ACUTE ANTILIPOLYTIC EFFECTS OF CORTICOSTERONE IN ADIPOCYTES

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ABSTRACT

Background: Individuals with elevated glucocorticoid (GC) levels tend to develop excess central adiposity; however this opposes the well known catabolic actions of GCs. Evidence suggests that in the long term GCs increase lipolysis, however they may also have acute inhibitory effects. **Purpose:** We investigate the acute effects of GCs on adipocytes and potential mechanisms of action. **Methods:** 3T3-L1 adipocytes were treated with corticosterone (Cort; 0-100μM) for 0-4 hours to analyze effects of Cort alone, or in combination with known lipolytic stimulants. **Results:** Cort produced a dose dependent decrease in basal, as well as stimulated lipolysis. These effects were most potent at doses ≥10μM, doses that represent those likely found within the tissue. Decreased PKA activity and reductions in the phosphorylation of HSL and perilipin appear responsible for Cort's antilipolytic effects. **Conclusions:** GCs have acute inhibitory effects on adipocyte lipolysis, and this may play a role in GC related adipose accumulation.

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LIST OF ABBREVIATIONS

5' Adenosine Monophosphate-Activated Protein Kinase (AMPK)
Adipose Triglyceride Lipase (ATGL)
Adrenocorticotropin Hormone (ACTH)
Bovine Serum Albumin (BSA)
Corticosterone (Cort)
Corticotropin Releasing Hormone (CRH)
Cortisol Binding Globulin (CBG)
Cyclic Adenosine Monophosphate (cAMP)
Diacylglycerol lipase (DAG)
Dulbecco's Modified Eagle Medium (DMEM)
Enzyme-linked Immunosorbant Assay (EIA)
Extracellular Signal-Regulated Protein Kinases (ERK)
Fatty Acid (FA)
Fetal Bovine Serum (FBS)
Glucocorticoid (GC)
Glucocorticoid Receptor (GR)
Glucocorticoid Response Element (GRE)
Growth Hormone (GH)
Hypothalamic-Pituitary-Adrenal (HPA)
Hormone Sensitive Lipase (HSL)
Lipoprotein Lipase (LPL)
Mitogen Activated Protein Kinase (MAPK)
Mitogen Activated Protein Kinase phosphatase-1 (MPK-1)
Monoacylglycerol (MAG)
Monoacylglycerol lipase (MGL)
Non-Esterified Fatty Acid (NEFA)
Phosphatidylinositol 3-Kinase (PI3-K)
Phosphodiesterase (PDE)
Protein Kinase A (PKA)
Triacylglcyerol/Triglyceride (TAG)
Type 2 Diabetes Mellitus (T2DM)
11-dehydrocorticosterone (11DHC)
11ß-hydroxysteroid Dehydrogenase (11ß-HSD)

INTRODUCTION 1

Adipose tissue plays an important regulatory role in substrate metabolism by storing and liberating energy as the body requires. During times when food is plentiful and activity is low, energy is stored within adipose tissue in the form of triglycerides. When an energy deficit occurs, such as during exercise or fasting, triglycerides are broken down and substrates (fatty acids and glycerol) are released into circulation. This release helps to supply the organism's energy demands. Balance usually exists between these two states, but dysregulation can occur, leading to either an excessive storage of fat within adipose tissue or excessive depletion in states of catabolism. One of the key regulators of energy flux within adipocytes are the stress hormones; glucocorticoids (i.e. cortisol in human, corticosterone in rodents); and catecholamines (i.e. epinephrine). In humans (55) and in rodents (43), stress and/or stress hormones have been widely cited as contributing to excess adiposity, particularly in the abdominal regions, despite the observations that these hormones have lipolytic actions in isolated conditions (74). This accumulation of adipose tissue, especially in the visceral adipose depot, is associated with many diseases, including type 2 Diabetes Mellitus (T2DM). Understanding the effects of stress hormones on the regulation of adipose tissue metabolism is critical in determining why these clinical links between stress and/or stress hormones and excess adiposity exist. There are several ways in which stress hormones could increase adipose accumulation; elevated caloric intake, increased adipogenesis or lipogenesis, or alterations in lipid breakdown. Our investigation will focus on the role of glucocorticoids on lipolysis, a

topic with conflicting findings in the literature, and how this may affect lipid accumulation.

LITERATURE REVIEW 2

2.1 Glucocorticoids and the Stress Response:

2.1.1 HPA Axis Activation:

Regardless of whether a stressor is psychological or physiological in nature, the body responds in much the same way. The brain perceives the stressor and begins a signaling cascade which results in the release of glucocorticoids (GC) (cortisol in humans and coticosterone (Cort) in rodents) and catecholamines (epinephrine) from the adrenal gland. Catecholamines are released from the adrenal medulla as a result of direct sympathetic innervation, while activation of the hypothalamic-pituitary-adrenal (HPA) axis results in GC release. The HPA axis is activated when a stressor is perceived, and the response begins with the release of corticotropin releasing hormone (CRH) from the paraventricular nucleus in the hypothalamus. CRH travels to the anterior pituitary where it stimulates the release of adrenocorticotropin hormone (ACTH). The binding of ACTH to receptors in the adrenal cortex induces GC synthesis and release.

The release of stress hormones allows the body to maintain homeostasis during times of stress (61). When a stressor arises, the tissues increase their metabolic needs and thus the energy demands of the body are elevated. GCs and catecholamines act to supply this energy as they liberate energy substrates such as glucose, amino acids, glycerol and fatty acids (FA) from the tissues. This catabolic action is the typically accepted role of stress hormones. In terms of adipose tissue, it is believed that stress hormones liberate

energy substrates through increases in lipolysis. Although the lipolytic effects of catecholamines are well established, the effects of glucocorticoids on lipolysis is a matter of controversy (9, 18, 47, 74).

2.1.2 Glucocorticoids:

Cortisol and Cort are the main active GCs secreted by the adrenal cortex in humans and rodents, respectively, and their biological effects are thought to be identical. Each is synthesized from a cholesterol precursor in the adrenal cortex, and consists of 3 cyclohexane rings and a pentane ring, but differ by one hydroxyl group. Rodents lack 17αhydroxylase, an essential enzyme for the conversion of cholesterol to cortisol, and thus are unable to produce cortisol (34). Upon release from the adrenals, the lipophilic GCs circulate bound to cortisol-binding globulin (CBG) or albumin. These plasma binding proteins act as a buffer to regulate the amount of free GC in circulation; bound GCs are not biologically active. Less than 10% of circulating GC exists in a free form and it is this fraction that is biologically active and capable of producing effects (46). GCs bind to CBG through covalent bonds that allow for their release when circulating free GC levels are reduced, and this helps to maintain constant free GC levels.

Although GCs are most well known for their effects in the stressed state, it is now clear that even in unstressed conditions normal diurnal fluctuations in GCs occur that help to maintain regulation over a variety of physiological processes. GC levels are elevated in the morning and low in the evening for humans, while they are the opposite in nocturnal rodents. These fluctuations are important in the regulation of immune function, growth, metabolism and behavior. Normal cortisol levels are between 5-15.2μg/dl (~.15-

.5uM) in the plasma of healthy patients (32), and healthy rodents have shown normal plasma Cort values that peak around 300-1000ng/ml (~1-3μM) (8-11, 20). GC levels may be increased for several reasons: elevated stress levels, exogenous GC treatment, or due to Cushing's Syndrome. Individuals with this syndrome may also have increased GC reactivation within certain tissues (central adipose, liver and muscle), potentiating the effects there.

Considerable evidence over the last 15 years suggests that GC levels within select tissue may by quite different than that circulating in the plasma (70). When GCs are released, both active and inactive forms of the hormone are sent into circulation. Inactive GCs, cortisone in humans and 11-dehydrocorticosterone (11DHC) in rodents, travel freely in the plasma (i.e. unbound to carrier proteins), and the tissues possess varying levels of activating enzymes that are able to convert between the active and inactive forms. Two microsomal enzymes, collectively referred to as the 11β-hydroxysteroid dehydrogenase (11βHSD) system, interconvert receptor-active cortisol and inert cortisone, and Cort and 11DHC. Through intracellular GC amplification or inactivation, 11βHSD represents an additional regulatory step prior to active GCs binding to their intracellular receptors. 11β-HSD type 1 (11β-HSD1) is found predominantly in the liver, brain, skeletal muscle and adipose tissue where it activates inactive cortisone and 11DHC. This amplifies the amount of active GC in the given tissue, and increases GC's actions there. 11β-HSD type 2 (11β-HSD2) is found predominantly in the kidney where it works to inactivate GCs, potentially acting as a protective mechanism to prevent tissue overexposure. Although it is difficult to adequately measure tissue levels of GCs,

Masuzaki et al. have suggested that adipose tissue may have GC levels that are at least 10-15 times that of circulating levels due to the actions of 11βHSD1 (43).

2.1.3 Stress Hormones' Mechanisms of Action:

The mechanisms of action for catecholamines and GCs are quite different. While catecholamines have immediate effects of short duration, the effects of GCs may take 6 hours to appear and last longer (46). Catecholamines have their effects by binding to G-protein coupled beta-adrenergic receptors on the cell membrane. This binding initiates an intracellular signaling cascade and allows for immediate and non-genomic effects. GCs, in contrast, are primarily believed to have genomic effects, either activating or repressing gene transcription, and these effects are thought to be mediated through the ubiquitously located intracellular glucocorticoid receptors (GR) (79). By altering protein expression GCs' effects are thought to take time to develop, but will last even when the hormone is no longer present (17). It is possible, however, that GCs also have non-genomic effects, immediate actions that do not affect gene transcription and may involve changes in phosphorylation states of some key signaling molecules (reviewed in (26)). Although non-genomic effects have been described in other tissues for a variety of functions they are not well understood in adipose tissue, and have not been investigated in regards to lipolysis, and thus will be a focus of this thesis work.

2.1.3.1 The Glucocorticoid Receptor and Traditional Genomic Mechanisms:

Most of GCs' effects are believed to be genomic, occurring due to alterations in gene transcription, and are regulated by the GR (reviewed in (15)). When not bound to its ligand, GR exists in the cytoplasm as part of a multi-protein complex that includes heat

shock proteins and immunophilins. The GR contains a ligand binding domain as well as a DNA binding domain. Upon GC binding, GR undergoes a conformational change and dissociates from the protein chaperones before translocating to the nucleus. Once in the nucleus, GR binds to the DNA at GC response elements (GRE) and through the recruitment of co-activators or co-repressors GR can up regulate or down regulate the transcription of target genes. Once GC binds to the GR, this complex binds to the GRE immediately (36), however alterations in protein expression take hours to occur.

Although there has only been one gene for GR discovered, alternate splicing of mRNA produces many isoforms of GR with distinct transcriptional activities and characteristics (40). Tissues express different proportions of each isoform, and this may contribute to tissue specific GC effects (40). More research is needed to gain a better understanding of these different isoforms and their functions in each tissue.

