THE CESSATION OF DAILY EXERCISE AND CALORIC RESTRICTION CAUSES RAPID ADIPOSITY REBOUND AND GLUCOSE INTOLERANCE, FINDINGS THAT ARE ABOLISHED BY MIFEPRISTONE

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ABSTRACT

We tested the efficacy of mifepristone, a glucocorticoid (GC) receptor antagonist, on limiting adiposity rebound and preserving whole-body insulin sensitivity following the cessation of daily exercise and caloric restriction (CR). We calorically restricted male Sprague-Dawley rats and provided 24hr access to voluntary running wheels for three weeks followed by locking of the wheels and reintroduction to *ad libitum* feeding either with or without mifepristone (80 mg/kg/day) for one week. Cessation of daily running and CR increased HOMA-IR, visceral adipose mass as well as glucose and insulin area under the curve during an oral glucose tolerance test versus exercising rats ($p<0.05$). These findings were prevented or attenuated by daily mifepristone treatment during the post-wheel lock period. These findings suggest that elevations in GC action following regular exercise and CR promote rapid deterioration in metabolic control in healthy organisms and that this phenomenon can be inhibited by the GC receptor antagonist mifepristone.

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LIST OF ABBREVIATIONS

6PG	6-phosphogluconolactone
11β-HSD	11β-hydroxysteroid dehydrogenase
ACTH	Adrenocorticotropic hormone
aP2	Adipocyte fatty acid-binding protein
apoE	Apolipoprotein E
ATGL	Adipose triglyceride lipase
AUC	Area under the curve
CR	Caloric restriction
CPT1	Carnitine palmitoyltransferase 1
C/EBP-β	CCAAT/Enhancer-Binding Protein-β
CD36	Cluster of differentiation 36
CRH	Corticotropin-releasing hormone
FATP1	Fatty acid transport protein 1
FPG	Fasting plasma glucose
FPI	Fasting plasma insulin
FFA	Free fatty acid
GC	Glucocorticoid
G6P	Glucose-6-phosphate
GLUT4	Glucose transporter 4
GR	Glucocorticoid receptor
H6PDH	Hexose-6-phosphate dehydrogenase
HOMA-β	Homeostasis model assessment for β-cells
HOMA-IR	Homeostasis model assessment for insulin resistance
HbA1c	Hemoglobin A1c
HDL	High-density lipoprotein
HPA	Hypothalamic-pituitary-adrenal
HSL	Hormone sensitive lipase
IR	Insulin receptor
IL	Interleukin
KO	Knockout
LPL	Lipoprotein lipase
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
MetS	Metabolic Syndrome
MGL	Monoacylglycerol lipase
MHO	Metabolically healthy obese

mtGPAT	Mitochondrial glycerol-3-phosphate acyltransferase
MONW	Metabolically obese normal weight
NCEP ATP-III	National Cholesterol Education Program Adult Treatment Panel-III
OLETF	Otsuka Long-Evans Tokushima Fatty
PPG	Post-prandial glucose
PR	Progesterone receptor
PPARγ	Peroxisome proliferator-activated receptor gamma
TG	Triacylglycerol
TNF-α	Tumor necrosis factor alpha
UCP2	Uncoupling protein 2
WL	Wheel lock

INTRODUCTION 1

Being overweight or obese are substantial risk factors for increasing mortality and morbidity due to cardiometabolic disease. Unfortunately, epidemiological studies have demonstrated that the prevalence of excess body weight has increased globally over the past 30 years (1, 2). Additionally, a paralleled rise in obesity-related pathologies including impaired fasting glucose and type 2 diabetes mellitus has occurred (3). An important contributor to the development and progression of these conditions is physical inactivity. Currently is it estimated that 31% of the world's population is not meeting physical activity recommendations (4). A sedentary lifestyle can lead to cardiovascular disease, type 2 diabetes, colon and breast cancer, dementia and depression. This clustering of diseases has been termed the 'diseasome of physical inactivity' (5), which has been directly related to 6-14% of all deaths from non-communicable diseases worldwide (6, 7). Although increased physical activity can prevent or delay such consequences and improve metabolic health, a key component to its success as an intervention is adherence. A well-documented deterioration of whole-body insulin sensitivity, glucose tolerance, and visceral fat mass rebound occurs rapidly following reduced physical activity in humans (8-13) and rodents (14-19). This strongly supports the notion that *regular* physical activity is critical in improving or maintaining health. It also provides insight into possible physiological reasons behind the difficulty that overweight and obese individuals often face when attempting to lose weight or maintain weight loss in the long term. Although these rapid detrimental repercussions of reducing physical activity have been identified, a unifying set of mechanisms underlying this

response has yet to be determined. The goal of this thesis is to explore the possibility that rapidly transitioning from a high to low level of daily physical activity sets the stage for rapid and deleterious adiposity rebound and impaired metabolic health by altering intracellular stress hormone metabolism.

LITERATURE REVIEW 2

2.1 Physical inactivity

Human beings are not designed to lead physically inactive lives. Prior to the development of agriculture and the domestication of animals, success of locating and obtaining food depended on physical activity (20). The supply of food was inconsistent (21) and ancient hunter-gatherers cycled through periods of feast and famine, which was interjected with necessary physical activity (22). In this setting, it is believed that the human genome evolved to ensure metabolic adaptations that corresponded to this cyclical environment (23). Thus, to ensure survival, triacylglycerol (TG) synthesis was maximized during feast periods to ensure optimal storage in adipose tissue for the subsequent prolonged periods of fasting. In today's society, sedentary lifestyles and easy access to calorically-dense foods have been proposed to stall the feast-famine cycle and favour an even larger and possibly unhealthy storage of fuel (22). An unhealthy accumulation of fuel storage which is not abated by subsequent physical activity or famine may precipitate the development of chronic disease (22). Two factors which are believed to play important roles in the progression of chronic disease due to physical inactivity and poor dietary habits are insulin resistance and visceral adiposity.

2.1.1 Insulin Resistance

Insulin resistance and glucose intolerance are linked to obesity and metabolic syndrome (MetS) and are hallmarks in the progression towards the development of type 2 diabetes. Because insulin resistance is an antecedent event in the progression from

metabolic impairments to overt type 2 diabetes it is often measured to assess risk and to guide management in both research and clinical settings (24). It has been suggested that the link between obesity and insulin resistance is causal (25). The rationale for this connection comes from both human and rodent studies demonstrating that loss and gain of weight correlate well with improvement and impairment of insulin sensitivity, respectively (26-28). However, the well-documented existence of metabolically healthy obese (MHO) and metabolically obese normal weight (MONW) (29, 30) individuals adds a puzzling complexity to the relationship between insulin sensitivity and body weight. As will be discussed in greater detail later, a substantial contributor to the development of insulin resistance is visceral adiposity (31).

2.1.1.1 Bed rest studies

Numerous studies have demonstrated impairments to insulin sensitivity (whole-body and skeletal muscle), after acutely lowering daily physical activity. In humans, following three to ten days of bed rest in both trained and untrained healthy subjects there is an increase in fasted blood glucose and insulin levels, homeostasis model assessment for insulin resistance (HOMA-IR), as well as insulin and glucose AUC in response to an oral or intravenous glucose tolerance test (11-13). To help understand the mechanisms behind these deleterious effects, researchers have conducted hyperinsulinemic-euglycemic clamps and reported reductions in both whole-body and leg glucose uptake following short-term bed rest (32, 33). This coincides with reports of a 16-17% reduction in glucose transporter 4 (GLUT4) content in the gastrocnemius and vastus lateralis muscles of healthy men following six to nineteen days of bed rest (34, 35). Evidence also

suggests that following acute bed rest the liver remains sensitive to insulin, as no changes are found in rate of hepatic glucose production in response to insulin stimulation compared to pre-bed rest values in the same individuals (33). Thus, the changes that occur to insulin sensitivity following acute bed rest are tissue-specific and skeletal muscle may be the most substantially affected tissue.

2.1.1.2 Reduced step count studies

Studies conducted with a less extreme reduction of physical activity have utilized reduced ambulation rather than inducing strict bed rest. Importantly, similar metabolic responses have been found. These reduced-ambulation studies in free-living adults are considered to be more representative of realistic fluctuations in daily activity, since individuals do not typically transition to chronic bed rest. Thus, data from reduced-ambulation studies may be most informative of realistic changes to human health. Although guidelines for physical activity commonly recommend ≥ 10,000 steps daily (36), the mean number of steps taken daily by non-exercising American adults is approximately 6,000 ± 1,200 (37). Appropriately, a sudden shift in daily ambulation from the upper end of the spectrum (~10,000 steps) to the lower end (~2,000 steps) is considered to be the most insightful model of simulating sedentary behaviour in humans. Acutely transitioning from ≥ 10,000 to 1,500-5,000 steps for 3 to 14 days in healthy humans results in an increased AUC for insulin and c-peptide following an oral glucose challenge (8, 9), increased post-prandial glucose (PPG) following a mixed meal (8), a 17% reduction in glucose infusion rate during a hyperinsulinemic-euglycemic clamp, as well as a reduced insulin-stimulated $pAkt^{thr308}$/total Akt ratio (10). Importantly, elevated

PPG has been recognized as a stronger risk factor for adverse cardiovascular complications and all-cause mortality than both fasting glucose and hemoglobin A1c (HbA1c) (38, 39).

2.1.1.3 Rodent wheel lock studies

Dr. Frank Booth and colleagues have pioneered the 'wheel lock' model to study the metabolic impairments that ensue following reduced daily physical activity in rodents. This model involves giving rodents access to voluntary running wheels, which they exercise on intermittently throughout the day, typically running from 2-20 kilometers per 24 hours for three to six weeks (14-19). The wheels are then locked and the transition from regular daily wheel running to sedentary behaviour is studied from a metabolic perspective. Although the daily running distances in these rodents can be extensive, it is being performed entirely voluntarily and it is argued that voluntary wheel running in rodents is relatively comparable to the upper end of the spectrum for daily ambulatory activity in humans (40). Impressively, only 53 hours into the wheel lock period after having exercised daily for three weeks, there is a (~40%) reduction in insulin-stimulated 2-deoxyglucose uptake by the epitrochlearis muscle versus rats that had their wheels locked for only 5 hours. Surprisingly, the 53 hour wheel lock group had 2-deoxyglucose uptake values that were comparable to those of rats that had never exercised. Thus, the improvement in insulin sensitivity gained from daily exercise for three weeks is lost within the first few days following the cessation of daily exercise. This reduction in glucose transport coincided with 29 to 34% reductions in insulin receptor (IR) protein, IR^{tyr} phosphorylation, Akt^{ser473} phosphorylation, as well as GLUT4 protein

content (19). Interestingly, it has been demonstrated that isolated epididymal adipocytes retain their insulin sensitivity at 29 and 53 hours following wheel lock in rats (14). Thus, similar to data from humans, insulin signalling is impaired in a tissue-specific manner shortly following a transition to reduced levels of daily physical activity. Skeletal muscle insulin sensitivity appears to be the most rapidly impaired in this model, whereas adipose tissue insulin sensitivity is not altered in the short-term. This selective insulin resistance in skeletal muscle and retained insulin sensitivity in adipose tissue, in combination with hyperglycemia and hyperinsulinemia, may underlie the skeletal muscle atrophy and adipose mass growth following cessation of regular voluntary wheel running. To date, no studies have directly measured liver insulin sensitivity using the wheel lock model. However, data from humans investigating acute physical inactivity indicate that the liver does not become insulin resistant quickly after the initiation of sedentary behaviour whereas the skeletal muscle does (33).

Ultimately, whole-body and skeletal muscle insulin sensitivity, as well as glucose tolerance, are consistently compromised following reductions to daily physical activity in both humans and rodents. This strongly indicates that fluctuations in daily physical activity significantly contribute to the day-to-day maintenance of proper glycemic control. For example, a single bout of running or cycling for 45 minutes following ten days of bed rest significantly improves and nearly restores glycemic response to baseline measurements (41). Thus, there is a substantial plasticity of insulin sensitivity and glucose tolerance in response to changes in physical activity. These and other studies demonstrate the ease with which healthy individuals can cycle through periods of glucose

tolerance and intolerance and thus be exposed to transient periods of hyperglycemia and hyperinsulinemia. Importantly, evidence from both healthy and type 2 diabetic humans suggests that extensive blood glucose oscillations can have more damaging effects than sustained hyperglycemia on endothelial cell function and generation of reactive oxygen species (42), a phenomenon known to play a significant role in the pathogenesis of type 2 diabetes and its associated complications (43).

2.1.2 Adiposity Tissue Depots: Subcutaneous vs. Visceral Fat

A large amount of subcutaneous adipose tissue has minimal to no detrimental metabolic effect and may even provide protection against chronic disease (44). However, there exists strong epidemiological evidence identifying visceral adiposity as a major risk factor for cardiovascular disease (45), insulin resistance (46, 47) and type 2 diabetes (48). Importantly, visceral fat appears to play a causative role in these metabolic abnormalities as its excision leads to dramatic improvements in hepatic and peripheral insulin sensitivity and glucose tolerance in rats (49, 50). Interestingly, transplantation of subcutaneous adipose tissue into the abdominal cavity improves insulin sensitivity, reduces body weight and total body fat and improves fasting glucose (51). These data indicate that it is not the location of visceral fat that matters most per se, but that there are distinct biological differences between visceral and subcutaneous fat depots. Indeed, there is great variance between gene expression profiles between visceral and subcutaneous depots, mostly in genes participating in glucose homeostasis, lipid metabolism, insulin action (52), and inflammatory cytokines (53). The purported mechanisms responsible for the pathophysiological effects of visceral fat growth include

increased release of free fatty acids (FFAs), cortisol and adipose tissue-derived cytokines including interleukin 1 (IL1), IL6, tumor necrosis factor alpha (TNF-α) and resistin (54, 55).

Two key lifestyle factors that regulate storage, mobilization, and metabolism of lipids are physical activity and food intake. In a twelve week randomized controlled study, both supervised aerobic exercise three times weekly and a hypocaloric diet resulted in a significant reduction in total body fat, which was accompanied by a ~20-30% reduction of visceral fat mass (56). Similar findings of substantial reductions to visceral fat mass by exercise intervention have been reported by others (57-59) and summarized in a meta-analysis more recently (60). However, one of the most challenging components of weight loss is maintenance of a reduced body mass. Following weight loss, weight regain to baseline values has been reported to occur in 50 to 97% of obese individuals within 2-5 years of the initial weight loss, and this coincided with not adhering to recommended physical activity guidelines (61, 62). Importantly, in response to physical inactivity the greatest relative increase in adipose tissue occurs within the visceral depots (63). Thus, the detrimental consequences of insufficient physical activity may be mediated or perpetuated through alterations to body fat distribution.

2.1.2.1 Adipose mass rebound

Altered lipid metabolism has been proposed to contribute to the rapid adiposity rebound that occurs following reduced physical activity (40, 62). Following two weeks of reducing daily step count from ~10,000 to ~1,500 in humans there is an ~7% increase in visceral fat and an ~3 % reduction in lean mass (9, 10). Yet, during this inactive period a

significant reduction in body mass index was also found (9). This suggests that the observed increase in visceral fat was not an indiscriminate result of being in a positive energy balance due to reduced physical activity. Rather, it suggests that a guided redistribution of energy substrates occurred, promoting the storage of excess lipids viscerally despite a mild (1.2 kg) total body weight loss (9). This data coincides with the rapid increase in visceral fat mass that also occurs following wheel lock in rodents. As early as 53 hours following three weeks of daily running there is an ~25% and ~50% increase in relative epididymal and omental fat, respectively in rats (14). Furthermore, daily voluntary running in rodents significantly lowers body fat percentage and total body fat but one week following wheel lock both indices are rapidly restored to levels found in animals that remained sedentary throughout the study (17). Importantly, this rapid visceral adipose tissue accumulation may contribute to the metabolic impairments that occur following reduced physical activity as excess visceral adiposity is well-established as a contributor to the development of cardiometabolic disease (47).

The mechanism underlying this rapid visceral fat growth remains to be determined. Considering the tissue-specific alterations to insulin sensitivity shortly following reduced physical activity whereby adipose tissue (14) and the liver (33) remain sensitive to insulin whereas skeletal muscle does not, it is plausible that in combination with hyperglycemia and hyperinsulinemia this may directly contribute to a rapid increase in adipose tissue as well as liver lipid content. Interestingly, despite a rapid up-regulation in precursors which lead to increased lipid content including fatty acid synthase, acyl-CoA carboxylase and malonyl-CoA, there is no significant increase in liver triglyceride

content one week following cessation of voluntary wheel running in Otsuka Long-Evans Tokushima Fatty (OLETF) rats (64). This coincides with data from an earlier report which found that eight weeks of treadmill running followed by two weeks of sedentary behaviour did not significantly increase liver TG content in female Sprague-Dawley rats versus rats that had remained sedentary throughout the study (65). However, an increase in liver lipid content was found in the same study following six weeks of sedentary behaviour (65). Thus, the immediacy and type of physiological response to reduced physical activity is tissue-specific whereby the skeletal muscle rapidly develops resistance to insulin, a fatty liver develops over the course of several weeks and adipose mass grows substantially within days.

