THE ACUTE ROLE OF GLUCOCORTICOIDS DURING EXERCISE ON LIPID METABOLISM

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ABSTRACT

Glucocorticoids (GCs) play a key role in regulating lipid metabolism. However, various studies have found conflicting results on whether they are predominantly lipolytic or lipogenic, likely due to differences in models, exposure times and which tissues were being examined[1]. While GCs are produced endogenously in response to stress, they are also potent anti-inflammatory agents and often consumed exogenously as a treatment for inflammatory conditions[2]. For this reason, it is necessary that we have a clear understanding of their physiological role in the regulation of metabolism. Despite this, little is known about their immediate response, especially in conjunction with exercise. For this study, Wistar rats were randomly divided into four groups: sedentary + vehicle (SV), sedentary + corticosterone (SC), exercise + vehicle (EV), and exercise + corticosterone (EC). All animals received an i.p. injection of their treatment (t = -15 min) and exercisers performed a 45-minute exercise on metabolic treadmills. All tissue collection occurred at t = +45 min. SC animals had more fluctuation in their respiratory exchange ratio (RER) over time compared to SV. In exercise groups, no differences in RER were observed. Non-esterified fatty acid (NEFA) levels were not significantly different between groups after one hour and lipolytic enzyme activity analyses indicated that SC animals had enhanced enzyme activity compared to EC, but were not different from SV (p = 0.047). Analyses indicate that GCs may acutely induce lipolysis when sedentary, but not in an exercise state.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

5' AMP	5' adenosine monophosphate
11 β -HSD	11 beta-hydroxysteroid dehydrogenase
AC	Adenylyl cyclase
AFABP	Adipocyte fatty acid binding protein
ANS	Autonomic nervous system
AQP7	Aquaporin-7
α -AR	Alpha adrenergic receptor
ACTH	Adrenocorticotropic hormone
ATGL	Adipose triglyceride lipase
β-AR	Beta adrenergic receptor
BG	Blood glucose
cAMP	Cyclic adenosine monophosphate
CBG	Corticosteroid-binding protein
CGI-58	Comparative gene identification-58
CLAMS	Comprehensive Lab Animal Monitoring System
CO_2	Carbon dioxide
CS	Cushing's syndrome
DAG	Diacylglycerol
DEX	Dexamethasone
EC	Exercise corticosterone (*treatment group*)
EV	Exercise vehicle (*treatment group*)
G6Pase	Glucose 6-phosphatase
GC	Glucocorticoid
GH	Growth hormone
Gi	G-inhibitory
GR	Glucocorticoid receptor
Gs	G-stimulatory
HFD	High fat diet
HPA	Hypothalamus-pituitary-adrenal
HSL	Hormone sensitive lipase
I	Insulin
IL	Interleukin
IMCL	Intramuscular lipid content
IR	Insulin receptor
IRS	Insulin receptor substrate
LPL	Lipoprotein Lipase
MAG	Monoacylglycerol
MetS	Metabolic Syndrome
MGL	Monoacylglycerol lipase
MR	Mineralocorticoid receptor
NEFA	Non-esterified fatty acid
O_2	Oxygen
PDE-3B	Phosphodiesterase 3B
PDK-1	Phosphoinositide-dependent kinase-1

PEPCK	Phosphoenolpyruvate carboxykinase
pHSL	Phosphorylated HSL
Pi-3K	Phosphatidylinositol-3 kinase
POMC	Pro-opiomelanocortin
PKA	Protein kinase A
PKB	Protein Kinase B
PLIN	Perilipin
PP-1	Protein phosphatase-1
RER	Respiratory exchange ratio
SC	Sedentary corticosterone (*treatment group*)
SV	Sedentary vehicle (*treatment group*)
TAG	Triacylglycerol
VCO_2	Volume of carbon dioxide
VO_2	Volume of oxygen
VO_2Max	Maximum volume of oxygen uptake
WAT	White adipose tissue

1.0 INTRODUCTION

Glucocorticoids (GCs) are hormones released from the adrenal glands and one of their predominant roles is to provide fuels to be used as energy in a state of stress[3]. Their release is regulated by the hypothalamic-adrenal-pituitary (HPA) axis and rises significantly with a stress response, although, they are also released at a low concentration throughout the day in a diurnal pattern where they are highest before waking up and lowest going to bed[4]. In addition to their endogenous release, they are also potent anti-inflammatory agents and are often supplemented to treat various conditions. Since the 1950s, GCs have been a predominant treatment for inflammation, either for an acute injury, or for chronic conditions including arthritis or asthma[5]. However, the rise in their use has shed light on some of their less desirable metabolic consequences, specifically when used over a chronic period. Various rodent models have also revealed that hypercortisolemia results in adiposity and associated comorbidities, including fatty liver, hepatic steatosis, elevated fasting glycaemia and insulin resistance[6,7]. These effects are also seen in Cushing's syndrome (CS), a condition of chronic hypercortisolism, often caused by enhanced stimulation of the HPA axis[8,9]. Despite their ability to induce adiposity, GCs enhance lipolysis, the breakdown of TAGs to NEFAs and glycerol, through increasing the transcription of various lipolytic enzymes (ATGL and HSL), as observed in various cell culture and rodent models[10,11]. Most of these studies have examined the chronic adaptations, but their immediate actions have not been studied extensively *in vivo*. Interestingly, cell culture models using 3T3-L1 cells have found that a very acute exposure (1 hour) to GCs exerts the opposite effects on lipid

metabolism, impairing lipolytic actions[12]. This impairment was further increased as concentration of GCs was increased.

Aerobic exercise is also considered a pro-lipolytic trigger, and being a physiological stressor, it induces a rise in catecholamines, immediately followed by an increase in GCs. It is often assumed that during exercise, GCs are involved in increasing the mobilization of fuels, specifically glucose and non-esterified fatty acids (NEFAs)[13], yet the acute interaction between elevated GCs and aerobic exercise on metabolism has yet to be thoroughly examined. In order to gain a better understanding of the immediate actions of GC on metabolism *in vivo*, and a better understanding of the physiological lipid response with exercise, we designed a model that examines the acute (1 hour) effects of GCs on lipid metabolism in both a resting and exercising state to get a better understanding of the specific actions that would occur with a stress response or a one time treatment with glucocorticoids.

2.0 LITERATURE REVIEW

2.1 ENERGY SUBSTRATE METABOLISM

i. Overview of Energy Substrate Metabolism

Metabolism comprises all of the chemical processes within a cell, tissue or organism that are required to sustain life. These processes are tightly controlled with various regulators and feedback signals in order to maintain cellular homeostasis[14]. Additionally, cells have the ability to respond to ever-changing environments and adapt to a number of factors including lifestyle (diet, physical activity, stress), environmental (temperature, altitude), age, gender and genetic components[15–21].

Although there are many aspects to consider when studying metabolism, energy substrate metabolism focuses on utilizing fuels for energy, specifically looking at the 3 major macronutrients: carbohydrates (CHOs), lipids and proteins. It is important to note that in normal physiological conditions, proteins contribute a negligible amount to cellular energy. For this reason, this review will be predominantly focused on glucose and lipid metabolism, with special attention being placed on lipids. All tissues in the body require energy to function, but the three major tissues that influence fuel oxidation are: skeletal muscle (which has the greatest influence on fuel selection), liver, and adipose tissue. Dysregulation in any of these tissues results in significant metabolic consequences.

ii. Carbohydrate Metabolism

Due to the increasing prevalence of insulin resistance and type 2 diabetes, a lot of research has been focused on glucose metabolism and regulatory factors. Glucose metabolism is predominantly regulated through insulin signalling, which increases

glucose uptake into tissues[22]. It does this through inducing the translocation of the glucose transporter, GLUT4, to the cell membrane to allow an influx of glucose into the cell[23]. Additionally, GLUT4 translocation may occur through an insulin-independent pathway when stimulated by muscle contraction, such as with exercise[24]. Once glucose enters the cell, it may be stored as glycogen; otherwise, it may undergo glycolysis and be converted to pyruvate. From here, it may be oxidized and used as a fuel for energy, either through aerobic respiration in the mitochondria, or anaerobically in the cytosol. Unlike the other major macronutrients, the body does not have a very large storage capacity for CHOs and its metabolism is regulated in a tissue-specific manner[25]. If systemic levels are elevated, the pancreas will release insulin to facilitate glucose uptake in tissues and the liver will convert excess glucose into glycogen until it needs to be utilized again[26]. Conversely, if circulating levels are low, the pancreas will release glucagon to signal the liver to convert glycogen into glucose.

iii. Lipid Metabolism

Adipose tissue is a functional metabolic tissue that affects thermogenesis, energy storage, metabolic regulation, mitochondrial biogenesis and immune system stimulation through the release of cytokines[27,28]. It is influenced by nutrition, satiety level, neuroendocrine factors and the circadian rhythm[29,30]. There are two types of adipose tissue, brown and white. Brown adipose tissue's primary function is to convert energy into heat[27,31]. White adipose tissue (WAT) is more abundant in adults and serves as a major energy reserve in mammals[32]. WAT is maintained through a balance between two processes, lipogenesis and lipolysis. Lipogenesis is the process of fatty acid synthesis in which triacylglycerol (TAG) is stored to be utilized later[33]. This process is favoured

4

when there is a positive energy balance, such as in a post-prandial state when insulin (a potent lipogenic agent) is high, or in a resting state when energy expenditure is low[34]. One way in which insulin influences lipogenesis is by increasing lipoprotein lipase (LPL), an enzyme involved in up-taking NEFAs into the cell to either be stored or oxidized. At the same time, insulin also inhibits lipolysis by reducing the activity of various lipolytic enzymes within the adipocytes themselves.

Lipolysis is the contrary process; it involves the hydrolysis of TAG to allow them to be mobilized and released into the blood as NEFAs[32]. In addition to glucose, NEFAs are a major fuel source that may be oxidized and used for energy via mitochondrial oxidative metabolism[34]. For this reason, lipolysis is dominant when there is a deficit in energy, such as in a fasted state or during exercise, when the body requires additional fuel.

Healthy individuals are able to balance these processes and maintain a healthy amount of body fat. However, for various reasons, metabolic deregulation sometimes occurs and may cause excessive accumulation of adipose tissue and an increases risk of metabolic aberrations and associated comorbidities[29].

iv. The Lipolytic Pathway

Lipolysis is regulated through many factors, but the greatest known moderators of the process are catecholamines[35] (Fig. 1.1). Circulating catecholamines, epinephrine (Epi) and norepinephrine (NE), bind to the β-adrenergic receptors (β-ARs) of adipocytes; this leads to the activation of adenylyl cyclase (AC) through interacting with the G-stimulatory (Gs) coupled protein[36]. AC increases cyclic adenosine monophosphate (cAMP) production, which leads to protein kinase A (PKA) activation[36]. PKA has two

roles in promoting the lipolytic pathway, while simultaneously blocking the anti-lipolytic pathway. PKA activation inhibits insulin action by blocking phosphatidylinositol-3 kinase (PI-3K) activity, which ultimately inhibits the anti-lipolytic effects of insulin to reduce intracellular cAMP content[37]. In addition, PKA activation has two modes of action to directly promote lipolysis; it triggers the phosphorylation of both perilipin (PLINs) and hormone sensitive lipase (HSL)[38]. PLINs are a family of enzymes that suppress lipolysis in a basal state, yet facilitate PKA stimulated lipolysis[39,40]. PLINs are structural proteins that coat the lipid droplet and once phosphorylated, assist in translocating phosphorylated HSL from the cytosol to lipid droplets, where phosphorylated HSL can facilitate lipolysis[41]. Additionally, it has been suggested that PLIN may be involved in positive feedback of PKA to increase the activation and further stimulate lipolysis[40]. In addition to assisting in the translocation of phosphorylated HSL (pHSL), the phosphorylation of perilipin also frees comparative gene identification 58 (CGI-58), a protein bound to perilipin. Once free, CGI-58 is able to activate adipose triglyceride lipase (ATGL) on the lipid droplet. ATGL, HSL, as well as monoacylglycerol lipase (MGL), are the primary lipases responsible for the breakdown of TAG to NEFAs in the lipid droplet (Fig. 1.2). The first step in the process involves ATGL, the rate-limiting enzyme, which converts TAG to diacylglycerol (DAG), releasing one NEFA in the process. From here, phosphorylated HSL converts DAG to monoacylglycerol (MAG), releasing an additional NEFA. Lastly, MGL cleaves MAG to be broken down into glycerol and one last NEFA[32]. The NEFAs and glycerol are then returned to the cytosol and are released back into circulation. Adipocyte fatty acid

