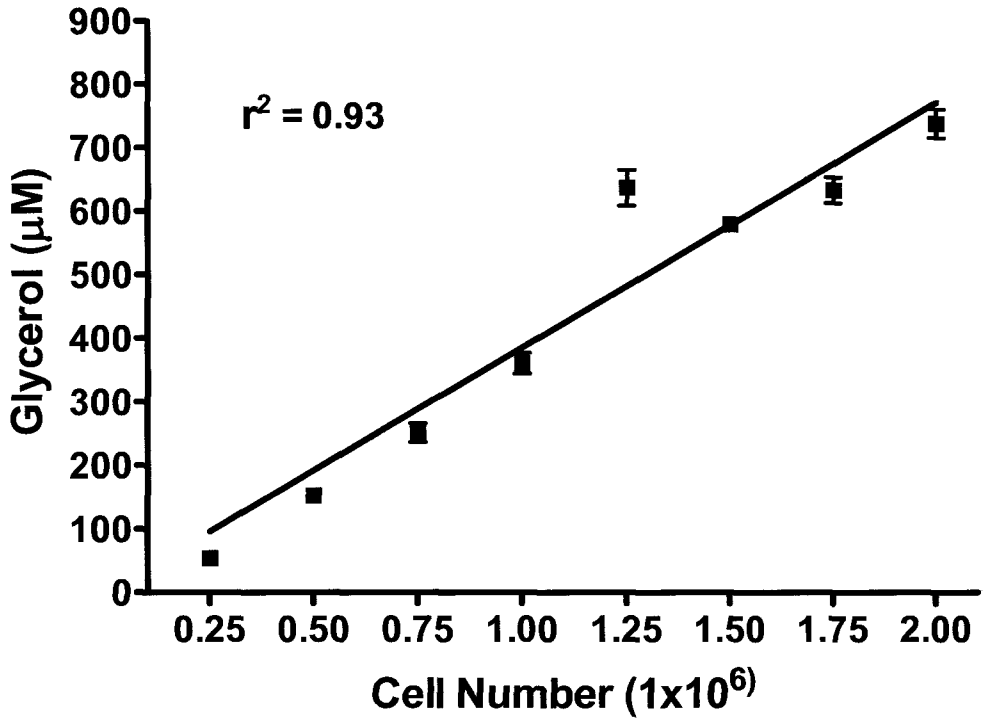


Figure 2: The relationship between the number of adipocytes and lipolytic rates in primary epididymal adipocytes stimulated with 1 μM corticosterone.
An increasing number of adipocytes were incubated in 3ml of media containing 1 μM corticosterone to determine the optimal lipocrit ratio for lipolytic experiments. The correlation between cell number and lipolytic rates remain linear between 25000 and 200000 adipocytes, with the exception of the 125000 point. This was considered an outlier and subsequent experiments utilizing primary adipocytes used 100000 cells in 3ml of media. Values are expressed as means ± SEM, n=3.