2.1.2.2 Lipogenesis, adipogenesis, and lipid oxidation

An identified mechanism underlying adiposity rebound in both humans and rodents includes an increased rate of lipid synthesis. In untrained healthy humans, one month of bed rest results in a 35% increase in plasma TG concentrations following a standardized meal versus lean untrained controls (66). Additionally, a larger 2- to 4-fold increase in post-prandial whole-body lipogenesis occurs within one week following bed rest (67). In rodents, as early as 10 hours following wheel lock, there is a ~3- to 4-fold rise in epididymal TG synthesis when compared to sedentary animals, and this elevated synthesis remains until 53 hours post-WL (14). This parallels findings of a 47% increase in plasma TG as well as a ~3-fold and 48% increase in mitochondrial glycerol-3-phosphate acyltransferase (mtGPAT) expression and activity, respectively, 10 to 53 hours post-WL (15). The protein mtGPAT is crucial for the acylation of glycerol-3-phosphate

to lysophosphatidic acid and subsequent TG synthesis (68). Thus, there appears to be a rapid increase in lipid synthesis immediately following initiation of sedentary behaviour and these elevations can persist when sedentary behaviour is prolonged. Interestingly, the main contribution to this immediate (≤ 53 hours) adiposity rebound in wheel-locked rodents was mediated by an 18% increase in mean adipocyte cell volume as opposed to increased cell number (15). However, more recent studies that have tracked the wheel lock period for up to one week, reported that the rapid increase in epididymal, omental, retroperitoneal, as well as perirenal fat mass was primarily due to hyperplasia of adipocytes rather than hypertrophy (16, 18). Of particular relevance to this thesis project, the process of adipogenesis is critically regulated by glucocorticoids (69, 70) and the release of these stress hormones, or the activation of them within certain cells, may play an important role in the rapid deterioration of metabolic health and adipose tissue growth following cessation of regular exercise.

To date, most studies investigating changes to lipid oxidation following reduced physical activity have focused on either whole-body or skeletal muscle measurements. A robust 2.5-fold reduction in fat/carbohydrate oxidation ratio and a ~90% reduction in basal whole-body lipid oxidation have been found in both men and women seven days into bed rest (67). After 1 month of bed rest a significant ~30% reduction in whole-body lipid oxidation remains following a standardized meal (66, 71). This coincided with significant reductions in cluster of differentiation 36 (CD36), lipoprotein lipase (LPL), carnitine palmitoyltransferase 1 (CPT1), fatty acid transport protein 1 (FATP1) mRNA, as well as reduced CD36 protein within the vastus lateralis (66, 71). These molecular

factors contribute to either the hydrolysis or uptake of lipids and thus a reduction in their content may contribute downstream to the impaired capacity for fat oxidation in skeletal muscle that follows reduced daily physical activity. It remains to be determined if there is an alteration in lipolysis/lipid oxidation rates within adipose tissue following cessation of regular exercise. However, given the robust visceral adipose mass accretion that occurs after the end of regular exercise, it is likely that rates of lipogenesis and adipogenesis dominate over lipid breakdown.

An important shortcoming to some of these studies is that food intake following reduced physical activity is often not controlled. Kump & Booth (14) allowed *ad libitum* feeding throughout six weeks of voluntary wheel running as well as during the sedentary wheel lock period and found that exercised rodents ate more than sedentary animals throughout the protocol. Thus, it was uncertain if the increased adipose mass was due solely to a reduction in physical activity or was contributed to by excess caloric intake. To test the hypothesis that inactivity, independent of caloric intake, could induce an increase in fat pad mass, a follow-up study used two sets of wheel-locked animals: a) given *ad libitum* access to food and b) pair-fed to match sedentary controls. Importantly, pair-feeding throughout the study did not prevent the adipose mass gain. A significant 34 and 91% increase in epididymal and retroperitoneal fat, respectively, still occurred one week after wheel lock when compared to rats that were euthanized 5 hours after wheel lock (16). Thus, it appears that cessation of regular exercise directly contributes to adiposity rebound in this model.

2.2 Glucocorticoids

2.2.1 Synthesis, secretion and action

GCs are a class of steroid hormones that are endogenously secreted in both a stress-induced and circadian fashion from the adrenal cortex and are the end-product of the hypothalamic-pituitary-adrenal (HPA) axis. Stimulation of the HPA axis first results in the secretion of corticotropin-releasing hormone (CRH) from the paraventricular nucleus within the hypothalamus. CRH travels into the hypophysial portal system to the anterior pituitary and stimulates the release of adrenocorticotropic hormone (ACTH). Once secreted into the blood, ACTH travels to the adrenal glands and ultimately causes the synthesis and release of GCs (cortisol in humans and corticosterone in rodents). GC secretion is pulsatile and circulating concentrations are highest in the morning and lowest in the evening in diurnal mammals. In contrast, nocturnal mammals have their nadir in the morning and highest levels in the evening. GCs circulate systemically and regulate many biological functions including metabolism, development, growth and apoptosis (72). Because GCs are steroid derived hormones and thus lipophilic, they circulate predominantly bound to carrier proteins, including cortisol binding globulin and albumin (73, 74). Only unbound or 'free' GCs are able to enter cells and this is completed by passively diffusing across cell membranes. Once inside a cell, active GCs bind to the glucocorticoid receptor (GR), which induces a conformational change and leads to the dissociation of accessory proteins including heat shock proteins and immunophilins (75). This GC-bound GR then translocates to the nucleus where it may interact either directly or indirectly with DNA via GC response elements or transcription factors, respectively. Once the GR binds to the targeted region of DNA it will either increase (transactivation)

or decrease (transrepression) gene transcription (76). GCs are primarily catabolic in action and as such, the genes which they activate primarily regulate hepatic gluconeogenesis, muscle proteolysis, as well as adipose tissue lipolysis (77) (Figure 1) (see a further discussion on GCs and lipolysis below). Local tissue exposure depends on circulating concentrations of GCs, the level of expression of GRs, as well as on the activity and content of the pre-receptor enzyme 11β-HSD1. This enzyme catalyzes the conversion of inactive cortisone to active cortisol in humans and is expressed in a variety of tissues including the liver, adipose tissue and skeletal muscle (78). The counterpart, 11β-HSD2, is located primarily in the distal nephron of the kidney and catalyzes the conversion of active cortisol to inactive cortisone in humans to protect against GR binding with the mineralocorticoid receptor to have aldosterone-like renal effects (78).

2.2.2 Actions on Lipolysis

Adipose tissue is the body's largest reservoir of energy and it plays a crucial role in the regulation of energy homeostasis. During periods of energy deprivation or greater energy expenditure such as caloric restriction or exercise, respectively, adipocytes favor hydrolysis rather than storage of TGs (Figure 2A). Subsequently, adipocytes release FFAs for oxidation in other tissues such as skeletal muscle, liver, and the heart for the provision of energy (79). The molecular machinery of lipolysis consists primarily of three lipases; adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL) and monoacylglycerol lipase (MGL). Both ATGL and HSL are regulated by additional interacting proteins and secondary messengers which are primarily regulated by catecholamines, natriuretic peptides and insulin (80). However, glucocorticoids (GCs)

have also been identified as notable contributors to adipose tissue lipolysis (Figure 2A). What limits the status of GCs as a major regulator of lipolysis is the inconsistency of their effects. This inconsistency has been attributed to the duration of exposure, the concentration, the experimental model used, as well as the type of GC (81, 82). Twenty-four to 48 hours of exposure to dexamethasone, cortisone, cortisol and corticosterone have all shown to increase lipolysis in mature adipocytes (83, 84). This occurs in tandem with increased mRNA expression of ATGL and total HSL as well as increased content of ATGL and greater phosphorylation of HSL on the activating sites Ser563 and Ser660 (83, 84). Additionally, the activity of protein kinase A (PKA) and cyclic adenosine monophosphate (cAMP) content are also elevated following 24 hours of exposure to dexamethasone. This occurs with a concurrent reduction in the expression of the cAMP-inhibitor phosphodiesterase 3B (PDE3B). Lastly, this group also found that dexamethasone reduced the content and increased phosphorylation of perilipins in a dose- and time-dependent manner (84). While catecholamines rapidly induce lipolysis (acting within seconds to minutes), the lipolytic action of glucocorticoids takes several hours. An increase in FFA and glycerol release has been found as early as 16 hours following dexamethasone administration in isolated rat adipocytes (84) and within 6 hours in humans infused with hydrocortisone (85, 86). Importantly, GC-induced lipolysis is abolished by co-treatment with the GR antagonist mifepristone (83, 84). Thus, the action of GCs on lipolysis is a delayed response that acts through the GR, regulates the expression and protein content of lipolytic enzymes and ultimately becomes significant only after several hours of exposure. This is supported by data showing that GCs, acting through the GR, regulate the expression of the transcription factors peroxisome

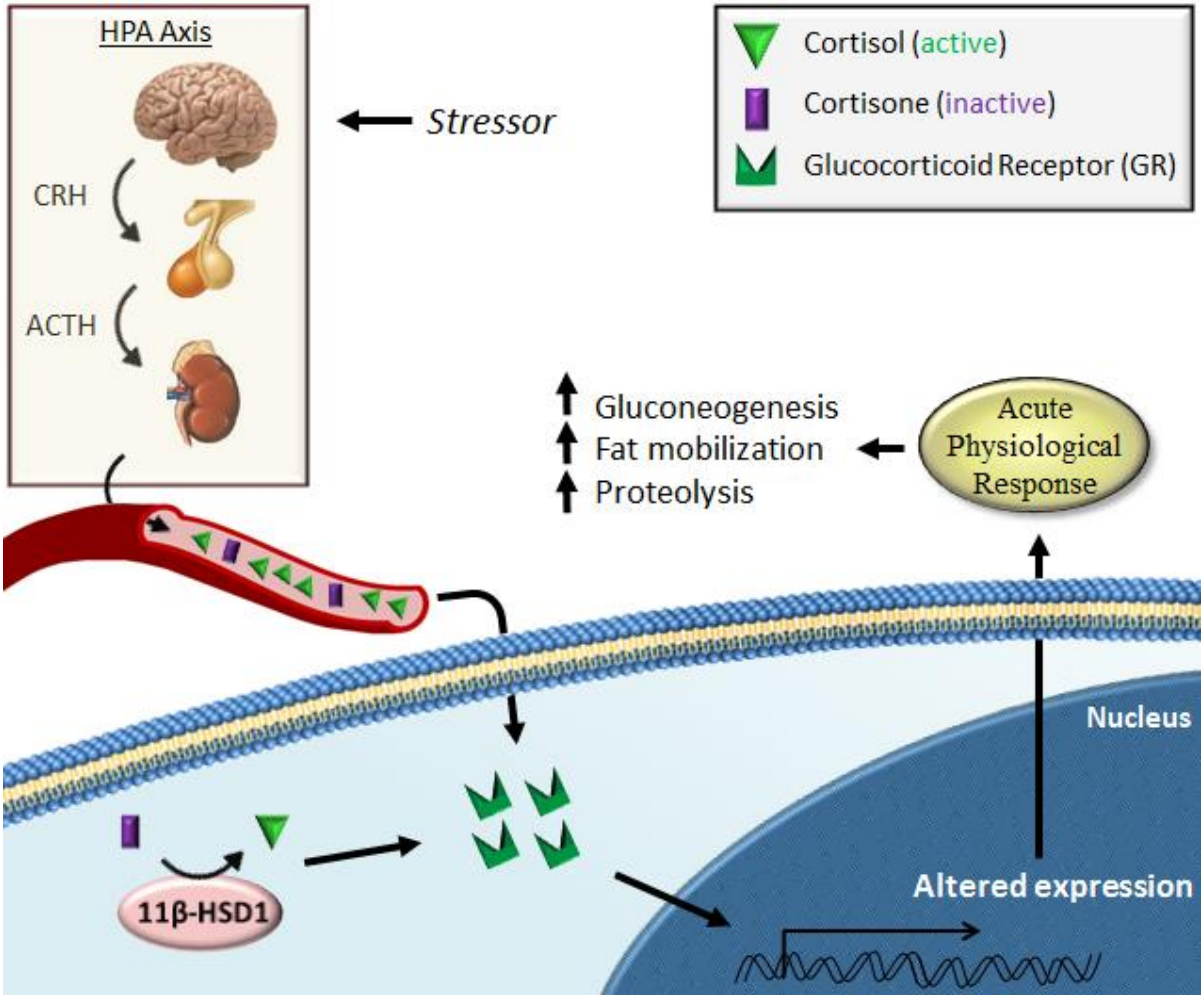

Figure 1. Glucocorticoid physiology. In response to a stressor the hypothalamus secretes CRH which leads to a cascade of ACTH from the anterior pituitary followed by GCs (cortisol in humans) secretion from the adrenal cortex. Cortisol enters cells via passive diffusion and bind to the GR. The GR may then translocate to the nucleus, bind to glucocorticoid response elements and alter the mRNA expression of its target genes. GCs induce gluconeogenesis, fatty acid mobilization and proteolysis. The ultimate effect is an elevation of blood glucose levels and metabolites which can be used for the provision of energy.

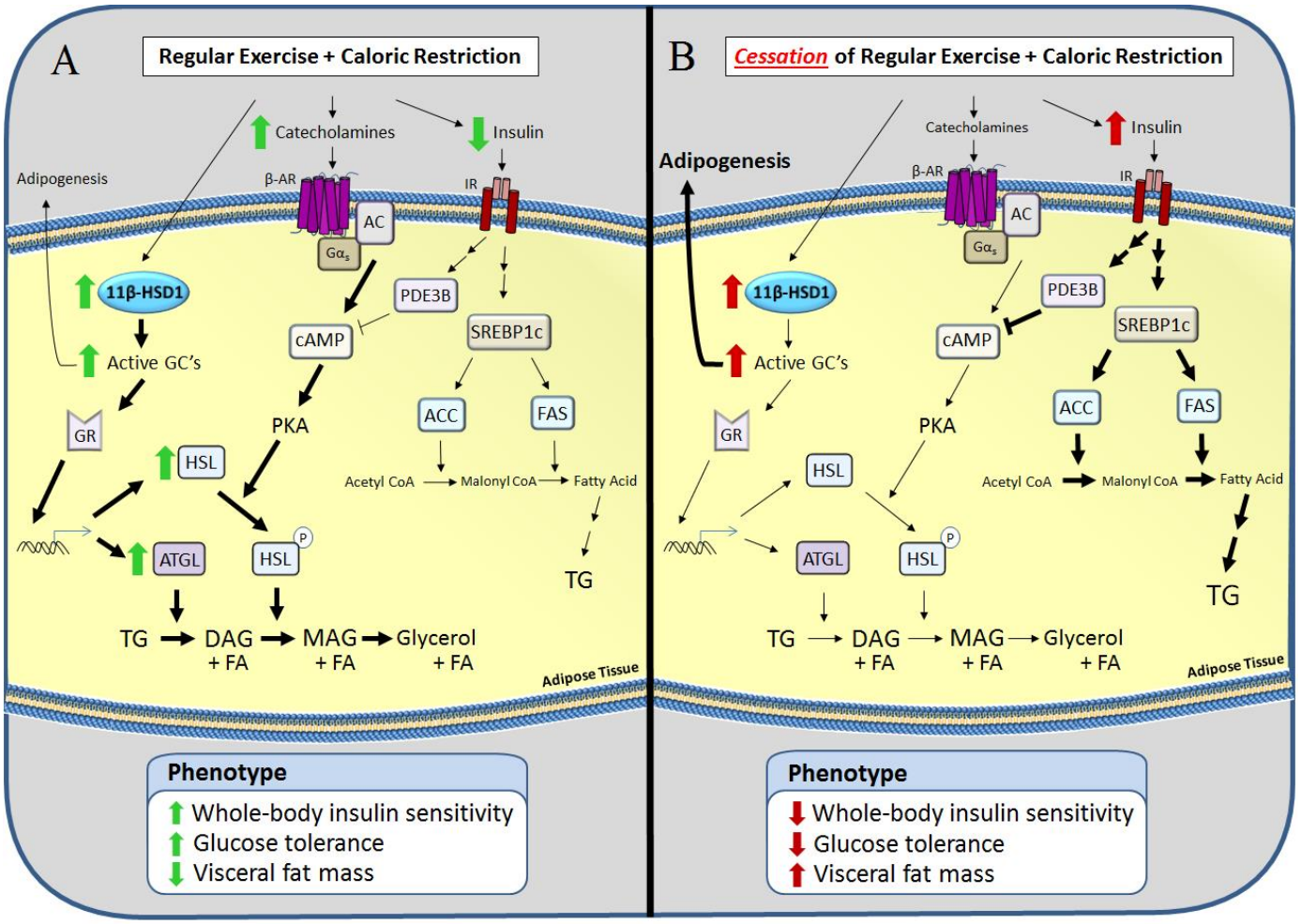

Figure 2. Glucocorticoids, exercise and caloric restriction affect lipid turnover. (A) Regular exercise and caloric restriction lead to increases in fat mobilization from adipose tissue by increasing the content and activity of glucocorticoid-inducible lipolytic proteins and limiting the antilipolytic effect of insulin. Regular exercise increases 11β-HSD1 content and thus intracellular GC exposure (B) Cessation of regular exercise and reintroduction to *ad libitum* food intake lead to a rapid increase in visceral fat mass, impaired glucose tolerance and whole-body insulin sensitivity. Rapid adipose tissue growth is believed to be due to inhibition of lipolysis and stimulating of lipogenesis which is exacerbated hyperinsulinemia due to the development of insulin resistance. Sustained elevations in GC exposure stimulate hyperplasia of visceral adipose tissue.

proliferator-activated receptor gamma 1 and 2 (PPARγ1 and PPARγ2) (86), which regulate the mRNA expression and protein content of ATGL both in vivo and in vitro (87).

Individuals with Cushing's syndrome have been documented as having a higher rate of lipolysis in adipose tissue than healthy subjects (88). A consequence of this, especially relevant under the threat of chronic exposure to GCs, is an increased efflux of FFAs from adipocytes that can both induce and exacerbate liver and whole-body insulin resistance (89, 90). Despite the clear impact that GCs can have on increasing the potential for lipolysis by increasing lipase expression, their precise roles *in vivo* remain conflicting as they have also been shown to increase adipogenesis and lipogenesis in adipose tissue. In support of this, ten days of treatment with corticosterone in Sprague-Dawley rats has been shown to significantly increase epididymal adipose mass despite an increased rate of basal lipolysis in isolated adipocytes. Interestingly, these rodents also exhibited a 60% reduction in visceral adipocyte size and a 2.5-fold increase in number of adipocytes per mg of tissue, suggesting hyperplasia as the major contributor to adipose mass growth (83). These findings are in contrast to four weeks of dexamethasone treatment in Wistar rats which resulted in increased adipocyte diameter, suggesting hypertrophy (91). The discrepancies between these studies may be due to the type of GC used, the duration of exposure, or the different rodent models. Thus, GCs are capable of concurrently inducing lipolysis and a growth in adipose tissue mass which may be contributed to by hypertrophy and/or hyperplasia.