binding protein (AFABP) shuttles the NEFAs out of the adipocyte and Aquaporin-7

(AQP7) facilitates the release of glycerol into circulation.
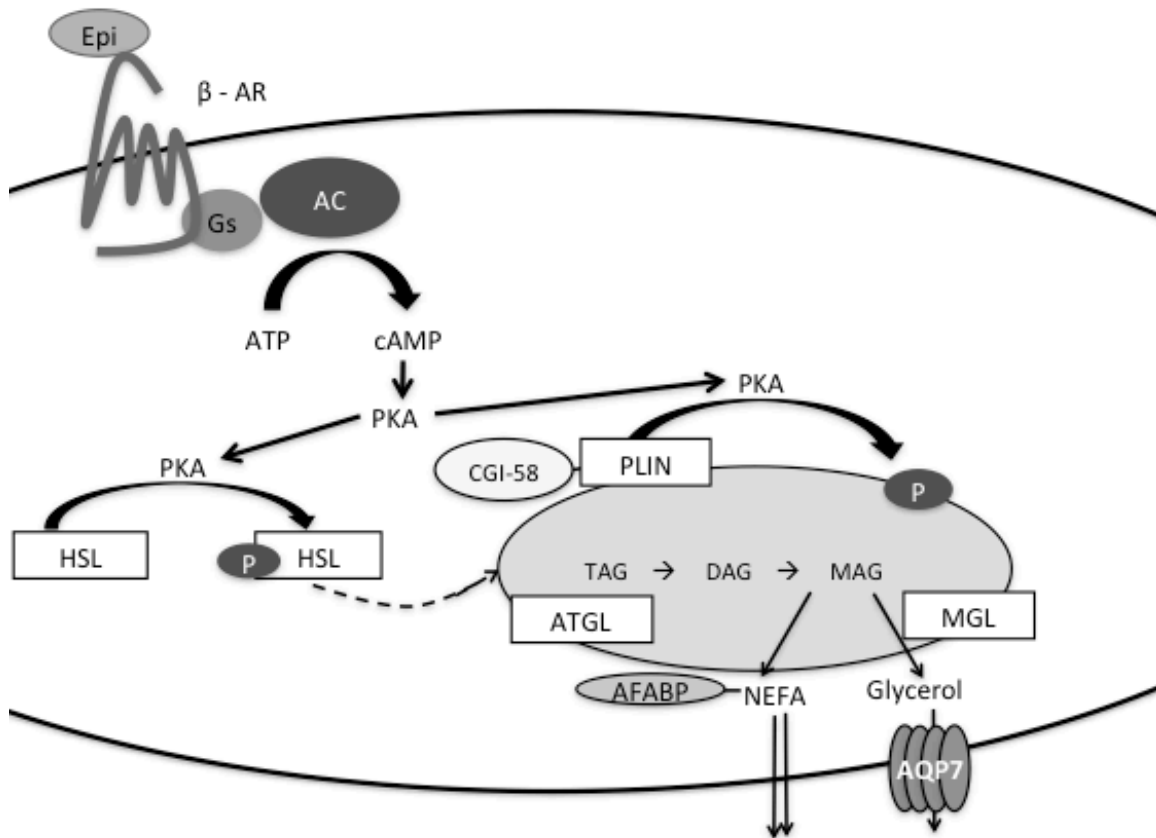
Figure 1.1, The lipolytic pathway within the adipocyte. Catecholamines (epinephrine or norepinephrine) act at the beta-adrenergic receptor (β-AR) to stimulate adenylyl cyclase (AC), which converts adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP). cAMP phosphorylates protein kinase A (PKA), which phosphorylates hormonse sensitive lipase (HSL) and perilipin (PLIN). PLIN translocates HSL to the lipid droplet. Within the lipid droplet, triacylglycerols (TAGs) are broken down into 3 Non-esterified fatty acids (NEFAs) + 1 glycerol. NEFAs leave the cell with the assistance of adipocyte fatty acid binding protein (AFABP) and glycerol is released by aquaporin-7 (AQP7).
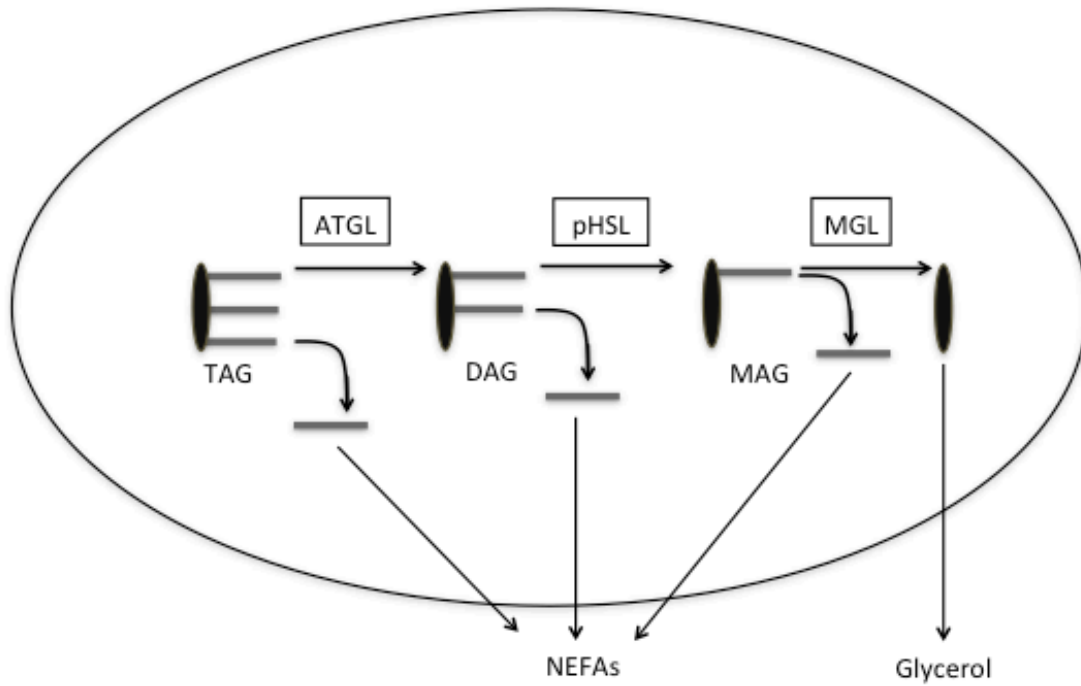
Figure 1.2, Triacylglycerol breakdown within the lipid droplet. Triacylglycerol (TAG) is broken down into diacylglycerol (DAG) by adipose triglyceride lipase (ATGL). In this step, one non-esterified fatty acid (NEFA) is released. Phosphorylated-hormone sensitive lipase (pHSL) breaks down DAG into monoacylglycerol (MAG), releasing one NEFA in the process. Monoacylglycerol lipase (MGL) breaks down MAG to one NEFA + one glycerol.

v. Anti-Lipolytic Pathway

The lipolytic pathway may also be inhibited by various hormones, most notably, insulin. When insulin binds to the insulin receptor (IR), it activates tyrosine kinases, which causes an interaction with insulin receptor substrates 1 and 2 (IRS-1, IRS-2). This leads to the phosphorylation of Pi-3K[42]. Pi-3K goes on to activate of phosphodiesterase 3B (PDE-3B), an enzyme that catalyzes cAMP breakdown to 5' adenosine monophosphate (5' AMP), limiting the amount of PKA phosphorylation, thereby inhibiting all the downstream actions in the lipolytic pathway. In addition, insulin is involved in the phosphorylation of protein phosphatase-1 (PP-1), an enzyme that dephosphorylates HSL, preventing its action[42,43]. For a visual understanding of this pathway, refer to Fig. 2.

Catecholamines (Epi and NE), the greatest known stimulators of lipolysis, may also inhibit the pathway if it binds to the alpha-adrenergic receptor (α-AR) of the adipocyte. While the β-AR stimulates lipolysis, the α-AR initiates the opposite action and phosphorylates Protein Kinase B (PKB), a downstream target of Pi-3K[37]. The binding of catecholamines to one isoform of these receptors over the other is determined by the affinity and abundance of each receptor at the tissue level. For example, in humans, α-ARs outnumber β-ARs in the subcutaneous fat and therefore these tissues have an impaired lipolytic response[44,45]. The ratio of α-AR:β-AR is adaptable, for instance, the ratio is increased with a high fat diet and causes obesity in rats via hyperplasia[45,46]. The anti-lipolytic effects of this receptor are clear when their actions are blocked in adipocytes. Treatment with either isoproterenol, or epinephrine elicited a spike in glycerol release[47]. Treatment with yohimbine, an α-AR antagonist, elicited a similar

response in stimulating glycerol release, comparable to the β-AR agonist treatments, indicating that a blockade of these receptors enhances lipolysis [47].
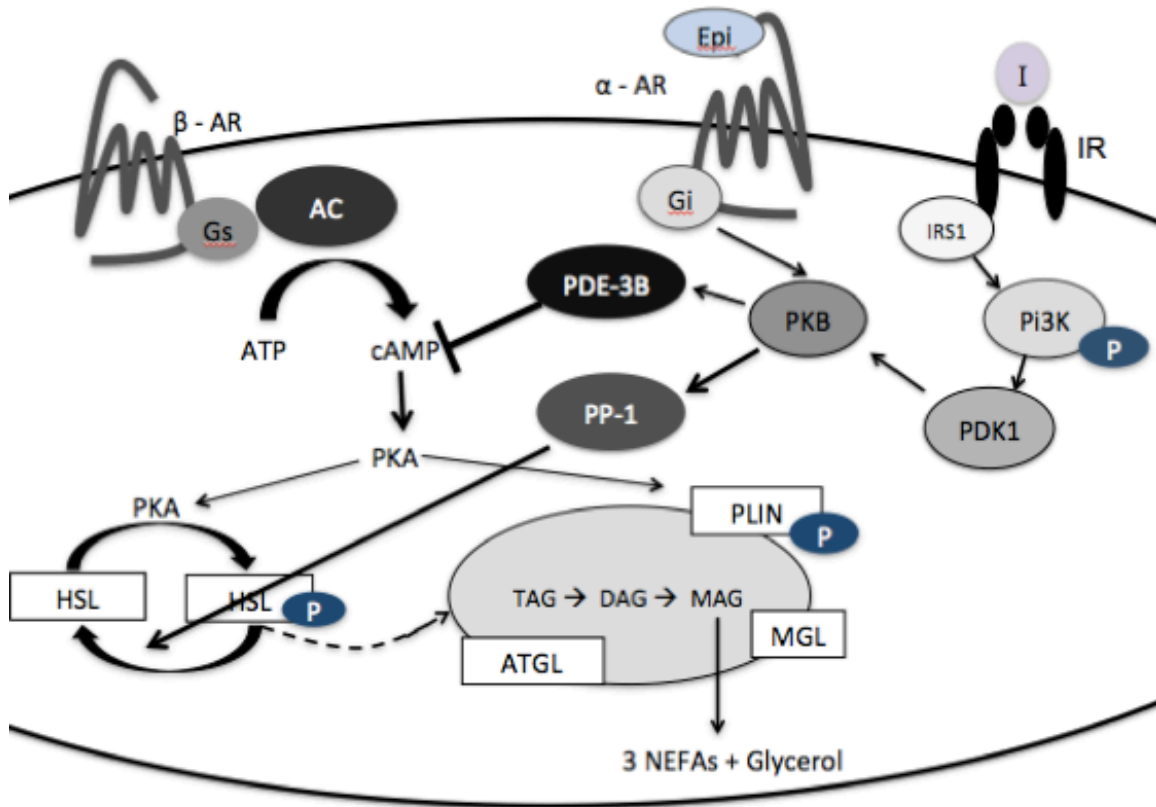
Figure 2. The anti-lipolytic pathway Insulin acts at the insulin receptor to interact with insulin receptor substrate-1 (IRS-1). IRS-1 phosphorylates phosphatidylinositol-3 kinase (Pi3K), which activates phosphoinositide-dependent kinase-1 (PDK1). PDK1 phosphorylates protein kinase B (PKB), which activates phosphodiesterase 3B (PDE-3B), which breaks down cyclic adenosine monophosphate (cAMP) to inactive 5'AMP. Epinephrine may stimulate the α-AR to also increase PKB phosphorylation, leading to enhanced PKB phosphorylation and increased activation of PDE-3B.

vi. Measures of Substrate Metabolism

Determining the oxidation of substrates is beneficial in studying metabolic responses. In normal conditions, proteins contribute a negligible amount to energy metabolism, especially during exercise, and for this reason, most research focuses on the balance between CHO and lipid use. In animals, radio-tracers have been used to determine tissue utilization of various substrates[48–50] and in humans; stable isotopes have been used[49,51,52]. Additionally, substrate metabolism at the specific tissue level has been measured by examining the arterio-venous difference in substrates and their markers, such as glucose, glycerol or NEFAs, to determine the utilization of an individual tissue[53]. A major limitation of these methods is their invasiveness. For this reason, the respiratory exchange ratio (RER), (VCO_2/VO_2), is the most commonly used method to estimate the contribution of CHOs and lipids during a steady state (either during resting state, or a steady submaximal exercise)[13]. The ratio uses indirect calorimetry to determine substance type and oxidation rate by measuring of oxygen (O_2) consumption and carbon dioxide (CO_2) production[54].

The basis of this relationship is that lipids and CHOs differ in their O_2 production when being metabolized. The range for RER values is from $0.7 - 1.0$, with the highest percentage of fat oxidation giving a value of 0.7, whereas a greatest percent of CHOs being oxidized is indicated by a value close to 1.0[54]. For an average resting individual that consumes a mixed diet, the RER would be in the range of 0.82-0.86. Substrate utilization at rest is determined predominantly by fuel availability. Elevated NEFAs enhance fatty acid oxidation while simultaneously impairing glucose utilization through

impairing insulin signalling[55], whereas a diet high in CHOs favours CHO oxidation[25,56].

2.2 Hormonal Influence on Energy Metabolism

i. Overview of GC Metabolism in Normal Physiology

Glucocorticoids (GCs) are naturally occurring hormones released from the adrenal cortex under the influence of the hypothalamus-pituitary-adrenal (HPA) axis[57]. Along with catecholamines, GCs are released as part of the fight-or-flight response during stress. The HPA axis is activated by the secretion of corticotropin-releasing hormone (CRH) from the hypothalamus which activates pituitary pro-opiomelanocortin (POMC)[58] gene transcription to increase adenocotropin hormone (ACTH) in the anterior pituitary gland. ACTH signals the adrenal glands to release GCs from the adrenal cortex[58]. In healthy individuals, release fluctuates throughout the day in a diurnal pattern, with secretion being highest upon awakening (morning) and lowest in the evening, although there is also a constant low concentration basal release [1]. This pattern is reversed in nocturnal animals (e.g. rodents). Release may also fluctuate in response to environmental stressors and food intake[59]. In addition to their endogenous release, cortisol and the various derivatives (e.g. dexamethasone, prednisolone, hydrocortisone) may also be consumed exogenously for their known anti-inflammatory properties to treat inflammation or autoimmune diseases[60].