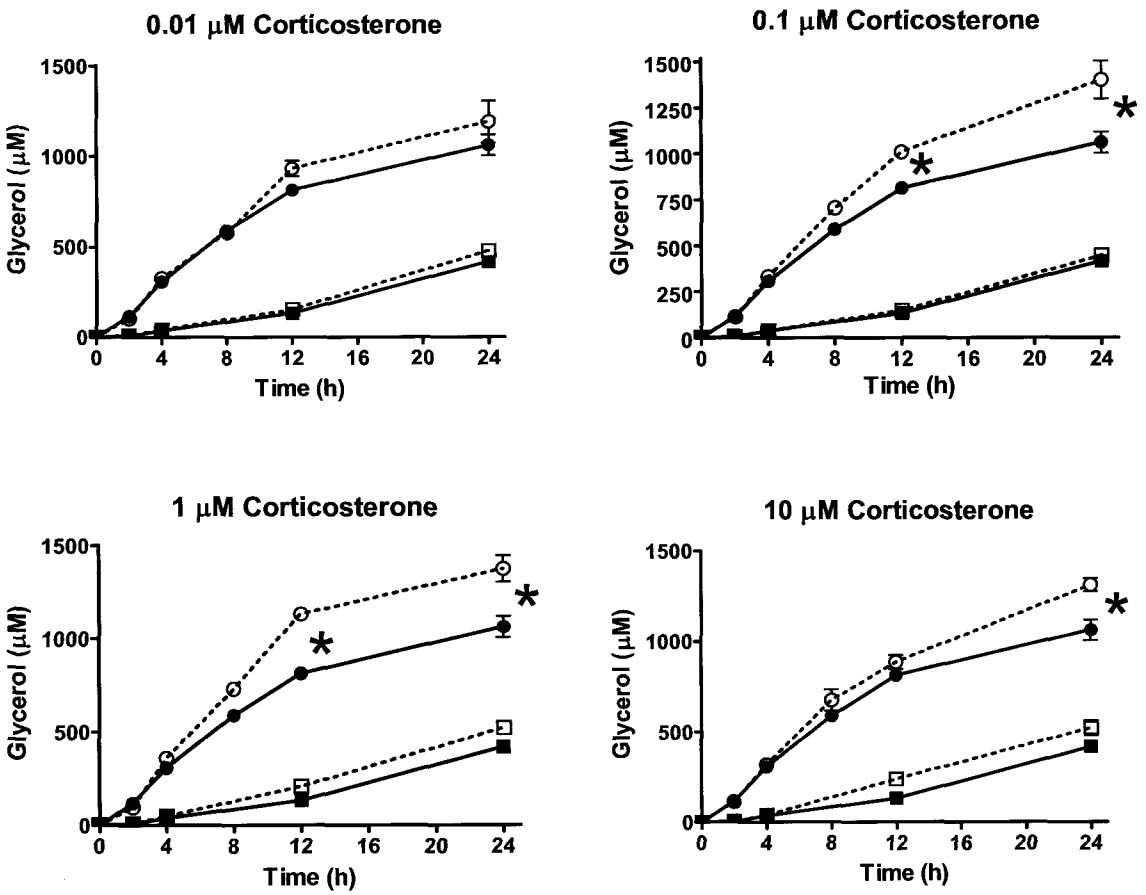


Figure 3: Time course of lipolysis in primary rat epididymal and subcutaneous primary adipocytes with various concentrations of corticosterone.
Corticosterone did not alter lipolytic rates in subcutaneous adipocytes at any time point. In epididymal adipocytes, corticosterone did not alter lipolytic rates at any concentration with an incubation time of 8 hours or less. Corticosterone concentrations of 0.1 and 1 μM significantly increased lipolytic rates at 12 hours. Corticosterone concentrations of 0.1, 1 and10 μM increase lipolytic rates at 24 hours. ○ – CORT-epididymal, ● – CON-epididymal, □ – CORT-subcutaneous, ■ – CON-subcutaneous. Values are expressed as means ± SEM, n=5, * - $P<0.05$ vs. control cells.

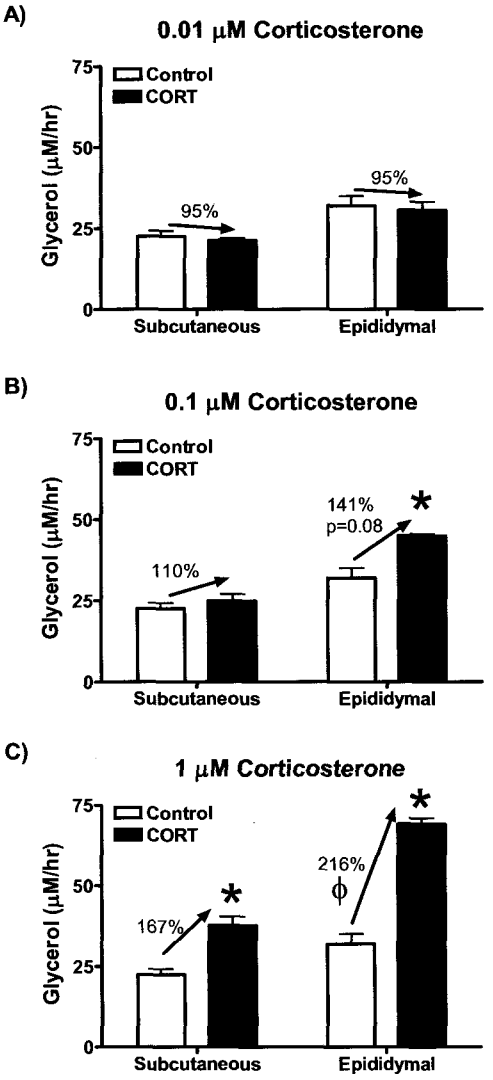


Figure 4: Basal lipolysis following corticosterone treatment in rat primary adipocytes