2.2.3 Conditions/consequences of excess glucocorticoid exposure

2.2.3.1 Cushing's syndrome

First described by Harvey Cushing in the beginning of the 20th century (92), Cushing's disease is caused by overproduction of ACTH due to a tumor on the pituitary gland and it is the most common cause of Cushing's syndrome (93). Cushing's syndrome refers to a pathological excess of GCs in the body, regardless of the cause. Excess exposure to GCs, as is found in individuals with Cushing's syndrome, leads to glucose intolerance, hypertension, muscle wasting and central adiposity growth (94).

The primary goals of treating Cushing's syndrome include elimination of hypercortisolism, reversal of comorbidities, and long term disease control (93). The first-line treatment of choice is trans-sphenoidal pituitary surgery where the ACTH-secreting adenoma is removed. Second-line treatments are comprised of radiotherapy, bilateral adrenalectomy, and medical therapy. Radiotherapy is a well-established therapy used in cases of recurrent or persistent Cushing's syndrome or where surgery is contraindicated. However, the greatest weaknesses of this therapeutic strategy are the substantial risk of hypopituitarism (93) and the mean time to remission being 2-3 years (95, 96). In contrast, bilateral adrenalectomy is a permanent treatment that provides immediate control of hypercortisolism. However, this option is recommended only in patients with intolerance to medical therapy, sustained hypercortisolism despite medical therapy, or as an alternative to long-term medical therapy after failure of radiotherapy (93). Patients undergoing this procedure resultantly require life-long glucocorticoid and mineralocorticoid replacement therapy (97).

Medical therapy for Cushing's syndrome is mostly advised in patients who are either awaiting the full benefits of radiation therapy to take effect, in need of better control of their hypercortisolism prior to surgery, or have ACTH-dependent hypercortisolism where surgery is contraindicated due to unknown origin of the tumor (98). Medications includes inhibitors of steroidogenesis which act by blocking one or multiple steps in glucocorticoid synthesis, neuromodulatory agents which inhibit ACTH secretion from the pituitary gland, as well as the type II GR antagonist mifepristone (also known by the names RU486 and Korlym™) (98).

2.2.3.2 Metabolic syndrome and obesity

MetS is a cluster of disease characteristics that are associated with insulin resistance and include hypertension, dyslipidemia, hyperglycemia and abdominal obesity (99). Although various health organizations have defined MetS differently (100), it remains a useful tool in identifying individuals at a greater risk of developing both cardiovascular disease and type 2 diabetes. One of the most widely used definitions is the one from the National Cholesterol Education Program (NCEP) Adult Treatment Panel-III (ATP-III). According to this definition, MetS is diagnosed when at least three of the following criteria are met: blood pressure $\geq$130/85 mmHg, high-density lipoprotein (HDL) <1.04 mM in men and <1.29 mM in women, TG $\geq$ 1.7 mM, fasting glucose $\geq$ 6.1 mM, and a waist circumference >102 cm in men and >88 cm in women (101). One of the major drawbacks to the current MetS definitions is that they do not directly encompass visceral adiposity. Although waist circumference is a better predictor of metabolic disease than body mass index, it is unable to distinguish between visceral and

subcutaneous fat. Importantly, visceral obesity has become increasingly recognized as the most prevalent feature underlying insulin resistance and MetS (102-104). However, the mechanisms that link causality between visceral obesity and MetS are not fully understood.

A striking similarity of metabolic abnormalities exists between individuals with MetS, Cushing's syndrome (94) or those on exogenous GC treatment (105). These individuals have an increased propensity for weight gain, visceral adiposity, and type 2 diabetes. These similarities were the basis for the hypothesis that perhaps systemic hypercortisolism played a significant role in the pathogenesis of MetS and obesity. Indeed, it has been demonstrated that circulating cortisol levels are higher in both overweight individuals and those with MetS versus healthy subjects (106-110). However, others have found no correlation between either circulating or urinary free cortisol in obese individuals (111-115).

An additional component which contributes to tissue exposure to GCs, 11β-HSD1, has become a target of much research as it is capable of increasing GC activity in the periphery despite any change to circulating concentrations. This effect has been labelled 'Cushing's disease of the omentum' (116). Furthermore, recent data suggests that this enzyme may be the major regulator of tissue-specific effects of excess GC exposure. It was found that adipose-specific 11β-HSD1 knockout (KO) mice administered corticosterone in their drinking water were resistant to developing glucose intolerance, hypertension, hyperinsulinemia, hepatic steatosis, and increased adiposity (117).

2.2.3.3 11β-hydroxysteroid dehydrogenase type 1

The enzyme 11β-HSD consists of two isoforms each with distinct functions. 11β-HSD1 is a low-affinity nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reductase expressed in key metabolic tissues including adipose tissue, muscle, and liver. 11β-HSD1 is positioned within the endoplasmic reticulum membrane. It catalyzes the generation of active GCs by reducing cortisone to cortisol (in humans) in the cytosol and oxidizing NADPH to $NADP^+$ within the ER lumen (118). The cofactor NADPH is generated within the lumen by the enzyme hexose-6 phosphate dehydrogenase during the conversion of glucose-6-phosphate to 6-phosphogluconolactone (Figure 3). This process leads to an amplification of intracellular active GC exposure. In contrast, 11β-HSD2 is a high-affinity nicotinamide adenine dinucleotide (NAD^+)-dependent dehydrogenase, expressed primarily in mineralocorticoid target tissues including sweat and salivary glands, colonic epithelium, and the distal nephron, that catalyzes the reverse reaction resulting in the conversion of active GCs to their inert 11-keto acid forms, thereby reducing intracellular exposure (78).

2.2.3.3.1 Adipose tissue overexpression

Evidence has been accumulating for nearly 20 years in support of a role of 11β-HSD1 in the pathogenesis of MetS and visceral obesity in particular. An early paper investigating this topic of intracellular hypercortisolism discovered that 11β-HSD1 is more abundant in omental than subcutaneous fat (116). Additionally, 11β-HSD1 mRNA, protein, and activity are significantly greater in visceral adipose tissue from obese humans and rodents compared to lean individuals (119, 120). This is suggestive of a role of local GC

amplification in the pathogenesis of obesity and MetS. In accordance with this, selective adipose tissue overexpression of 11β-HSD1 in mice results in the development of many features of MetS including insulin resistance, glucose intolerance, dyslipidemia, hypertension and visceral obesity (121, 122). Under the control of the adipocyte fatty acid-binding protein (aP2) promoter, these mice present with a mild 2- to 3-fold increase in 11β-HSD1 activity exclusively within adipose tissue. Importantly, this increase is comparable to that found in obese humans and is thus a physiologically relevant model (123). These mice are obese and hyperphagic, have significantly increased serum TG, increased adipose lipoprotein lipase gene expression, serum TNF-α, and elevated portal vein corticosterone and FFAs (121). This portal drainage of elevated FFAs and corticosterone is believed to contribute to the development of hepatic insulin resistance (124).

2.2.3.3.2 Liver overexpression

The phenotype of mice overexpressing 11β-HSD1 exclusively within the liver diverges from that of the aP2 promoter model. This model is under the control of the human apolipoprotein E (apoE) promoter and results in a 2- to 5-fold elevated hepatic 11β-HSD1 activity. Interestingly, these mice present with milder systemic insulin resistance and remain glucose tolerant. They develop dyslipidemia, hepatic steatosis, and hypertension, but demonstrate no difference in adipose tissue mass when compared to wild type mice (125). These data suggest that elevated 11β-HSD1 hepatic expression may relate to the pathogenesis of fatty liver, hypertension, and insulin resistance, although without obesity as may occur in MONW individuals. The difference in capacity to induce

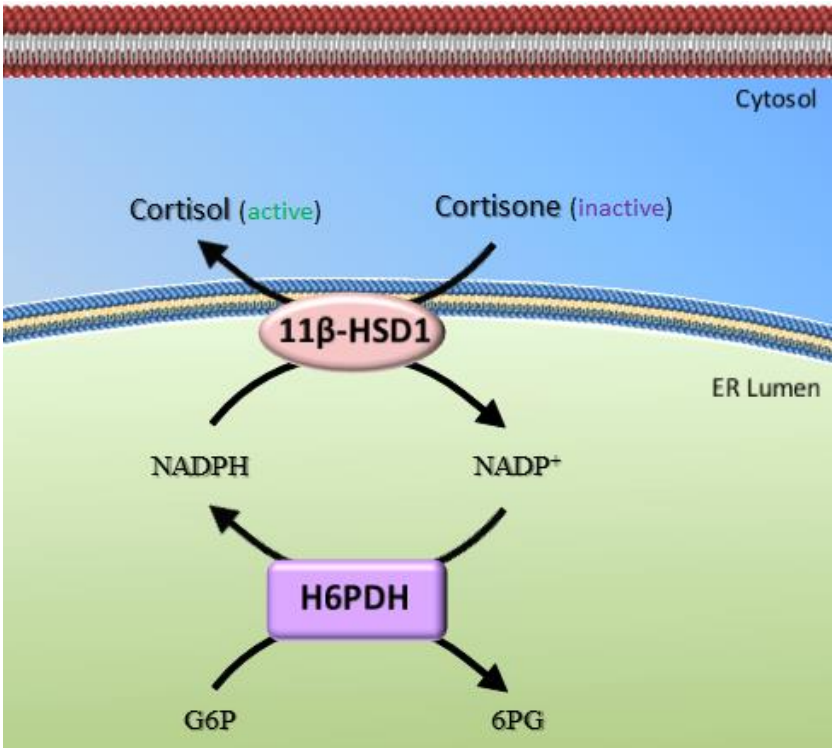

Figure 3. Generation of active GCs by 11β-HSD1. 11β-HSD1 has oxoreductase activity and catalyzes the conversion of inactive cortisone to active cortisol in humans (11-dehydrocorticosterone to corticosterone, in rodents). Generation of active cortisol occurs via the reduction of cortisone to cortisol in the cytosol and oxidization of NADPH to $NADP^+$ within the ER lumen (118). The cofactor NADPH is generated within the lumen by the enzyme hexose-6 phosphate dehydrogenase (H6PDH) during the conversion of glucose-6-phosphate (G6P) to 6-phosphogluconolactone (6PG). This process leads to an amplification of intracellular exposure to active GCs.

obesity suggests that adipose tissue exposure to GC has a greater impact on energy-homeostasis than the liver.

Although 11β-HSD1 is more abundant in intra-abdominal compared to subcutaneous depots (116) and the subsequent pathophysiological consequences are well-established, evidence suggests that increased 11β-HSD1 mRNA, protein, and activity in subcutaneous fat also has a positive correlation with BMI, fasting insulin, and visceral and total body fat (126, 127). Furthermore, 11β-HSD1 content in skeletal muscle also appears to be an important regulator of glucocorticoid-induced insulin resistance (128). Thus, there are multiple tissues that are detrimentally affected by increasing 11β-HSD1 content. However, the relative contribution towards developing MetS remains incompletely understood.

2.2.3.3.3 Knockout studies

Due to the strong connection between 11β-HSD1 activity and features of MetS, inhibition of local GC production has become a desirable therapeutic target. Over the past twenty years genetic studies investigating 11β-HSD1 knockout have found that these rodents display a reduced risk for the development of obesity and MetS. Mice with global 11β-HSD1 deletion secrete increased amounts of corticosterone and ACTH, develop adrenal hyperplasia, and upon fasting they have attenuated activity of the gluconeogenic enzymes phosphoenolpyruvate carboxykinase and glucose-6-phosphatase (129, 130). Compared to wild type mice they also present with reduced plasma TG, increased HDL, and enhanced glucose tolerance even on a chow diet (131). When placed on a high-fat diet, these knockout mice are resistant to becoming obese (132). Following eighteen

weeks of high fat feeding, 11β-HSD1 KO mice accumulate less visceral fat and do not gain as much body weight despite greater food intake. In addition, they are protected from development glucose intolerance and maintain greater insulin sensitivity Moreover, 11β-HSD1 KO resulted in elevated adipose tissue gene expression of adiponectin, PPARγ, and uncoupling protein-2 (UCP-2), as well as reduced adipose tissue gene expression of resistin and TNF-α, suggesting greater adipose tissue insulin sensitivity and energy dissipation (132). Similarly, adipose tissue-specific inactivation of GCs by overexpression of 11β-HSD2 also protects against high-fat diet-induced obesity (133). Following twenty-one weeks of high-fat feeding, these transgenic mice exhibited increased gene expression of adiponectin, PPARγ and UCP2, and reduced resistin (133). Thus, reduced intracellular exposure to active GCs by either 11β-HSD1 knockout or overexpression of 11β-HSD2 lead to and increased ratio of insulin-sensitizing to desensitizing factors. Furthermore, both 11β-HSD1 KO and 11β-HSD2 overexpression protected against the development of glucose intolerance and insulin resistance in response to a high-fat diet (132, 133).

Chronic inflammation within adipose tissue has also been identified as a common feature of metabolic disease and obesity (134). Altered FFA and adipokine release can impair insulin signaling within the liver and skeletal muscle (135, 136) and is linked to the recruitment of proinflammatory cytokines particularly within visceral fat depots (137). GCs are well-known for their potent anti-inflammatory effects (138) and thus it is plausible that elevated 11β-HSD1 would lead to increased active GCs and subsequently reduce inflammation within adipose tissue. However, mice with 11β-HSD1 KO actually

present with reduced levels of genes participating in the differentiation, proliferation and adhesion of immune cells, including T cells and macrophages (139). In fact, several inflammatory conditions including inflammatory bowel disease, colitis, and atherosclerosis have been associated with increased rather than reduced 11β-HSD1 expression (140-142). The proinflammatory cytokines TNF-α and IL1β are induced by nutrient excess and can subsequently induce 11β-HSD1 expression within adipocytes (143). Thus, inflammation may play a role in the initial augmentation of 11β-HSD1 and subsequently lead to local GC excess.

2.3 Mifepristone (RU486) and 11β-HSD1 inhibitors

Both mifepristone and 11β-HSD1 inhibitors act to block GC signaling by preventing GCs from binding to the GR or reducing the intracellular conversion to active GC's, respectively. Mifepristone is a non-selective competitive antagonist for the GR, the progesterone receptor (PR), but not the mineralocorticoid receptor (144). It is able to bind to the GR with an affinity 4-fold greater than dexamethasone and 18-fold greater than cortisol (145). Upon binding, it inhibits the subsequent transcriptional activity of the GR and thus reduces the physiological effects of GCs. One of the first published reports of the clinical efficacy of mifepristone in humans was its use in a patient with Cushing's syndrome. Over a nine week period, daily oral mifepristone administration resulted in the resolution of some consequences of their hypercortisolism including hypertension and hyperglycemia (146). Interestingly, this individual also had reduced central adiposity following treatment despite no change in body mass (146). Thus, GR antagonism appears to counteract the preferential redistribution of energy substrates being stored viscerally

due to hypercortisolism. Mifepristone also ameliorates whole-body and skeletal muscle insulin resistance caused by high-fat feeding (147) and exogenous GC administration in rats (148). These improvements are paralleled by reduced fed and fasted plasma glucose, fasted insulin, fasted FFAs, as well as resolution of glucose intolerance and ectopic fat deposition in skeletal muscle and the liver (148). More recently, mifepristone administration in mice fed a high-fat diet led to increased plasma content and mRNA expression of adiponectin from visceral fat (149). This was also paralleled by improvements in insulin sensitivity (149).

Of note, GCs upregulate the expression of 11β-HSD1 mRNA in adipocytes (150), preadipocytes (151), and the liver (152) by acting indirectly through the transcription factor CCAAT/Enhancer-Binding Protein-β (C/EBP-β) (153). Mifepristone downregulates both the mRNA expression and protein content of 11β-HSD1 in adipose tissue and the liver (148, 154). Thus, mifepristone may be considered as an indirect 11β-HSD1 inhibitor. Inhibition of 11β-HSD1 has proven to be of great benefit in rodents. The potent small molecule inhibitor, compound 544, binds to 11β-HSD1 with >100 and >450-fold selectivity versus mouse and human 11β-HSD2, respectively (140). Use of this compound improved insulin resistance and insulin, glucagon and glucose levels in mouse models of type 2 diabetes (140). It also reduced body mass, visceral fat mass and serum lipid levels in models of diet-induced obesity and atherosclerosis. Additionally, it almost completely abolished the progression of aortic plaque deposition in apoE-deficient mice, a model of atherosclerosis (140). In humans, daily administration of the 11β-HSD1 inhibitor INCB13739 for twelve weeks in patients with type 2 diabetes reduced HbA1c (-

0.6%), HOMA-IR, fasting plasma glucose, and mildly reduced body mass (155). In those patients who were hyperlipidemic at baseline, plasma triglycerides, low density lipoprotein (LDL) cholesterol and total cholesterol were all reduced (155). Currently, there are numerous 11β-HSD1 inhibitors that are in development, with some progressing to phase I and II clinical studies (138). However, excitement for pharmaceutical development as a treatment for type 2 diabetes has diminished somewhat lately due to the relatively mild improvements in primary outcomes (i.e. fasting plasma glucose, blood pressure), complexities of 11β-HSD1 physiology, and competition from other glucose-lowering agents on the market (156). There are also naturally occurring compounds that demonstrate a potent and selective capacity to inhibit 11β-HSD1. Curcumin and epigallocatechine-3-gallate (derived from green tea), both phenolic compounds, have demonstrated inhibition of rodent and human 11β-HSD1, respectively (157, 158). Daily administration of curcumin reduced fasted plasma glucose, triglycerides, total cholesterol and LDL cholesterol in obese high-fat fed rats (158). Collectively, the data from GR and 11β-HSD1 inhibition studies indicate a great capacity to reduce cardiometabolic risk, symptoms of type 2 diabetes, Cushing's syndrome, and MetS.