Once in circulation, they may exist in two forms in humans: firstly, inactive and bound to corticosteroid-binding protein (CBG) and otherwise, they may be active and circulate unbound[61]. In humans, cortisol is the active form (in rodents, corticosterone) and cortisone is inactive (in rodents, 11-dehydrocortcicosterone)[1]. If active, GCs may

readily cross the plasma membrane and may bind to the intracellular glucocorticoid receptor (GR)[61], a steroid nuclear receptor that is found in abundance throughout the body. The mineralocorticoid receptor (MR) may also be activated by GCs in some tissues, specifically found in the distal nephron, colon and sweat glands[34]. Activation of these receptors may be amplified by the pre-receptor 11 beta- hydroxysteroid dehydrogenase type 1 (11β-HSD-1)[34], an oxo-reductase enzyme that catalyzes the conversion of inactive cortisone into active cortisol[62]. 11β-HSD-1 is predominantly expressed in liver, adipose, bones, nervous system, muscles and lungs[63,64]. Conversely, GR activation may be down-regulated via the activation of 11β-HSD-2, a dehydrogenase pre-receptor that converts cortisol back into cortisone[65]. Through GR activation, GCs play a role in regulating metabolism[66], influencing growth, and impacting the immune system[67]. The implications of excess GCs have been extensively studied, although it is not entirely clear whether some of their actions are a result of direct genomic changes or are the indirect consequence of influencing other hormones, including epinephrine, insulin or glucagon. In addition to interacting with the GR, they are also believed to influence other hormones that may elicit a number of responses[66]. Hypercortisolemic models have demonstrated that elevated GCs result in insulin resistance, increased protein catabolism, elevated plasma glucose and lipolysis[34,60]. In a short-term stressed state, these responses are quite adaptive, increasing substrate availability for energy via the mobilization of glucose, lipids and amino acids. However, these responses are also seen with a chronic exposure to GCs and if sustained over a long period become maladaptive and are associated with insulin resistance and obesity[68]. For this reason, various signalling pathways tightly control GC regulation to meet the

demands of the body and provide feedback in order to adapt to changing environments[69].

ii. GCs and fuels (CHO, Protein, Lipids)

GCs play a role in influencing all of the major fuel sources. They are potent inducers of hyperglycaemia through specific actions within the various major metabolic tissues. They influence various counter-regulatory hormones including, insulin, catecholamines and glucagon, to alter fuel metabolism [70–72].

GCs decrease insulin signalling and therefore reduce glucose uptake in the muscle, which is one of the most influential tissues on glucose metabolism[73–75]. In the liver, GCs also induce insulin resistance. This in turn results in increases in gluconeogenesis via the increased expression of PEPCK and G6Pase, thereby enhancing hepatic glucose output[72,76,77]. In the pancreas, GC overexposure results in impairments in insulin secretion[78,79]. Lastly, in adipose tissue, GCs reduce the responsiveness to insulin through decreasing IRS-1 phosphorylation and reducing PKB activity[80]. Studies examining the *short term* effects of GCs on adipose cells using 3T3-L1 cells (30 min Dexamethasone (DEX)) have shown that GCs induce immediate effects on adipose by inhibiting the insulin response[81]. They do this by impairing receptor signalling, although they do not change receptor expression[81]. Insulin receptor expression is altered, however, with a *long term* exposure to GCs (24-hr DEX) through GR-mediated reductions in IRS-1 and PKB concentrations in rat adipocytes[82]. In tissues with decreased expression of GR and thereby reduced GC actions, there is an association with increased insulin sensitivity in humans[83].
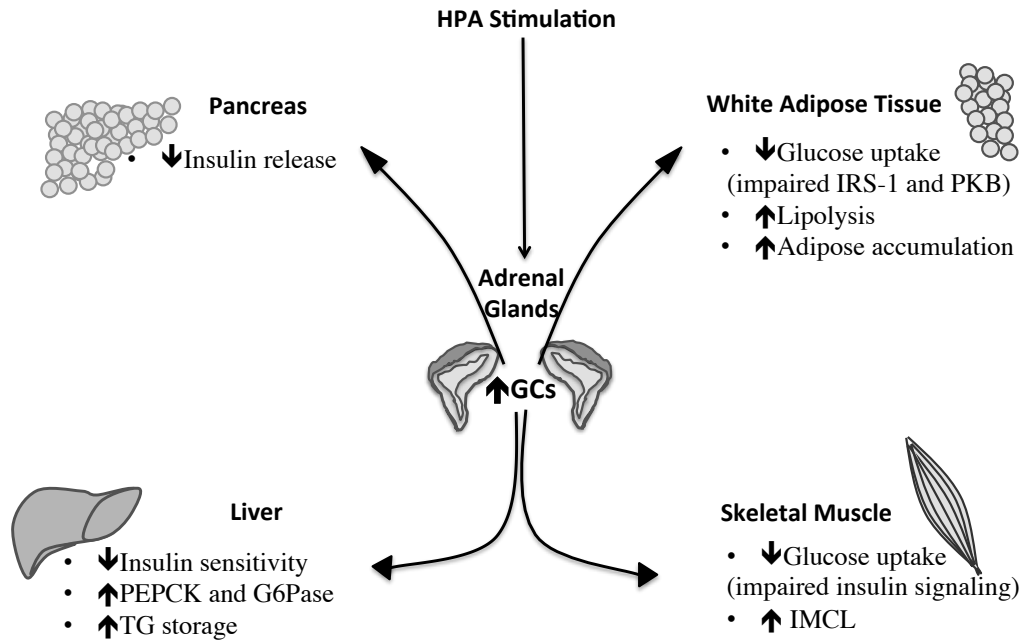
Figure 3.0. Metabolic effects of chronic GC exposure at the various metabolic tissues. HPA stimulation results in glucocorticoid (GC) release from the adrenal glands. Once in circulation, GCs increase blood glucose (BG) through increasing gluconeogenesis (GNG) in the liver via increasing GNG enzyme activity (Phosphoenolpyruvate carboxykinase (PEPCK) and Glucose 6-phosphatase (G6Pase), while impairing insulin release and insulin sensitivity at the various tissues. In adipose, insulin signalling is reduced through impaired insulin-receptor substrate (IRS-1) and protein kinase B (PKB) activity. In muscle, glucose uptake is reduced due to impaired insulin signalling. The pancreas has reduced beta cell function and decrases the amount of insulin output. GCs increase fatty acid release (lipolysis) and promote lipid storage in the adipose depot itself, as well as in liver (triglyceride (TG)) and muscle (intramyocellular lipid content (IMCL)).

iii. Dual Roles of Glucocorticoids in Lipid metabolism

The actions of GCs on lipid metabolism have been extensively studied through various models and species. Several early studies used an adrenalectomy model on rodents supplemented with GCs exogenously, either to physiological or supraphysiological levels. From here, various *in vitro* models using isolated adipocytes have been used to try and examine specific mechanisms. *In vivo* murine models, as well as human studies have been used to examine GC overexposure. Studies have utilized both exogenous GC treatments, as well as examined disease states where there is a chronic elevation in GC release, such as in Cushing's syndrome. Conversely, the effects of impaired GCs have been studied by examining conditions with impaired GC release, such as Addison's disease, or by using GR blockers, such as RU-486 [84,85].

The lipolytic actions of GCs have been confirmed in various studies, both *in vivo* and *in vitro*, by increasing the rate of lipolysis through increases in lipolytic enzymes and receptor changes [10,86]. However, in certain circumstances, such as with very acute cell culture models, glucocorticoids are found to be anti-lipolytic and potentially even lipogenic, making their role in lipid metabolism quite perplexing[12,87]. Some of the differences in lipid metabolism response are related to variation between models (i.e., *in vivo* versus *in vitro*), concentration and duration of exposure, differences between species or depots, as well as some confounding effects due to the presence or absence of other regulatory hormones (e.g. Epi, insulin, growth hormone (GH) etc.).

2.3 Models of GC Manipulation and Lipid metabolism

i. Adrenalectomy Models

Many of the early studies examining the role of glucocorticoids on lipid metabolism used adrenalectomized rodents. The main benefit of this model is that it eliminates the endogenous release of both GCs and epinephrine, either of which may be supplemented back with exogenous treatment. Adrenalectomized rats had impaired lipolysis due to reduced β-AR concentration, as well as by reduced coupling with AC. This impairment was found to be corrected with short-term Dexamethasone (DEX) treatment (5mg/kg, 24-48hr) through increases in β-AR concentration [88]. However, once again some studies did not find this effect. In an adrenalectomized rat model, treatment with GCs did not have any enhancements in lipolysis unless treated in combination with growth hormone (GH)[77]. When treated with both, the enhancement in lipolysis was believed to be due to an increased sensitivity to epinephrine (either by increased β-AR concentration or enhanced AC coupling), as measured by an increase in cAMP[77].

ii. Cell Culture Models

Cell culture models using isolated adipocytes from animal or humans, or 3T3-L1 cells, an established pre-adipocyte cell line that is derived from mice, have been very useful in studies examining the regulation of enzymes with GCs. They have also been useful in determining the specific effects of GCs without the interaction of other hormones[89]. Many of these models have demonstrated that GC treatment enhances lipolytic capacity. Isolated 3T3-L1 cells exposed to DEX for 24 hours had increased expression of both ATGL and HSL, key lipolytic enzymes[10,90], as well as enhanced PKA activity[11]. Additionally, DEX treatment (30-100 nM, 24 hours) on primary rat adipocytes down-regulated the expression of PDE-3B (mRNA and content), thereby

impairing the antilipolytic actions of insulin and further enhancing cAMP production to thereby promote lipolysis[11]. This was accompanied with an increase in glycerol release, indicating enhanced lipolysis had occurred. A study examining the cumulative effects of DEX (0.016ug/ml) in combination with GH (1.0 ug/mL) in isolated rat adipocytes, found that there was increased glycerol and fatty acid release 2 hours following treatment (4 hour treatment in a 2 mL volume)[91]. Interestingly, neither GH nor DEX alone produced a significant effect on NEFA release in isolated adipocytes, however, DEX did increase lipolysis in isolated rat fat pads[91]. These effects on lipolysis were countered when isolated adipocytes were treated with insulin in combination with either DEX, or DEX + GH[91]. Xu and colleagues found that 4-hour treatment with DEX was sufficient enough to note a slight increase in NEFA release, but not glycerol, a by-product of lipolysis in isolated adipocytes (10-100nM). Following 16-24 hours, release of both glycerol and NEFAs was significantly increased in a time dependent manner in this model[11]. It has previously been reported that GC responses are not notable until approximately 4-8 hours [11,42], likely the time required to see responses of stimulating the GR. When treated with GR-antagonist RU-486, glycerol release was suppressed, further indicating that these were genomic, receptor mediated adaptations[11].

There is reason to believe that GCs exhibit very different immediate, non-genomic immediate actions. Interestingly, 3T3-L1 cells *acutely* exposed to corticosterone (1 hour), have a decreased rate of glycerol release compared to controls[12]. At this exposure time, increasing the concentration of corticosterone resulted in further decreases in glycerol

release, indicating anti-lipolytic actions in an acute setting[10]. Further investigation of these potential non-genomic actions is required in an *in vivo* model.

iii. Human and In Vivo Rodent Models

Alterations in GC concentrations in vivo have been extensively studied in both *in vivo* animal models and in humans, but similarly to cell culture studies, most research has focused on the chronic adaptations. *In vivo* models have been beneficial for examining the interaction between GCs and various regulatory hormones, most notably, insulin.

In rodents, implantation of a corticosterone pellet (300 mg) resulted in an increase in lipolysis when measured after 10 days, despite an increase in visceral adiposity[10]. Studies examining short-term GC over-exposure (~6 hours) in humans also elicited a lipolytic response[92]. Healthy men that received an infusion of hydrocortisone had increased glycerol in the interstitial and femoral adipose tissue, as well as systemic glycerol when measured up to 6 hours after infusion[92], comparable to the expected time of genomic effects to occur in cell culture models. Interestingly, in a study using healthy subjects that had hydrocortisone infused to achieve plasma cortisol concentrations of 1500–1700nmol/L, it was observed that 4-5 hours following treatment there was an increase in lipolysis in the subcutaneous adipose tissue of the limbs. However, in the abdominal subcutaneous adipose, there was a decrease in NEFA release and HSL activity, suggesting a potential anti-lipolytic mechanism in this depot [93]. This study provided insight to the potential depot specific differences in GC responses, as observed in the phenotype of individuals with chronic hypercortisolemia (i.e.; Cushing's syndrome (CS)). This condition results in increased abdominal adiposity, while muscle and adipose is wasted in the limbs. Studies on CS patients have demonstrated that they

have reduced lipolytic activity, as indicated by impaired NEFA turnover[94,95]. Additionally, obese individuals have local elevations in GC activity through increased GR and 11β-HSD-1 content in adipose tissue, indicating that GCs are involved in promoting adipose storage[96].

As mentioned earlier, although some short-term exposure studies (6-48 hours) have been performed, the immediate, non-genomic actions have not been as extensively studied in an acute *in vivo* model. In rodents, a handling-stress model has been used as an effective way to induce a stress response and it has been found that 165 minutes following stress results in an efflux in NEFA release[97]. However, these studies are limited because it is difficult to determine if these changes are the result of GC elevations, or the more immediate actions of Epi and NE that are also released in a stress response. Additional investigation is required to further understand the immediate actions of GC.

2.4 GCs in the Subcutaneous versus Omental/Visceral Depots

Excessive accumulation of body fat is known to increase the risks of metabolic abnormalities and the distribution and type of fat are also important factors that contribute to potential complications. Excessive android adiposity (abdominal accumulation), in combination with increased visceral fat poses an increased risk for associated metabolic complications, such as hyperglycaemia, insulin resistance and elevated triglycerides[93,94]. Individuals with a gynoid distribution (increased lipid accumulation in gluteo-femoral region) have a larger proportion of subcutaneous fat and increased accumulation of this type of fat is not associated with metabolic aberrations.