2.1.3.2 Non-Genomic Effects of Glucocorticoids:

Although genomic effects through the GR remain the primary mechanism for GC action, a great deal of evidence now points to the fact that non-genomic mechanisms of action may also exist. Non-genomic effects are those that do not involve changes in gene transcription. In a review by Haller et al. criteria for classifying an effect as non-genomic are described (26). They state that an effect can confidently be described as non-genomic if it 1) occurs rapidly (<5min), 2) is insensitive to blockade of the GR and 3) is insensitive to inhibition of protein synthesis and gene transcription. Haller et al. also review the proposed mechanisms for these non-genomic actions. They suggest that GCs may act in several ways and cause effects through interactions with 1) membrane lipids,

to alter fluidity of the membrane, 2) membrane proteins, altering the function of ion channels and in particular altering Ca^{2+} transport, or neurotransmission, 3) intracytoplasmic proteins, to alter activity of protein kinases, and 4) protein-protein interactions that occur when GCs bind to the GR and cause disassociation of proteins from the receptor complex (26). It is speculated that upon dissociation, the chaperone proteins may undergo protein-protein interactions within the cytosol to stimulate signaling cascades (12). It is also thought that the phosphorylation of GR may be involved in its non-genomic effects (29). GR mediated, non-genomic effects would be sensitive to GR blockade, but would not be affected by the inhibition of transcription (6). Haller et al. also suggest that non-genomic effects may be more obvious with high doses of GCs or in a stimulated state.

Non-genomic effects of GCs have not been previously studied in adipocytes with regards to lipolysis, however, they have been suggested to occur in other cellular functions. For example, GCs are known to affect glucose utilization and transport within adipocytes, and previous work by Livingston et al. found that with high doses of dexamethasone (25-100 uM), glucose transport was significantly reduced within 1 minute of hormone treatment, suggesting that non-genomic actions were mediating the effect (39). Indeed, these authors hypothesized that inhibition of glucose transport comes as a result of GCs' interactions with plasma membrane lipids, although no evidence was provided to substantiate this hypothesis. It has also been suggested that GCs may promote adipogenesis through non-genomic mechanisms (73). In this case, GCs effects were independent of transcription but involved the GR; inhibition of GR with RU486

removed GCs effects, suggesting that non-genomic effects were likely occurring through GR associated protein interactions (73).

2.2 The Stress and Excess Adiposity Link:

Although stress hormones are traditionally thought of as catabolic and lipolytic in nature, they are linked to increases in adipose mass. A recent meta-analysis of longitudinal studies has concluded that perceived psychological stress is a significant, albeit small, risk factor for excess weight gain in humans (68). The link between GCs and excess adiposity is well established clinically, and is clearly demonstrated in individuals with Cushing's Syndrome (66) or those on exogenous corticosteroid treatment (44). These individuals display increased weight gain, visceral adiposity and are at increased risk for developing type 2 Diabetes Mellitus (T2DM). Adipose tissue specific increases in GC levels also lead to increased fat accumulation, further suggesting that GCs have a role in the development of obesity (13, 43).

Increasing levels of 11βHSD1 that produce elevated GC levels in the adipose tissue are also linked to obesity. Elevated levels of 11βHSD1 are found in the adipose depots of obese individuals (13, 53), and increasing 11βHSD1 in a transgenic rodent model leads to increases in GC levels and subsequent increases in adipose tissue mass (43). It has also been reported that both 11βHSD1 (4) and GR levels (43, 54) are higher in visceral compared to subcutaneous adipose depots, with visceral adipocytes showing a greater ability to bind GCs (64). This suggests that GCs have a larger impact in the visceral depot (4) and suggests reasons for the site specific adiposity that results in individuals with elevated GC levels.

Although obvious in the clinical setting, the link between stress hormones and adipose accumulation is difficult to understand physiologically as GCs have long been accepted as lipolytic hormones (17-19, 37, 65, 74). There are several other ways in which adipose accumulation could be increased by GCs. Adipose tissue mass may be accumulated by either hypertrophy or hyperplasia. Increased synthesis and storage of lipids may occur and cause existing adipocytes to hypertrophy, while hyperplasia may occur with increased adipogenesis, the differentiation of preadipocytes into mature adipocytes (3). Indeed, adipose stromal cells, preadipocytes, are stimulated to differentiate into mature adipocytes by both cortisol and dexamethasone in a dose dependent fashion (27). In fact, it appears that GCs are required to induce the differentiation of both human adipose stromal cells as well as 3T3-L1 preadipocytes (27, 49). An increase in adipogenesis is a likely effect of GCs, but if adipogenesis was the sole cause for adiposity, then one would expect that individuals with elevated GC levels would have many, small adipocytes due to hyperplasia. In contrast, evaluation of adipose morphology in Cushing's patients shows that they have enlarged, hypertrophic adipocytes (55), and the same occurs in rodents treated with exogenous GCs (56). Therefore, GCs must also stimulate hypertrophy, through either increased synthesis and storage or decreased breakdown, in addition to hyperplasia.

GCs may promote hypertrophy of the adipocytes in several ways. Research has shown that elevated HPA activity and GC levels tend to increase food intake (67), with a greater number of calories coming from high fat, sweet foods (16). This would increase the amount of circulating triglycerides and FAs available for uptake into the adipose

tissue. GCs may also increase the availability of FAs for adipose uptake by increasing the activity of lipoprotein lipase, the enzyme responsible for the breakdown of circulating triglycerides. GCs do increase the activity of LPL (2, 23, 48) which would suggest that there would be increased FA uptake and storage in adipocytes. How GCs affect lipid storage is less well known, but it appears they may increase lipogenesis (45, 69, 71), but decrease FA re-esterification (30). If GCs do posses strong lipolytic effects they would be expected to oppose these increases in fat uptake, and limit adipose accumulation. Anti-lipolytic effects on the other hand would promote hypertrophy. The following sections will summarize the conflicting effects, and proposed mechanisms of action, of GCs on lipolysis.

2.3 Lipolysis:

2.3.1 Lipase Enzymes:

Lipolysis, the break down of stored lipids, is governed by the lipase enzymes that control the stepwise breakdown of triglycerides (Fig 1). Lipids are stored in the adipose tissue as triacylglycerol (TAG), also referred to as triglycerides, and are composed of 3 fatty acids attached to a glycerol backbone. Lipases break down TAGs resulting in the liberation of FAs and the glycerol molecule. With the removal of one FA, TAGs are converted to the lipid intermediate diacylglycerol and with the removal of two FAs become monoacylglycerol. Adipose triglyceride lipase (ATGL) is predominantly responsible for the conversion of TAGs to diacylglycerol and the release of one FA. Hormone sensitive lipase (HSL) is also capable of breaking down TAGs to

diacylglycerol, but to a lesser extent than ATGL, and is the main lipase involved in the conversion of diacylglycerol to monoacylglycerol. In the basal state, HSL is found in the cytoplasm and has little lipolytic activity. In the stimulated state however, HSL translocates to the lipid droplet and is able to have a great impact on lipolysis (the details of stimulated lipolysis will be discussed further below). Monoacylglycerol lipase is involved in the final stage of lipolysis and is responsible for the breakdown of monoacylglycerol to glycerol and the third FA. Liberated FA can be released from the adipocyte or re-esterified into triglycerides for storage. Glycerol however, must be released as adipocytes do not contain glycerol kinase, an enzyme necessary to phosphorylate and trap it within the cell. Monitoring the amount of glycerol released provides an effective means to monitor lipolysis.

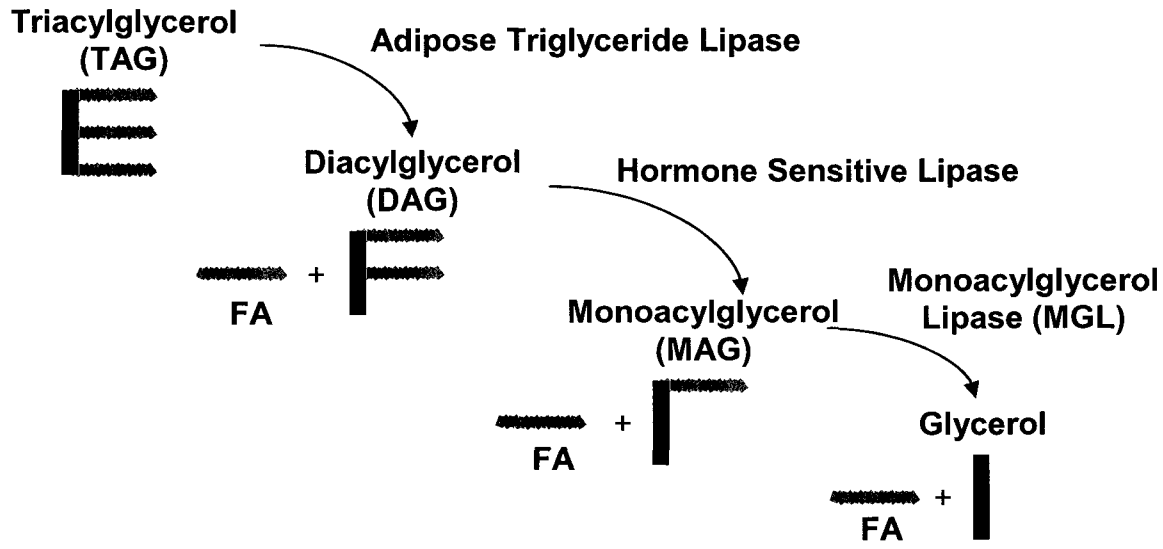

Figure 1: Lipolysis - The stepwise breakdown of lipids by the lipase enzymes
The breakdown of TAGs during lipolysis. The lipase enzymes ATGL, HSL, and monoacylglycerol lipase are responsible for the stepwise breakdown of TAG and the liberation of FAs and glycerol.

2.3.2 ß-Adrenergic Stimulated Lipolysis:

There are several upstream regulators of lipolysis that can affect the efficiency of the lipase enzymes (Fig 2). One of the better defined pathways regulating lipolysis is the ß-adrenergic pathway. Catecholamines are strong stimulators of the ß-adrenergic pathway, and it is through this upstream regulatory pathway that they increase lipolysis in a dramatic fashion. These ß-adrenergic effects on lipolysis occur quickly and allow for a large increase in lipolysis in a short amount of time. Their stimulatory effect is stronger in the visceral adipose tissue than in the subcutaneous (57). Catecholamines bind to ß-adrenergic receptors and initiate a signaling cascade that increases the activity of adenyl cyclase and results in increased cyclic AMP (cAMP) levels. cAMP then activates protein kinase A (PKA) which phosphorylates downstream targets. PKA phosphorylates HSL on Ser659, Ser660, and Ser563, allowing it to translocate to the lipid droplet and initiate its lipase activity (Fig 3) (42). In the basal state, HSL is phosphorylated on Ser565 and has little lipolytic activity. It appears that upon stimulation, HSL is first phosphorylated on Ser660, allowing translocation to the lipid droplet to occur (42). Following translocation, HSL is dephosphorylated at Ser565, and is phosphorylated at Ser563; these two sites cannot be phosphorylated simultaneously (42). PKA also phosphorylates perilipin, a protein that is associated with the lipid droplet in the basal state and impedes lipase access and activity. The expression of perilipin protein is necessary to allow the translocation of HSL, and with phosphorylation of perilipin HSL's lipolytic activity is enhanced (62). It is also suspected that the phosphorylation of perilipin allows for the

release of comparative gene identification 58 (CGI-58), a protein that may then associate with ATGL and increase its lipolytic activity (77,60).