2.4 Physical activity, 11β-HSD1 and glucocorticoids

GC exposure in response to physical activity appears to be both tissue- and temporally-specific. A significant reduction in liver GR protein content occurs following seven weeks of either treadmill or swim training in rats (159, 160). Skeletal muscle demonstrates a reduced content in both GR as well as 11β-HSD1 in response to four weeks of voluntary wheel running (161). Interestingly, it appears that following two

weeks of exercise there is an initial increase in adrenal sensitivity to ACTH, resulting in significantly more circulating GCs in response to an ACTH challenge. Yet, after eight weeks of regular exercise this effect is attenuated and a similar response is found to that of sedentary rats (162). Thus, with long-term exercise there is a trend of reduced tissue sensitivity to GCs that may be preceded by an initial enhancement relative to sedentary subjects.

Our laboratory studies the mechanisms of the protective effects of exercise on the development of MetS and how regular exercise affects GC signaling. Previously we have demonstrated in various rodent models that daily exercise increases intracellular visceral adipose tissue exposure to GCs, as demonstrated through elevated content and activity of 11β-HSD1 and elevated GR protein (161, 163, 164). Importantly, excess exposure to GCs within adipose tissue, mediated by overexpression of 11β-HSD1, has been well established as an inducer of hyperinsulinemia, hyperglycemia and growth of visceral fat. However, the rodents from our studies remained insulin sensitive, glucose tolerant, and had reduced visceral adiposity. Thus, increased GC exposure within adipose tissue can be associated with both impaired and improved metabolic health. Booth and colleagues have demonstrated the rapid impairments in insulin sensitivity and growth of visceral adipose tissue that occurs after cessation of regular physical activity in rodents (9, 14, 16). Strikingly similar consequences are found in response to chronically elevated exposure to GCs. Thus, it is plausible that elevations in GC exposure in visceral adipose tissue, which appear to be non-detrimental in association with regular exercise, may predispose

individuals to rapid adipose tissue growth, impaired insulin sensitivity, and glucose intolerance once regular exercise is stopped (Figure 2B).

2.5 Summary

In summary, reducing daily physical activity is detrimental for metabolic health, although the mechanism for this deterioration in health is not entirely clear. Numerous studies investigating transient physical inactivity (bed rest, reduced ambulatory activity, cessation of regular exercise) for various lengths of treatment (3-60 days in human studies and 5-173 hours in rodent studies) consistently demonstrate similar metabolic impairments with rapid rebound in adipose mass and a rapid deterioration in skeletal muscle insulin sensitivity. Thus, despite the specific intervention and the debated relevance of each on being insightful to human health, the reproducibility and similarity of their consequences are striking. Additionally, these metabolic impairments are believed to perhaps precipitate the development of cardiometabolic disease. GCs are capable of stimulating lipolysis, adipose tissue growth (primarily visceral), and impairing metabolic health. Currently, it remains to be determined whether GCs contribute to the susceptibility behind this rebound adiposity phenomenon, rapid impairments in glucose tolerance, and insulin sensitivity that occurs following cessation of regular physical activity.

RATIONALE AND OBJECTIVES 3

3.1 Rationale

The acute transition from high to low daily physical activity is an understudied area of research. It is well established that reducing daily physical activity results in impaired insulin sensitivity, glucose tolerance, and a rapid increase in visceral adiposity that occurs in tandem with increased rates of lipid synthesis and adipocyte hyperplasia. Strikingly similar findings are produced by excess exposure to GCs. Chronic exposure to GCs, as is found in individuals with Cushing's syndrome, result in numerous metabolic impairments including but not limited to insulin resistance, impaired glucose tolerance, hypertriglyceridemia, and pronounced visceral obesity (121, 165, 166). Furthermore, GCs facilitate the process of adipogenesis; the conversion of preadipocytes to adipocytes (69, 167, 168).

Previously our laboratory has demonstrated in various rodent models that daily exercise increases intracellular visceral adipose tissue exposure to GCs, as demonstrated through elevated content and activity of the pre-receptor enzyme 11β-HSD1 as well as through elevated GR protein (161, 163, 164). Yet, these rodents remained insulin sensitive, glucose tolerant, and had reduced visceral adiposity. Thus, increased GCs can be associated with both impaired and improved metabolic health. We believe that sustained elevations in GC exposure in visceral adipose tissue, which appear to be non-detrimental in association with regular exercise, may predispose individuals to the well-

documented rapid adiposity rebound, impaired insulin sensitivity and glucose intolerance that occur following reduced daily physical activity.

3.2 Objectives

The objectives of this thesis were to demonstrate if there is a link between GC exposure and the rapid deterioration of metabolic control that follows cessation of regular exercise. We aimed to determine if the reductions in glucose tolerance, insulin sensitivity, and rapid adipose tissue growth that occurs following the cessation of regular exercise and caloric restriction could be attenuated by using a GC receptor antagonist, mifepristone (RU486, Korlym^{TM}), after exercise and caloric restriction is stopped.

3.3 Hypotheses

We hypothesized that sustained elevations in GC exposure, which appear to be non-detrimental in association with regular exercise, may influence the rapid adiposity rebound and reduction in insulin sensitivity that occurs upon cessation of regular exercise. Furthermore, we anticipated that blocking the action of GCs upon initiation of sedentary behaviour prevents, reverses, or attenuates these detrimental obesogenic consequences.

MANUSCRIPT 4

CESSATION OF DAILY EXERCISE AND CALORIC RESTRICTION CAUSES RAPID ADIPOSITY REBOUND AND GLUCOSE INTOLERANCE IN YOUNG MALE RATS, FINDINGS THAT ARE ABOLISHED BY MIFEPRISTONE

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Tables: 1
Figures: 9

CONTRIBUTION BY THE AUTHORS

This thesis project was designed by myself, Dr. Jacqueline Beaudry and Dr. Michael Riddell. I performed all western blotting, glucose tolerance tests, blood glucose sampling, saphenous vein blood collections and assays for insulin and corticosterone. I also completed all data and statistical analysis including the creation of all of the figures and tables. Emily Dunford completed the succinate dehydrogenase staining of the tibialis anterior. Jacklyn Pivovarov assisted with the western blotting data. Dr. Jacqueline Beaudry prepared the mifepristone and vehicle for gavaging. Dr. Hazel Hunt and Dr. Joseph Belanoff provided our laboratory with mifepristone and provided input on the interpretation of data. Dr. Michael Riddell is the principal investigator and supervisor of this project.

Introduction

Overweight and obesity are substantial risk factors for increasing mortality and morbidity due to cardiometabolic disease. Unfortunately, epidemiological studies have demonstrated that the prevalence of excess body weight has increased globally over the past 30 years (1, 2). Additionally, a paralleled rise in obesity-related pathologies including impaired fasting glucose and type 2 diabetes mellitus has occurred (3). An important contributor to the development and progression of these conditions is physical inactivity. Currently is it estimated that 31% of the world's population is not meeting physical activity recommendations (4). A sedentary lifestyle can lead to cardiovascular disease, type 2 diabetes, colon and breast cancer, dementia and depression. This clustering of diseases has been termed the 'diseasome of physical inactivity' (5), which has been directly related to 6-14% of all deaths from non-communicable diseases worldwide (6, 7). Although increased physical activity can prevent or delay such consequences and improve metabolic health, a key component to its success as an intervention is adherence. A well-documented deterioration of whole-body insulin sensitivity, glucose tolerance, and visceral fat mass rebound occurs rapidly following reduced physical activity in humans (8-13) and rodents (14-19). This strongly supports the notion that *regular* physical activity is critical in improving or maintaining health. It also provides insight into possible physiological reasons behind the difficulty that overweight and obese individuals often face when attempting to lose weight or maintain weight loss in the long term. Although these rapid detrimental repercussions of reducing physical activity have been identified, a unifying set of mechanisms underlying this

response has yet to be determined. The acute transition from high to low daily physical activity is an understudied area of research. To date, it is known that reducing daily physical activity results in impaired insulin sensitivity, glucose tolerance, and a rapid increase in visceral adiposity that occurs in tandem with increased rates of lipid synthesis and adipocyte hyperplasia. Strikingly similar findings are produced by excess exposure to glucocorticoids (GCs). Chronic exposure to GCs, as is found in individuals with Cushing's syndrome, result in numerous metabolic impairments including but not limited to insulin resistance, impaired glucose tolerance, hypertriglyceridemia, and pronounced visceral obesity (121, 165, 166). Furthermore, GCs facilitate the process of adipogenesis; the conversion of preadipocytes to adipocytes (69, 167, 168).

We have previously demonstrated in various rodent models that daily exercise increases intracellular visceral adipose tissue exposure to GCs, as demonstrated through elevated content and activity of the pre-receptor enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) as well as through elevated GR protein (161, 163, 164). Yet, these daily exercised rodents remained insulin sensitive, glucose tolerant, and had reduced visceral adiposity. Thus, increased GC exposure can be associated with both impaired and improved metabolic health. We propose that sustained elevations in GC exposure in visceral adipose tissue, which appear to be non-detrimental in association with regular exercise, may predispose individuals to the rapid adiposity rebound, impaired insulin sensitivity and glucose intolerance that occur rapidly following reduced daily physical activity.

To study this phenomenon, a ‘wheel lock’ experimental design was used. This involves providing access to voluntary running wheels to rodents for several weeks followed by locking of the wheels for various periods of time. Booth and colleagues have contributed much of the existing research on this model (14-19). To date, their observations have paralleled many of the findings from bed rest and reduced ambulation studies in humans, which have documented rapidly impaired glucose tolerance, insulin sensitivity, and increased visceral fat accumulation (8-13). However, no attempt has been made to investigate how and if these impairments can be abolished during the wheel lock period. We hypothesized that by blocking exposure to GCs during the initiation of sedentary behaviour these metabolic impairments may be improved. A corollary of this investigation would be the elucidation of crucial intracellular signaling events that underlie the physical inactivity-induced impairments to metabolic health.

Methods

Ethics Statement

This study was carried out in accordance with the recommendations of the Canadian Council for Animal Care guidelines and has been approved by the York University Animal Care Committee (Protocol # 2013-6).

Rodent Treatment and Experimental Design

Thirty-six young male Sprague-Dawley rats (Charles River Laboratories, 50-75 g upon arrival) were individually housed after acclimatization to a humidity (50-60%), temperature (22-23°C), and light-controlled (12 h: 12 h light-dark cycle) room. Rats were

randomly assigned to one of four different treatment groups: sedentary, control runners, placebo post-wheel lock (post-WL), or mifepristone post-WL (n=8-10 per group). We measured *ad libitum* food intake in sedentary rats for 28 days and found a daily average intake of 30.7 ± 1.7 g during this period (Supplementary Fig. 2C). For the first three weeks, control runners, placebo post-WL, and mifepristone post-WL were given 24 h access to voluntary running wheels and were placed on a calorically-restricted diet of 15 g/day of standard rodent chow (Purina Labdiet, 5012, St. Louis, Missouri). The sedentary animals (normal cage ambulation) were placed on the same calorically-restricted diet. At the end of the third week, the placebo and mifepristone post-WL groups had their running wheels locked and were reintroduced to *ad libitum* feeding for one additional week. During this final week, the placebo and mifepristone post-WL groups received an oral gavage of either mifepristone (80 mg/kg/day) or a placebo (Vehicle). All groups were euthanized by decapitation; sedentary and control runners at the end of the third week for use as pre-wheel lock comparisons; placebo and mifepristone post-WL at the end of the fourth week.

Plasma Analysis

Plasma was isolated from blood collected via saphenous vein bleed during an oral glucose tolerance test to measure insulin (Cat #90060, Crystal Chem, Downer's Grove, Illinois) at select time points, as well as on the morning of day 0, 10, 20 and 25 at approximately 0800 h to measure basal corticosterone (Cat # 07120102, MP Biomedicals, Solon, Ohio). Mixed trunk blood was also collected at the time of animal death (decapitation). All samples were collected in potassium-EDTA coated microvette

capillary tubes (Sarstedt, Des Grandes Prairies, Montreal, Québec, Canada, Cat #16.444.100), centrifuged at 15,000 rpm for 5 minutes, aliquoted into polyethylene tubes and stored at -80°C until further analysis.

Western Blotting

Fifty micrograms of protein lysate from epididymal adipose tissue was run on a 10% (ATGL, HSL, pHSL^{ser660}, LPL and GR) or 12% (11β-HSD1) SDS-page gel and transferred to a PVDF membrane (Bio-Rad, Canada). Membranes were blocked in 5% powdered milk and Tris-buffered saline with Tween 20 (TBST) at room temperature for 1 hour. Membranes were then incubated overnight in primary antibody at 4°C (ATGL, 1:500, sc-50223, Santa Cruz Biotechnology, Dallas, TX; HSL, 1:1,000, sc-25843, Santa Cruz Biotechnology; pHSL^{ser660}, 1:1000, Cat#4126, Cell Signaling, Beverly, MA; LPL, 1:1500, ab137821, Abcam, Cambridge, MA; GR, 1:1000, sc-8992, Santa Cruz Biotechnology; 11β-HSD1, 1:1,000, Cat#10004303, Cayman Chemical Company, Ann Arbor, MI). The membranes were then washed with TBST and incubated with goat anti-mouse (1:10,000, ab6789, Abcam) or goat anti-rabbit (1:10,000, ab6721, Abcam) secondary antibodies for 1 hour at room temperature. Membranes were then washed and imaged. Images were detected on a Kodak In vivo FX Pro imager and molecular imaging software (Carestream Image MI SE, version S.0.2.3.0, Rochester, New York) was used to quantify protein content. For loading controls, α-tubulin (1:40,000, ab7291, Abcam) was used for 11β-HSD1 and β-actin (1:20,000, ab6276, Abcam) was used for ATGL, HSL, LPL and GR.

Drug Administration

Mifepristone was initially dissolved in dimethyl sulfoxide (DMSO) as a stock solution (0.2 g/mL). The mixture was then dissolved further in a vehicle composed of 0.1% Tween 80 and 0.5% hydroxypropyl methyl cellulose in double distilled water. The dilution was adjusted in order to provide a dose of 80 mg/kg body mass of mifepristone in a 2 mL/kg body mass solution. This dosing strategy, which is considered to be a high dose, was selected based on dosages used in previous rodent studies (169). Comparatively, a high dosage for humans is 25 mg/kg body mass (170). The placebo post-WL group received a solution of DMSO + vehicle (placebo) and the mifepristone post-WL group received mifepristone + vehicle. Mifepristone and placebo solutions were administered once daily via syringe and oral gavage tube (Cat #FTP-18-75, Instech, Plymouth, Pennsylvania). Importantly, mifepristone administration did not alter food intake compared to the placebo post-WL group.

Oral glucose tolerance test

All rats were fasted overnight and administered an oral glucose tolerance test (OGTT; 1.5 g/kg body mass) via gavage immediately prior to euthanization. Blood glucose was measured at t=0, 5, 15, 30, 60, 90, 120 minutes via a handheld glucometer (Bayer, Contour, New York). Blood samples to measure insulin were taken at t=0, 15, 30, 60, 120 minutes. Glucose and insulin AUC was then measured according to each individual rat's fasting values.

HOMA Analysis

Homeostasis model assessment has been described in detail previously (171). Briefly, for insulin resistance (HOMA-IR) was calculated as follows: (Fasted Glucose (mM) x Fasted Insulin (mU/L)/22.5). This calculation primarily reflects the relationship between hepatic glucose output, insulin secretion, and hepatic resistance to insulin in the basal state. Homeostasis model assessment for β-cells (HOMA-β) was calculated as follows: (20 x Fasted Insulin (mU/L)/(Fasted Glucose (mM)-3.5). This calculation indicates basal pancreatic β-cell function in response to fasted glycemia.

Statistical Analysis

All data was analyzed using an appropriate one- or two-way ANOVA with a criterion of $p<0.05$. All significant differences for ANOVA testing will be evaluated using a Tukey HSD post-hoc test (GraphPad Prism version 6.03). In each bar graph, a different letter denotes a significant difference between groups. All data are mean ± SEM.

Results

Cessation of daily running and caloric restriction reduces skeletal muscle mass

Daily running with caloric restriction for three weeks significantly increased soleus and adrenal mass in the control runners versus all other groups ($p<0.05$, Table 1). One week of sedentary behaviour and *ad libitum* food intake following the three weeks of daily exercise and caloric restriction caused a significant reduction in relative plantaris and gastrocnemius mass, sedentary versus placebo post-WL ($p<0.05$, Table 1). Daily mifepristone treatment attenuated the reductions in plantaris and gastrocnemius masses.

Cessation of daily running and caloric restriction causes rapid body mass gain and is attenuated with mifepristone treatment

All groups that ran during the first three weeks (control runners, placebo and mifepristone post-WL) had significantly lower body mass versus the sedentary group following 13 days of daily running and remained lower until wheel lock (day 21) ($p<0.05$, Fig. 2A). All groups were provided with 15 grams of standard rodent chow daily during the first three weeks of the study. However, relative daily food intake was significantly lower in the sedentary group from day 16 until wheel lock ($p<0.05$, Fig. 2B) because these animals weighed more than the other groups. In the post-WL period, the placebo post-WL group gained body mass at a faster rate versus the mifepristone post-WL group and this resulted in a significantly greater final body mass (placebo post-WL (318 ± 8.9 g), mifepristone post-WL (290 ± 6.0 g), $p<0.05$, Fig. 2A and B). A reduction in weight gain has also been found with mifepristone administration in mice fed a high-fat diet when compared to mice only receiving vehicle (169). No differences were found in running distances between the three running groups (Fig. 2C and C').