As discussed, the phenotype of individuals with hypercortisolemia is unusual. GCs induce adiposity through stimulating adipogenesis, as well as enhancing adipocyte hypertrophy[100]. Microarray studies have determined that GCs have a powerful effect on influencing gene networks that regulate human adipose tissue, promoting lipid accumulation in abdominal adipose tissue (both omental and subcutaneous)[101]. Looking at the phenotype of these individuals with chronically elevated GCs, it is clear there is some altered regulation of adipose tissue and that different adipose depots elicit difference responses. Increases in omental (i.e. visceral) adipose tissue are associated with elevated cortisol, androgens and reduced GH [102]. As stated, subcutaneous adipose of the extremities is diminished in these individuals. Various studies have examined the potential mechanisms for these differences [8,96,101–103]. Considering that in conditions such as metabolic syndrome or obesity, systemic GC levels may remain in the normal range, one of the major hypotheses is that the specific depots are susceptible to tissue-specific enhanced activation of GCs through increased GR or 11β-HSD-1 expressions [104,105].

11β -HSD1 content has been established as an important predictor of adiposity[103]. Higher levels of 11β-HSD-1 and cortisol content have been found in omental compared to subcutaneous adipose tissue in women with excess abdominal adiposity[96]. Both omental and subcutaneous adipose tissue were extracted from healthy individuals and exposed to elevated DEX (20 nmol/l in 1mL) and insulin, which was chosen to replicate a stress response. 11β-HSD-1 mRNA content and activity was enhanced in the omental adipose tissue, indicating a role in which GCs contribute to visceral obesity[106]. This visceral-specific up-regulation of 11β-HSD-1 activity is also seen in Zucker rats, a

diabetic rodent model[107]. In the visceral versus subcutaneous adipose of obese patients, there is increased 11β-HSD-1 and cortisol content[103]. Additionally, this depot had an increased expression of enzymes involved in fatty acid metabolism when compared to the subcutaneous depot in both obese and healthy subjects[103]. Increased local activation of GCs is accompanied by increased GR density in omental compared to subcutaneous human adipose tissue and more GC binding occurs in intra-abdominal adipose[83,108]. When examining RNA sequencing on abdominal subcutaneous adipose from patients with Cushing's disease, it was found that GCs also play a role in up-regulating lipogenic genes[109].

Adipocytes treated with DEX have an enhanced capacity to uptake free fatty acids via DEX-induced increases in LPL activity, a known lipogenic protein. The increase in LPL activity more pronounced in omental compared to subcutaneous adipocytes, further contributing to a potential mechanism for GCs to promote central adipocyte hypertrophy[110].

Contrary to the impaired insulin sensitivity that GC administration causes in visceral adipose tissue and muscle, short-term GC administration (14 hour hydrocortisone infusion of 0.2mg/kg*hr) enhances insulin sensitivity in subcutaneous adipose tissue[8]. This is also seen in subcutaneous adipocytes treated with DEX[111]. Considering that GCs induce insulin resistance, it is plausible that the enhanced lipolytic activity in this depot may be an indirect effect of GCs through impairing insulin signalling.

2.5 GC Association with Dysregulation and Metabolic Disease

Despite the benefits being a potent anti-inflammatory agent, chronic overexposure of GCs results in CS—a clinical condition associated with metabolic complications,

including central adiposity, muscle wasting, insulin resistance, hepatic steatosis, and dyslipidemia. The most common etiologies of CS are pituitary adenomas (most frequently), which increase the endogenous production of ACTH, but adrenal hyperplasia or adrenocortical tumours also account for many cases [112,113]. The excess production impairs the feedback to the HPA axis. Additionally, CS may result from excess exposure to exogenous corticosteroids or synthetic GCs, usually from oral administration, but also, less frequently, may occur from topical exposure[114].

Metabolic syndrome (MetS) is a multifactorial condition characterized by a cluster of risk factors including, increased waist circumference, hyperglycaemia, dyslipidemia, hypertension and obesity[115–117]. Many phenotypical and symptomatic similarities have been observed between MetS and CS, leading many to hypothesize that cortisol may be involved in the pathophysiology of MetS[68,118]. The physical Cushingoid features are distinct, with excessive android obesity and very thin extremities. While the wasting at the extremities is a feature that is unique to Cushing's, the excessive abdominal obesity is similar to that of a MetS patient. In the liver, patients with adrenal cortical incidentalomas and CS have reduced HDL and cholesterol levels accompanied with elevated triglycerides, resulting in dyslipidemia[119]. Additionally, elevated cortisol is consistently associated with insulin resistance [120]. It has also been found that type 2 diabetes is associated with variations in 11β-HSD-1 gene expression, indicating increased local activation of GCs in the tissues[121].

In addition to the metabolic consequences, there is also an association between psychological symptoms of depression and anxiety, and central obesity, insulin resistance and cardiovascular morbidities[120,122]. In obese children, symptoms of anxiety and

depression correlate with elevated salivary cortisol levels throughout the day[123]. In men, cortisol metabolites were increased with depression and central obesity[124].

The reduced muscle mass in CS patients impairs their exercise capacity and even if exercise is performed, there is an impaired ability to lose weight. Fortunately, the negative metabolic effects of this condition are countered when treated with RU-486, a GR antagonist. It was found that with RU-486, CS patients have significant improvements in metabolic outcomes, such as decreased HbA1c and reduced waist circumference[125].

2.6 Lifestyle Influences on metabolism

Although there are genetic components that contribute to metabolism, environmental factors significantly influence metabolic health and regulation. There are a number of environmental factors that influence this relationship; including, sleep patterns (altered circadian rhythm), temperature, diet and activity. Metabolic homeostasis is achieved through a balance of energy input being met with fuel utilization, so for this reason two of the most influential factors of energy balance are diet (energy input) and physical activity (energy output).

i. Diet

In addition to the basic input versus output requirements, macronutrient intake must also balance oxidation[25]; for this reason, dietary choices, in addition to the overall calories, have a significant impact on energy metabolism. Unbalanced diets with significant elevations of a particular macronutrient leads to metabolic aberrations and dysregulation[126]. For example, high fat diets (HFDs) significantly increase adiposity and are a main factor in inducing the development of type 2 diabetes, as seen in various

human and rodent studies[127–129]. Increases in adipose accumulation not only affects adipose metabolism, but also impacts how the body processes other substrates. HFDs impair insulin sensitivity via impairing GLUT4 mRNA and protein content in adipocytes, leading to less glucose uptake and oxidation[130].

Additionally, diets relying predominantly on protein may provide insufficient levels of CHOs, and protein must be converted to glucose in a process termed protein sparing [131,132]. Eating a balanced diet rich in nutrients is recommended to maintain a healthy metabolism.

ii. Exercise

Exercise has a major influence on energy substrate metabolism. Changes occur during exercise, but the body also adapts to influence metabolism following the completion of the activity. During exercise, there is an increased metabolic demand for energy to sustain muscle activity, which places the body in a state of energy deficit and stress.

Depending on the type and duration of the activity, the body adjusts its fuel utilization to meet the workload demand. Various studies have examined how fuel selection is manipulated by exercise type and intensity though assessing changes in RER [54,133]. At the onset of a moderate intensity (40-60% VO_2Max) endurance exercise, an immediate rise can be seen in RER, but as the exercise continues and exceeds approximately 30 minutes, RER decreases, indicating an increase in the reliance on lipid oxidation[54]. While high intensity exercise favours CHO oxidation from blood glucose and muscle glycogen stores, as CHOs yield the most energy (in the form of adenosine triphosphate) per each molecule that is oxidized, the optimal type of exercise to utilize

lipids as the predominant fuel is low-intensity endurance exercise, below 50% of VO_2Max[49]. In addition to the type of exercise, other factors influence fuel selection with physical activity. For instance, trained athletes have a shift in RER to utilize lipids more readily than non-trained individuals, even at rest [134].

iii. Aerobic Exercise and Hormonal Regulation

It is well known that exercise has numerous benefits in improving metabolism, reducing adiposity and increasing muscle mass. These changes are the result of physiological adaptations that occur with activity. Acutely, a bout of exercise is a stressor to the body. Activating the stress response as an adaptable response in order to maintain homeostasis and meet the increased metabolic demand. The stress response triggers the immediate activation of the Autonomic Nervous System (ANS) in what is known as the "fight-or flight" response[135] and a signal is sent down the spinal cord to trigger the release of NE and adrenal glands to release a flux of Epi. The hypothalamus also induces the cascade of signals to also promote the release of GCs from the adrenals[135]. Although there are two major types of exercise, resistance and continuous (aerobic) or a combination of the two, this review will only be focusing on the physiological response for continuous/aerobic forms of exercise.

Both catecholamines and GCs influence metabolism directly, as well as by altering the regulation of other neuroendocrine hormones. Epinephrine, a major catecholamine, has been shown to trigger glycogenolysis in muscle to allow glucose to be used as a fuel[136]. While epinephrine inhibits insulin, it enhances the ability of the muscle to take up plasma glucose through contraction mediated glucose uptake, (insulin-independent)[137]. Following an exercise bout, however, there is also an enhanced

sensitivity to insulin that lasts up to 48 hours. This further mediates glucose uptake (insulin-dependent) [138–141]. In addition to these effects, interleukin-6 (IL-6) is increased during exercise from working muscles, partly under the influence of epinephrine. IL-6 is involved in increasing GLUT4 protein content in white adipose tissue following exercise to enhance glucose uptake[142].

Epinephrine is the major hormone involved in initiating the mobilization of free fatty acids to be available to be used as a fuel. During exercise, epinephrine acts on the β-AR to trigger the lipolytic pathway to release NEFAs to the plasma where working muscles are then able to use them as fuel to be oxidized[44]. Contrary to their lipolytic role, epinephrine is also the preferred amine to the α2-AR, which promotes anti-lipolytic actions[143]. Additionally, IL-6 indirectly increases lipolysis in glycolytic muscles and various studies have demonstrated that a single bout of continuous exercise reduces intramyocellular lipid content (IMCL) [144].

iv. Role of GCs in Aerobic Exercise

In a state of exercise, GCs have typically been shown to be quite beneficial in improving exercise performance and assisting in maintaining homeostatic regulation. During exercise, it has been found that rats that received corticosterone took significantly longer to reach exhaustion than those that did not, demonstrating that the corticosterone enhanced the endurance capacity of the rats by increasing the amount of time it took to reach exhaustion[145].

Exercise induces a rise in GCs most significantly when working at high intensities[146]; however, in a resting state, chronically trained individuals have reduced GC levels[147,148]. In addition to the amount of GCs in circulation increasing during

exercise, tissues also increase their sensitivity to GCs [149]. In muscles, this may be done to help reduce the inflammatory reaction caused during exercise by reducing cytokine synthesis, thereby limiting the amount of damage at the muscle during exercise[149]. Twenty-four hours following exercise, tissue sensitivity to GCs has been shown to decrease[149–151], which may help in preventing excessive catabolism to muscle that may otherwise be observed with an overload of GCs.

In terms of their role in metabolism with exercise, GCs cause a rise in circulating glucose by increasing glycogenolysis at the liver, while reducing the muscular sensitivity to glucose[152]. These adaptations may be used as a mechanism to prevent hypoglycaemia with exercise from occurring. The presumed role of GCs in lipid metabolism, as cited in various textbooks, is that they are lipolytic to allow NEFAs to be used as a fuel[152,153]. Considering their role in impairing insulin signalling, however, this has not actually been studied *in vivo* during exercise and further research is required to determine if they have alternative acute actions.

2.7 Clinical Relevance/Gaps in the literature

GCs have been prescribed for many years as a potent anti-inflammatory agent, both for acute injuries as well as chronic conditions, such as rheumatoid arthritis and some forms of cancer. Considering the detrimental effects of chronic hypercortisolism, examining the immediate effects further would be useful in helping to understand their specific actions and implications on metabolism. The most curious uncertainty about GCs is the perplexing role they play on lipid metabolism and further investigation is required to better understand their function. Acutely, they are believed to enhance systemic lipolysis as well as NEFA uptake, although this may be due to the associated rise in

catecholamines with a stress response. The need to examine the interaction of GCs and exercise is also intriguing. GCs fluctuate during exercise and influence various other hormones, leading to serious alterations in metabolism. This information would be of interest to many athletes that use GCs for injuries, providing insight on their potential influence on athletic performance.

3.0 RATIONALE AND OBJECTIVES

3.1 Background and Rationale

Glucocorticoids are naturally occurring steroid hormones that rise in concentration during situations of stress, starvation and exercise. GCs promote insulin resistance and induce epinephrine release from the adrenal medulla[154]. They play a perplexing role in lipid metabolism and may exert pleotropic effects. They are typically stated to be "lipolytic" by most physiological textbooks [153] but the mechanism of action for this effect is somewhat unknown. Elevations in GCs have been shown to be associated with epinephrine and norepinephrine secretion and lower insulin sensitivity, both of which may help facilitate lipolysis [38,155]. Previous research from our lab supports the lipolytic actions of GCs, but only when GCs are elevated long enough to increase the gene transcription of ATGL and/or HSL[10]. It is also apparent in 3T3-L1 cell culture models that GCs induce acute antilipolytic actions, particularly at higher dosages of exposure[12]. This latter point supports the clinical observation that hypercortisolemia is strongly associated with central obesity [156]. Although several studies have examined the chronic effects of glucocorticoids on body adiposity and insulin sensitivity[60,62,81], less is known about their immediate action on adipose tissue metabolism. For this reason, we designed a rodent exercise model to examine the acute role of glucocorticoids in relation to lipid metabolism once activated by a stress response, in this case, exercise. The model will assess molecular and in vivo markers of adipose tissue lipolysis as well as catecholamine and insulin levels following acute GC treatment and hopefully gain insight as to which mechanism plays a more dominant role in lipid regulation.

3.2 Purpose

The purpose of this study is to gain insight into the role of glucocorticoids in lipid metabolism and to determine if an acute dose will induce a lipolytic response once activated by exercise. Additionally, this study will examine the extent to which glucocorticoids influence insulin concentration and the phosphorylation of lipolytic enzymes, as well as determining if these relationships are influenced by exercise. The acute effects of glucocorticoids have previously been examined in our lab *in vitro*, this model will assess the role in an *in vivo* setting.

3.3 Hypothesis

We hypothesize that acutely elevated levels of GCs (corticosterone) will attenuate the lipolytic responses seen at rest and with moderate exercise.