The ß-adrenergic pathway can be inhibited at several stages, to cause decreases in lipolysis. Insulin is one such anabolic hormone that inhibits lipolysis through the activation of phosphodiesterase (PDE). PDE decreases lipase activity through the hydrolysis of cAMP. There are many isoforms of PDE, but PDE3B is the predominant isoform associated with lipolysis in adipocytes due to its high affinity for cAMP (41). PDE3B is activated as a result of phosphorylation of the Ser302 residue (52). By binding to the insulin receptor, insulin begins a cascade of events that results in the activation of phosphatidylinositol 3-kinase (PI3-K) and protein kinase B (PKB/Akt) which ultimately phosphorylate and activate PDE3B and lead to decreases in lipolysis (51, 72).

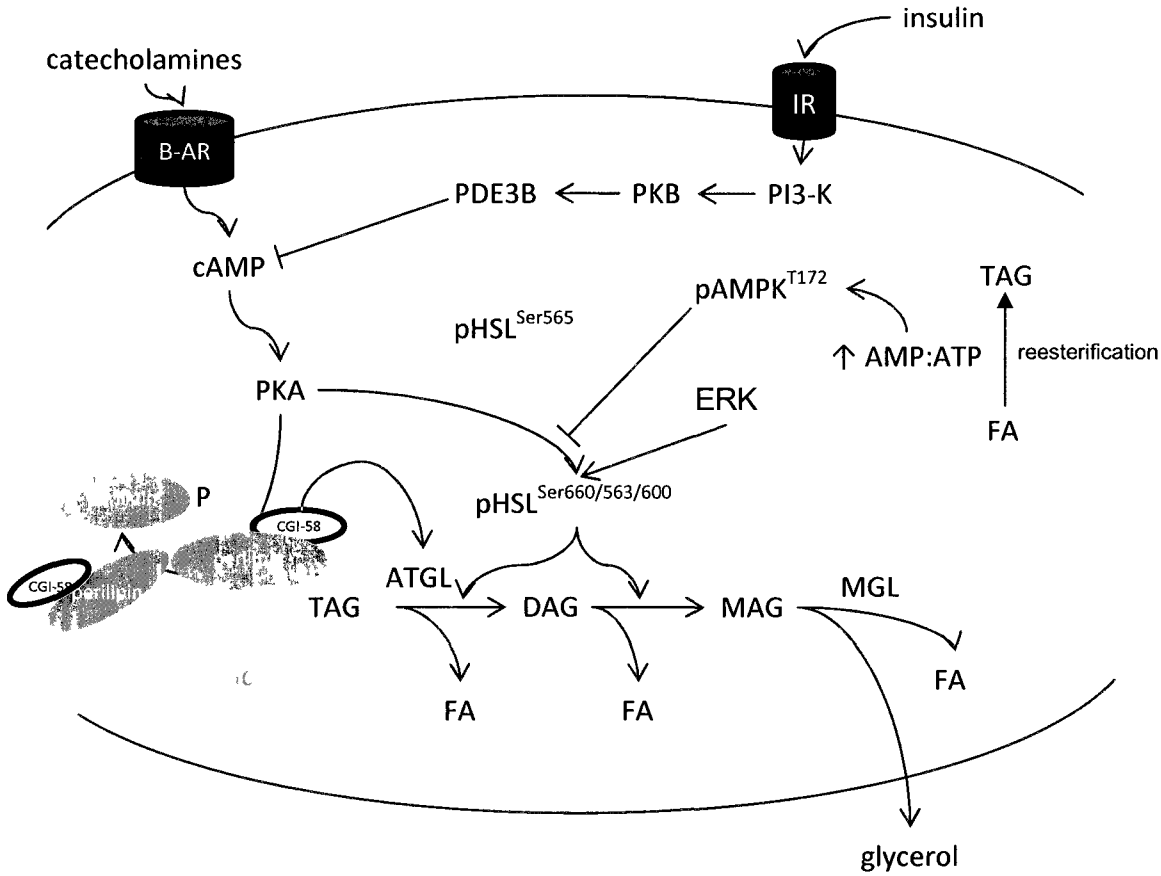

Figure 2: Regulators of lipase activity
There are many upstream regulators that function to stimulate or inhibit lipolysis by altering the activity of the lipase enzymes. Catecholamines stimulate lipolysis through cAMP and PKA-dependent mechanisms. PKA activation results in the stimulatory phosphorylation of HSL on Ser660 and Ser563 and the dephosphorylation of inhibitory Ser565. PKA also phosphorylates perilipins, further increasing HSL's lipolytic activity. Phosphorylation of perilipin also promotes the disassociation of comparative gene identification 58, allowing it to associate with ATGL and increase lipase activity. ERK is also capable of stimulating lipolytic activity by phosphorylating HSL on Ser600. With elevated lipase activity, there is an increase in the breakdown of TAG and the release of glycerol and FAs. Insulin inhibits this lipolytic pathway by activating PDE3B, and thus hydrolyzing cAMP and reducing the activation of PKA. AMPK is also able to inhibit lipolysis by inhibiting stimulatory phosphorylation of HSL. AMPK is activated following increased lipolysis and FA re-esterification to reduce futile FA cycling.

2.3.3 AMPKs Role in the Regulation of Lipolysis:

The ß-adrenergic pathway can also be inhibited further downstream by AMPK's inhibition of HSL's lipolytic activity (1). This inhibition acts as a negative feedback mechanism to reduce the costly and futile cycling of FA release and re-esterification that occurs with elevated lipolysis. Acute increases in AMPK cause decreases in lipolysis as a result of differential HSL phosphorylation (1). As mentioned above, stimulated lipolysis involves the phosphorylation of HSL at Ser660 and Ser563, and the dephosphorylation of the inhibitory site Ser565 (14, 42). In the short term, elevations in AMPK activity lead to increases in $pHSL^{ser\ 565}$, and decreases in $pHSL^{ser\ 660}$ and $pHSL^{ser\ 563}$ causing decreased activity of HSL and reduced hydrolysis of lipids (1) (Fig 3). AMPK is activated by phosphorylation on the Thr172 residue in response to increased lipolysis which is most likely an indirect result from an increased AMP:ATP ratio that follows the reesterification of lipids (24). This would suggest a negative feedback mechanism to reduce the energy demands of unnecessary lipolysis.

2.3.4 Mitogen Activated Protein Kinase Stimulated Lipolysis:

Although ß-Adrenergic stimulation via PKA is the most well known pathway for stimulated lipolysis, evidence also suggests a role for mitogen activated protein kinases (MAPK). Increased activation of the MAP kinases, ERK-1/2, may occur through a cAMP dependent mechanism (38, 63) and this activation may account for a portion of ß-Adrenergic stimulated lipolysis. ERK is able to increase the lipolytic activity of HSL by phosphorylating the Ser600 residue, an additional stimulatory site (25) (Fig 3). Although ERK's activation of HSL is sufficient to increase lipolysis, this increase is small in

comparison to that of PKA activation. This is likely due to the fact that ERK does not have the ability to concurrently phosphorylate perilipin, and thus limits HSL's lipolytic activity (22). Due to ERKs ability to stimulate lipolysis similarly to the classic ß-Adrenergic stimulation, it's involvement in GC stimulated lipolysis is of interest.

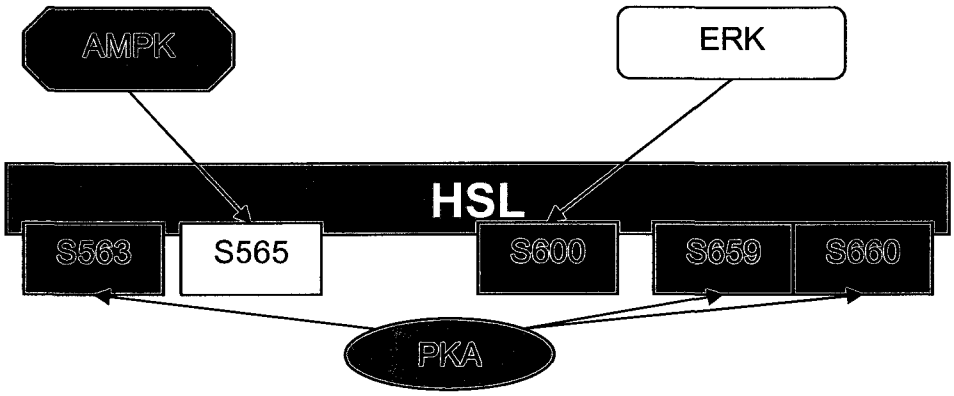

Figure 3: Phosphorylation of HSL by upstream regulators
HSL is phosphorylated on several serine residues by its upstream regulators. AMPK stimulates the phosphorylation of Ser565 to inhibit stimulatory phosphorylation of Ser563. ERK stimulates activity by phosphorylating HSL on Ser600, while PKA stimulates phosphorylation of Ser563, Ser659 and Ser660 to increase lipolytic activity.

2.4 Glucocorticoids and Lipolysis:

2.4.1 Contradictions within the Literature:

As discussed above, GCs have been traditionally believed to be catabolic hormones that increase lipolytic rates in order to provide energy substrates during times of stress. Therefore, much of the literature has focused on the lipolytic effects of GCs. Previously it was thought that GCs could potentiate the lipolytic effects of other hormones, such as catecholamines or growth hormone, but had little effect on their own (37). More recently, however, GCs have been shown to directly stimulate lipolysis in

adipocytes, although the time required for this effect appears to be a major determining factor (74). Although much of the literature has focused on the lipolytic effects, inhibitory effects of lipolysis have also been found in response to GC exposure (8, 9, 47, 58). Indeed, previous work in our lab suggests that GCs can have both a stimulatory and inhibitory effect, depending on the dose and duration of exposure (8, 9).

Some of the contradictory findings may result from the varied models used to study the effects of GCs on adipocytes. A summary of the models and findings can be found in Table 1. The dose and duration of GC exposure, type of GC (synthetic or natural), along with type of adipocyte (primary adipocyte, cell line etc) used, appear to have differential effects on lipolytic activity. GCs effects on adipocytes are commonly studied in *in vitro* models using either primary adipocytes taken from visceral (Lamberts et al., 1975; Xu et al., 2009) or subcutaneous (Fain et al., 1971; Ottosson et al., 2000) depots of humans and rodents, or in 3T3-L1 differentiated adipocytes (8, 9). Many studies have used the synthetic GC, dexamethasone, in their treatments rather than the physiologically active cortisol and Cort. Dexamethasone acts by binding to GR as cortisol and Cort do, however it is more potent and could have some differential effects. Hence, studies using cortisol and Cort are of more physiological relevance and are lacking within the literature.

Table 1: Studies investigating the effects of GCs on lipolysis.