Subcutaneous and epididymal primary adipocytes were treated with various concentrations of corticosterone (0.01-1 μM) for 24 hours, washed, and resuspended in media to assess basal lipolysis for 1 hour. (A) A concentration of 0.01 μM corticosterone did not alter basal lipolytic rats in either subcutaneous or epididymal cells. Pooled epididymal data indicated higher lipolytic rates compared to pooled subcutaneous data ($P<0.05$). (B) A concentration of 0.1 μM corticosterone did not alter the basal lipolytic rats in subcutaneous adipocytes, but did increase basal lipolysis in epididymal adipocytes ($P<0.05$). The percent increase in basal lipolysis in the epididymal cell in responsed to corticosterone tended to by greater than the percent increase in subcutaneous ($P=0.08$). (C) A concentration of 0.1 μM corticosterone increased basal lipolytic rates in both subcutaneous and epididymal adipocytes ($P<0.05$). The effect of corticosterone (i.e. percent increase) was greater in epididymal adipocytes compared to subcutaneous ($P<0.05$). Values are expressed as mean ± SEM, n=5, * - $P<0.05$ vs. control cells, same depot, θ – $P<0.05$, percent increase in basal lipolysis following corticosterone treatment, in epididymal vs. subcutaneous adipocytes.

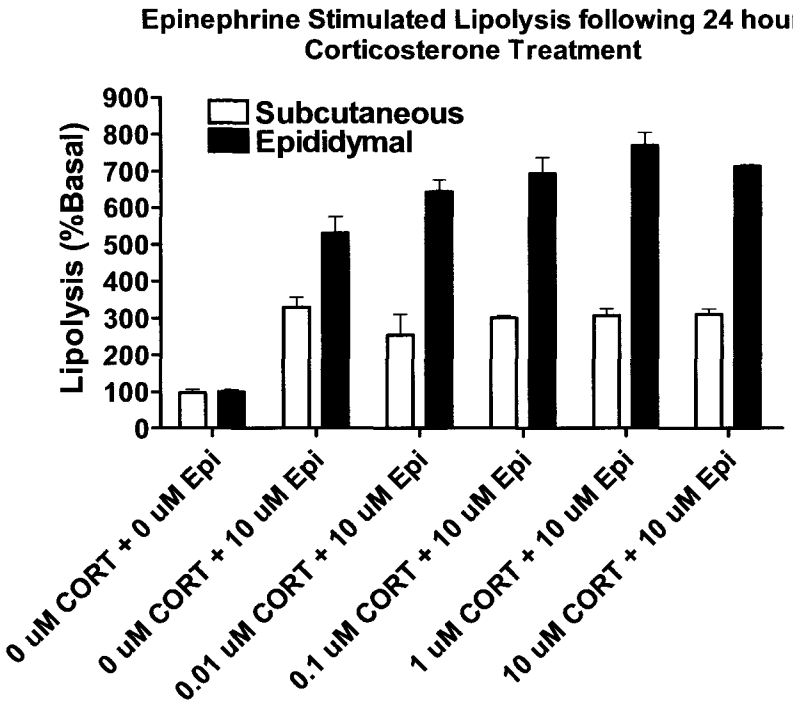


Figure 5: Epinephrine Stimulated Lipolysis following 24 hour corticosterone treatment in rat primary adipocytes.

Subcutaneous and epididymal adipocytes were incubated for 24 hours in various concentrations of corticosterone (1-10 μM), after which, the cells were washed and resuspended in media containing 10 μM epinephrine for 1 hour. Epididymal cells responded to the epinephrine with greatly lipolytic rats compared to the subcutaneous cells at all concentrations of corticosterone pre-treatment ($P<0.001$). Corticosterone pre-treatment did not affect the subcutaneous adipocyte response to epinephrine. A trend was found for an effect of corticosterone pre-treatment on the epididymal response to epinephrine. Specifically, 1 μM corticosterone pre-treatment tended to increase the response to epinephrine compared to control cells ($P<0.07$). Values are expressed as mean ± SEM, n=4.