4.0 MANUSCRIPT

THE ACUTE ROLE OF GLUCOCORTICOIDS DURING EXERCISE ON LIPID METABOLISM[1]

INTRODUCTION

Glucocorticoids (GCs) are hormones that are released from the adrenal medulla at low levels throughout the day and their concentration significantly increases with a stress response[154]. However, in cases with increased stimulation of the adrenal response, circulating levels of GCs are elevated and they disrupt metabolic homeostasis. Despite their known benefits as potent anti-inflammatory agents, they have been proven to cause detrimental effects when chronically elevated, resulting in obesity, hyperglycaemia and insulin resistance. This has been observed in various rodent models in which glucocorticoids are increased exogenously, or in human studies of hypercortisolemia, where GCs were either given as treatment, or were naturally elevated, such as in the medical condition, Cushing's syndrome.

With regard to lipid metabolism, GCs are typically stated to be "lipolytic" in most physiological textbooks [153], in that they are believed to increase the flux of substrates, yet the mechanism of action for this supposed effect is unknown. Elevations in GCs have been shown to be associated with increased catecholamine secretion and reduced insulin sensitivity, which are potential mechanisms in which they may facilitate lipolysis [38,155]. Cell culture studies using 3T3-L1 cells support the lipolytic actions of GCs, but

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Key terms: Adipose, Glucocorticoids, Lipolysis, Exercise, Metabolism, Non-esterified fatty acids

this is only the case when elevated long enough to increase the gene transcription of ATGL and/or HSL[157]. It is, however, also apparent in these models that GCs induce acute antilipolytic actions, particularly at higher dosages of exposure[12]. This latter finding is more supportive of the clinical manifestations of chronic hypercortisolemia and its association with central obesity[156]. Several studies have demonstrated that chronically elevated glucocorticoids have profound effects on increasing body adiposity while impairing insulin sensitivity[158], However, their immediate actions on lipid metabolism have not been specifically demonstrated *in vivo*. For this reason, we designed a rodent exercise model to examine the acute role of glucocorticoids in relation to lipid metabolism once activated by a stress response—in this case, exercise. The model was designed to assess molecular and *in vivo* markers of adipose tissue lipolysis, as well as glycemic markers following acute GC treatment with the objective of hopefully gaining a better understanding of lipid regulation with exercise.

METHODS

Ethics Statement

The following study was been approved by the York University Animal Care Committee (*Protocol # 2015-3*) and was carried out in accordance with the regulations of the Canadian Council for Animal Care guidelines.

Experimental Design

Sixty male Wistar rats (Charles River Laboratory, approximately 225-250 grams upon arrival) were used in this study. Rats were acclimated to the York University vivaria for one week after arrival and were housed in a humidity and temperature controlled

room in a 12 hour : 12 hour light-dark cycle. All animals had access to a standard chow ad libitum diet. Following the one-week acclimation, rats were individually housed and randomly assigned to one of 4 treatment groups. A schematic of this protocol is represented in Fig. 1.1. Animals were either assigned to an exercising or sedentary protocol, and from here they were further separated to receive either Vehicle (25% DMSO in saline), or Corticosterone (25 mg/kg body weight in 25% DMSO in saline). All groups were acclimated to running treadmills for 3 days within a one-week period. Intensity and duration were increased in each acclimation session. On the first day of acclimation, rats ran for 10 minutes at a pace of 10 meters/minute with 0% incline. On the second day, the speed was increased to 20 meters/minute with a 0% incline for 10 minutes. On the final acclimation day, they ran for 20 minutes at 20 meters/minute with 0% incline.

After the one-week acclimation, the experimental protocol commenced (Fig. 1.2). Approximately 4 animals underwent the experimental protocol each day with two overlapping at a time. Experiments were performed in the morning when basal corticosterone levels were lowest. For this protocol, each animal received a saphenous blood draw, which was collected as a pre-treatment measure (t = -25min) and at this time, blood glucose was also measured. Approximately 15 minutes following this, animals received an intraperitoneal (i.p.) injection of their treatment (t = -15 min), either the vehicle solution, or corticosterone. Immediately following the injection, animals were placed in Comprehensive Lab Animal Monitoring System (CLAMS) treadmills, which allowed the measurement of the respiratory exchange ratio (RER) for each animal. A period of 15 minutes elapsed before turning on the treadmills in order to establish a stable

basal reading of ventilation and RER. Once this equilibration period was complete (t = 0 min), the treadmills were turned on for the exercise groups to 20m/min, 0% incline, and remained on for 45 minutes. The sedentary animals remained in the CLAMS treadmills for this 45-minute period, but the running belt was turned off. For both groups, RER was measured throughout.

Immediately after the 45-minute exercise (or 45 minute sedentary period), animals were removed from the treadmills and each received a tail prick in order to measure post-treatment blood glucose. Directly following this, animals were placed in an induction box filled with isoflourane and oxygen, which was used as an anaesthetic. Once fully unconscious, animals were euthanized and the renal vein was cut to allow for the collection of a post-treatment blood sample. Additionally, a fragment of the liver was dissected for additional blood collection from the trunk. Concurrently, tissues (peritoneal adipose depots) were collected and immediately placed in microtubes and put into liquid nitrogen. Tissues and plasma samples were then transferred to storage at -80°C to be preserved for future assays.

Drug Preparation

Our vehicle was prepared as a stock solution of 25% dimethyl sulfoxide (DMSO) in saline. Corticosterone (Sigma-Aldrich, Cat # C2505) was also prepared in a stock solution, where the corticosterone was dissolved into the vehicle (0.005 mg/ml) and sonicated immediately before use. Each animal in this group received 25mg/kg body weight, which was considered to be a moderately high yet safe dose that had been previously published in other studies [159,160].

Plasma Analyses

Plasma was collected in potassium-coated EDTA microvette capillary tubes (Sarstedt, Cat #16.444.100). Blood was collected from the saphenous vein for the pre-treatment blood draw and during euthanization from the renal vein for the post-treatment measures. Immediately after collection, 10μL of Trasylol was added as a proteinase inhibitor before samples were placed on ice. Samples were then centrifuged for 5 minutes at 15,000 rpm before plasma was pipetted out into polyethylene tubes. Samples were placed back on ice before being transferred to -80°C storage where it remained until used for later analysis. Plasma was used for various analyses including non-esterified fatty acid, glucagon and insulin concentrations, which were assessed using enzyme-linked immunosorbent assays (ELISAs) (Wako, Cat #999-34691; Mercodia, Cat #10-1281-01; Crystal Chem, Cat #90060) and corticosterone concentration was assessed using a radioimmunoassay (RIA) (MP Biomedicals, cat#07-120103).

Western Blotting

Radioimmunoprecipitation assay (RIPA) buffer (150mM NaCL, NP-40, sodium deoxycholate, SDS, 50mM Tris base) was supplemented with protease inhibitor cocktail (Sigma-Aldrich, Cat #P8340) and phosphatase inhibitor cocktail (Sigma-Aldrich, Cat #P0044). The solution was added to adipose tissue samples (approximately 50mg) at a concentration of 10mL/mg. Samples were homogenized using a D2400 Homogenizer (Diamed) for 6 cycles of 45 seconds. Samples were then placed on ice for 10 minutes and then centrifuged at -4°C for 10 minutes at 14,000 rpm. Supernatant was pipetted out and transferred to microtubes. Protein concentrations were determined using a commercially

available bicinchoninic acid assay (BCA) and samples were stored at -80°C until later use.

Thirty micrograms of protein from the various fat depots was run on a 10% SDS-page gel before being transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Canada). Membranes were then blocked for 1 hour at room temperature in 5% skim milk dissolved in Tris-buffered saline with Tween 20 (TBST). Next, membranes were left to incubate in their primary antibody at a concentration of 1/1000 (pHSL, tHSL, Perilipin-A, PKA-substrate indicator) or 1/10,000 (beta-actin) overnight at 4°C. The next morning, the primary antibody was removed and membranes were washed in TBST for 3 cycles of 10 minutes. Membranes were then incubated in their secondary antibody for 1 hour at room temperature. The secondary antibodies used were goat-anti-mouse when beta-actin was the primary antibody (1:10,000, ab6789, Abcam, Cat #ab6789), or goat-anti-rabbit for all other primary antibodies (1:10,000, Abcam Cat #ab6721). Secondary antibodies were diluted into in the 5% skim milk in TBST. Membranes were again washed in TBST for 3 cycles of ten minutes. All membranes were images using a Kodak In vivo FX Pro imager, with Carestream used as the molecular imaging software.

Statistical Analyses

All data was represented as mean ± standard deviation. Two-way ANOVA analyses were used to compare differences between groups and a cut off of $p < 0.05$ was considered to be significant. For significant values, Tukey's post-hoc test was used to determine differences between specific groups (treatments and activity).
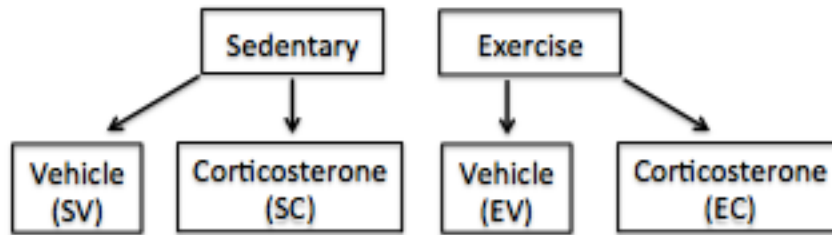
Figure 1.1 – Animal treatment groups. Animals were randomly divided into one of 4 groups. The first two groups were sedentary and received either vehicle (SV) (25% DMSO in saline) or corticosterone (SC) (25 mg/kg). The other animals were exercisers and were also subdivided to receive either vehicle (EV) (25% DMSO in saline) or corticosterone (EC) (25 mg/kg).

Figure 1.2 – Acclimation protocol to treadmills. All animals (exercise and sedentary groups) were acclimated to the treadmills in the week leading up to the day of experiments in order for them to be able to adequately perform the exercise on the day of experiment. On day 1, animals exercised at a pace of 10 m/min for 10 minutes. They had a rest day and on day 3 were acclimated again, but the speed was increased to 20 m/min. This exercise lasted for 20 minutes. Animals had an additional rest day before performing their final acclimation training on day 5, where they exercised at 20 m/min for 30 minutes.
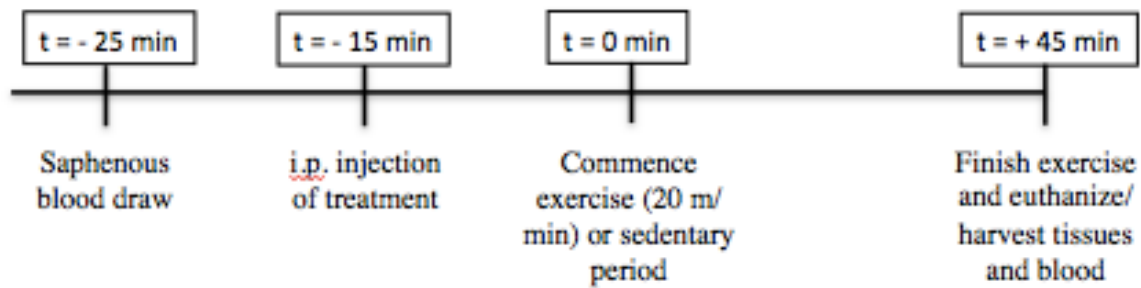
Figure 1.3 – Experimental protocol. Animals underwent a saphenous blood draw at t = -25 min. At t = -15 min, animals received an i.p. injection of their treatment. Animals were immediately placed inside the metabolic treadmills. At t = 0 min, after a basal RER value was established, exercise commenced for EV and EC groups. At this time, SV and SC animals remained on the inactive treadmills with them turned off. At t = +45 min, animals were euthanized and tissues and post-treatment blood samples were collected.

RESULTS

Plasma corticosterone is significantly increased one hour following corticosterone injection. (Fig. 2) In order to test our model and ensure that our corticosterone was sufficiently increased in our groups, a RIA kit was used to measure plasma corticosterone concentrations. Plasma corticosterone concentration was elevated in the groups that received an i.p. injection of corticosterone compared to vehicle controls. There was a main effect of corticosterone treatment to increase plasma corticosterone levels (p<0.0001), as well as an interaction between corticosterone treatment and activity (p<0.0001). Corticosterone trended towards being significantly greater than vehicle in a sedentary state (350.48 ± 63.56 ng/mL, to 600.01 ± 43.59 ng/mL, p = 0.052) and when exercising EC was significantly greater than the EV control (376.54 ± 43.51 ng/mL , 748.01 ± 115.63 ng/mL , p = 0.0005).

Corticosterone fluctuates in RER at rest, but does not have an effect with exercise. (Fig. 3) In the sedentary groups, there was a significant interaction between time and treatment (p = 0.0033) in RER values, but no main effect of treatment or time. While the sedentary group did not fluctuate much throughout the duration of the experiment, the corticosterone group had an initial rise in RER up until t = +10 min, before it proceeded to decline, indicating a shift towards favouring lipid oxidation, which continued until the end of the experiment (t = +45 min). There was no significant interaction between treatment and time in the exercising groups; however, there was a significant effect of time (p<0.0001). Both vehicle and corticosterone-treated animals had a comparable rise in RER after exercise commenced, then gradually declined for the remainder of the exercise.

Non-esterified fatty acid concentration is not significantly altered after 1 hour of corticosterone treatment. (Fig. 4) As a primary measure of lipolysis, an ELISA kit was used to measure NEFA concentration before animals received treatment (t = -25 minutes), as measured from the saphenous vein, as well as at t = +45 minutes, with blood collected from the trunk during euthanization. Pre-treatment values were used to express a range of normal NEFA concentrations for healthy controls. There were no differences in NEFA concentrations between sedentary and exercising groups, additionally, there was no significant effect of corticosterone treatment.