Reference	Adipose Model	Dose/type GC	Time	Lipolysis	Suggested Mechanisms
Fain et al. (18)	Isolated parametrial adipocytes –rats	Dexamethasone 0.016μg/ml (0.04μM)	4 hours	↑FFA release ↔ glycerol release	Altered transcription
Fain et al. (17)	Isolated parametrial adipocytes - rat	Dexamethasone 0.1μg/ml (2.5μM)	4 hours	↔ glycerol	↔ cAMP
Lamberts et al. (37)	Isolated epididymal adipocytes -rat	Dexamethasone 0.1μg/ml (2.5μM)	4 hour	↔ glycerol	↔ cAMP
Samra et al. (58)	In vivo Human	Hydrocortisone 1.5μM	Acute IV infusion	↑ overall NEFA release ↓ NEFA veno-arterial difference in abdominal	↑ LPL activity ↑ peripheral lipase activity ↓ visceral lipase activity
Ottosson et al. (47)	Isolated subcutaneous abdominal adipocytes - human	Hydrocortisone 1μM	3 days	↓ basal lipolysis ↓ β-adrenergic stimulated lipolysis	↓production or ↑elimination of cAMP
Xu et al. (74)	Isolated epididymal adipocytes - rat	Dexamethasone 0.1μM	24 hours	↑FFA release after 4 hours ↑glycerol release after 16 hours	↑HSL and ATGL transcription ↓PDE3B expression ↑cAMP and PKA activity ↑perilipin and HSL phosphorylation
Campbell et al. (9)	3T3-L1 adipocytes	Corticosterone 1μM 100μM	48 hours 48 hours	↑glycerol release ↓glycerol release	↑HSL and ATGL transcription ↓cAMP activity

2.4.2 Genomic Effects of Glucocorticoids Result in Increased Lipolysis:

As mentioned previously, the majority of research has focused on the mechanisms of increased lipolysis, either by GCs direct stimulation of adipocytes or by potentiating the effects of other hormones (45, 69, 71). GCs effects may be genomic, long term effects that result from altering gene transcription, or may be non-genomic, immediate actions that do not affect transcription and may involve changes in phosphorylation states. The normally understood role of GCs involves genomic effects; GCs bind to the GR and alter gene transcription. That being the case, it is suspected that GCs function through the upregulation or downregulation of proteins along the lipolytic pathway or by regulating the transcription of lipases themselves. Much of the literature agrees that GCs increase lipolysis in mature adipocytes as a result of increased transcription and expression of the lipase proteins ATGL and HSL (8, 9, 19, 65, 74) . These increases are typically seen when a low dose of GC (<1uM) is given for a duration of several hours (4-48hrs) (9, 74). Due to the time it takes for transcription and protein expression to occur, these effects are not seen immediately and it takes hours for lipolysis to be increased (17, 65).

An early study by Fain et al., utilizing dexamethasone, was unable to find changes in lipolysis with dexamethasone alone, but found that adipocytes incubated with dexamethasone, in the presence of growth hormone (GH), had increases in lipolysis after a lag period of 1-2 hours (17). This lag period is in contrast to the rapid increase in lipolysis seen with catecholamines through the β-adrenergic pathway. It became clear

that these effects were occurring due to changes in protein synthesis as the use of RNA and protein synthesis inhibitors decreased lipolysis. About 90% of RNA synthesis stimulated by GH and dexamethasone is completed after 2 hours of incubation (19) suggesting that short term exposure can affect lipolytic rates due to transcription regulation, however these effects will not be seen immediately.

Longer exposure (24-48hours) of adipocytes to GCs alone can directly increase lipolysis through changes in protein expression. Xu et al. found that FA release was increased from adipocytes treated with dexamethasone after a lag period of 4-6 hours, with maximal FA release occurring after 32 hours of incubation (74). This increase was accompanied by increased transcription of the lipases HSL and ATGL (74). Moreover, ATGL and HSL protein expression was also increased in the adipose tissue of rats treated with dexamethasone (74). Similar to this, Campbell et al. found increased mRNA expression of both ATGL and HSL and increased protein expression of ATGL in 3T3 adipocytes treated with Cort for 48 hours, with corresponding increases in lipolysis (9). These studies suggest that GCs, both dexamethasone and Cort, are able to stimulate lipolysis by upregulating transcription and expression of lipases with long term incubation. Changes in protein expression take time to occur; hence we would not expect to see changes in lipolysis immediately with the addition of GCs, if this is the only mechanism of action.

2.4.3 Glucocorticoids' Effects on the β-adrenergic Lipolytic Pathway:

GCs' effects on the β-adrenergic pathway have also been studied, albeit to a limited extent, to determine if they can affect lipase activity and have acute immediate effects on lipolysis similar to what is observed with catecholamine exposure. Although several research groups have investigated this pathway (9, 19, 74), contradictory findings have been reported and no clear consensus exists. In addition, any changes to this pathway have been attributed to changes in the protein expression of upstream regulators with long term GC treatment, rather than to acute non-genomic actions.

As cAMP is the main regulator of the β-adrenergic stimulated lipolysis pathway it is often the first target of investigation. Increases in cAMP levels with GC treatment would suggest a mechanism for increased lipolysis, however, few studies have seen this to be the case. Although there is evidence that GCs decrease PKB and PDE expression and activity (5), and would thus be expected to cause increases in cAMP levels, such increases are not often found with GC treatment alone (17, 37). In one case, primary adipocytes treated with dexamethasone *in vitro* did produce an increase in cAMP levels (74). These increases in cAMP concentrations were associated with decreases in PDE protein expression, as well as increased PKA activity. As would be expected with increases in PKA, these authors observed increased phosphorylation of HSL and perilipin. A surprising finding, however, was that this phosphorylation did not promote the translocation of HSL to the lipid droplet, as would be expected to occur with β-adrenergic agonists and with increased lipolysis. Thus, the mechanisms of how GCs might stimulate the β-adrenergic pathways to promote lipolysis are still not clearly defined.

2.4.4 Glucocorticoids' Anti-Lipolytic Effects:

Adding to the uncertainty of lipolytic mechanisms is evidence that GCs may also have an antilipolytic role (7-9, 47, 58). Treating 3T3-L1 adipocytes with Cort at levels that would be found in the plasma (<1μM) for 24-48 hours does produce increases in lipolysis as previously demonstrated, however if Cort is removed from the media following the 48 hour incubation period, lipolysis levels increase further during a one hour basal period. If Cort concentrations are increased beyond 1uM, to levels that may be present in the tissue due to the actions of 11βHSD1, decreases in lipolysis are observed after 48 hours (9). When these high doses of Cort are removed from the media, increases in lipolysis are observed as with the lower doses. These results suggest that Cort is able to upregulate the expression of lipases and proteins along the lipolytic pathway, and these are the effects that remain after the removal of the hormone. However, if Cort is present, its effects may be inhibitory. Very little research has investigated this potential antilipolytic effect of GCs, but is important when considering GC induced adipose accumulation.

An *in vivo* study looking at FA efflux from subcutaneous abdominal adipose tissue found similar decreases in lipolytic rate when subjects were given elevated levels of GCs (58). Although total arterial levels of FA are increased with treatment, the adipose venous FA levels are decreased compared to controls suggesting decreased lipolysis in the abdominal adipose tissue. These authors hypothesize that LPL activity and potential increases in lipolysis in the peripheral tissue may account for the overall

increase in FA while there are site specific decreases in lipolysis in the abdominal region. It is also possible that site specific increases in FA uptake are occurring, however, as suggested previously, it is believed that GCs reduce re-esterification and thus would limit re-uptake (21, 30). Similar decreases in lipolysis were also found when abdominal subcutaneous adipose tissue was cultured, treated with cortisol for 3 days, and then allowed to undergo basal or stimulated lipolysis (47). It was speculated by the authors that GCs were able to either decrease the production of, or increase the elimination of, cAMP in order to produce this response, although measurements of cAMP levels were not conducted in that study. Decreases in lipolysis, particularly in specific adipose depots may contribute to excess adipose accumulation. Greater investigation is needed on this topic however as mechanisms of action are not clearly defined and it contradicts the traditionally accepted lipolytic role of GCs.

2.4.5 Possible Non-genomic effects on lipolysis:

With regards to lipolysis, non-genomic effects have not been previously explored. There are several ways that the previously mentioned non-genomic mechanisms of action could affect lipolysis. Through interaction with the plasma membrane or membrane proteins, GCs may alter membrane permeability and ion transport and alter the levels of intracellular Ca^{2+} (26). Increased intracellular Ca^{2+} levels have been shown to inhibit both basal and stimulated lipolysis (78), and suggest that GC interactions with membrane bound proteins and altered Ca^{2+} transport could affect lipolysis. It is suspected that increased Ca^{2+} levels reduce lipolysis due to increased PDE transcription and thus

reduced cAMP levels and PKA activity (28). Previous research has linked pre-incubation of GCs with increased intracellular Ca^{2+} levels, however, with this increase a concomitant increase in lipolysis was observed (33). This link between Ca^{2+}, GCs and lipolysis is not well established.

GC interactions with cytoplasmic proteins are of considerable interest as many proteins that may be non-genomically regulated by GCs are directly linked to lipolysis; GCs have been shown to alter the phosphorylation of MAPK and protein kinases (26). As mentioned previously, both MAPK and PKA are able to phosphorylate HSL to increase lipolytic activity, and thus this may be a potential mechanism for lipolytic control by GCs. It has been suggested that GCs inhibit the activation of ERK-1/2 genomically by increasing the expression MAPK phosphatase-1 (MPK-1), and by decreasing proteasomal degradation of MPK-1 (31). Decreased ERK-1/2 activation is a potential mechanism by which GCs could reduce lipolysis, however since they involve changes in protein expression they may take time to arise. On the other hand, non-genomic increases in ERK1/2 phosphorylation with GC treatment have also been observed in other tissues, and could suggest a mechanism for GC induced lipolysis (59).

How interactions between GCs and membrane lipids, or the GR protein complex itself, may affect lipolysis is not known, but these interactions may have potential effects on lipolysis as well. Non-genomic mechanisms of GCs are not well understood in regards to adipocyte and lipolysis, however it seems that they may play a role in acute effects of GCs.

2.5 Summary:

Acute increases in catecholamines and GCs during the fight or flight response are essential to maintain homeostasis during the stressor. While this is an adaptive and necessary response, chronic stress can be maladaptive and is linked to increases in obesity and metabolic diseases. Research points to elevated GC levels as the cause of this adipose accumulation. GCs appear able to affect lipid metabolism within these adipocytes directly, although the mechanisms by which this occurs are unclear. It is also possible that there are differential effects on the adipocytes depending on the level of GC present and the duration of exposure. Evidence suggests that GCs are able to upregulate the expression of lipase enzymes, such as ATGL and HSL, to increase lipolysis, while other studies suggest that when GCs are present in high amounts within the tissue they may inhibit lipolysis and increase lipid accumulation. It may be that GCs have both genomic and non-genomic effects that cause differential acute and long term results. It seems likely that all mechanisms exist, but some may play larger roles than others, depending on the adipose depot in question and the level and duration of exposure. Understanding adipose tissue metabolism has become vital to dealing with the obesity epidemic, and the treatment and prevention of obesity related diseases such as T2DM.

RATIONALE AND OBJECTIVES 3

Rationale:

A recent study in our lab has observed that Cort has dual effects in adipocytes, promoting increases in lipolysis with Cort exposure for 48 hrs, but also having acute inhibitory effects. It is the goal of this thesis work to further explore the inhibitory effects of Cort on adipocyte lipolysis, and to determine potential mechanisms of action for altered lipolytic rate.

Objectives:

We wish to assess the acute effects of Cort on adipocyte lipolysis by studying short term exposure (0-4 hours), and to assess the effects of Cort in combination with known lipolytic stimulants, such as isoproterenol. We will investigate the activation of HSL, as well as upstream regulators of stimulated lipolysis, cAMP and PKA, to determine Cort's mechanisms of actions.

Hypothesis:

We hypothesize that Cort will act through non-genomic mechanisms to reduce lipase activity and lipolysis, in both the basal and stimulated state, in 3T3-L1 adipocytes. We believe that these effects will be similar in visceral adipose tissue, suggesting possible reasons for GC induced increases in adipose accumulation in this depot.