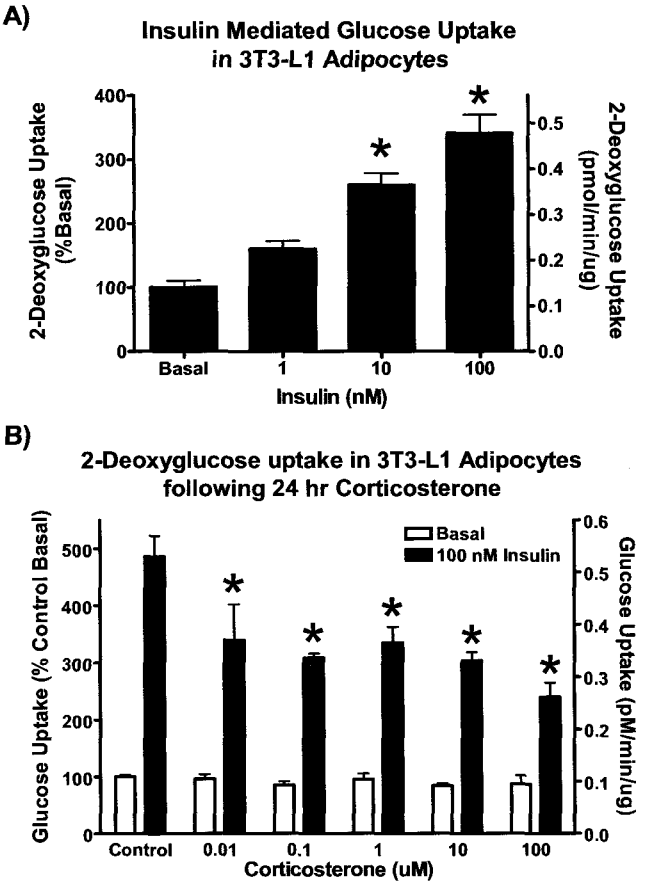


Figure 6: Glucose Uptake in 3T3-L1 adipocytes

(A) Fully differentiation 3T3-L1 adipocytes were serum-starved for 3 hours and then incubated with various concentrations of insulin to stimulate glucose uptake. A concentrations dependent increase in glucose uptake was seen with increasing concentrations of insulin ($P<0.05$). (B) Fully differentiation 3T3-L1 adipocytes were treated with various concentrations of corticosterone for 24 hours, serum-starved for 3 hours, and incubated with 100 nM insulin to stimulated glucose uptake. Pre-treatment with corticosterone did not alter basal glucose uptake, but did decrease insulin-stimulated glucose uptake at all concentrations of corticosterone ($P<0.05$). Values are expressed as mean ± SEM, n=3, * - $P<0.05$ vs. control.