Peritoneal lipolytic enzyme activity in corticosterone treated animals is affected by activity. (Fig. 5) Western blot analyses were performed on the peritoneal fat depot to determine the activity of the major lipolytic enzymes in the fat pad. All measurements were analyzed using tissues collected one hour following corticosterone treatment (t = +45 minutes). Phosphorylated HSL was made relative to total HSL in order to get a measure of its activity and a phosphorylated PKA substrate-indicating antibody was made relative to total perilipin in order to assess perilipin activity. All samples are represented as a fold change from the control (SV) on the gel they were run on. Both HSL and perilipin activity were not significantly different from the sedentary control (SV). However, perilipin activity in the SC was significantly increased compared to the EC group (p = 0.0469).

Blood glucose is not altered with 1 hour of corticosterone treatment, but rises with a 45-minute exercise. In order to determine if corticosterone significantly alters blood glucose, blood was collected from the saphenous vein prior to injection at t = -25 minutes (pre-treatment values were used to express a range of normal glycemic concentrations for

healthy controls, indicated as grey shaded area, Fig. 6.1). Additionally, blood glucose was measured by a tail prick at t = +45 minutes. Corticosterone did not alter blood glucose relative to vehicle one hour following injection. It did, however, significantly rise with exercise for both treatment groups (SV; 6.82 ± 0.70, SC; 6.08 ± 0.69, EV; 10.3 ± 1.57, EC; 10.8 ± 1.27, $p < 0.0001$).

Plasma insulin concentration is not significantly altered with corticosterone treatment or 45 minutes of exercise. (Fig. 6.2) GCs are consistently associated with the development of insulin resistance with chronic exposure, but their immediate interactions have not yet been examined. No significant different occurred between any of the groups. All insulin values were measured using plasma from trunk blood (t = +45 minutes). Pre-treatment values were used to express a range of normal insulin concentrations for healthy controls, as indicated by the grey shaded area.

Plasma glucagon increases with corticosterone treatment at rest, but no difference in exercising animals. (Fig. 6.3) The rapid effects of glucocorticoids on glucagon are unknown, so to examine this an ELISA kit was used to assess plasma glucagon measurements. There was a main effect of treatment ($p = 0.0032$), but not activity. In a sedentary state, plasma glucagon in corticosterone treated animals was approximately 3-fold higher than sedentary controls (SV; 20.23 ± 3.81, SC; 53.45 ± 8.65 pmol/L, p <0.0226). There were no detectable changes between the corticosterone versus vehicle treatment in exercising animals.

DISCUSSION

Glucocorticoids are consistently considered to be lipolytic due to their ability to increase the expression of lipolytic enzymes. Acute models of hypercortisolemia have shown that GCs induce lipolysis in isolated adipocytes, in rodents and in humans, yet taken together with the increased adiposity that occurs with hypercortisolemia or CS patients, makes the picture less clear. Additionally, it has also been found that CS patients have reduced NEFA turnover compared to controls[94]. Also, while some studies have found conflicting data, these were not found to be significant[95]. Currently, *in vivo* studies examining the rapid, non-genomic effects of GCs are lacking in vivo. Recent work in cell culture models indicate that it is possible that GCs initially induce anti-lipolytic effects after as little as 30 minutes to one hour, as indicated by a reduction in glycerol release with corticosterone treated 3T3-L1 cells[12]. In another model, DEX treatment did not increase free fatty acid release in isolated adipocytes, while it did when examining the entire fat pad[91]. For this reason, we designed our model to determine if the same response would be seen *in vivo*. Our treatment successfully altered the amount of circulating corticosterone between groups (Fig. 2). When comparing our sedentary groups, there was more fluctuation with corticosterone treatment in RER response. GC treated animals had an initial rise in RER up until t = +10 min, before shifting towards a decrease in RER until the end of the experiment (Fig. 3A). This shift towards a decrease in RER indicates a favouring of lipid oxidation over CHO utilization[56]. This drop in RER did not reach significance when compared to the SV, but it would be beneficial to examine determine if this group would reach significance after increasing the number of subjects. When examining NEFA concentrations, however, there was no change 1 hour

following corticosterone injection (Fig. 4). Considering that this was a static measurement of assessing NEFA content, it is possible that the flux of NEFAs is altered with corticosterone, but is perhaps matched by increases in oxidation, maintaining the plasma NEFA content comparable to controls. Despite this, there was an increase in perilipin (but not HSL) activity in the SC compared to the EC group, appearing to have an increased phosphorylation in the peritonenal adipose tissue. It is important to note, however, that this trend did not reach significance when compared to the SV group. Previously, it has been reported that it requires 4-6 hours before seeing the effects of GCs, due to the delay required to see any receptor mediated genomic changes[11,42]. However, this trend would suggest a possible immediate non-genomic response to increased lipolysis by enhancing lipolytic enzyme phosphorylation. It is still unclear, however, if these changes were a direct response to increased GCs, or if they were an indirect effect of other hormones that may have altered regulation by GCs. Catecholamines, for example, is often associated with a rise in GCs as they are both released as part of the stress response [3]. Epinephrine and norepinephrine are known to have more immediate effects than GCs. Exercise studies comparing healthy controls to paraplegic individuals (impaired SNS activity) have demonstrated that catecholamine release may be the driving inducer of a lipolytic response with exercise[161]. This may not give the full picture though; treatment with propranerol, a β-AR blocker, only partially inhibits lipolysis during exercise[162]. Further investigation into the specific mechanisms involved in this increase in lipolytic action during exercise is still required.

As exercise is marked by a sharp increase in metabolic demand, it places the body in a state of stress. A rise in fuel mobilization occurs in order to meet these demands. For

decades, it has generally been accepted that GCs induce a rise in FFAs during exercise[13,152], however, no study has objectively measured this and any increases in NEFAs or glucose may be due to the immediate actions of catecholamines with exercise. Our data indicates that there is no reason to believe that corticosterone is lipolytic in an acute state of exercise. No differences in RER were observed when comparing EV to EC during exercise (Fig. 3B). A trend towards an initially reduced RER with corticosterone treatment was observed. After further examination of all baseline RER points (Fig. S3.0), it was determined that there was an effect of both treatment and time, but there were no specific time points in which the groups different. Additionally, there were no significant differences between plasma NEFA concentrations of EV versus EC animals (Fig. 4). Although we expected a rise in NEFAs with exercise compared to the sedentary controls, our results indicated no change between the groups. This is potentially due to the fact that any increase in mobilization of NEFAs may have been matched by oxidized in the working muscles, thereby maintaining plasma levels at a normal volume. Looking at the lipolytic activity of the adipose tissue further confirms that enhanced lipolysis is not occurring with the moderate to high dosage of corticosterone treatment (20mg/kg) in this time frame. If anything, we observed that exercise is impairing any lipolytic activity that may have been induced by corticosterone treatment at rest, as indicated by significantly reduced PLIN phosphorylation in the EC versus SC group. This change was not observed when analyzing HSL activity. When compared to the control exercisers (EV), there were no differences in phosphorylation of either PLIN or HSL, indicating that any effects of corticosterone may be masked with exercise. As expected, there was an effect of exercise compared to the sedentary vehicle group to increase lipolytic enzyme activity.

Consistently GCs are associated with an increase in hyperglycaemia. Chronic models of hypercortisolemia result in increased fasting blood glucose and insulin resistance. It has previously been reported that a 3-day treatment of dexamethasone (2mg/day) in humans results in a dramatic increase (55-110%) in glucagon secretion[163] and rodent models have determined that these adaptations are likely due to changes in alpha cell mass[79]. Once again, less data is available on the immediate effects of GCs, specifically with exercise. Our data demonstrated that short-term treatment with GCs had no effect on blood glucose, although there was an increase with exercise (Fig. 6.1). Plasma insulin was unaffected by corticosterone treatment and surprisingly also unaffected by exercise (Fig. 6.2). Normally, in rodents and in humans, insulin levels drop with prolonged exercise while counter-regulatory hormone levels rise [164,165]. In the sedentary groups, EC increased glucagon compared to EV, but no differences were observed in glucagon between the exercise groups. This data indicates that it is plausible that GCs increase blood glucose concentrations through inducing a rise in glucagon at rest (Fig. 6.3), but it is possible that our protocol did not allow for glucagon to stimulate and increase in blood glucose, and perhaps we would seen this increase if we increased our protocol to measure up to t = +120 minutes. According to our RER data, the SC at the end of the experiment appeared to be relying more heavily on FFA oxidation, so it is not likely that enhanced oxidation of the CHOs are responsible for the lack of changes observed in BG at rest.

Taken all together, it is likely that corticosterone may induce immediate lipolytic actions at rest. Our data determined that corticosterone treated animals had more variability in RER when sedentary, potentially mediated through altered lipolytic enzyme

activity. It may also increase glycaemia, but these changes may take longer to occur, considering that plasma glucagon, but not blood glucose concentrations were increased. In an acute exercise state, it appears that there are no differences in lipolytic actions with GC treatment, considering that no differences were observed in oxidation after exercise between EV and EC and there was reduced PLIN activity in EC compared to SC. Further examination at additional time points would be beneficial to further understand the immediate actions of GCs.
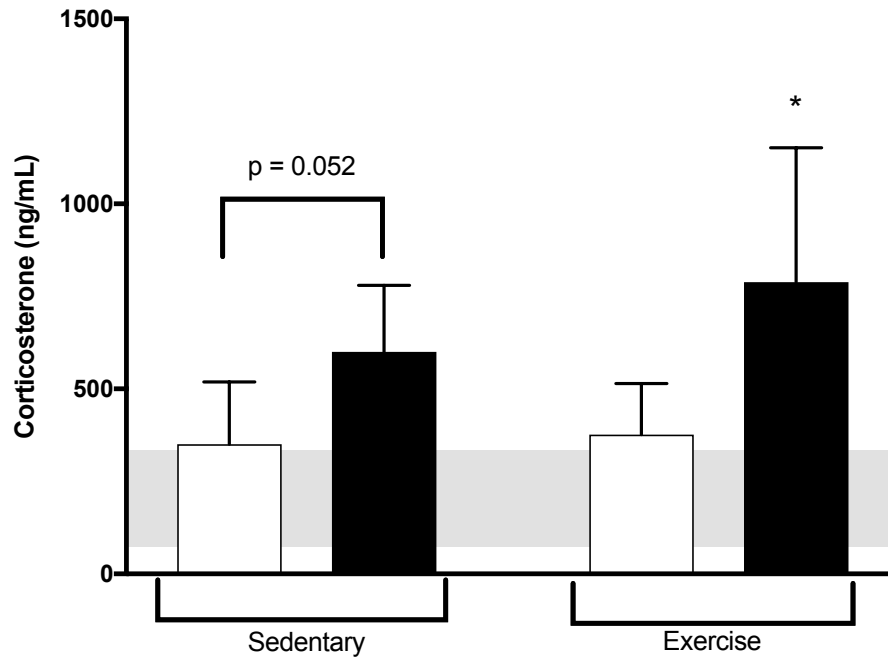
FIGURES