MANUSCRIPT 4

ACUTE ANITLIPOLYTIC EFFECTS OF CORTICOSTERONE IN ADIPOCYTES

Running Title: The antilipolytic effects of corticosterone

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Keywords: glucocorticoids, adipocyte, lipolysis, corticosterone, adipose tissue

Contributions of Authors:

This project was conceived and designed by myself, Dr. Michael Riddell, Dr. Jonathon Campbell and Dr. David Wright. I performed all experiments in the project, which consist of cell culture techniques to grow and treat 3T3-L1 adipocytes, adipose tissue organ culture, western blotting, and the various assays for glycerol, NEFA, cAMP etc. Dr. Jonathon Campbell provided a great deal of assistance in teaching me various lab and cell culture techniques, and Dr. David Wright provided assistance with the adipose tissue organ culture technique. Dr. Michael Connor provided assistance with cell culture and western blotting techniques. Analysis of data was done by myself with the input of all authors.

Dr. Riddell is the principal investigator and supervisor of this project. The first draft of this paper was written by myself, with the final draft including revisions and recommendations from the thesis supervisory committee.

Introduction:

Stress hormones (i.e. catecholamines, glucocorticoids) are thought to be key regulators of lipid metabolism within adipocytes, mobilizing fuel for the fight or flight response. While catecholamines are well established regulators of lipolysis, liberating fatty acids and glycerol via increases in lipase activity, the effects of glucocorticoids (GC) on lipolysis is much more controversial. GCs have long been cited to increase lipolysis within isolated adipocytes, through changes in protein expression (7, 9, 18, 74). However, clinically the opposite appears true, since elevations in both endogenous and exogenous GCs are associated with elevations in adipose accumulation and central obesity (56). The mechanisms for the paradoxical catabolic and anabolic actions of GCs are unknown, however, lipid accumulation and adipocyte hypertrophy could occur due to an increase in uptake and storage of fatty acids, or due to a decrease in lipolysis. Although much evidence has been provided to support GC's lipolytic effects, there is also evidence for anti-lipolytic effects that would favour lipid storage (47, 58). Our study investigates these potential anti-lipolytic effects of GCs.

Lipids are stored within adipocytes in the form of triacylglycerol (TAG). Lipolysis, the stepwise hydrolysis of stored TAG, is governed by the lipase enzymes, primarily ATGL and HSL (60). The rate of lipolysis may be increased or decreased by altering the expression of the main lipase proteins, or altering their activity through upstream regulators; mainly, via increases in cAMP and subsequent activation of protein kinase A (PKA). HSL is the lipase most known for direct hormonal stimulation, with PKA induced phsophorylation of HSL on Ser660, Ser659 and Ser563, and

dephosphorylation of Ser565 stimulating translocation to the lipid droplet and lipolytic activity (42). PKA also directly phosphorylates perilipin, a protein associated with the lipid droplet, which further increases HSL's lipolytic activity (62). Through the phosphorylation of perilipin, PKA can also indirectly activate ATGL as it allows perilipin to release comparative gene identification 58 (CGI-58), a protein that may then associate with ATGL and increase its lipolytic activity (60, 77). AMPK and MAPK are also able to affect the activity of HSL by increasing phosphorylation at Ser565 and Ser600 respectively

The traditional mechanism for GC action is genomic in nature; GCs bind to the glucocorticoid receptor (GR) and subsequently alter the transcription of target genes. Previous research suggests that GCs stimulate lipolysis in a genomic fashion, through increased expression of both ATGL and HSL with long term treatment; however investigation of the effects on cAMP and PKA activity has produced conflicting results (9, 19, 47, 74), and suggests that other mechanisms of action may also exist. Recent work in our lab suggests that corticosterone (Cort), the naturally occurring GC in rodents, plays a dual role, with long term, genomic effects that increase lipase expression and lipolysis, as well as acute inhibitory effects that may contribute to adipose accumulation (9).

Our current study investigates these acute antilipolytic effects in greater detail and works to clarify mechanisms of GC induced adiposity with the use of an *in vitro* model. Acute Cort exposure produces antilipolytic effects that lead to reductions in both basal and stimulated lipolysis in isolated adipocytes. These effects are non-genomic and occur

through reductions in PKA activity and resultant reductions in HSL and perilipin stimulatory phosphorylation. Such inhibitory effects would favour lipid accumulation and thus point to mechanisms for GC induced adiposity.

Methods:

3T3-L1 Adipocytes:

3T3-L1 fibroblasts (ATCC, Manassas, Virginia; lot #58432133) were differentiated as previously described (9). In brief, fibroblasts were seeded in 6 well plates and maintained in high glucose Dulbecco's Modified Eagle Medium (DMEM; Wisent, St. Bruno, Quebec, cat #319005CL) that was supplemented with 10% fetal bovine serum (FBS; Wisent, cat #080150), DMEM-FBS. Two days post-confluence fibroblasts were treated with DMEM-FBS containing 500uM isobutylmethyxanthine (IBMX; Sigma, Oakville, Ontario cat #I7018), 500nM dexamethasone (Sigma cat #D4902), and 200pM insulin (Humalog, Lilly, Toronto, Ontario cat#VL-7516) to induce differentiation, and were maintained in this media for 6 days. Cells were then placed in DMEM-FBS containing 200pM insulin for 2 days. Prior to experimentation, cells were maintained in DMEM-FBS for 48 hours. Plates were kept in an incubator at 37°C with 5% CO_2, and the media was changed every 48 hours. All media also contained 1% Antibiotic/Antimycotic solution (Wisent cat#450-115-EL).

Adipose Tissue Organ Culture (ATOC):

250 mg of visceral (epididymal) adipose tissue was harvested from Sprague-Dawley rats (Charles River, Montreal, Quebec), minced into 5mg pieces, and cultured in 7.5ml of Medium 199 (Sigma cat #M4530) supplemented with 500uM dexamethasone and 200pM

insulin and 1% antibiotic/antimycotic. After 24 hours, media was switched to M199 supplemented with 1% antibiotic/antimycotic and 3.5% bovine serum albumin (BSA; Bioshop, Burlington, Ontario cat #ALB001). Tissue was incubated in this media for 1 hour to establish control conditions, after which time 100 μM of Cort was added.

Lipolysis:

Following differentiation, 3T3-L1 adipocytes were treated with Cort alone, or in combination with known stimulators of lipolysis or inhibitors of GC action. In each case, glycerol release was used as the main marker of lipolysis. Media was sampled at baseline, during and/or following treatment and glycerol was measured with the use of a commercially available kit (Sigma, Cat#FG0100). Fatty acid, non-esterified fatty acid (NEFA), release was also measured with the use of a commercially available kit (Wako, Richmond, Virgina, cat#999-34691). Cort (Sigma cat #C2505), RU486 (mifepristone; Sigma cat#M8046), actinomycin D (Sigma cat#A9415), and forskolin (Sigma cat#F3917) were dissolved in dimethyl sulfoxide (DMSO), and isoproterenol (Sigma cat#I2760) was dissolved in H_2O, prior to use. To assess the effects of Cort alone, Cort was added to phenol free DMEM, supplemented with 3.5% FBS, to produce the given concentrations. First, the long term effects of Cort were investigated. Adipocytes were treated with DMEM containing 100μM Cort for 48 hours. Glycerol release was also monitored during a 1 hour basal period (without the presence of Cort or β-adrenergic stimulants in the media) following the 48hr Cort treatment; media was removed, cells were washed once in warm PBS, then maintained in DMEM containing 3.5% FBS. Similar experiments were conducted to monitor short term effects with 4 hours of Cort treatment

followed by a period of basal lipolysis. To determine the dose dependent effects, adipocytes were incubated in DMEM containing 0, 1, 10, 50 and 100 μM Cort for 30 minutes. Dexamethasone was also substituted for Cort to compare the effects of the synthetic glucocorticoid over the short term. We chose a dose of 0.1 μM dexamethasone as this has been shown to have equivalent lipolytic effects as 1 μM Cort with 24 hours of exposure (74), and 100 μM dexamethasone to assess effects at higher doses. In order to determine whether short term effects were happening through the GC receptor (GR), adipocytes were co-incubated with Cort in DMEM as described above, along with 10 μM of RU486, a GR inhibitor. Adipocytes were treated with 1uM actinomycin D, a transcription inhibitor, in combination with Cort to determine whether these acute effects involved gene transcription.

To further assess the inhibitory effects of Cort and its involvement in the β-adrenergic pathway, adipocytes were first stimulated with 100nM isoproterenol in Krebs Ringer buffer (125mM NaCL, 4.7mM KCl, 1.2 $MgSO_4$, 2.5mM $CaCl_2$, 1mM KH_2PO_4, 25mM Hepes, 5mM D-glucose) supplemented with 3.5% BSA, for 30 minutes to increase lipolysis, and then Cort was added to the media at the given concentrations. Cort's effects were monitored below the level of the β-adrenergic receptor by initially stimulating lipolysis with 1uM of forskolin, an adenyl cyclase agonist, in Krebs Ringer buffer with 3.5% BSA, and then Cort was added to the media.

cAMP Assay:

Following treatment, media was removed and 271μl of 0.1M HCl was added to each well of the 6 well plate. Adipocytes were incubated in HCl for 10 minutes at room

temperature. Cells were then scraped and centrifuged for 10 min at 14,000 x g. The pellet was discarded and supernatant was collected and frozen. Samples of the supernatant were diluted 5 fold and cAMP was measured in the whole cell lysate with the use of a commercially available EIA kit (Cayman Chemicals, Ann Arbor, Michigan, cat#581001). Protein concentrations were measured using the Bradford method and cAMP values are expressed as pmol of cAMP per gram protein.

Protein Expression:

Following treatment, cells were lysed in adipose lysis buffer (150mM NaCL, 20mM Tris, 2mM EDTA, 0.5% triton, supplemented with protease inhibitor cocktail (Sigma cat#P8340) and phosphatase inhibitor 2 cocktail (Sigma cat#5726)), scraped from the plate, sonicated for 20 sec, and centrifuged at 14,000 x g for 10 min at 4°C. Protein concentrations were determined by the Bradford method for each sample. Equal amounts of protein were separated with SDS-PAGE (8 or 10% polyacrylamide gel), then transferred to a PVDF membrane. 5% BSA was used to block the membrane for 1 hour at room temperature, followed by an overnight incubation in the following primary antibodies at 4°C: total HSL, $pHSL^{Ser565}$, $pHSL^{ser\ 563}$ and $pHSL^{ser660}$ (Cell Signaling, Danvers, Massachusetts, cat#4107, 4137, 4139, 4126), total and pAMPK (Cell Signaling, cat#2793, 2531) as well as total and phosphorylated ERK 1/2 (Cell Signaling cat #4695, 4370) were measured to determine their activation. The use of a phospho-PKA substrate antibody (RRXS*/T*; Cell Signaling cat#9624) allowed for the measurement of PKA activity. This same antibody was used to measure perilipin phosphorylation, utilizing the band that appears at 62kDa and corresponds to the molecular weight of perilipin. Total

perilipin protein was also measured (Cell Signaling cat#9349). The membrane was then incubated in the appropriate secondary antibody in 2.5% BSA for 1 hour at room temperature. Blots were visualized with chemiluminescence (GE Healthcare, Bai d'Urfe, QC) with the use of the Carestream imaging system. Relative band intensity was corrected for loading using GAPDH (Abcam, Cambridge, MA cat#9484) or α-tubulin (Abcam cat#7291) as loading controls, and set relative to the control for each 6 well plate. Band intensity was measured with the use of the Carestream Molecular Imaging software.