11β-HSD1 increases in visceral adipose tissue in response to daily running and is reduced by mifepristone

Three weeks of daily running increased 11β-HSD1 protein content 5-fold in epididymal fat (control runners versus sedentary, $p<0.05$, Fig. 3). 11β-HSD1 remained elevated one week following the cessation of daily exercise and caloric restriction in the placebo post-WL group versus sedentary. ($p<0.05$, Fig. 3A). Daily mifepristone administration significantly reduced 11β-HSD1 content during the post-WL period

(mifepristone post-WL versus control runners, placebo post-WL, $p<0.05$, Fig. 3). GR protein content did not differ between any groups in epididymal fat (Fig. 3B).

Mifepristone does not alter markers of lipolysis in visceral adipose tissue

Three weeks of daily running increased $pHSL^{ser660}$ and total HSL 2-fold, and ATGL 4-fold in control runners versus sedentary in epididymal fat ($p<0.05$) (Fig. 4A, B and C). One week of sedentary behaviour and *ad libitum* food intake reduced $pHSL^{ser660}$ ~7-fold, total HSL 2-fold and ATGL 4-fold versus control runners ($p<0.05$) The protein content of $pHSL^{ser660}$, total HSL and ATGL were unaltered by mifepristone administration. $pHSL^{ser660}$ was also significantly reduced in placebo and mifepristone post-WL below levels of the sedentary group ($p<0.05$). Three weeks of daily running caused a small but non-significant reduction in LPL content in epididymal fat versus the sedentary group. Cessation of daily running and caloric restriction caused a 2-fold increase in LPL content and was unaltered by mifepristone treatment ($p<0.05$) (Fig. 4D).

Cessation of daily running and caloric restriction causes glucose intolerance, insulin resistance, and is attenuated by mifepristone

One week of sedentary behaviour and *ad libitum* food intake following three weeks of daily wheel running and caloric restriction impaired glucose tolerance and raised blood glucose levels both in the fasted state (Fig. 6A) and at multiple time points during an OGTT (Fig. 5A). The cumulative area under the curve (AUC) for the glucose concentration response to oral glucose gavage significantly increased 1.8-fold in the placebo post-WL group (238.3 ± 16.3) versus control runners (133.1 ± 25.8) ($p<0.05$). This impairment in glucose tolerance was prevented in the mifepristone post-WL group

(139.2 ± 11.2) ($p<0.05$ versus placebo post-WL, Fig. 5A'). The glucose AUC of the placebo post-WL group was the same as rats that were sedentary and had *ad libitum* food intake for the entire study (sedentary ad lib, 229 ± 19.8) (Supplementary Fig. 3B), despite significantly less cumulative food intake in the placebo post-WL group (Supplementary Fig. 2). Thus, the improvements in glucose tolerance in response to daily wheel running and caloric restriction are eliminated after only one week of sedentary behaviour and reintroduction to *ad libitum* feeding.

There were greater insulin concentrations both in the fasted state (Fig. 6B) and during an OGTT (Fig. 5B) in the placebo post-WL group compared to all other groups. The placebo post-WL group (193.2 ± 33.8) demonstrated an 8-fold increase in insulin AUC versus control runners (23.4 ± 6.4) and this elevated response, suggesting insulin resistance, was attenuated in the mifepristone post-WL group (72.1 ± 9.2) ($p<0.05$ versus placebo post-WL, Fig. 5B').

Cessation of daily running and caloric restriction impairs homeostasis model assessment and is prevented by mifepristone

Homeostasis model assessment of insulin resistance (HOMA-IR) primarily reflects hepatic insulin resistance in the basal state and takes into account the fasted plasma glucose (FPG) and fasted plasma insulin (FPI) values. There was a significant increase in the placebo post-WL group for fasting glucose (6.12 ± 0.29 mM) and insulin (1.89 ± 0.28 ng/mL) concentrations versus control runners (4.4 ± 0.22 mM glucose, 0.25 ± 0.05 ng/mL insulin) ($p<0.05$; Fig. 6A, $p<0.01$; Fig. 6B). These increases were attenuated in the mifepristone post-WL group (4.94 ± 0.19 mM glucose, 0.60 ± 0.11

ng/mL insulin) and were significantly lower than in the placebo post-WL group ($p<0.05$). The placebo post-WL group (14.88 ± 2.31) had an extensive 10-fold increase in insulin resistance versus control runners (1.46 ± 0.36) and this was abolished in the mifepristone post-WL group (3.66 ± 0.77) ($p<0.01$, Fig 6D).

Homeostasis model assessment of β-cell function (HOMA-β) measures the insulin secretory function of β-cells and also takes into account FPG and FPI. There was a small but non-significant increase in relative β-cell function in the placebo post-WL group (1.59 ± 0.23) versus control Runners (0.76 ± 0.20) (Fig. 6C). These data indicate that insulin resistance in the fasted state occurs following cessation of daily running and caloric restriction, it is uncompensated by alterations in β-cell function, and it can be prevented by antagonism of GRs by mifepristone.

Rapid adiposity rebound occurs following cessation of regular caloric restriction and exercise and is attenuated by mifepristone

Three weeks of voluntary running significantly reduced relative fat mass in perirenal (0.196 ± 0.02 g), epididymal (0.86 ± 0.09 g), and inguinal (0.30 ± 0.06 g) depots in control runners versus sedentary (perirenal; 0.42 ± 0.07 g, epididymal; 2.64 ± 0.30 g, inguinal; 1.88 ± 0.22 g) ($p<0.05$, Fig. 7A, B, and C). One week of sedentary behaviour and *ad libitum* feeding following three weeks of daily running and caloric restriction resulted in a significant rebound in relative adiposity in perirenal (0.91 ± 0.04 g), epididymal (4.48 ± 0.17 g), and inguinal (3.28 ± 0.32 g) depots in the placebo post-WL group versus control runners ($p<0.05$, Fig. 7A, B, and C). This relative adiposity rebound phenomenon was significantly attenuated in the perirenal (0.64 ± 0.07 g) and

inguinal (2.11 ± 0.20 g) depots in the mifepristone post-WL group versus placebo post-WL ($p<0.05$, Fig. 7A and C). Mifepristone also attenuated relative epididymal fat mass in placebo versus mifepristone post-WL ($p=0.07$). This suggests that GC signaling in the post-WL period may contribute to increasing adipose tissue mass and this can be reduced by a GR antagonist. The combination of daily running plus caloric restriction versus only caloric restriction for three weeks did not result in any differences in relative adiposity following the one week sedentary *ad libitum* period (Supplementary Fig. 4). Thus, the addition of exercise did not predispose rats to further adiposity rebound versus caloric restriction alone.

Basal corticosterone is increased by exercise and caloric restriction

Corticosterone measurements were made on day 0 before the rats were divided into respective groups and a basal AM value of 117.3 ± 28.3 ng/mL was found for all of the rats (Fig. 8). Ten days into the protocol there was a significant increase in AM corticosterone concentrations in all the runner animals combined (359.6 ± 54.0 ng/mL) versus day 0 values ($p<0.05$) and a trend for an increase in the sedentary animals (288.7 ± 98.5 ng/mL). On day 20, both sedentary (390.8 ± 40.5 ng/mL) and all runners (336.3 ± 48.2 ng/mL) had significantly greater AM corticosterone concentrations versus day 0 values ($p<0.05$). Cessation of regular exercise and caloric restriction significantly reduced basal corticosterone in both the placebo post-WL (48.0 ± 25.8 ng/mL) and mifepristone post-WL group (56.4 ± 18.1 ng/mL) versus sedentary and all runners on day 20 ($p<0.05$, Fig. 8). Despite antagonism of GC receptors in the mifepristone post-WL group after

cessation of regular exercise and caloric restriction there was no difference in corticosterone versus placebo post-WL.

Oxidative capacity improves with exercise but is not altered by Mifepristone

Skeletal muscle oxidative capacity was qualitatively assessed by succinate dehydrogenase histology in the tibialis anterior (Fig. 9). Exercise increased the intensity of staining for succinate dehydrogenase indicating improved oxidative capacity and mitochondrial content. One week of sedentary behaviour and *ad libitum* feeding did not appear to result in reduced mitochondrial content as staining intensity in the placebo and mifepristone post-WL groups remained similar to the control runners.

Discussion

In the current study, we have demonstrated that one week of sedentary behaviour and *ad libitum* food intake following three weeks of daily voluntary wheel running and caloric restriction resulted in a rapid impairment in insulin sensitivity, glucose tolerance and a rapid rebound in adipose tissue growth. A major finding from this study was that daily oral administration of mifepristone, a GR antagonist, attenuated, prevented, or reversed all of the detrimental metabolic effects that were observed. Whole-body insulin sensitivity and glucose intolerance were preserved, significantly less adipose tissue was gained and body mass was lower despite no difference in food intake versus the placebo post-WL group (Fig. 2). Interestingly, this reduced quantity of adipose mass in the mifepristone treated group did not appear to be related to increased lipase expression following mifepristone treatment, but rather it was associated with a reduction in 11β-HSD1 content in visceral adipose tissue. These findings suggest that elevations in GC

action following regular exercise and caloric restriction promote rapid deterioration in metabolic control in healthy organisms and that this deterioration can be inhibited by a GC receptor antagonist.

Insulin Sensitivity

A novel finding of this study is that these rapid impairments in whole-body insulin sensitivity that occur following reduced daily physical activity and increased food intake appears to be influenced by stress hormones. One week of reduced physical activity and *ad libitum* food intake following three weeks of daily exercise and caloric restriction caused a striking impairment in glucose tolerance and whole-body insulin sensitivity. During the oral glucose tolerance test in our model, the placebo post-WL group exhibited a 1.8-fold and 8.3-fold increase in glucose AUC and insulin AUC, respectively versus control runners (Fig. 5). Additionally, fasting blood glucose was nearly 2 mM higher, fasting insulin was 7-fold higher and insulin resistance, as measured by HOMA-IR, was 7-fold higher in placebo post-WL versus control runners (Fig. 6). Daily administration of mifepristone prevented hyperglycemia and hyperinsulinemia during the oral glucose tolerance test (Fig. 5) as well as reduced fasting blood glucose and insulin versus placebo post-WL (Fig. 6). Additionally, the increase in HOMA-IR found in the placebo post-WL group was prevented in the mifepristone post-WL group. Collectively, these findings support that factors which regulate GC action, including circulating abundance of GCs, 11β-HSD1, and the GR may play a significant role in the rapid development of insulin resistance that ensues following reduced physical activity and excess caloric intake.

In particular, we found that the placebo post-WL group had a 5-fold greater abundance of 11β-HSD1 protein in epididymal fat versus mifepristone post-WL although no difference was found in GR protein content (Fig. 3). 11β-HSD1 increases intracellular GC availability via the conversion of inactive cortisone to active cortisol in humans (11-dehydrocorticosterone to corticosterone in rodents). Overexpression of 11β-HSD1 within adipose tissue increases intracellular GC abundance, promotes visceral adiposity, whole-body insulin resistance, glucose intolerance, as well as increased serum FFAs and triglycerides (121). Importantly, studies have shown that GCs and insulin stimulate both the activity and expression of 11β-HSD1 (116, 172, 173). This feed-forward phenomenon of GCs and 11β-HSD1 has been demonstrated both in vivo and in vitro (174, 175). This is an important characteristic of the regulation of 11β-HSD1 as the development of hyperinsulinemia, which we observed following reduced physical activity, could initiate a cycle of increased 11β-HSD1, further visceral intracellular hypercortisolism followed by exacerbation of insulin resistance. Furthermore, we found a 3-fold increase in circulating corticosterone throughout the first three weeks in all of the exercising rats (Fig. 8). Thus, immediately prior to the sedentary *ad* libitum period, there was systemic hypercortisolism. In combination with the hyperinsulinemia, this may have contributed to the increased abundance of 11β-HSD1 protein which was observed. This is supported by our finding that blockage of GC action with mifepristone significantly reduced 11β-HSD1 and prevented fasted hyperinsulinemia.

What remains puzzling is the function of this increased 11β-HSD1 protein content in visceral adipose tissue in wheel running rats. Despite the substantial evidence in

support of a role of elevated 11β-HSD1 in the pathogenesis of MetS and visceral obesity, we have found increased activity and content of 11β-HSD1 in visceral fat following regular exercise to be associated with improved metabolic health (161, 163, 164). Thus, increased intracellular visceral GC exposure correlates with both impaired and improved metabolic health. Whether the resulting phenotype is impaired or improved, metabolic health appears to be most closely related to the amount of daily physical activity undergone by the organism.

Both chronic exogenous and endogenous GC exposure are well known to induce insulin resistance in both humans and rodents (176-178) as well as contribute to the development of MetS (121). However, although GCs impair whole-body insulin sensitivity, this does not necessarily indicate impaired insulin action in all tissues. Previous studies by Booth and colleagues have found that impaired insulin sensitivity occurs in skeletal muscle but not adipose tissue shortly following wheel lock in rodents (14, 19). Impressively, only 53 hours post-WL after having exercised daily for three weeks, there is a significant 37% reduction in insulin-stimulated 2-deoxyglucose uptake by the epitrochlearis muscle when compared to values found in rats that had their wheels locked for only 5 hours (19). Resultantly, 53 hours after wheel lock, 2-deoxyglucose uptake was comparable to those of rats that had never exercised. This reduction in glucose transport was concurrently associated with a 29 to 34% reduction in insulin receptor (IR) protein, IR^{tyr} phosphorylation, Akt^{ser473} phosphorylation, as well as GLUT4 protein content in skeletal muscle (19). In contrast, isolated epididymal adipocytes remain insulin-sensitive at 29 and 53 hours following wheel lock in rodents (14).

Interestingly, several studies have found that both exogenous and endogenous GC exposure increase rather than impair insulin signaling in adipose tissue (179-181). For instance, despite inducing whole-body and hepatic insulin resistance, overnight intravenous hydrocortisone infusion in humans resulted in enhanced insulin action in adipose tissue (181). GCs either improving insulin sensitivity or failing to cause insulin resistance in adipocytes despite inducing whole-body insulin resistance and hyperinsulinemia could be a potential mechanism underlying the rapid growth of adipose tissue we observed due to excessive stimulation of lipogenesis. Thus, there are tissue-specific impairments at multiple levels within the insulin signaling pathway that occur shortly following initiation of physical inactivity and skeletal muscle appears to be the most detrimentally affected in terms of impaired insulin sensitivity.

Insulin sensitivity and glucose tolerance are consistently compromised following reduced daily physical activity. This strongly indicates that fluctuations in physical activity significantly contribute to the day-to-day preservation of glycemic control. Interestingly, a single bout of running or cycling for 45 minutes following ten days of bed rest significantly improves and nearly restores glycemic response to baseline measurements in humans (41). However, returning to normal ambulation without organized bouts of exercise does not restore glycemic response until two weeks after bed rest (182). This demonstrates the ease with which healthy individuals can cycle through periods of glucose tolerance and intolerance based on their current level of daily physical activity. Importantly, evidence from healthy humans suggests that extensive blood glucose oscillation can have even more damaging effects than chronic hyperglycemia on

endothelial cell function and generation of reactive oxygen species (42) and this may participate in the pathogenesis of type 2 diabetes (43).

Adiposity rebound

Not only does the quantity of visceral fat mass correlate closely with metabolic and cardiovascular complications (46, 47), but it also exhibits great plasticity in response to exercise and dietary interventions. We observed a 4- to 5-fold increase in relative adipose mass in perirenal and epididymal fat and a 10-fold increase in inguinal fat after one week of sedentary behaviour and *ad libitum* feeding following three weeks of daily wheel running and caloric restriction (Fig. 7). These data extend the findings by Booth and Thyfault (14) where as early as 53 hours following three weeks of daily running in rats there was a significant 25 and 48% increase in relative epididymal and omental fat, respectively. In another study by this group, daily voluntary running was shown to significantly lower body fat percentage and total body fat but one week following wheel lock both indices were rapidly restored to levels found in animals that had remained sedentary throughout the study (17). In our study, we found that blocking the action of GCs with mifepristone significantly attenuated this rapid adipose mass rebound phenomenon. This suggests that during the sedentary *ad libitum* period, GCs may collectively be acting in an adipogenic, lipogenic or anti-lipolytic manner either directly or indirectly to increase the storage of lipids within visceral adipose tissue. Consequently, mifepristone administration likely resulted in either less lipid production or increased lipid breakdown. In terms of lipolysis this is initially counterintuitive due to the well-established role of GCs on increasing lipolysis. Twenty-four hours to ten days of exposure to dexamethasone, cortisone, cortisol or corticosterone have all shown to

increase lipolysis in adult adipocytes (83, 84). This occurred in tandem with increased mRNA expression of ATGL and total HSL as well as increased protein content of ATGL and greater phosphorylation of HSL on the activating sites Ser563 and Ser660. In addition, this elevated rate of lipolysis was abolished by co-treatment with the GR antagonist mifepristone (83, 84). In the current study, we found a significant increase in total HSL, $pHSL^{ser660}$, and ATGL protein content in response to daily voluntary wheel running followed by a 2- to 4-fold reduction in their content after one week of sedentary behaviour and *ad libitum* feeding (Fig. 4). However, mifepristone administration did not alter their protein content versus the placebo group. Furthermore, we also found no obvious difference in relative oxidative capacity of skeletal muscle, as measured by succinate dehydrogenase histology, in placebo versus mifepristone post-WL (Fig. 9). Thus our data suggest that an increase in lipolysis within adipose tissue as well as increased oxidative capacity in skeletal muscle did not likely contribute substantially to the reduced adipose tissue rebound in the mifepristone treatment group.