Figure 2; Plasma corticosterone content. White box indicates vehicle treatment, shaded boxes indicate corticosterone. The light grey box denotes the range of the pre-treatment mean ± standard deviation. Pre-treatment plasma samples were collected via saphenous vein, post-treatment were collected from the trunk blood. A main effect of corticosterone treatment (P<0.0001) and an interaction between treatment and activity were observed (p<0.0001). * indicates p = 0.0005. SV, n = 7; SC, n = 13; EV, n = 10; EC, n= 10.
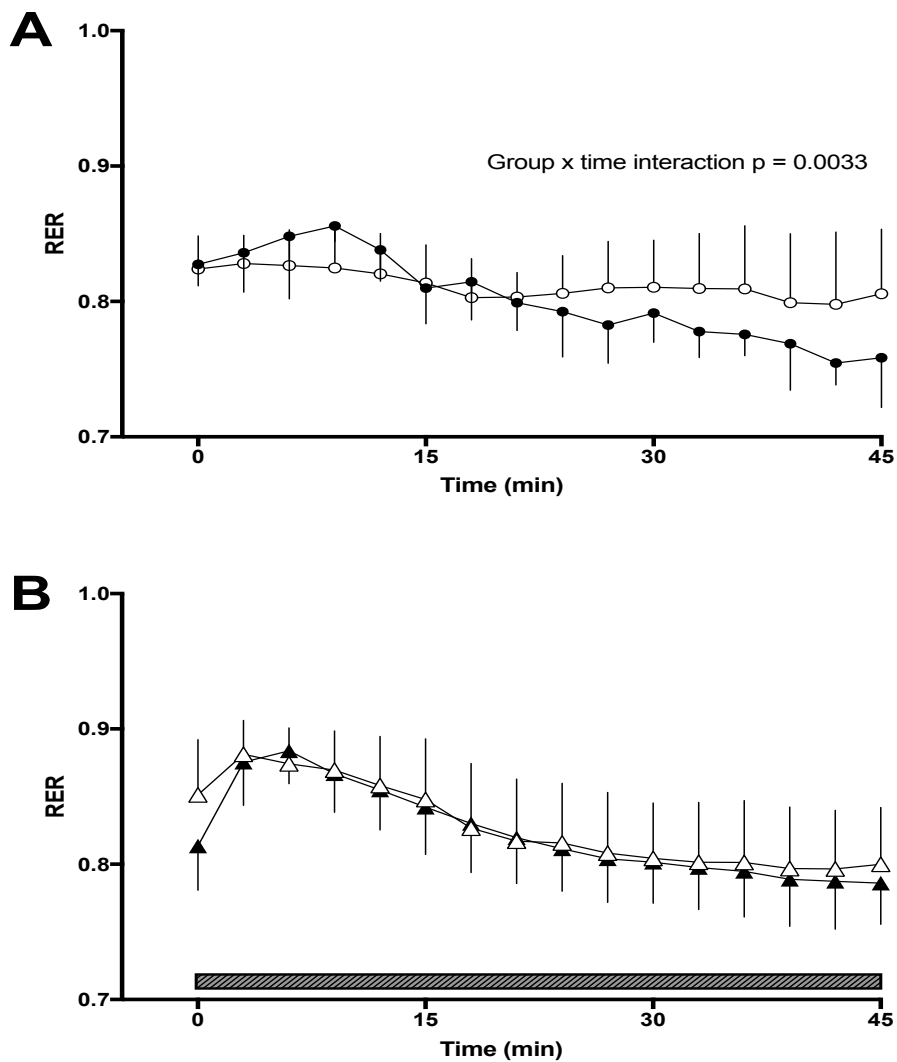
Figure 3.0; Respiratory exchange ratio; hashed box indicates exercise period. Corticosterone animals are indicated with black symbols, white symbols indicate vehicle. A) RER in sedentary animals. There was a significant interaction between treatment and time (p = 0.0033), but no main effect of treatment. B) RER with exercise. No effect of treatment or interaction between treatment and time occurred, but there was a significant effect of time (p<0.0001). SV, n = 6, SC, n = 4, EV, n = 10, EC, n = 10.
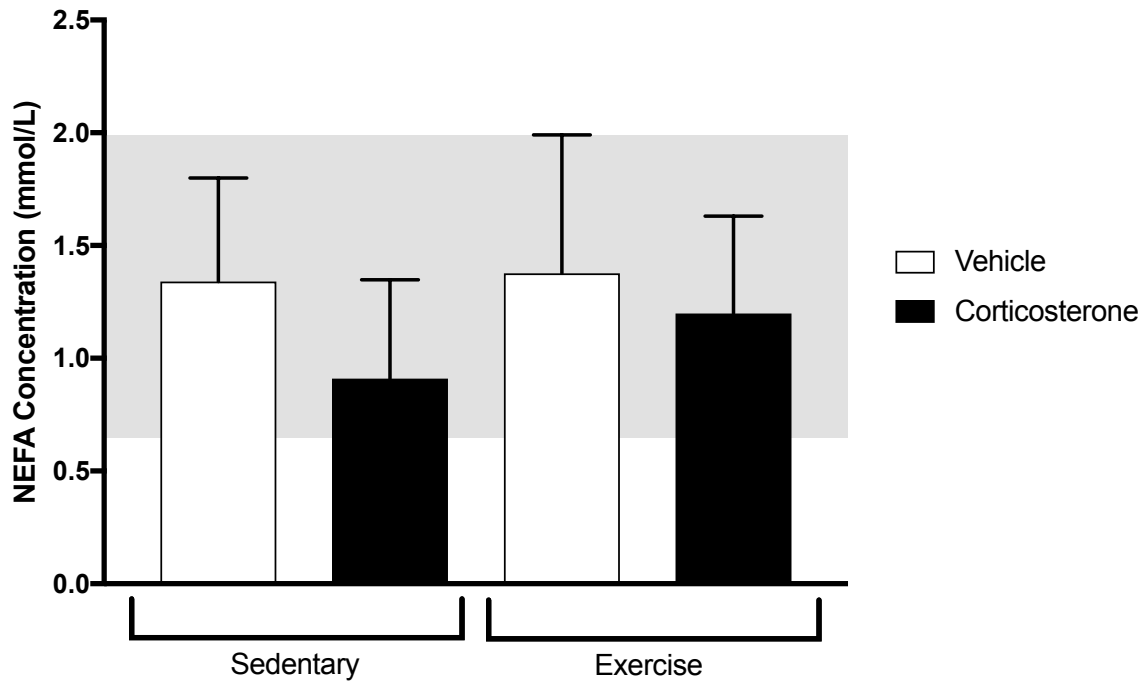
Figure 4.0. Plasma Non-esterified fatty acid content. White box indicates vehicle treatment, shaded boxes indicate corticosterone. The light grey box denotes the range of the pre-treatment average ± standard deviation. Pre-treatment plasma samples were collected via saphenous vein, post-treatment were collected from the trunk blood under isoflurane. There were no significant effects of treatment or activity. SV, n = 7; SC, n = 13; EV, n = 10; EC, n =10.
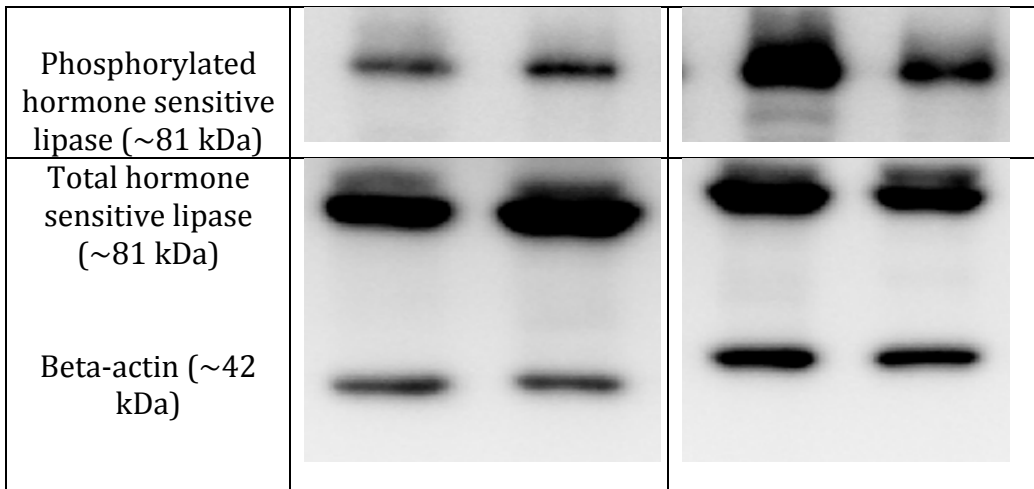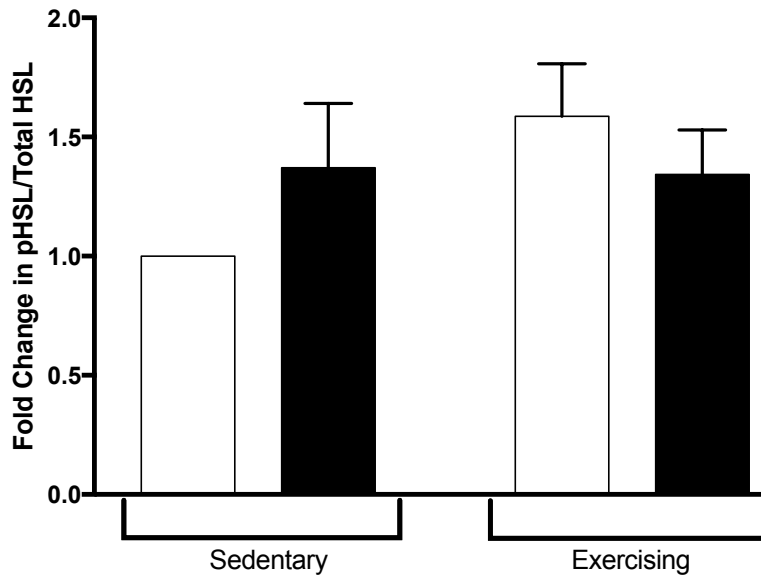
Figure 5.1. Lipolytic enzyme measures; Hormone sensitive lipase (HSL) Activity.
Lipolytic enzyme activities as measured by western blot. HSL activity was measured using a phosphorylated HSL antibody relative to total HSL in the peritoneal fat depot. White boxes indicate vehicle treatment, while shaded boxes indicate corticosterone. All samples are made relative to the sedentary control on the gel they were run on. There were no significant effects of treatment or activity. SV, n = 6; SC, n = 8; EV, n = 10; EC, n =10.

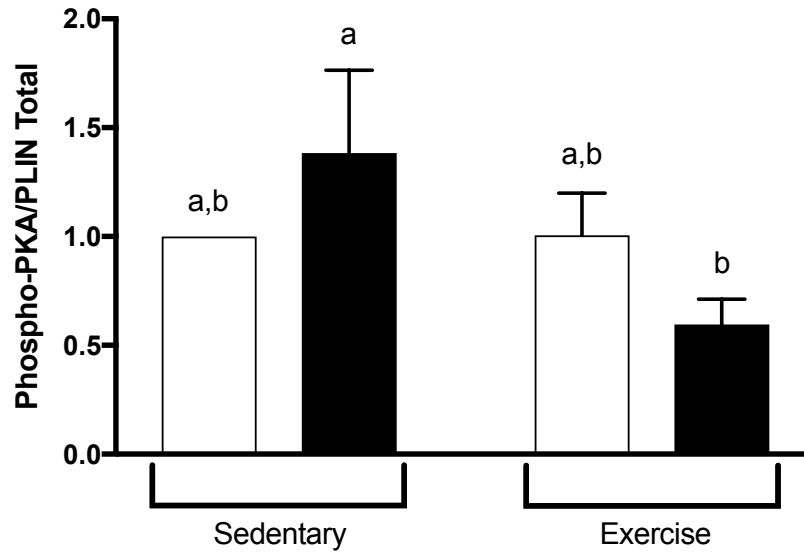**Figure 5.2. Lipolytic enzyme measures; Perilipin Activity.** Lipolytic enzyme activities as measured by western blot. Perilipin activity was measured using a phosphorylated PKA substrate indicator relative to total Perilipin in the peritoneal fat depot. White boxes indicate vehicle treatment, while shaded boxes indicate corticosterone. All samples are made relative to the sedentary control on the gel they were run on. SC was significantly different from EC (p = 0.047). SV, n = 6; SC, n = 8; EV, n = 10; EC, n =10.

Figure 6.1; Measures of Glycaemia - blood glucose concentrations. White box indicates vehicle treatment, shaded boxes indicate corticosterone. The light grey box denotes a normal blood glucose range, derived of the pre-treatment average ± standard deviation. Pre-treatment blood samples were measured during a blood draw from the saphenous vein and post-treatment were measured via tail-prick. There was significant effect of exercise, but not treatment (p<0.0001). SV, n = 7; SC, n = 13; EV, n = 7; EC, n = 9.

Figure 6.2. Measures of Glycaemia - plasma insulin concentrations. White box indicates vehicle treatment, shaded boxes indicate corticosterone. The light grey box denotes the range of the pre-treatment average ± standard deviation. Pre-treatment plasma samples were collected via saphenous vein, post-treatment were collected from the trunk blood under isoflurane. There were no significant effects of treatment or activity. SV, n = 7; SC, n = 13; EV, n = 8; EC, n = 8.
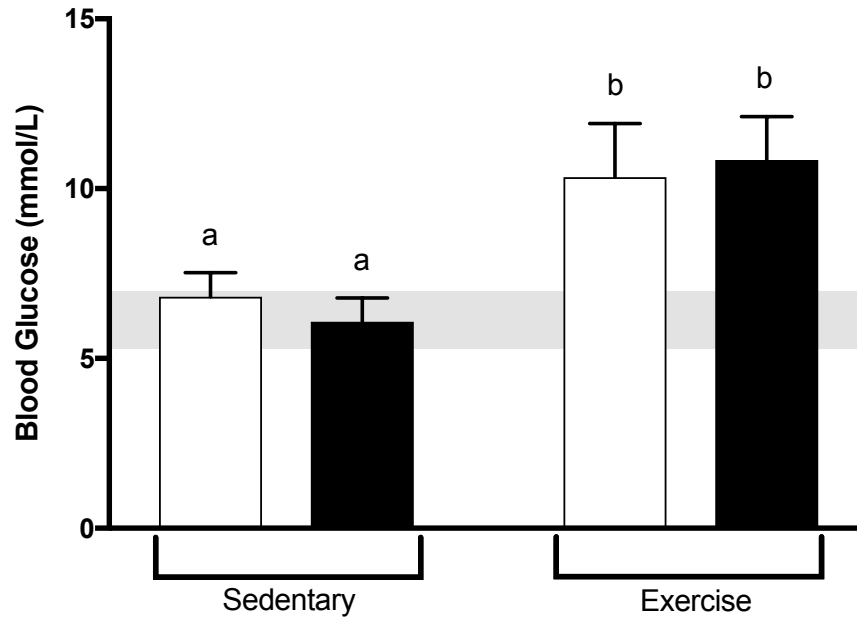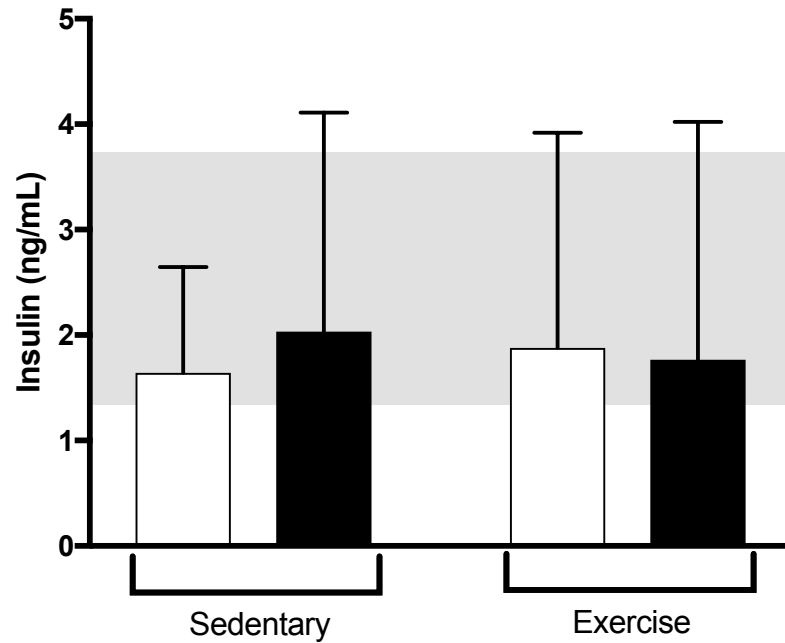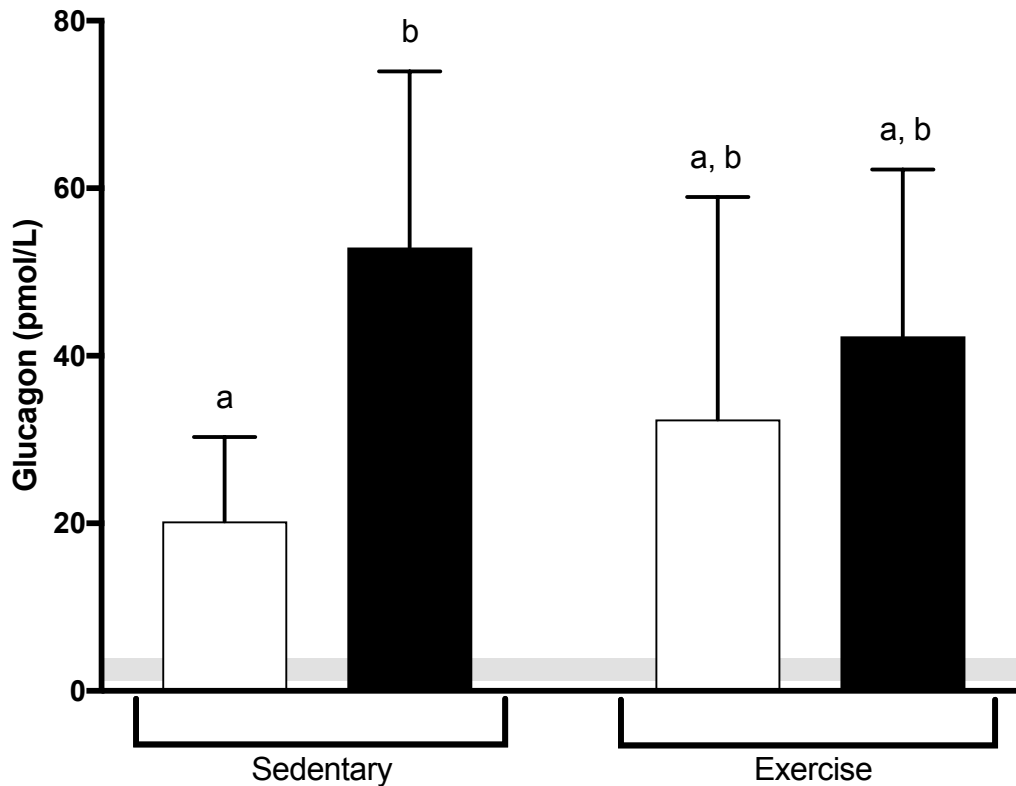
Figure 6.3. Measures of Glycaemia - plasma glucagon concentration. White box indicates vehicle treatment, shaded boxes indicate corticosterone. The light grey box denotes the range of the pre-treatment average ± standard deviation. Pre-treatment plasma samples were collected via saphenous vein, post-treatment were collected from the trunk blood under isoflurane. There was a significant effect of treatment (p = 0.0032), but not activity. SV was significantly lower than SC (p = 0.0226). SV, n = 7; SC, n = 13; EV, n = 8; EC, n = 8. .