Statistical Analysis:

Data are expressed as mean ± SEM. For each experiment the appropriate one-way or two-way ANOVA, or t-test was performed to compare differences between groups. When significant, a Fisher post hoc was used to determine specific differences. Statistica 6.0 software was used for all analysis.

RESULTS:

Cort acutely reduces lipolysis, effects that are non-genomic and are not mediated by the glucocorticoid receptor: In order to measure the effects of Cort on lipolysis, we monitored glycerol release from adipocytes and whole adipose tissue in the presence of Cort for 0-4 or 48 hours, and following the removal of Cort from the treatment media. As we have previously reported (9), long term (48hr) exposure of 3T3-L1 adipocytes with 100μM of Cort inhibits the release of glycerol by 30% ($p<0.05$) (Fig 1A). With the removal of Cort from the treatment media, basal glycerol release was elevated above levels of control ($p<0.05$) (Fig 1A). This suggests that while present Cort produces an

inhibitory effect that is acute in nature and easily reversible. To focus on the acute effects, adipocytes were treated with Cort for 0-4 hours. Adipocytes treated with 100μM Cort produced an immediate decrease in glycerol release which persisted throughout the 4 hour treatment, however with the removal of Cort, basal glycerol release was immediately normalized to levels of controls (Fig 1B). The inhibition of glycerol release is dose dependent with significant decreases seen with low and high doses of Cort with 30 minutes of exposure (Fig1C). NEFA release is also reduced with the addition of Cort: 100μM Cort produced a 10% decrease in NEFA release ($p<0.05$; Appendix Fig 1A). Epididymal adipose tissue behaved similarly to 3T3-L1 adipocytes: incubation in 100μM Cort for 2 hours produced a 30% decrease in glycerol release ($p<0.05$; Fig 1D). Dexamethasone, a synthetic GC commonly used in experiments to study various GC actions, did not produce significant decreases in glycerol release with 30 minutes of exposure, with either low (0.1μM) or high (100 μM) doses (Fig 1E). Together, these results show that Cort, but not dexamehtasone, inhibits lipolysis in an acute dose dependent fashion in 3T3-L1 adipocytes, a model that likely represents the anabolic effects of GCs in visceral (epididymal) adipose tissue.

The traditional mechanisms for GC action involve binding to the glucocorticoid receptor (GR) and subsequent up or down regulation of target gene transcription. 3T3-L1 adipocytes were treated with Cort in combination with either 10 μM of RU486, a GR inhibitor, or with 1μM actinomycin D, a transcription inhibitor, to determine if Cort's acute effects were occurring through the traditional genomic mechanisms. The main effect of Cort was not abolished with the addition of 10 μM RU486 (Fig 2A), a dose we

have shown reduces long term lipolytic effects (Appendix Fig 2A). Increasing the concentration of RU486 to 100μM was also unable to remove the inhibitory effects of Cort (Appendix Fig 2B). The inhibition of transcription via Actinomycin D, also did not effect the reductions in glycerol release produced by Cort either (Fig 2B). These results show that Cort's acute antilipolytic effects occur independent of receptor binding and do not involve changes in gene transcription.

Cort dramatically reduces stimulated lipolysis: To determine if Cort blunts stimulated lipolysis, which would also favor an anabolic action of GCs, adipocytes were treated with Cort in combination with isoproterenol, a β-adrenergic agonist, or with forskolin, the adenyl cyclase agonist. Both isoproterenol and forskolin alone stimulate lipolysis via increases in cAMP and PKA; isoproterenol does so through the β-adrenergic receptor, while forskolin acts downstream of this, directly stimulating adenyl cyclase. As expected, isoproterenol treatment significantly increased glycerol release, however the addition of Cort resulted in a dose dependent decrease in glycerol release compared to isoproterenol alone, and at doses of 50 and 100μM, Cort abolished the stimulatory effects of isoproterenol (Fig 3A; $p<0.05$). Reductions in stimulated glycerol release were not dependent on the order that isoproterenol and Cort were added, but did rely on the presence of Cort (Appendix Fig 3A-D). This treatment produced a similar pattern in NEFA release; increased NEFA release with isoproterenol and significant reductions in NEFA release with the addition of 50 and 100μM Cort ($p<0.05$; Appendix Fig 1). To determine whether Cort's inhibitory effects were happening at or below the level of the ß-adrenergic receptor, adipocytes were first treated with forskolin, and then Cort was added

to the media (Fig 3B). Forskolin produced a great increase in glycerol release (~400% vs. control, $p<0.05$), and the addition of Cort caused a dose dependent decrease in this stimulation; glycerol release was significantly decreased with doses of 50 and 100μM Cort ($p<0.05$ vs forskolin alone). Reducing the concentration of forskolin to reduce stimulated lipolysis did not alter the ability of Cort to reduce glycerol release (Appendix Fig 4). Together these results suggest that Cort is able to reduce stimulated lipolysis with effects occurring at or below the level of cAMP.

cAMP concentration is not altered by Cort: The literature has produced conflicting results as to GCs effects on cAMP with studies reporting increases (74), no change (17, 37) or decreases (9) in cAMP concentration following treatment with GC. We measured cAMP concentrations following 30 minutes of Cort treatment alone or in combination with isoproterenol. There were no changes in cAMP concentration with Cort treatment alone (Figure 4A) suggesting that the acute inhibition of lipolysis is not produced through changes in cAMP. The treatment of adipocytes with isoproterenol resulted in significant increases in cAMP concentrations, as would be expected from a ß-adrenergic agonist (Fig 4B; $p<0.05$), but the addition of Cort did not significantly alter cAMP concentration from that of isoproterenol alone. A full dose response shows that cAMP levels do not change with any dose of Cort in either the basal or stimulated state (Appendix Fig 5 and 6). It therefore does not appear that Cort is able to acutely affect lipolysis through changes in cAMP concentration.

Cort reduces PKA activity in the stimulated state, but cannot alter basal activity: PKA acts downstream of cAMP, increasing lipolysis through phosphorylation of both HSL and

perilipin. In line with cAMP concentration, there were no detectable changes in PKA activity when adipocytes were treated with Cort alone for 30 minutes (Fig 4C). Treatment of adipocytes with isoproterenol alone significantly increased PKA activity, and with the addition of Cort PKA activity was normalized to levels of controls (Fig 4D; $p<0.05$). A full dose response shows that Cort reduces isoproterenol stimulated PKA activity in a dose dependent fashion (Appendix Fig 5 and 6). This suggests that in the stimulated state, Cort's antilipolytic effects result from reductions in PKA activity.

HSL stimulatory phosphorylation is altered with Cort treatment, effects that are most prominent in the stimulated state: HSL activity is altered through the phosphorylation of multiple serine residues and is largely regulated by PKA. HSL has increased lipolytic activity when phosphorylated on Ser660 and Ser563, while phosphorylation of Ser565 reduces lipolytic activity. The treatment of 3T3-L1 adipocytes with Cort for 30 minutes did not alter the expression of HSL total protein (Fig 5A, 5B), $pHSL^{Ser565}$ (Fig 5C) or $pHSL^{Ser660}$ (Fig 5D). There was however a 26% decrease in HSL phosphorylation at Ser563 that matched reductions in glycerol release ($p<0.05$; Fig 5E), suggesting that altered HSL activity with GCs is likely responsible for the observed reductions in lipolysis.

As with treatment of adipocytes with Cort alone, neither treatment with isoproterenol and/or Cort altered total HSL protein expression (Fig 6B), and confirms that Cort is not affecting lipolysis through genomic changes in lipase expression. As expected, treatment of adipocytes with isoproterenol reduced HSL phosphorylation on Ser565 when compared to controls in a paired t-test (Fig. 6C; $p<0.05$). With the addition

of 100μM Cort, $pHSL^{Ser565}$ was normalized to levels of controls. Phosphorylation of HSL on Ser660 was increased with isoproterenol treatment as expected ($p<0.05$) and was decreased with the addition of 100 μM Cort compared to isoproterenol alone (Fig 6D; $p=.06$). As with $pHSL^{Ser660}$, phosphorylation of HSL on Ser563 was increased with isoproterenol treatment, and the addition of Cort significantly reduced phosphorylation compared to isoproterenol alone (Fig 6E; $p<0.05$). A full dose response for each treatment was also done (Appendix Fig 7 and 8) and confirmed that Cort's effects occur in a dose dependent fashion. Reduced stimulatory phosphorylation of HSL is in line with the reductions in PKA activity we have also reported, and suggests that reductions in HSL lipase activity may be responsible for reductions in lipolysis.

Perilipin phosphorylation is reduced with Cort treatment but expression is not changed: Perilipin, a protein associated with the lipid droplet, is important in the regulation of the lipase enzymes. Perilipin expression is necessary to facilitate the translocation of HSL to the lipid droplet (62), and phosphorylation of perilipin increases the activity of both ATGL and HSL (62, 77). Total perilipin expression was not altered with isoproterenol treatment or the addition of Cort (Fig 7B). Perilipin is a substrate of PKA with a molecular weight of 62 kDa. Perilipin phosphorylation was measured with the use of the PKA substrate antibody and the band that appears at 62kDa was analyzed for relative intensity. In line with decreased PKA activity, phosphorylation of perilipin is increased with isoproterenol treatment and significantly reduced with the addition of Cort compared to isoproterenol alone ($p<0.05$; Fig 7C). Reductions in perilipin

phosphorylation will produce reductions in lipase activity and contribute to reductions in lipolysis.

Neither AMPK nor ERK1/2 phosphorylation is significantly altered with Cort treatment: The phosphorylation of AMPK on Thr172 causes reductions in lipolysis via increases in $pHSL^{Ser565}$, while phosphorylation of ERK1/2 increases lipolysis via phosphorylation of HSL on Ser600. Each was analyzed following treatment with isoproterenol and Cort to determine if they were playing a role in Cort mediated reductions in lipolysis. pAMPK was elevated with isoproterenol treatment alone ($p<0.05$) but was not different from controls or isoproterenol alone when isoproterenol and Cort were combined (Figure 8B). pERK1/2 was elevated when compared to controls only using a paired t-test ($p<0.05$), as would be expected with ß-adrenergic stimulation, but the addition of Cort did not produce a significant difference in phosphorylation status from this or controls (Figure 8C). These results suggest that neither AMPK nor ERK is responsible for the reductions in lipolysis observed with Cort treatment.

DISCUSSION:

In this study, we have shown that Cort, the main GC in rodents, inhibits lipolysis in 3T3-L1 adipocytes as well as epididymal adipose tissue cultured ex vivo, through non-genomic mechanisms. By studying Cort's effects on both basal and stimulated lipolysis, we find that the inhibitory effects occur due to reduced PKA activity and decreased stimulatory phosphorylation of HSL and perlipin. These reduced lipolytic rates found in

this study reveal new mechanisms that likely help to explain the central obesity that develops in individuals with elevated GC levels (55, 56).

GCs have long been considered catabolic and lipolytic in nature (7, 9, 18, 74), however in some cases they have been found to inhibit lipolysis (47, 58). The present study, combined with our previous reports (9), suggest that Cort has paradoxical and dual roles: increasing lipolysis via genomic actions in the long term (9), but also having acute, non-genomic inhibitory effects when present at high doses. We have previously established that treatment of 3T3-L1 adipocytes with Cort for 48 hours increases lipolysis at low and moderate levels (0-10μM), but decreases lipolysis with high doses (10-250μM) (9). Placing adipocytes in treatment media without Cort following the 48hr exposure immediately removes the inhibitory effects and lipolysis dramatically increases compared to controls (Fig 1A), highlighting Corts paradoxical effects. Indeed, long-term increases in lipolysis with GCs have been shown in the literature previously by ourselves and others (9, 18, 74) and have been determined to exist primarily through increased transcription of lipase enzymes (9, 74). Based on this current study and the investigations by others (9, 74), we now propose that changes in protein expression explain the lasting, lipolytic effects following the removal of Cort, while the acute inhibiting effects required further investigation.