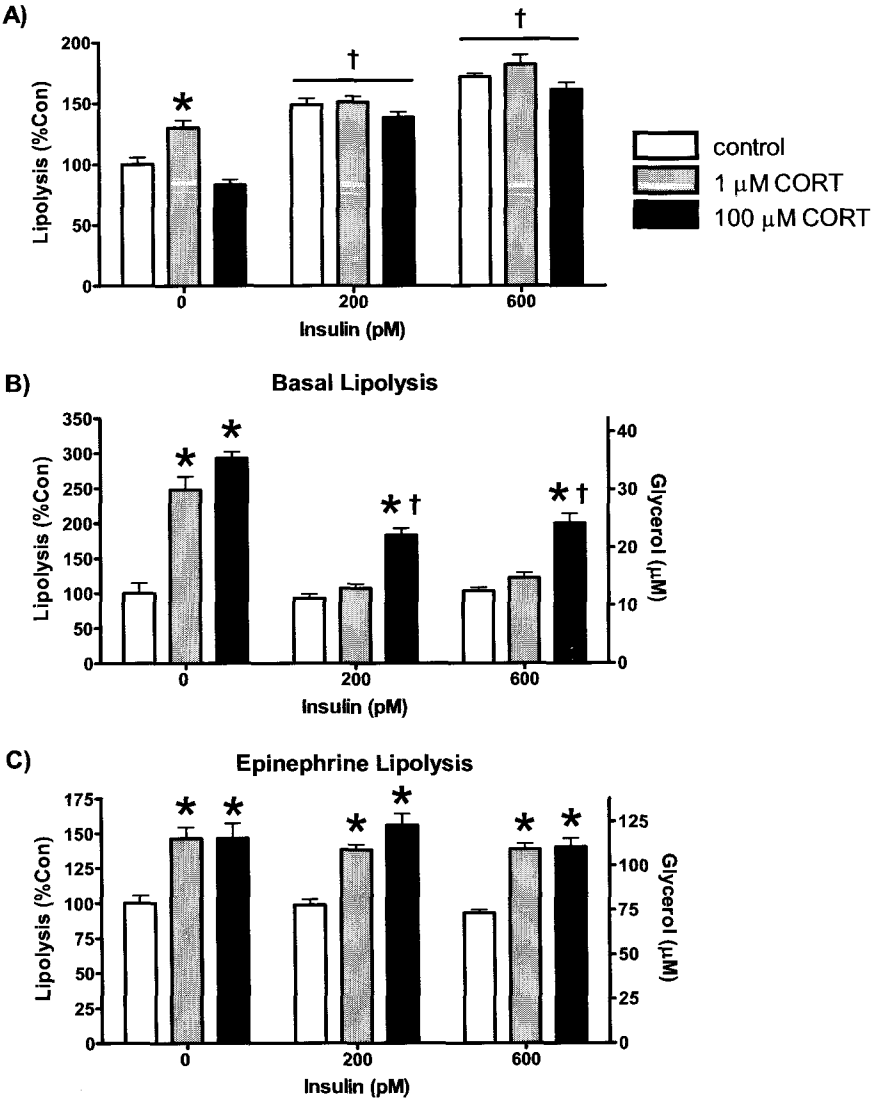


Figure 7: The effect of insulin on corticosterone-stimulated lipolysis, as well as basal- and epinephrine-stimulated lipolysis following corticosterone pretreatment in 3T3-L1 adipocytes.

(A) Cells were incubated in various concentrations of corticosterone (0, 1, and 100 μM) and various concentrations of insulin (0, 200, 600 pM) for 24 hours. Lipolytic rates were measured by the release of glycerol over the 24-hour period. Without insulin, 1 μM, but not 100 μM, increase lipolytic rates in a similar parabolic manner previous described. Both 200 and 600 pM insulin abolished this effect, however, did increase lipolytic rates at all concentrations of corticosterone compared to control cells (i.e. no corticosterone or insulin). (B) Pre-treatment with corticosterone increased basal lipolysis in a concentration-dependent manner, as previously described. Co-incubation with insulin abolished the increase in basal lipolysis with 1 μM corticosterone, and reduced the basal lipolytic rates with 100 μM corticosterone. (C) Co-incubation with insulin did not affect epinephrine-stimulated lipolysis following corticosterone treatment. Values are expressed as means ± SEM, n=6, * - $P<0.05$ vs. control cells in the same concentration of insulin, † - $P<0.05$ vs. cells treated with the same concentration of corticosterone and no insulin.

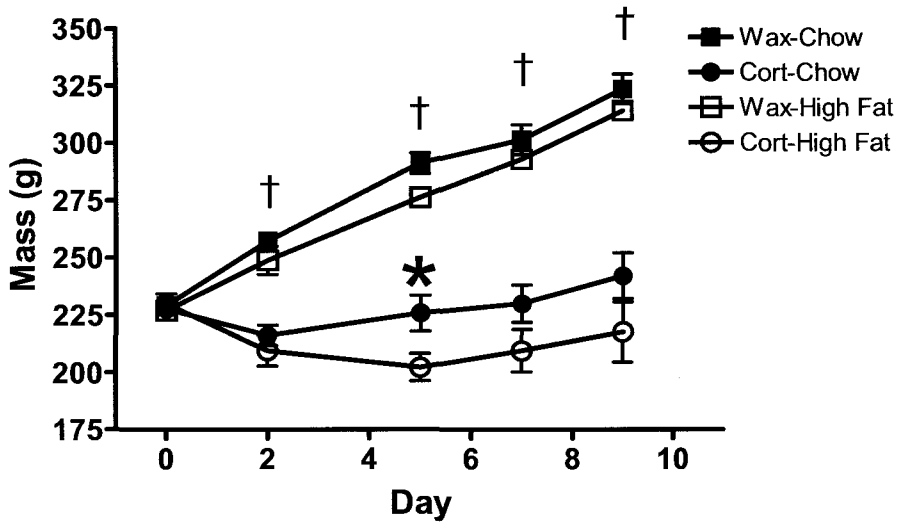


Figure 8: Body weights of rats in the CORT-high fat study
Male, Sprague-dawley rats were subcutaneously implanted with either 4x100mg wax or corticosterone pellets. Corticosterone treatment significantly impaired the increase in weight gain seen in the wax animals ($P<0.05$). Animals treated with corticosterone and given a high-fat diet weight significantly less than all other groups on day 6 ($P<0.05$) and did not achieve their baseline body weight by day 9. Values are expressed as means ± SEM, n=6, † - $P<0.05$, wax vs. corticosterone, * - $P<0.05$, cort-chow vs. cort-high fat.