Importantly, although GCs play a role in increasing lipolysis, they can also cause adipose tissue growth, particularly visceral, as well as induce insulin resistance and hyperinsulinemia. Within adipocytes, insulin stimulates the production of sterol regulatory element binding protein 1c which regulates the transcription of a variety of different lipogenic genes including fatty acid synthase, acyl-CoA carboxylase, and glycerol-3-phosphate acyltransferase (183). Insulin also potently inhibits basal and catecholamine-mediated lipolysis by activation of phosphodiesterase-3B and consequent catalysis of cAMP, reduced PKA activation and reduced stimulation of HSL. Therefore it

is plausible that the hyperinsulinemia in our study that was induced by cessation of daily wheel running and caloric restriction dominates lipid metabolism to result in excessive lipogenesis and inhibition of lipolysis. As early as 10 hours following wheel lock in rodents there is a ~3- to 4-fold rise in epididymal TG synthesis when compared to sedentary animals, an effect that remained elevated to a similar extent at 53 hours post-WL (14). Furthermore, there is a 1.5- and 3-fold increase in mtGPAT activity and expression, respectively, 10 to 53 hours post-WL (15). The protein mtGPAT is crucial for the acylation of glycerol-3-phosphate to lysophosphatidic acid and subsequent TG synthesis (68). Thus, there appears to be a rapid increase in lipid synthesis in adipose tissue immediately following initiation of sedentary behaviour and these elevations can persist when sedentary behaviour is prolonged.

This suggests that the attenuated growth of epididymal fat which occurred in the mifepristone post-WL group was not due to greater lipolysis in this depot. Due to the reduction in energy expenditure and hyperphagic food intake following cessation of daily running and reintroduction to *ad libitum* food availability in our study, it is likely that lipogenic processes dominate in adipose tissue. Future research should investigate whether mifepristone administration attenuated adipose tissue growth by limiting lipogenesis.

Importantly, GCs also stimulate adipogenesis (69, 167, 168). This is supported by data which found that despite a significant increase in adipose tissue lipolysis in rats treated for 10 days with corticosterone, they exhibited a 1.5-fold increase in epididymal adipose mass and a 2.5-fold increase in number of adipocytes per mg of tissue (83). Thus,

GCs can concurrently contribute to both the breakdown of lipids as well as the accumulation of new adipocytes. Interestingly, the main contribution to the immediate (≤ 53 hours) adiposity rebound in wheel-locked rodents is mediated by an 18% increase in mean adipocyte volume as opposed to increased cell number (15). However, more recent studies that tracked the wheel lock period for up to 1 week, reported that the rapid accrual of epididymal, omental, retroperitoneal, as well as perirenal fat was primarily due to hyperplasia of adipocytes rather than hypertrophy (16, 18). Future studies are needed to confirm if less adipose hyperplasia and TG synthesis occurs following treatment with a GR antagonist during a period of reduced physical activity and whether or not this contributes to the attenuated adipose tissue accumulation.

Conclusion

In conclusion, reducing daily physical activity in rodents rapidly promotes expansion of adipose tissue depots and a deterioration of whole-body insulin sensitivity and glucose tolerance. These findings coincide with data from human studies which have documented impaired insulin sensitivity, glucose tolerance and visceral fat expansion following reduced physical activity through either bed rest or reduced daily step count (8, 9, 11, 12). Thus, despite the specific intervention, the reproducibility and similarity of their consequences are striking. Consequently, adopting a lifestyle of intermittent periods of sedentary behaviour and fluctuating caloric intake may precipitate the development of cardiometabolic disease over time. We also demonstrate in this study, for the first time, that the actions of stress hormones during periods of reduced physical activity and increased caloric may be linked to the deterioration in metabolic health. At the very least,

many of the deleterious metabolic effects of stopping caloric restriction and exercise can be blocked or at least attenuated by the global glucocorticoid receptor antagonist mifepristone. Although the mechanisms for the benefits for mifepristone are not entirely clear, reductions in 11β-HSD1 expression in adipose tissue appear to be associated with improved metabolic health this animal model of cessation of daily exercise and caloric restriction. Further studies are needed to determine the molecular mechanisms underlying the contribution of stress hormones to this adiposity rebound phenomenon, rapidly impaired glucose tolerance and insulin sensitivity.

TABLES AND FIGURES 5

Table 1. Tissue masses

	Sedentary	Control Runners	Placebo post-WL	Mifepristone post-WL
Tibialis anterior	1.97 ± 0.05^{a}	1.95 ± 0.06^{a}	1.78 ± 0.02^{b}	1.79 ± 0.03^{b}
Plantaris	0.96 ± 0.04^{a}	0.90 ± 0.03^{ab}	0.86 ± 0.02^{b}	0.90 ± 0.02^{ab}
Soleus	0.45 ± 0.02^{a}	0.54 ± 0.02^{b}	0.45 ± 0.01^{a}	0.45 ± 0.02^{a}
Gastrocnemius	5.23 ± 0.13^{a}	4.96 ± 0.12^{ab}	4.67 ± 0.11^{b}	4.86 ± 0.11^{ab}
Liver	30.2 ± 0.36^{a}	30.7 ± 1.48^{a}	35.9 ± 1.25^{b}	37.9 ± 1.27^{b}
Adrenals	76.6 ± 3.44^{a}	102.3 ± 5.48^{b}	78.8 ± 4.32^{a}	75.3 ± 1.41^{a}

Skeletal muscle and liver masses relative to body mass (g/kg body mass); Adrenal mass relative to body mass (mg/kg body mass). Post-wheel lock (post-WL). Different letter indicates a significant difference ($p<0.05$). All data are mean ± SEM; n=8-10 for skeletal muscle and liver; n=3-4 for adrenals.

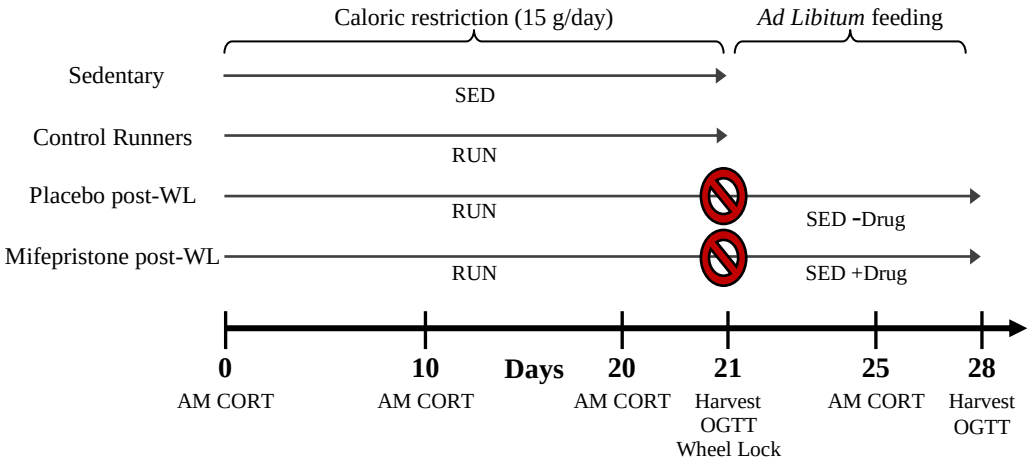


Figure 1. Experimental timeline. Sprague-Dawley rats were divided into 4 different groups; sedentary, control runners, placebo post-WL, and mifepristone post-WL. All groups were placed on a calorie restricted diet (15 g/day) and all rats except for the Sedentary group were given access to voluntary running wheels 24h/day for 21 days. Basal (AM) corticosterone (CORT) measurements were taken on days 0, 10, 20 and 25. On day 21, the placebo and mifepristone post-WL groups had their wheels locked, were reintroduced to *ad libitum* feeding, and were given daily oral gavage either with or without drug (mifepristone). The sedentary and control runner groups were harvested on day 21 and used at pre-wheel lock comparisons. The placebo and mifepristone post-WL groups were harvested on day 28, one week following cessation of regular exercise and caloric restriction. All groups had an oral glucose tolerance test (OGTT) administered on the day of harvest.

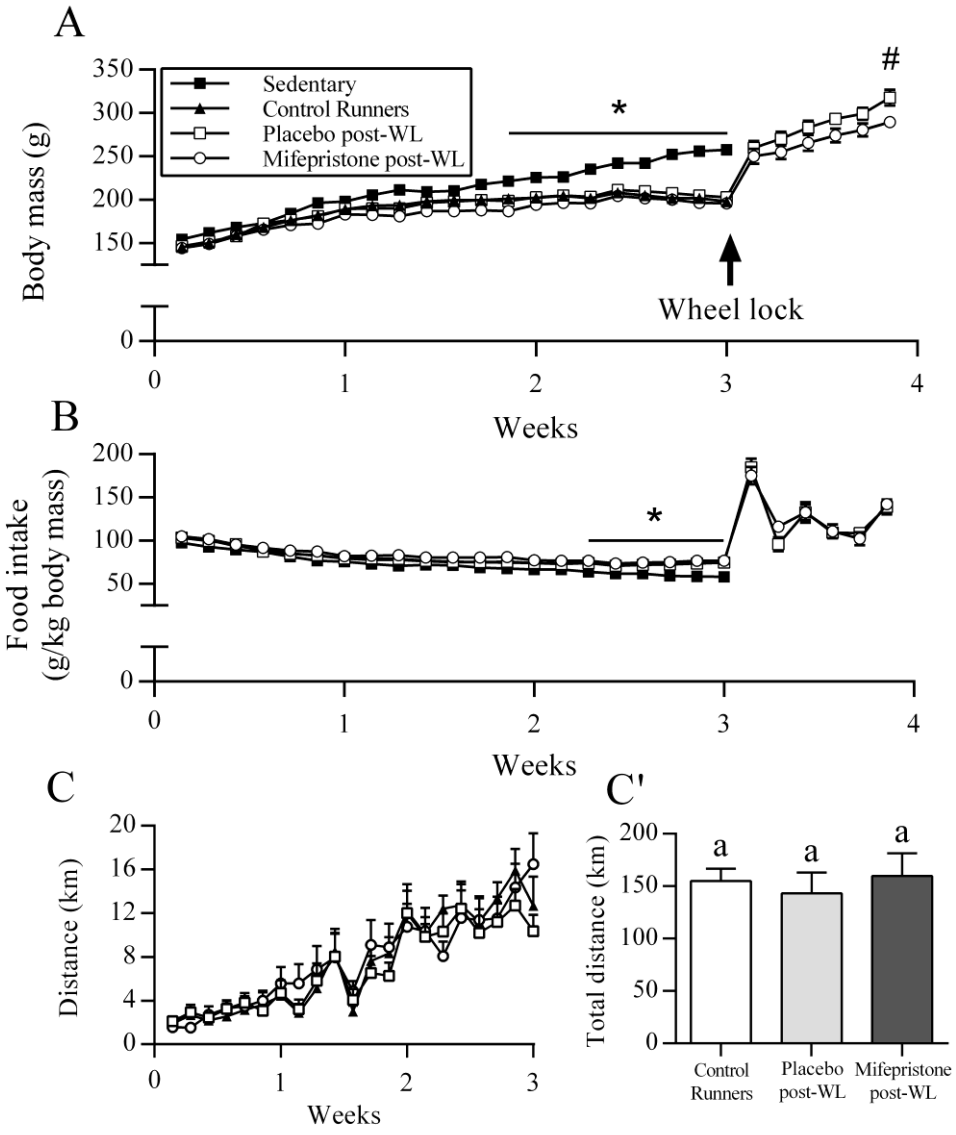


Figure 2. Body mass, food intake, and running data. (A) Body mass increases over time in all groups. All groups were calorie restricted during the first three weeks (15 g/day). At the end of the 3rd week, the placebo and mifepristone post-wheel lock (post-WL) groups had their wheels locked and were reintroduced to *ad libitum* feeding. The sedentary and control runner groups were harvested and used as pre-wheel lock comparisons. (B) Relative daily food intake during the caloric restriction (Week 1-3) and *ad libitum* (Week 4) periods. (C and C') Daily and total running distance throughout protocol in all 3 running groups. *, sedentary vs. all other groups ($p<0.05$). #, placebo post-WL vs. mifepristone post-WL ($p<0.05$). All data are mean ± SEM. n=8-10.

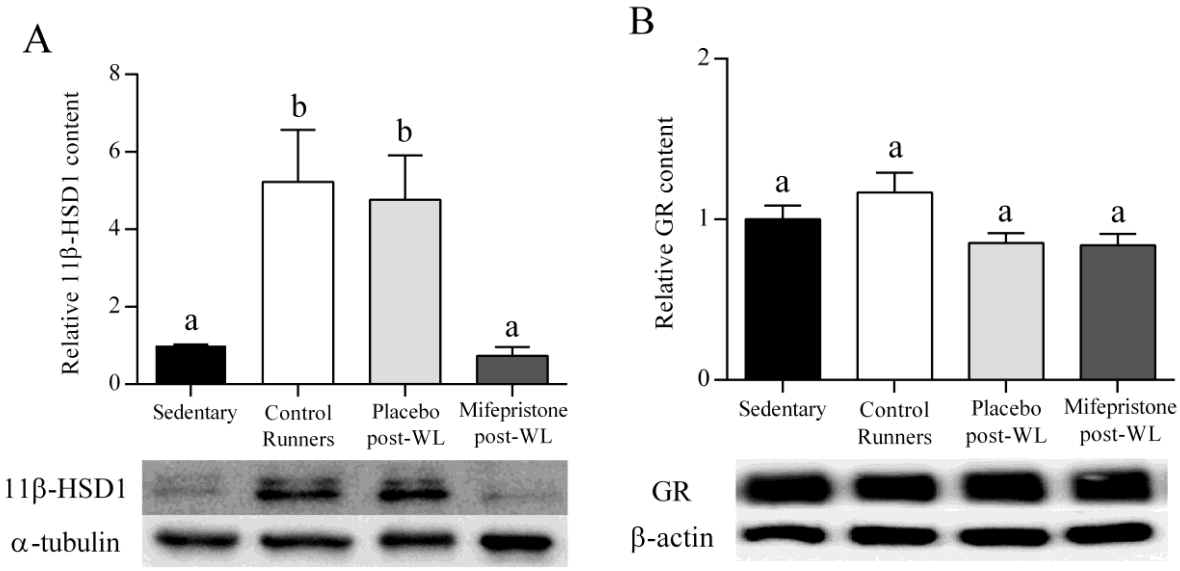


Figure 3. Visceral adipose tissue glucocorticoid exposure. Relative 11β-HSD1 and GR protein expression in epididymal fat. Post-wheel lock (post-WL). Different letter indicates a significant difference ($p<0.05$). All data are mean ± SEM. n=4-6.

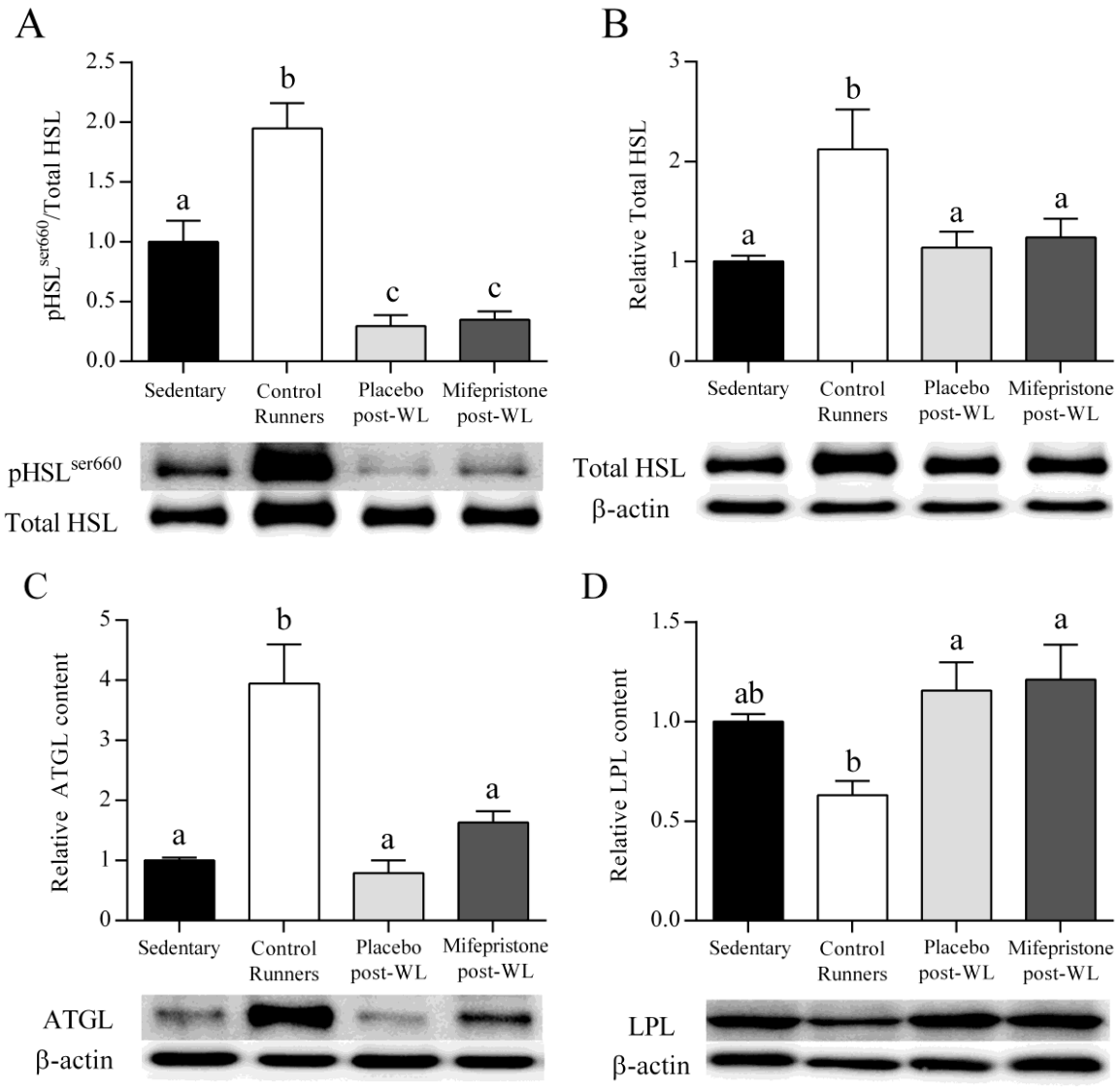


Figure 4. Visceral adipose tissue lipolytic markers. Relative pHSL^{ser660}, total HSL, ATGL and LPL protein expression in epididymal fat. Post-wheel lock (post-WL). Different letter indicates a significant difference ($p<0.05$). All data are mean ± SEM. n=5-8.