5.0 SUMMARY, LIMITATIONS AND FUTURE DIRECTIONS

Our findings indicate that it would be beneficial to take a closer examination at additional time points. For instance, tissue analysis was only taken one hour following treatment (t = +45 minutes), but further investigation is warranted at 25 minutes following injection to determine if there are any immediate anti-lipolytic actions occurring, as evidenced by the rise in RER, indicating a favouring of CHO oxidation. Additionally, it would be beneficial to carry out this study for another hour (total t = +120 minutes), and see if the trends in RER would continue and any changes would occur in the phosphorylation of lipolytic enzymes or plasma NEFA levels.

As well as this, no initial VO_2Max test was conducted to determine the exact intensity for each rat. Instead, a running speed that had been previously used in other studies as a light to moderate intensity was chosen[166]. While moderate exercise does not usually alter blood glucose in healthy controls after just 45 minutes of light/moderate activity, intense exercise has been shown to increase glycaemia[167]. Considering that there was an effect of exercise to increase blood glucose in our data, it is possible that these animals were running at a higher intensity than anticipated. For this reason, a preliminary VO_2Max test would be recommended if this model were to be repeated.

When examining RER, there were some trends towards significant differences between the sedentary groups, such as at t = +10 minutes (p = 0.079). A possible reason we did not find significance is that the sedentary corticosterone group was underpowered (n=4) and the addition of more animals in this group would be beneficial. In Fig, S2.0, we examined the metabolic parameters of only the all animals that we had RER data. All of these graphs followed the same pattern as the graphs examining the full number of

subjects for these groups. However, with this smaller n, there was no main effect of corticosterone to influence plasma glucagon (Fig. S2.0C).

While this was a successful model of examining the effects of a rapid increase in corticosterone *in vivo*, one of the most significant limitations of the study was that handling stress may have elicited a stress response in all animals, as indicated by a rise in corticosterone from the pre-treatment value (Fig. 2.0). These animals were not adrenalectomized and the i.p. injection of their treatment may have caused an increase in both corticosterone release, as well as epinephrine. Additionally, we could not prevent the rise in these hormones with exercise.

One of the most primary markers of lipolysis is NEFA release. Although an ELISA kit examining plasma NEFA concentration was performed, this only indicates a static measurement in the plasma. In order to properly examine the dynamics of lipid metabolism, it would be ideal to measure the constant efflux of NEFAs. This may be does by utilizing stable isotopes labelled glycerol or NEFAs, or by measuring the aterio-venous differences in NEFAs with a catheter[168]. Even with this method comes with some limitations; despite the accuracy of measuring flux, the aterio-venous difference can only be measured in subcutaneous tissue and could not provide information on omenal adipose lipolysis. In order to counter this, we used measures of oxidation (RER) and compared them with molecular measures of lipolysis (western blotting of lipolytic enzymes).

Overall, our study suggests it is possible that 1 hour following corticosterone treatment may enhance lipolytic activity and NEFA oxidation. Contrary to many exercise physiology textbooks, it appears that further elevating GCs during exercise does not

appear to be mediating any effects on lipolysis. Further research at additional time points and various intensities would be beneficial to gain a better understanding of the immediate actions of GCs.

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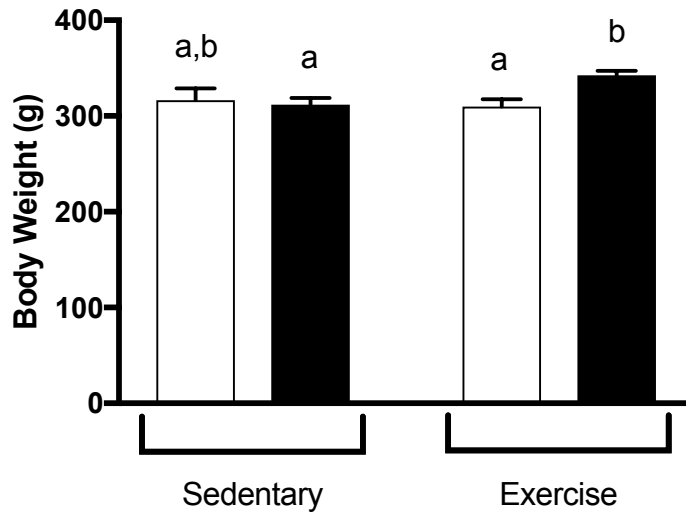
Figure S1.0; Body weight in grams on day of experiment. Vehicle animals are expressed as white box, corticosterone treated animals expressed as shaded box. There was a significant effect of treatment ($p = 0.03$). SC weighed significantly less than EC ($p = 0.004$). SV; $n = 7$, SC; $n = 13$, EV; $n = 10$, EC; $n = 10$.
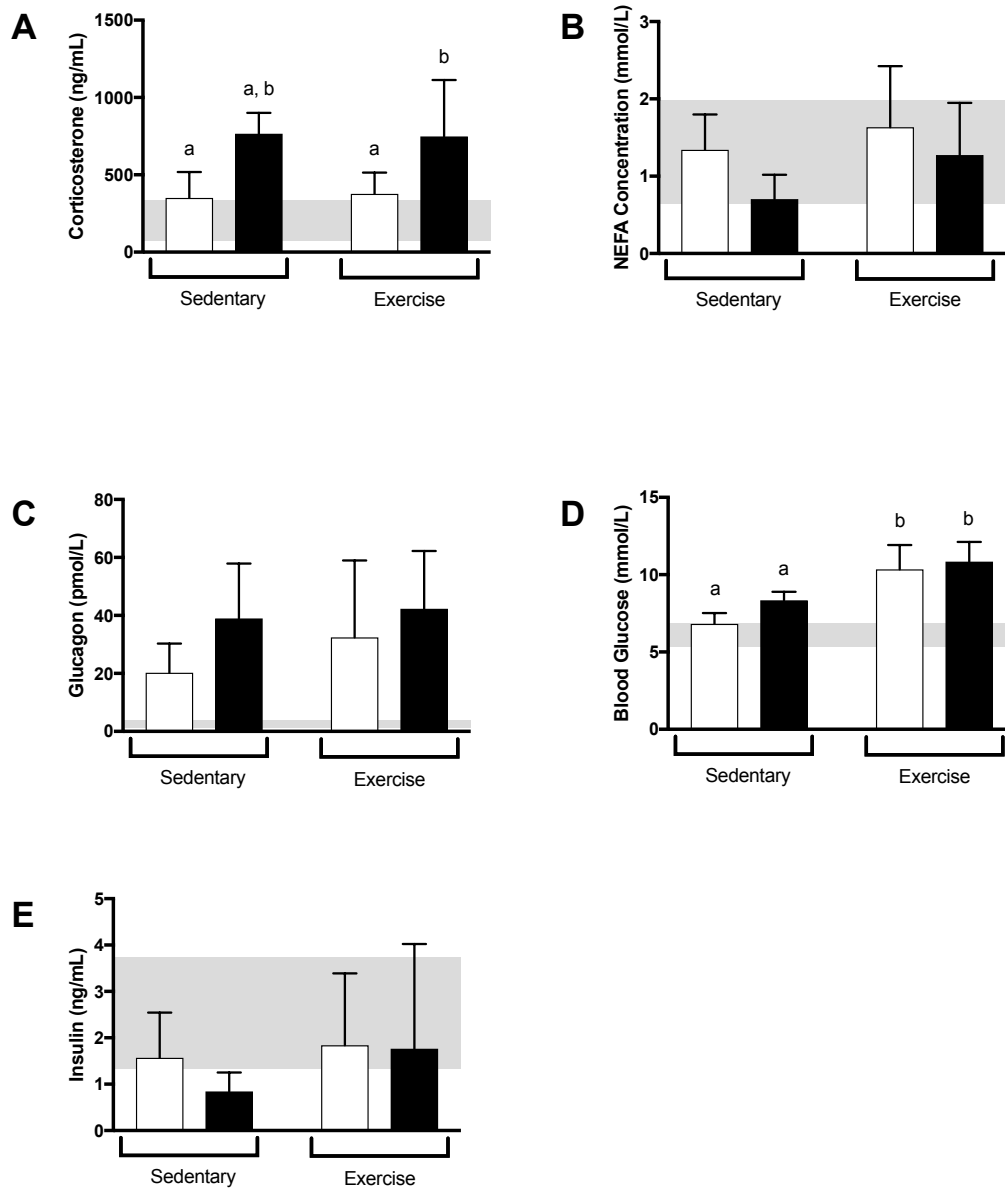
Figure S2.0; Metabolic Data for all animals in which RER data was collected. Vehicle animals are expressed as white box, corticosterone treated animals. Data is represented as mean ± standard deviation. The light grey box denotes the range of the pre-treatment mean ± standard deviation. A) Plasma corticosterone content. Main effect of treatment (p = 0.0003). B) Plasma non-esterified fatty acid content. C) Plasma glucagon content. D) Blood glucose content. Main effect of activity (p<0.0001). E) Plasma insulin content. SV; n = 7, SC; n = 4, EV; n = 10, EC; n = 10.
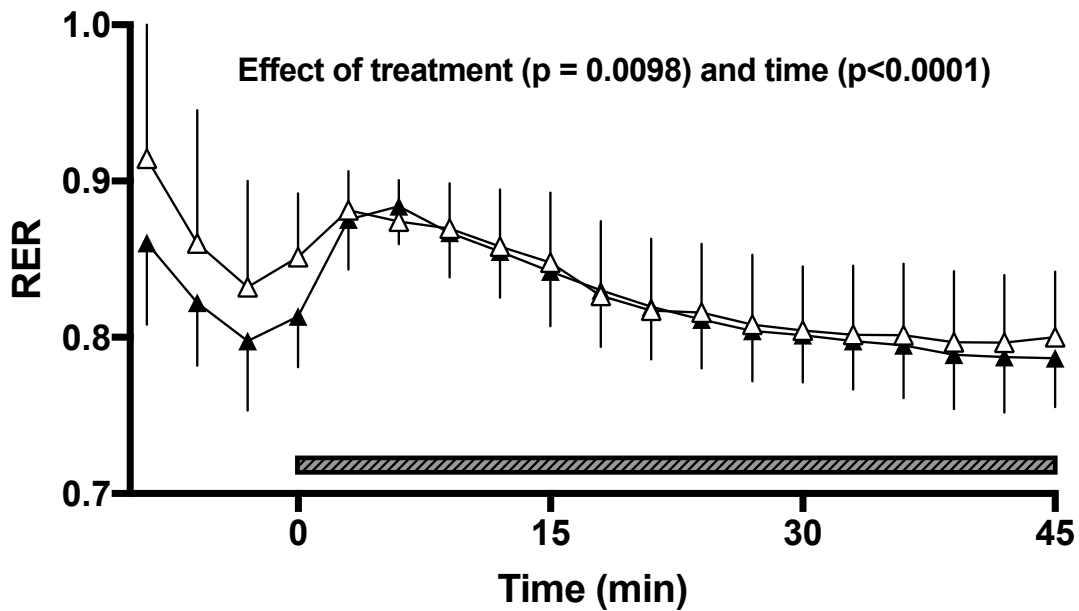
Figure S3.0; Respiratory exchange ratio for exercising animals at all time points; hashed box indicates exercise period. Corticosterone animals are indicated with black symbols, white symbols indicate vehicle. Data points represent mean RER over 3 minutes with standard deviation. There was an effect of treatment (p = 0.0098), and an effect of time (p<0.0001), but no interaction. EV, n = 10, EC, n = 10.