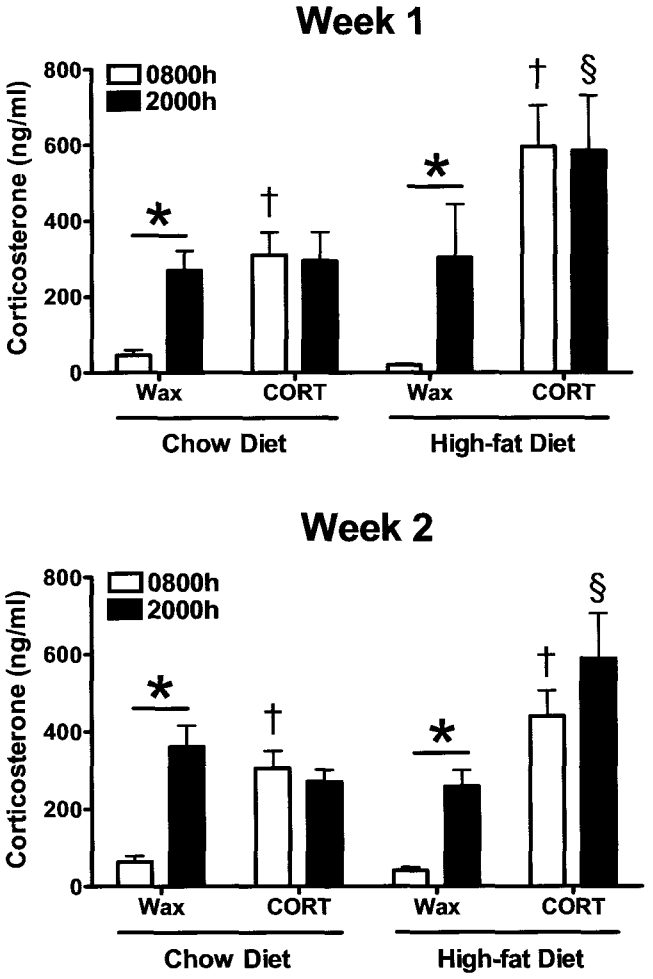


Figure 9: Diurnal corticosterone values in the CORT-high fat study
Animals receiving wax pellets displayed expected diurnal corticosterone patterns, with values high in the evening and low in the morning. Corticosterone treatment abolished the diurnal pattern in both chow and high-fat fed groups. There was an additive effect of corticosterone treatment and high-fat diet, with this group displaying the highest levels of circulation corticosterone. Values are expressed as means ± SEM, n=6, † - $P<0.001$ vs. wax-chow at 0800h, * - $P<0.001$, 0800h vs. 2000h, § - $P<0.01$ vs. cort-show 2000h.