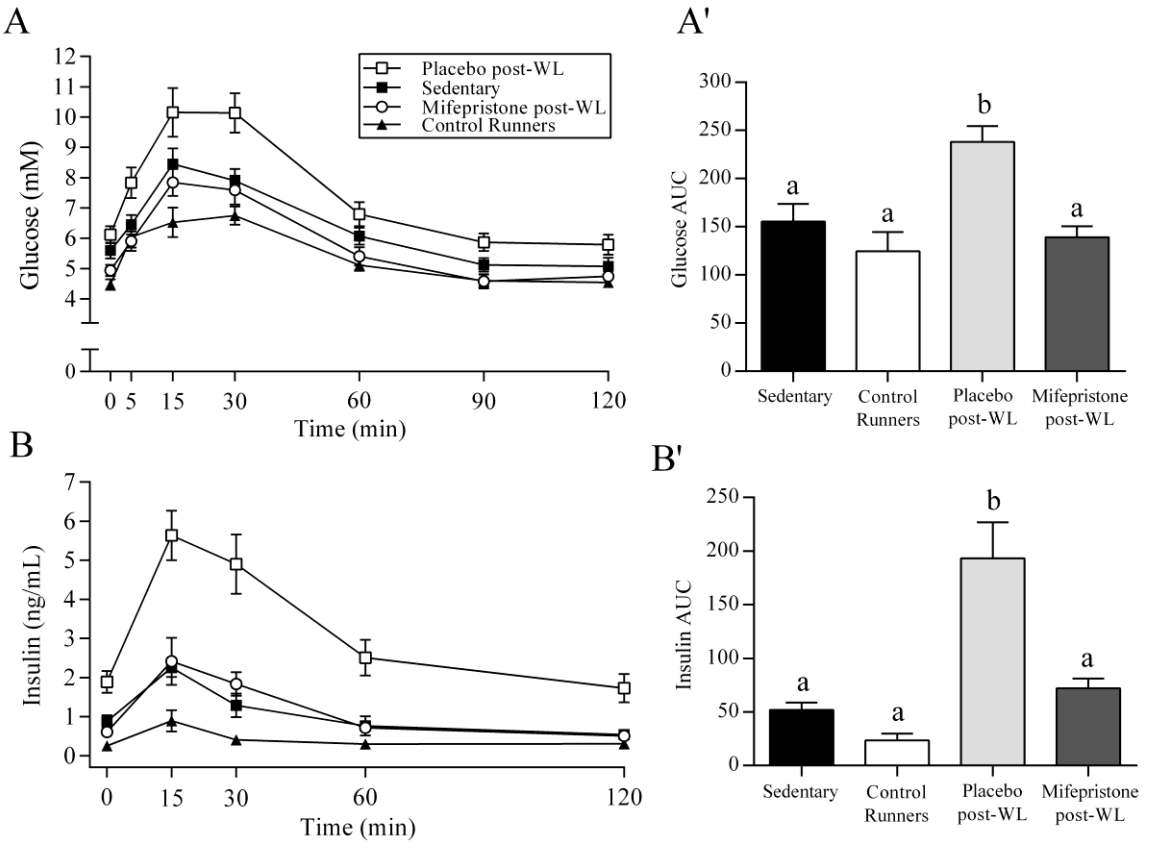


Figure 5. Glucose tolerance. All groups had an oral glucose tolerance test (OGTT) administered (1.5 g/kg body mass) at either the end of the 3rd (sedentary and control runners) or 4th week (placebo and mifepristone post-wheel lock (post-WL)) (A) Glucose concentrations (mM) in the fasted state (t=0) and at t=5, 15, 30, 60, and 120 minutes post oral glucose load. (B) Insulin concentrations (ng/mL) in the fasted state (t=0) and at t=15, 30, 60, and 120 minutes. (A' and B') Area under the curve (AUC) for glucose and insulin during the OGTT was calculated relative to the t=0 glucose or insulin value for each individual rat. Different letter indicates a significant difference ($p<0.05$). All data are mean ± SEM. n=8-10.

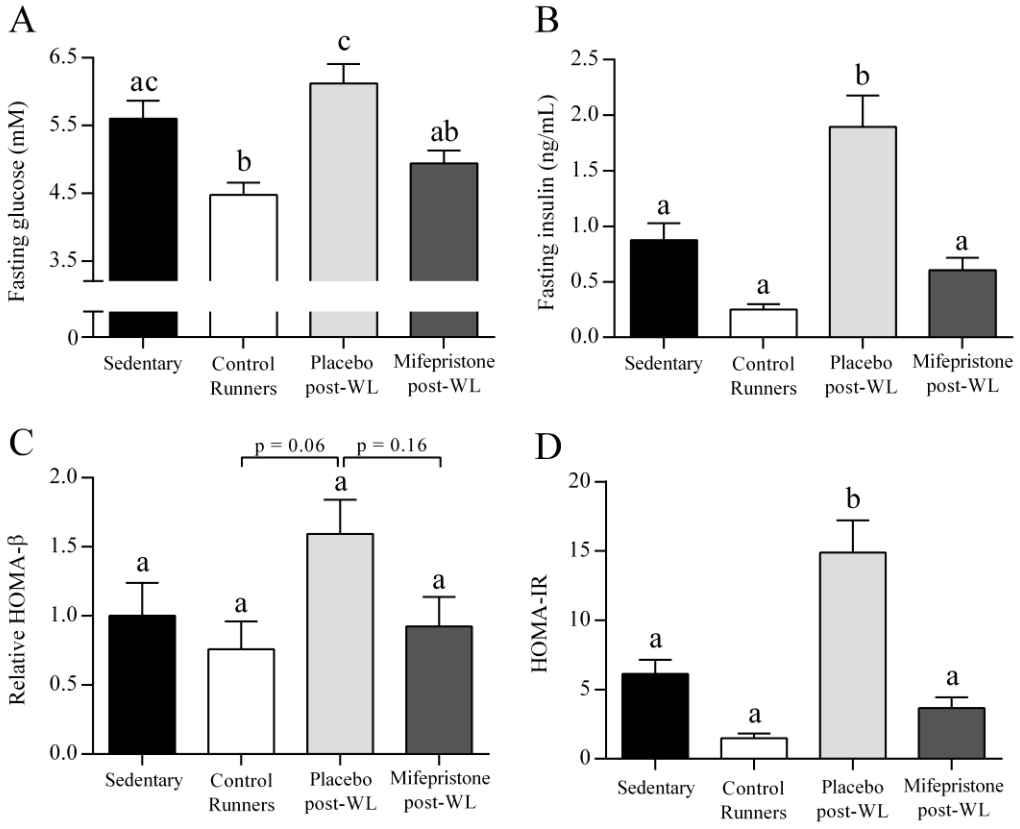


Figure 6. β-cell function and insulin resistance. (A and B) Fasted glucose and insulin values taken at the end of the 3rd (sedentary and control runners) or 4th week (placebo and mifepristone post-wheel lock (post-WL)). (C) Relative HOMA-β was calculated as follows: [20 x Insulin (mU/L)]/[Glucose (mM) – 3.5]. (D) HOMA-IR was calculated as follows: [Insulin (mU/L) x Glucose (mM)]/[22.5]. Different letter indicates a significant difference ($p<0.05$ for A, $p<0.01$ for B and D). All data are mean ± SEM. n=8-10.

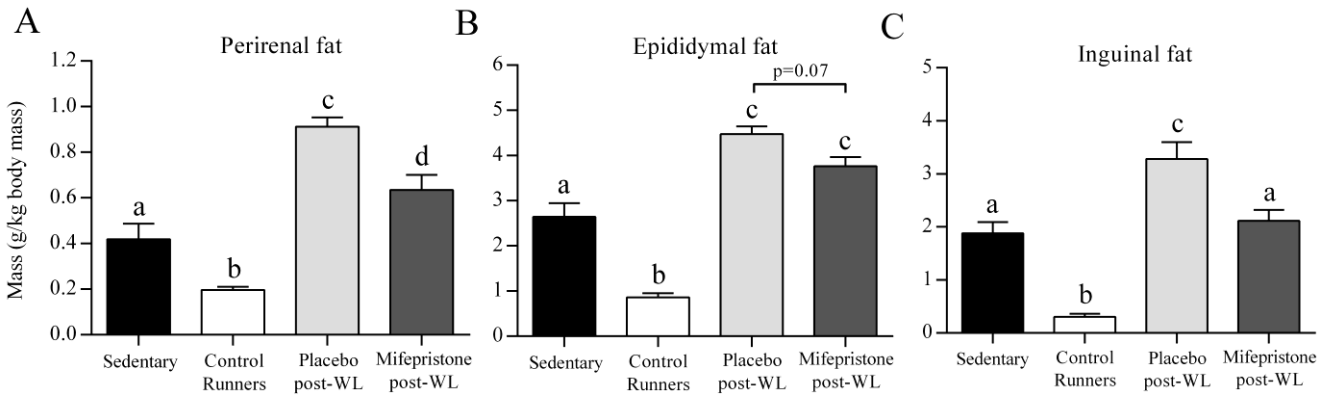


Figure 7. Adipose tissue mass. Perirenal, epididymal, and inguinal fat pads were harvested at the end of the 3rd (sedentary and control runners) or 4th week (placebo and mifepristone post-wheel lock (post-WL)). Different letter indicates a significant difference ($p<0.05$). All data are mean ± SEM. n=8-10.

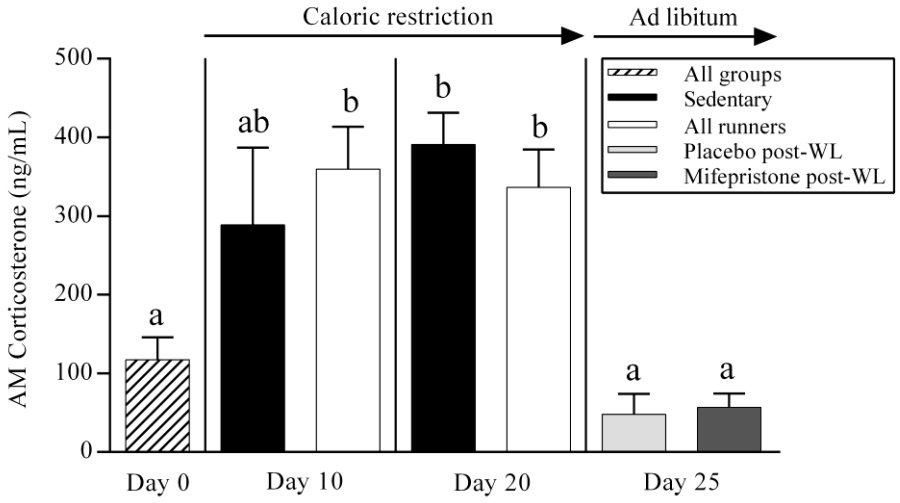


Figure 8. Basal corticosterone. AM corticosterone (0800 h) concentrations were collected on days 0, 10, 20 and 25 from the saphenous vein. Day 0 represents all rats before they were divided into separate groups. Day 10 represents the mid-way point of the caloric restriction ± daily voluntary running pre-wheel lock portion of the protocol. Day 25 represents the post-wheel lock (post-WL) period. Different letter indicates a significant difference ($p<0.05$). All data are mean ± SEM. n=3-4 for sedentary, placebo and mifepristone post-WL. n=11-15 for 'all groups' and 'all runners'.

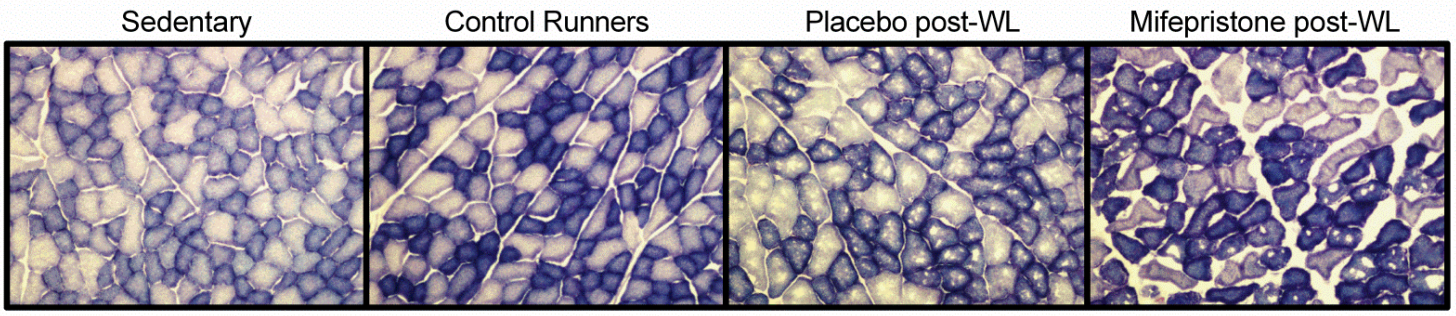


Figure 9. Oxidative capacity in skeletal muscle. Representative succinate dehydrogenase histology in the tibialis anterior.

SUMMARY AND FUTURE DIRECTIONS 6

Summary:

In this thesis project, we investigated the effects of cessation of regular voluntary wheel running and caloric restriction on the rapid development of glucose intolerance, insulin resistance and adipose tissue growth. Elevated 11β-HSD1 within adipose tissue is well-known to be associated with insulin resistance and central adiposity. In contrast, our laboratory has previously found elevations of 11β-HSD1 activity and content in visceral adipose tissue following regular exercise and this was associated with improvements in metabolic health. Based on this divergence of association of 11β-HSD1 with improved or impaired metabolic health, we hypothesized that sustained elevations in 11β-HSD1 may predispose animals to the rapid impairments in glucose tolerance, insulin sensitivity and adipose tissue growth that is found following cessation of regular exercise (Supplementary Fig. 5).

Confirming earlier studies, we found that daily voluntary wheel running increased 11β-HSD1 content in epididymal fat. Additionally, cessation of wheel running and caloric restriction caused glucose intolerance, insulin resistance and rapid adipose tissue growth. We found that daily oral administration of mifepristone, a GR antagonist, greatly attenuated the impairments found in glucose tolerance and insulin sensitivity. In addition, mifepristone reduced the amount of both adipose tissue growth and body growth that occurred following cessation of daily running and caloric restriction. This study has identified a potential role of GCs in being causative agents towards the impairment in

metabolic health that ensues after transitioning to lower amounts of daily physical activity and increased food intake.

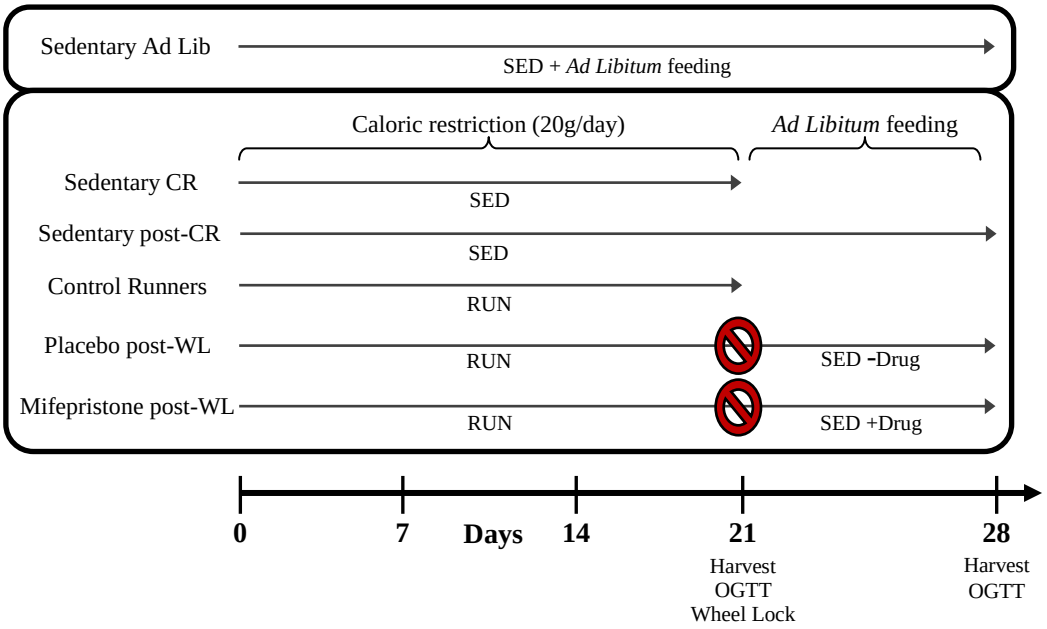
Limitations and Future Directions:

Importantly, mifepristone is a non-selective glucocorticoid receptor antagonist and it competitively binds with both the GR and PR (144). Because of this, we cannot rule out the influence of the PR in our study. Although we have identified a role of GCs in impairing metabolic health following reduced physical activity and elevated food intake, the specific mechanisms and which tissues are involved remains undetermined. In this study, we found that treatment with mifepristone caused a reduction in 11β-HSD1 within visceral adipose tissue. However, based on these findings we inferred that visceral adipose tissue in the placebo post-WL group had elevated production and concentration of GCs compared to the mifepristone group. We did not measure adipose tissue GC concentrations or release of GCs from adipose tissue into circulation. In terms of alterations in adipose tissue lipolysis in the different groups we inferred the relative content of lipolytic enzymes to correspond to relative lipolysis. To more accurately assess lipolytic rates and verify that it did not play a role in the attenuation of adipose tissue growth in the mifepristone post-WL versus placebo post-WL groups, future studies should analyze basal and/or stimulated lipolysis *ex vivo*. Additionally, considering that in the post-WL period of this study, the mifepristone and placebo groups were expending less energy and consuming greater calories per day, it may be more appropriate for future studies to investigate if mifepristone attenuated the production of new lipids via lipogenesis and/or adipogenesis. We also investigated skeletal muscle oxidative capacity