To isolate the acute effects, we chose to investigate changes in lipolysis during the first (0-4) hours of Cort exposure. Previous research has shown that although transcription changes occur soon after GC exposure, there are little alterations in protein expression during the first 2-6 hours, and thus genomic effects would not likely play a

role during this time period (18, 19, 74). We find that high doses (100μM) of Cort reduce glycerol release in 3T3-L1 adipocytes throughout a 4 hour exposure, effects that are readily reversible with the removal of Cort and highlight the acute nature of inhibition (Fig 1B). These effects were dose dependent with glycerol release reduced following 30 minutes of Cort exposure with low doses, (1μM) that represent those found in the plasma of healthy rodents (9-11, 20), and to a greater extent with higher doses (10-100μM) that represent those that would be found in diseased states and within the tissue due to the effects of 11β-HSD type 1, an enzyme that increases the concentration of Cort within the tissue by activating inactive GC (43) (Fig1C). Epididymal adipose tissue exhibits similar reductions in glycerol release when treated with Cort ex vivo (Fig 1E), and allows us to conclude that our 3T3-L1 adipocyte model is representative of the effects that occur in the visceral adipose tissue.

Cort's antilipolytic effects appear immediately and are not attenuated by the blockade of GR by RU486 (Fig 2A), or the inhibition of transcription by actinomycin D (Fig 2B). These are the three main characteristics included in the criteria typically used to classify an effect as non-genomic (26), and we thus conclude that Cort is causing these antilipolytic effects via non-genomic mechanisms, a concept not previously reported in adipocyte lipolysis, at least to our knowledge.

Importantly, we demonstrate in this study that the acute antilipolytic effects of Cort are not recapitulated by the synthetic steroid dexamethasone, which is often used in experiments examining the role of GCs on adipose tissue lipolysis (74). Cort and dexamethasone have been reported to have differential effects in some tissues (35, 76),

and it is possible that although dexamethasone binds to the GR and produces potent genomic, lipolytic effects (74), it does not produce the same acute, non-genomic antilipolytic effects. In accordance with this, we show that following 24 hours of steroid treatment, the antilipolytic effects persist with high doses of Cort exposure, while dexamethasone treatment, in fact, increases lipolysis at both low and high doses (Appendix Fig 9). These differential effects of synthetic and naturally occurring GCs, could suggest reasons for conflicting findings in the literature.

Prior stimulation of 3T3-L1 adipocytes with the ß-adrenergic agonist, isoproterenol before the addition of Cort further highlighted the antilipolytic effects. Catecholamines are a main regulator of lipolysis during a stress response and cause potent increases in lipolysis through elevations in cAMP and PKA activity via the ß-adrenergic receptor. Cort significantly reduced isoproterenol stimulated lipolysis in a dose dependent fashion (Fig 3A), a finding similar to that of Ottosson et al. who showed that with 3 days of exposure to the synthetic GC, hydrocortisone, human subcutaneous abdominal adipocytes have reduced lipolytic response to isoproterenol (47). Forskolin, a potent adenyl cyclase agonist also produces increases in lipolysis through increases in cAMP, but does not involve the ß-adrenergic receptor. Cort produced significant reductions in forskolin stimulated lipolysis (Fig 3B), pointing to the fact that Cort may be altering the activity of enzymes along this stimulatory pathway, at or below the level of cAMP.

cAMP concentrations have a large impact on lipolytic rate via PKA activation and subsequent phosphorylation of HSL and perilipin, a protein associated with the lipid

droplet. Previously it has been speculated that GCs could increase cAMP levels due to decreased transcription or activity of phosphodiesterase (PDE), which hydrolyzes cAMP (17, 37, 74), however most studies have been unable to demonstrate changes in cAMP concentration with GC treatment (17, 37). Similar to this, we did not find that cAMP levels were altered with exposure to Cort in either the basal state (Fig 4A), or after stimulation with isoproterenol (Fig 4B), thereby suggesting that Cort is not acutely affecting lipolysis through changes in cAMP concentration. Moving downstream of cAMP, we find that Cort reduces isoproterenol stimulated elevations in PKA activity (Fig 4D). Our cAMP and PKA findings do not follow the effects found by Xu et al. who report increased cAMP concentrations and PKA activity when primary adipocytes are treated for 24 hours with dexamethasone (74). This discrepancy in findings could be due to the fact that increases found in cAMP by Xu et al. were attributed to altered protein expression of PDE3B, genomic effects which are not present in our model.

PKA activity mediates lipolysis, primarly through its stimulatory phosphorylation of HSL and perilipin. HSL activity is increased with phosphorylation of Ser660, Ser563 and dephosphorylation of Ser565. In the basal state, even when no change in PKA activity was detected, phosphorylation of Ser563 was reduced by 25% with CORT treatment (Fig 5E), reductions that closely match those of glycerol release (Fig 1C), and suggests that altered HSL stimulation and activity is responsible for the decreased glycerol release. This result differs from those of Xu et al, who report increased stimulatory phosphorylation of HSL with 24 hours of dexamethasone treatment (74). Again this may result from the genomic decreases in PDE3B and increased cAMP they

have observed that are not present in our short term study. To confirm if the mechanism for reduced lipolysis with GC treatment is via alterations in HSL phosphorylation, we next examined the regulation of this key lipase in the stimulated state. HSL total expression was not changed with the treatment of isoproterenol and/or Cort, as would be expected in this short course of exposure (Fig 6B). In line with reduced PKA activity, Cort normalized HSL phosphorylation at inhibitory Ser565, while reducing phosphorylation of Ser660 and Ser563 (Fig 6C-E). These results are similar to those we have previously reported in 3T3-L1 adipocytes incubated in Cort for 48 hours prior to epinephrine stimulation (9), and suggest that even with long term exposure, at high doses, antilipolytic reductions in PKA activity and HSL stimulatory phosphorylation overpower the genomic stimulatory effects. Perilipin phosphorylation is also reduced with Cort treatment compared to isoproterenol alone (Fig 7C). Perilipin phosphorylation is important in mediating both ATGL and HSL activity during stimulation (62, 75) and reductions would also contribute to reductions in lipolysis.

Based on this study, it appears that reductions in PKA activity are the primary non-genomic mechanisms for Cort's ability to induce reductions in lipolysis. There are several ways through which GCs have been reported to induce non-genomic effects in other tissues of the body: changes to protein kinase activity (AMPK, MAPK, PKA), altered membrane permeability and ion transport into the cytosol, specifically changes to intracellular calcium levels, as well as protein-protein interactions mediated by GC binding to the GR (reviewed in (26)). The involvement of MAPK and AMPK were of considerable interest as the MAPK, ERK, is able to increase HSL activity, while

increased phosphorylation of AMPK causes acute inhibition of HSL activity (1). There are trends for increases in ERK1/2 phosphorylation with isoporterenol treatment and Cort (Fig 8C), however this would suggest increases in lipolysis and would not contribute to Cort's antilipolytic effects. Upon investigation we find that AMPK phosphorylation is increased with isoproterenol stimulation but normalized with Cort (Fig 8B). pAMPK acts as a negative feedback mechanism to decrease lipolysis and reduce costly cycling of FA release and reesterification. It therefore appears that pAMPK increases with isoporterenol stimulated lipolysis secondary to increases in lipolysis, but is unchanged with Cort when lipolysis is normalized. Changes in pAMPK therefore are not responsible for reduced HSL stimulatory phospohorylation and reductions in lipolysis. Non-genomic increases in calcium could also contribute to reductions in lipolysis as elevated calcium levels are reported to reduce cAMP concentration (78). Although we have not measured calcium levels directly, we have not observed significant changes to cAMP levels with treatment, and thus it seems unlikely that elevated calcium levels are responsible for these acute antilipolytic effects. Finally, as our effects were not inhibited with the blockade of GR (Fig 2A), it seems unlikely that Cort reduces lipolysis through a GR associated mechanism. It thus appears that reductions in PKA activity are the primary mechanism through which Cort is able to produce this non-genomic, anti-lipolytic effect.

While we agree with the past literature that GCs have lipolytic effects via increased expression of lipase enzymes (9, 74), we clearly show that Cort also has acute inhibitory effects that can mask its own lipolytic effects (Fig 1A). These antilipolytic

effects are most potent at high doses and last only while the hormone is present. We suspect that heightened levels and effects of GCs in the abdominal adipose tissue through elevated 11βHSD1 activity (4) may highlight the antilipolytic effects in this depot and result in site specific adiposity. Although we show that epididymal adipose tissue cultured ex vivo responds to Cort with reduced glycerol release, similarly to 3T3-L1 adipocytes, further research is needed to fully understand depot specific differences in GC levels and effects. Through reductions in PKA activity, Cort non-genomically reduces the stimulatory phosphorylation of HSL and perilipin and results in decreased NEFA and glycerol release. Lipid accumulation is favoured by increased uptake and storage of FA, and reduced breakdown of TAGs. GCs have been previously reported to increase FA availability and uptake into adipocytes (reviewed in (50)), and it seems likely that this, along with the reductions in lipolysis that we report, contributes to the adiposity that exists in individuals with consistently elevated GC levels.

FIGURES 5

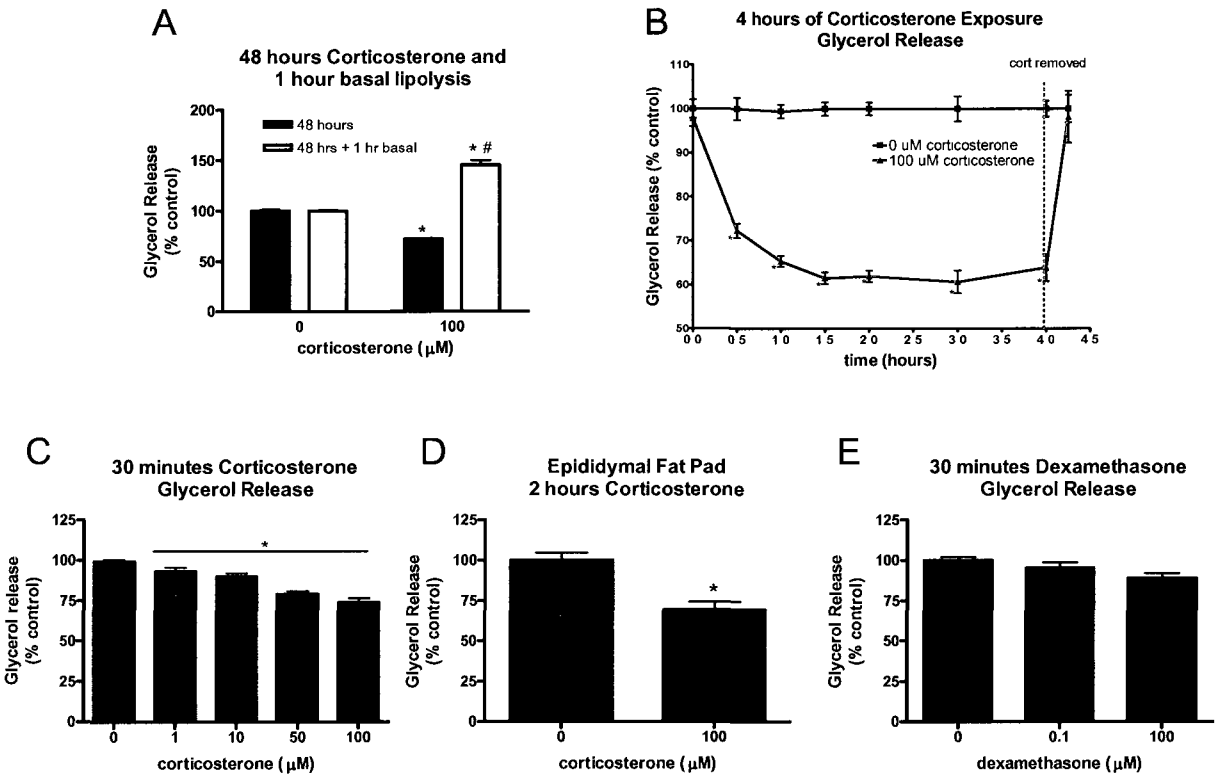


Figure 1: Cort has acute antilipolytic effects on 3T3-L1 adipocytes and epididymal adipose tissue cultured ex vivo.