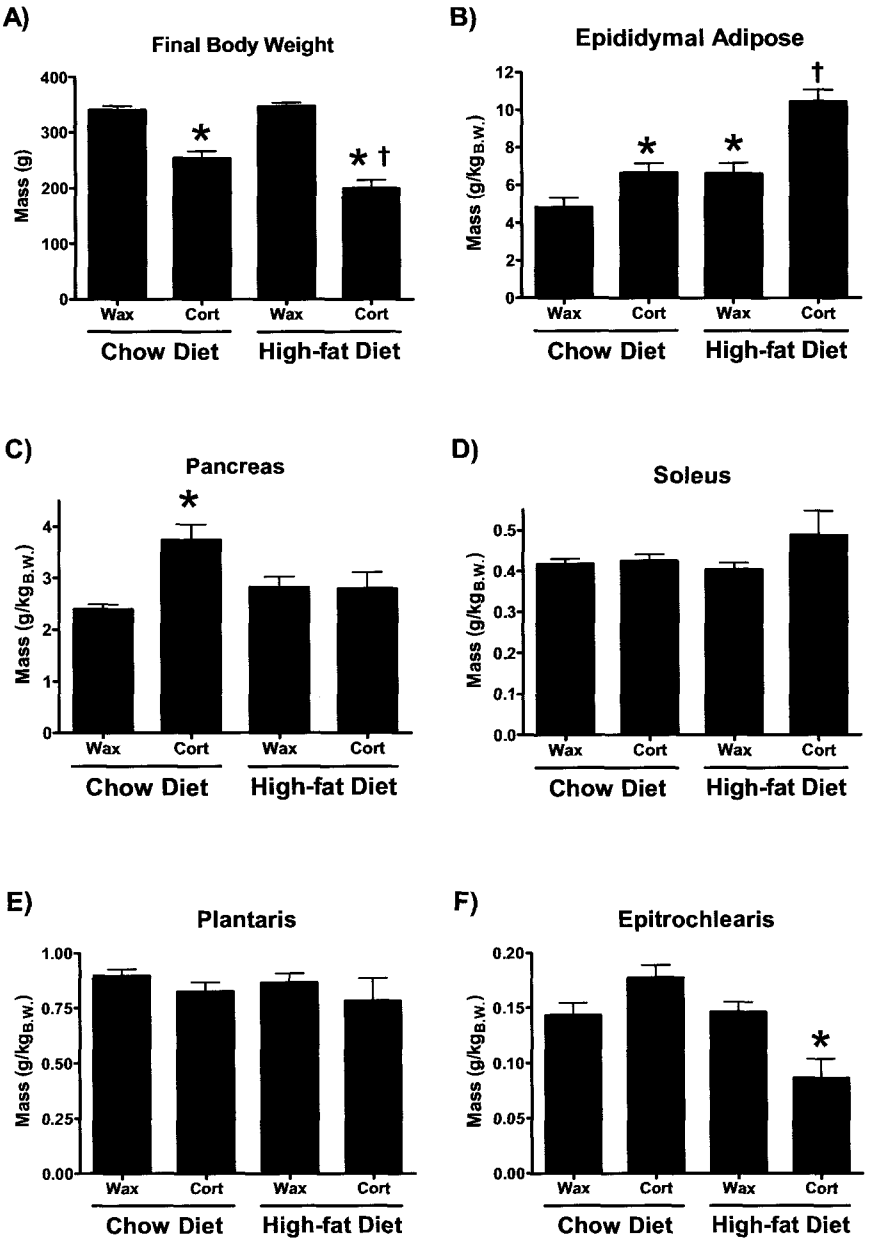


Figure 10: Body weight and tissue weights at euthanasia in the CORT-high fat study (A) Corticosterone treatment attenuated body growth in both chow and high-fat fed groups. Cort-high fat animals also weighed significantly less than cort-chow animals. (B) High-fat feeding and corticosterone treatment independently increase visceral adiposity compared to the wax-chow groups. Cort-high fat animals had significantly more visceral adiposity compared to all groups. (C) Corticosterone alone increased pancreas tissue weight compared to all other groups. (D) No differences were seen in soleus weight among the groups. (E). No differences were seen in plantaris weight among the groups. (E). Cort-high fat animals had significantly smaller epitrochlearis weights compared to all other groups. Values are expressed as means ± SEM, n=6, * - $P<0.05$ vs. wax-chow, † - $P<0.05$ vs. all groups.