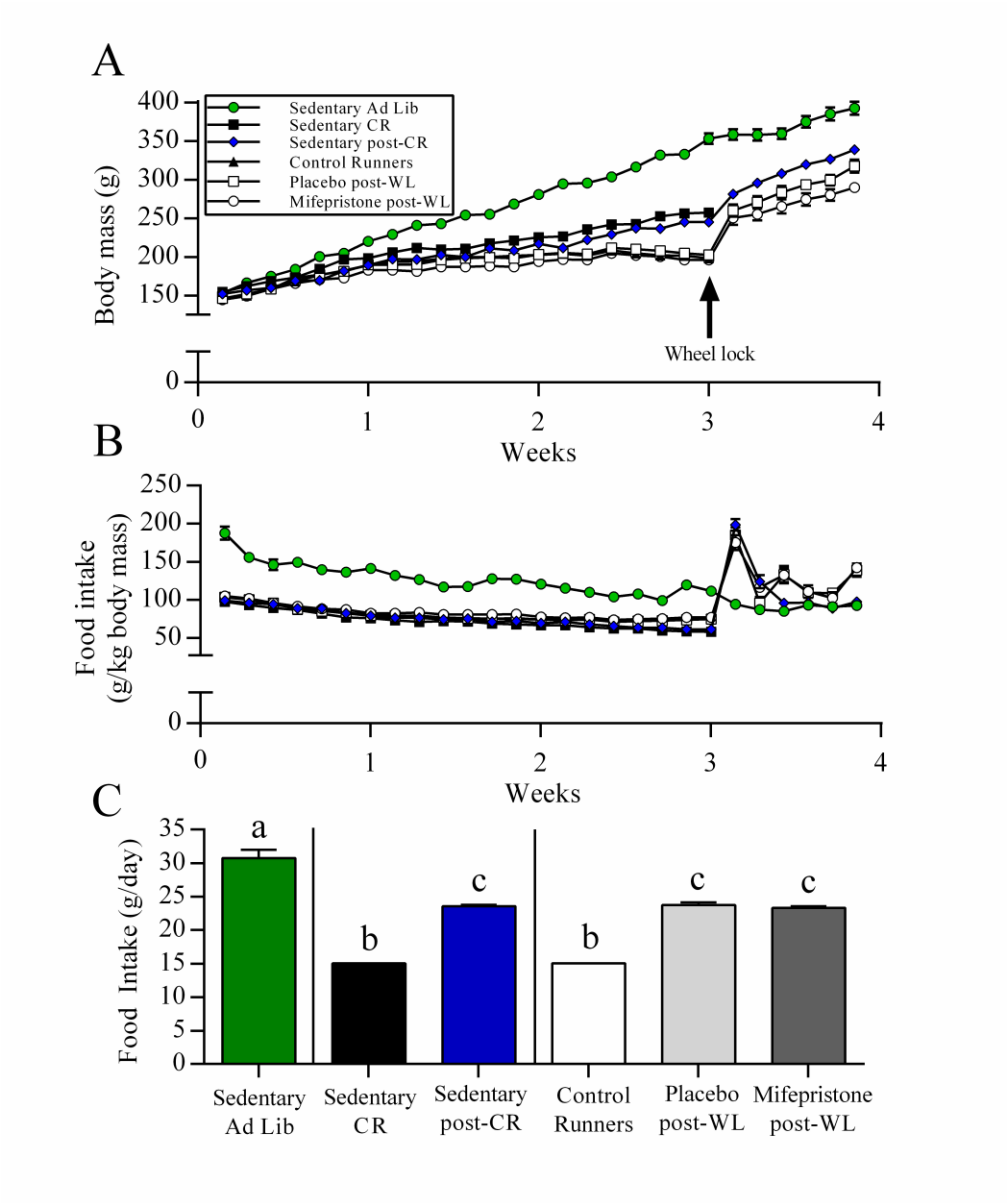
by only qualitative means. More analysis is needed to confirm if our findings of no visual differences in staining intensity between the mifepristone post-WL and placebo post-WL groups in SDH are valid. The data we have collected thus far points to 11β-HSD1 as a possible contributor to the rapid impairments in metabolic health and adipose tissue growth that occurs after transitioning from high to low levels of daily physical activity. To establish this connection, future studies should investigate if treatment with an 11β-HSD1 inhibitor also attenuates the impairments in metabolic health we observed.

APPENDIX 6

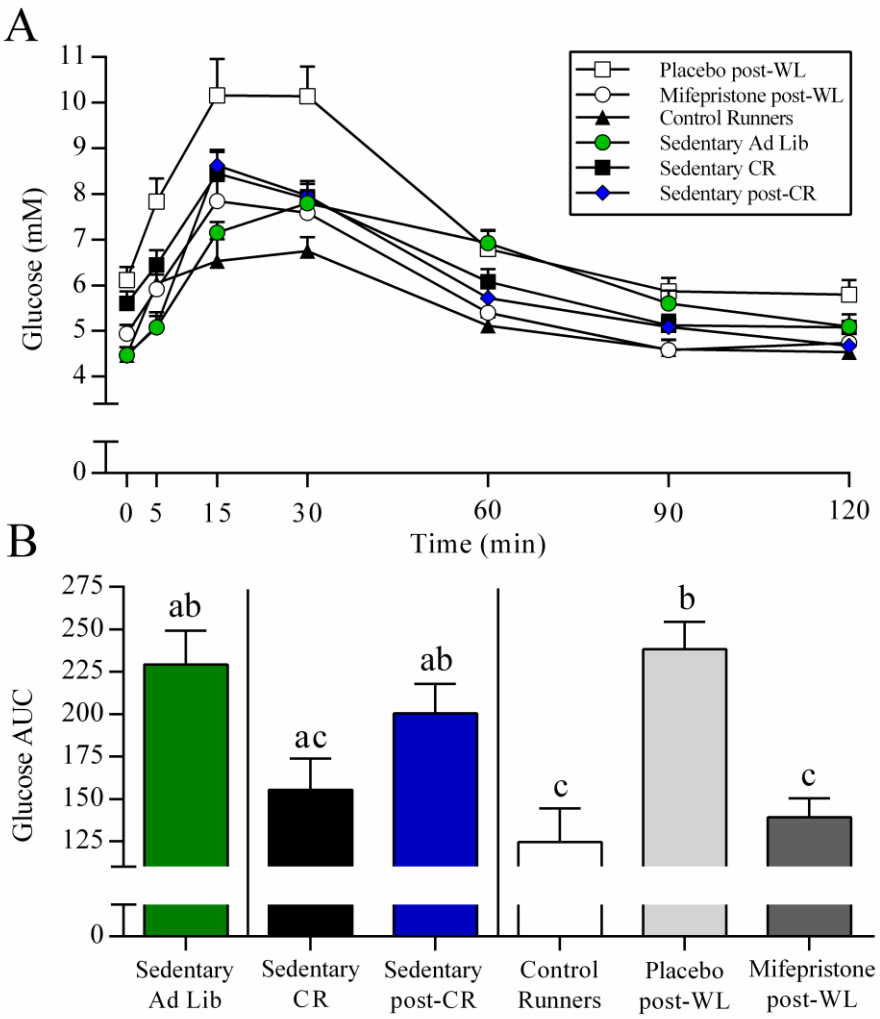
Appendix A: Supplementary Figures



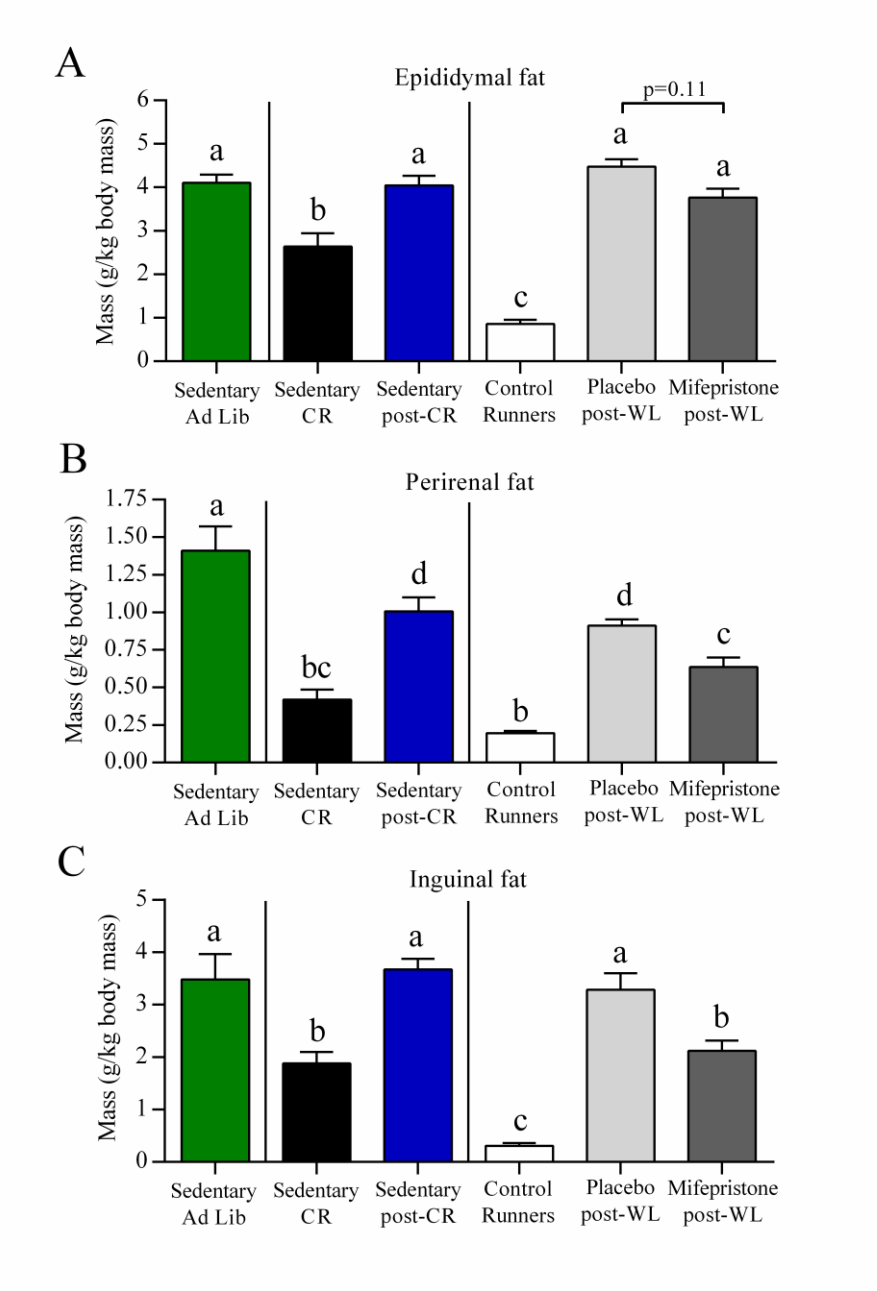
Supplementary Figure 1. Experimental timeline. Sprague-Dawley rats were divided into six different groups; sedentary ad lib, sedentary CR, sedentary post-CR, control runners, placebo post-wheel lock (post-WL), and mifepristone post-WL. All groups except sedentary ad lib were placed on a calorie restricted diet (15 g/day) with or without access to voluntary running wheels 24h/day for 21 days. On day 21, the sedentary post-CR, placebo and mifepristone post-WL groups were reintroduced to *ad libitum* feeding. Placebo and mifepristone post-WL had their wheels locked and were given daily oral gavage either with or without drug (mifepristone). The sedentary CR and control runner groups were harvested on day 21 and used as pre-wheel lock comparisons. The placebo and mifepristone post-WL groups were harvested on day 28, one week following cessation of regular exercise and caloric restriction. All groups had an oral glucose tolerance test (OGTT) administered on the day of harvest.



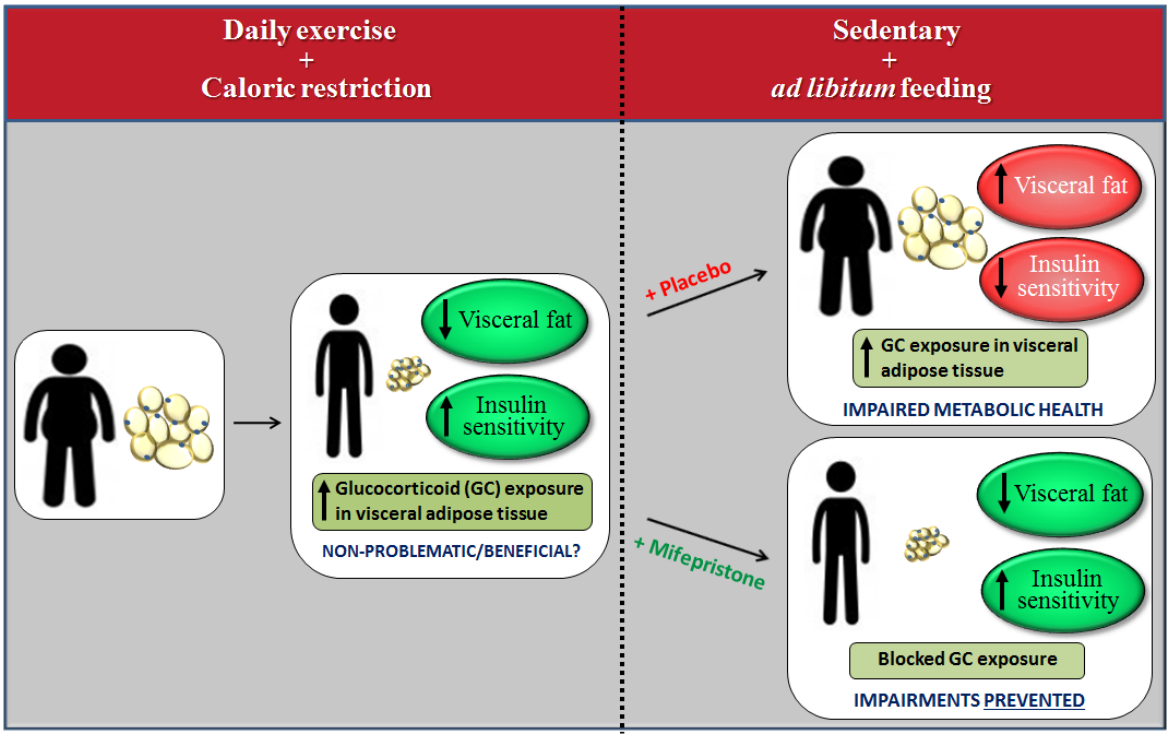
Supplementary Figure 2. Body mass, food intake, and running data. (A) Body mass throughout the study. (B) Daily fluctuations in food intake relative to body mass. (C) Average daily food intake. Post-wheel lock (post-WL). Post-caloric restriction (post-CR). Different letter indicates a significant difference ($p<0.05$) All data are mean ± SEM. n=5-10.



Supplementary Figure 3. Glucose tolerance. All groups had an oral glucose tolerance test (OGTT) administered (1.5 g/kg body mass) at either the end of the 3rd (sedentary CR and control runners) or 4th week (all other groups) (A) Glucose concentrations (mM) in the fasted state (t=0) and at t=5, 15, 30, 60, and 120 minutes post oral glucose load. (B) Area under the curve (AUC) for glucose during the OGTT was calculated relative to the t=0 glucose value for each individual rat. Post-wheel lock (post-WL). Post-caloric restriction (post-CR). Different letter indicates significant difference ($p<0.05$). All data are mean ± SEM. n=5-10.



Supplementary Figure 4. Adipose tissue mass. Perirenal, epididymal, and inguinal fat pads were harvested at the end of the 3rd (sedentary CR and control runners) or 4th week (all other groups). Post-wheel lock (post-WL). Post-caloric restriction (post-CR). Different letter indicates a significant difference ($p<0.05$). All data are mean ± SEM. n=5-10.



Supplementary Figure 5. Summary of findings. Daily exercise and caloric restriction led to reduced visceral fat, improved insulin sensitivity and increased GC exposure within epididymal fat. Cessation of regular voluntary wheel running and reintroduction to ad libitum food intake led to rapid gains in visceral fat, insulin resistance and glucose intolerance with sustained elevations of GC exposure within epididymal fat. With daily mifepristone treatment there was an attenuation of visceral fat growth, complete retention of glucose tolerance and insulin sensitivity, and GC exposure within epididymal fat was reduced.

Appendix B: Other Contributions

Journal articles published during the completion of this Master's thesis:

1. Beaudry JL, Dunford EC, **Teich T**, Zaharieva DP, Hunt H, Belanoff JK, Riddell MC. (2014) Effects of selective and non-selective glucocorticoid receptor II antagonists on rapid-onset diabetes in young rats. *PLoS One*. 9(3):e91248

2. Beaudry JL, D'souza A, **Teich T**, Tsushima R, Riddell MC. (2013) Exogenous glucocorticoids and a high-fat diet cause severe hyperglycemia and hyperinsulinemia and limit islet glucose responsiveness in young male Sprague-Dawley rats. *Endocrinology*. 154(9):3197-208

3. Karimian N, Qin T, Liang T, Osundiji M, Huang Y, **Teich T**, Riddell M, Cattral MS, Coy DH, Vranic M, Gaisano HY. (2013) Somatostatin receptor type 2 antagonism improves glucagon counter-regulation in biobreeding diabetic rats. *Diabetes*. (8):2968-77

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9. **Olsen RH, Krogh-Madsen R, Thomsen C, Booth FW, Pedersen BK.** Metabolic responses to reduced daily steps in healthy nonexercising men. *JAMA*. 299(11):1261-1263, 2008
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