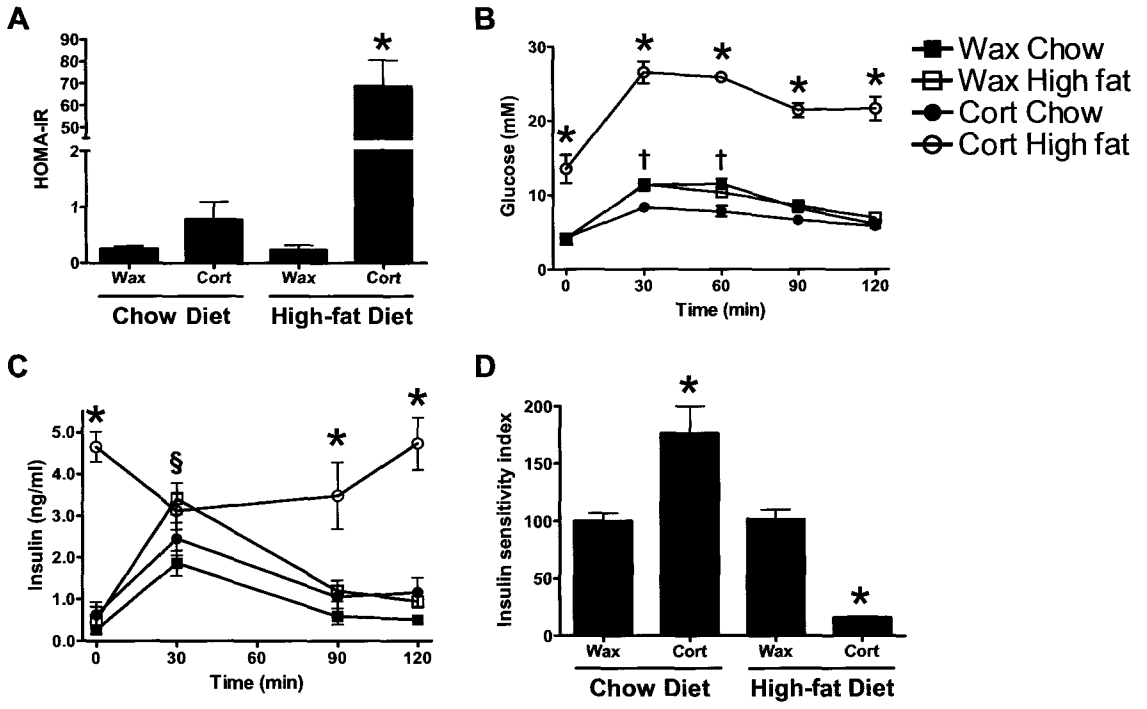


Figure 11: Oral glucose tolerance test on day 14 in the CORT-high fat diet study
On day 14, all animals underwent an oral glucose tolerance test. For this, the animals were fasted overnight and then gavaged with 2 mg/kg 50% dextrose. Fasted blood samples were taken prior to gavage, and blood samples were taken at 30, 60, 90, and 120 minutes following gavage. (A) HOMA-IR, a measure of insulin resistance, was calculated based on fasting glucose and fasting insulin, using the formula: [fasting insulin (pM) x fasting glucose (mM)]/135. Cort-high fat animal were significantly more insulin resistant than all other groups based on this test ($P<0.001$). (B) Cort-high fat animals had significantly higher glucose values during the entire oral glucose tolerance test ($p<0.001$). Interestingly, the cort-chow animal had lower glucose levels than all other groups at 30 and 60 minutes ($p<0.05$). (C) Cort-high fat animals had higher insulin levels at 0, 90, and 120 minutes ($P<0.001$). Both high-fat fed groups had high insulin values at 30 minutes compared to only the wax-chow group ($P<0.05$). (D) An insulin sensitivity index was calculated based on the area under the curve for both glucose and insulin during the oral glucose tolerance test, using the formula: $2/[(AUC_{glucose} \times AUC_{insulin}) +1]$. Cort-high fat animals were significantly more insulin resistance compared to all other groups, based on this calculation ($P<0.05$). Interestingly, the cort-chow animals were more insulin sensitive compared to all other groups ($P<0.05$).

C. Other Contributions

The following papers were published during the completion of this dissertation:

1. Kiraly MA, **Campbell JE**, Park E, Bates HE, Yue JT, Rao V, Matthews SG, Riddell MC, Giacca A, Vranic M. Volitional wheel running maintains euglycemia in association with decreased activations of cJUN NH2-terminal kinase and serine phosphorylation of IRS1 in the liver of Zucker diabetic fatty rats. Am J Physiol Endocrinol Metab. 2009 Dec 8. [Epub ahead of print]

2. Johnston AP, **Campbell JE**, Found JG, Riddell MC, Hawke TJ. Streptozotocin induces G2 arrest in skeletal muscle myoblasts and impairs muscle growth in vivo. Am J Physiol Cell Physiol. 2007 Mar;292(3):C1033-40.

3. Iscoe KE, **Campbell JE**, Jamnik V, Perkins BA, Riddell MC. Efficacy of continuous real-time blood glucose monitoring during and after prolonged high-intensity cycling exercise: spinning with a continuous glucose monitoring system. Diabetes Technol Ther. 2006 Dec;8(6):627-35.

4. Fediuc S, **Campbell JE**, Riddell MC. Effect of voluntary wheel running on circadian corticosterone release and on HPA axis responsiveness to restraint stress in Sprague-Dawley rats. J Appl Physiol. 2006 Jun;100(6):1867-75.

The following projects were completed during the completion of this dissertation and are currently in preparation for publication:

1. Gordon CS, Krause MP, **Campbell JE**, Cafarelli E , Hawke TJ , Riddell MC. Impaired Growth and Absolute Force Production in Skeletal Muscles of Young Pancreatectomized Rats: Effects Targeting Type II Fibres in a Model of Adolescent Type 1 Diabetes Myopathy.

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