A) 3T3-L1 adipocytes exposed to 100μM Cort for 48 hours have decreased glycerol release compared to control. When Cort is removed from the media, and cells are allowed to undergo basal lipolysis for one hour, glycerol release is immediately increased compared to controls (n=10). B) Adipocytes treated with 100μM Cort for 4 hours have decreased glycerol release and this decrease was normalized immediately with the removal of Cort. (n=6-12) C) Glycerol release is dose dependent; adipocytes treated with increasing doses of Cort for 30 minutes show a stepwise decrease in glycerol release ($n\geq17$). D) Epididymal adipose tissue cultured ex vivo responds similarly to 3T3 adipocytes with reduced glycerol release following treatment with 100μM Cort (n=8). E) Dexamethasone, a synthetic glucocorticoid did not produce significant decreases in glycerol (n=6). *p<0.05 vs control, #p<0.05 compared to 48hrs.

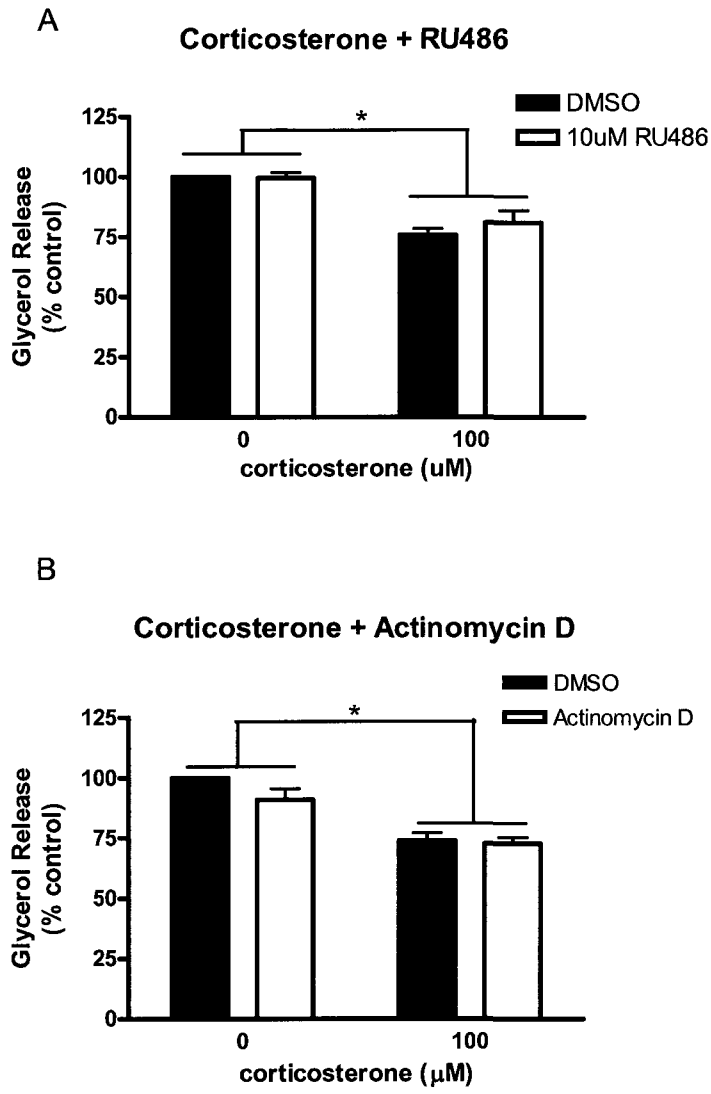


Figure 2: Cort's antilipolytic effects are not mediated by the glucocorticoid receptor, and are non-genomic.

3T3-L1 adipocytes were treated with or without 100 μM Cort in DMEM for 30 minutes as before, with either 10 μM RU486, a glucocorticoid receptor (GR) inhibitor, 1uM Actinomycin D, a transcription inhibitor, or an equivalent volume of DMSO as a placebo. A) The acute inhibitory effects of Cort were not attenuated with the addition of 10μM RU486 (n=4). B) The addition of 1μM actinomycin D did not alter Cort's anti-lipolytic effects (n=6). *main effect of cort, p<0.05

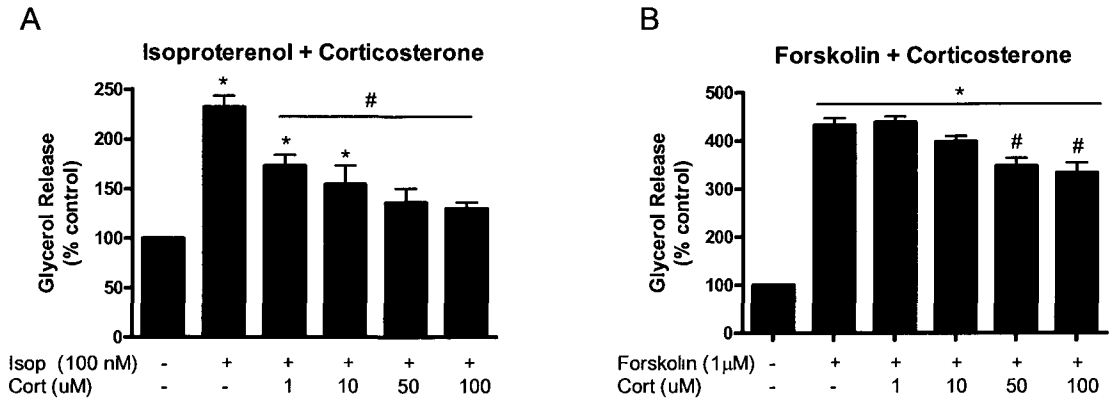


Figure 3: Corticosterone reduces stimulated lipolysis.
3T3-L1 adipocytes were treated with isoproterenol, a ß-adrenergic receptor agonist, or 1 μM forskolin, an adenyl cyclase agonist, in KREBS Ringer buffer for 30 minutes, then Cort was added for an additional 30 minutes. A) The addition of Cort to media containing 100nM isoproterenol significantly reduced glycerol release compared to isoproterenol alone in a dose dependent fashion (n=6). B) 1μM forskolin stimulated lipolysis to a greater extent than isoproterenol, however Cort produced inhibitory effects at doses of 50 and 100μM (n=4). This suggests that Cort's effects occur at or below the level of cAMP *p<0.05 vs. controls, #p<0.05 vs. isoproterenol or forskolin alone

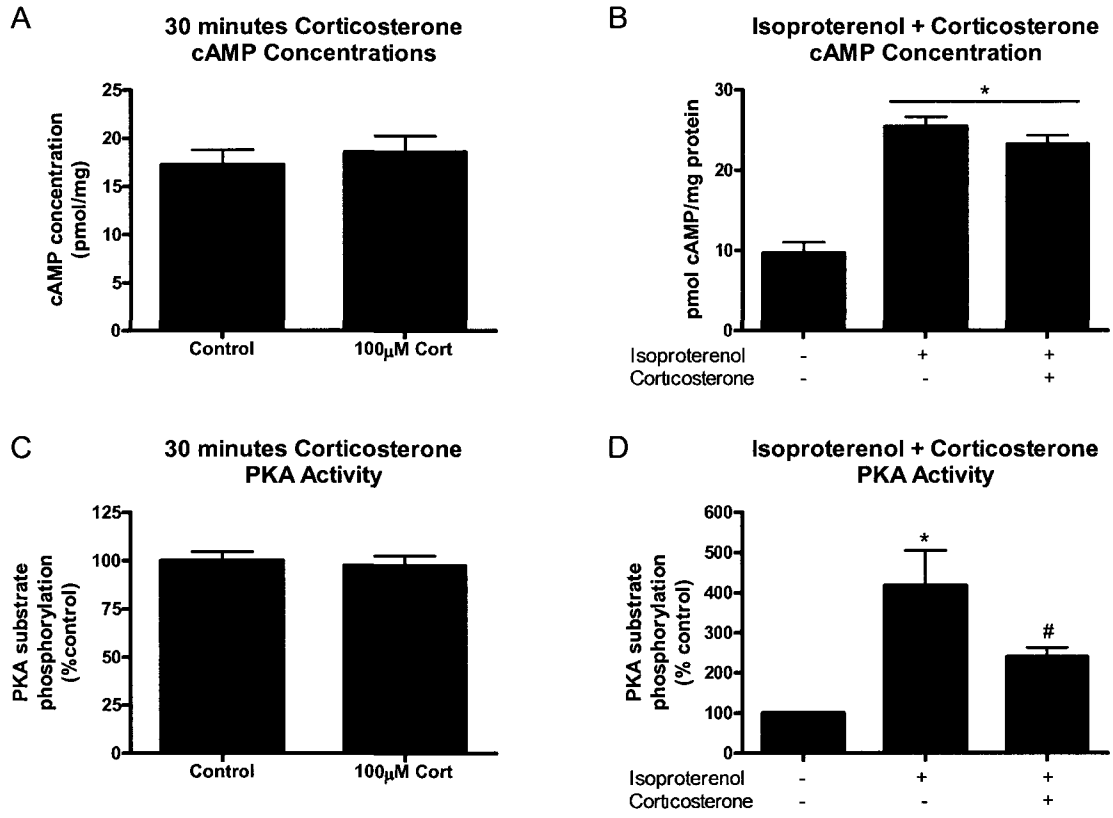


Figure 4: Cort reduces β-adrenergic stimulated increases in PKA but does not alter cAMP concentrations.

3T3-L1 adipocytes were treated with or without 100 μM Cort for 30 minutes, or with a combination of isoproterenol and Cort, and then collected for cAMP analysis and immunoblotting. There were no changes in cAMP concentration with Cort treatment alone (A; n=4-8), nor were there any changes in PKA activity (B; n=4-8). C) cAMP concentrations were elevated with isoproterenol treatment, and the addition of 100 μM Cort did not significantly alter cAMP concentrations (n=5-6). D) PKA activity was significantly increased with isoproterenol, and was normalized to levels of controls with the addition of 100 μM Cort (n=7). *p<0.05 vs. controls, #p<0.05 vs. isoproterenol alone

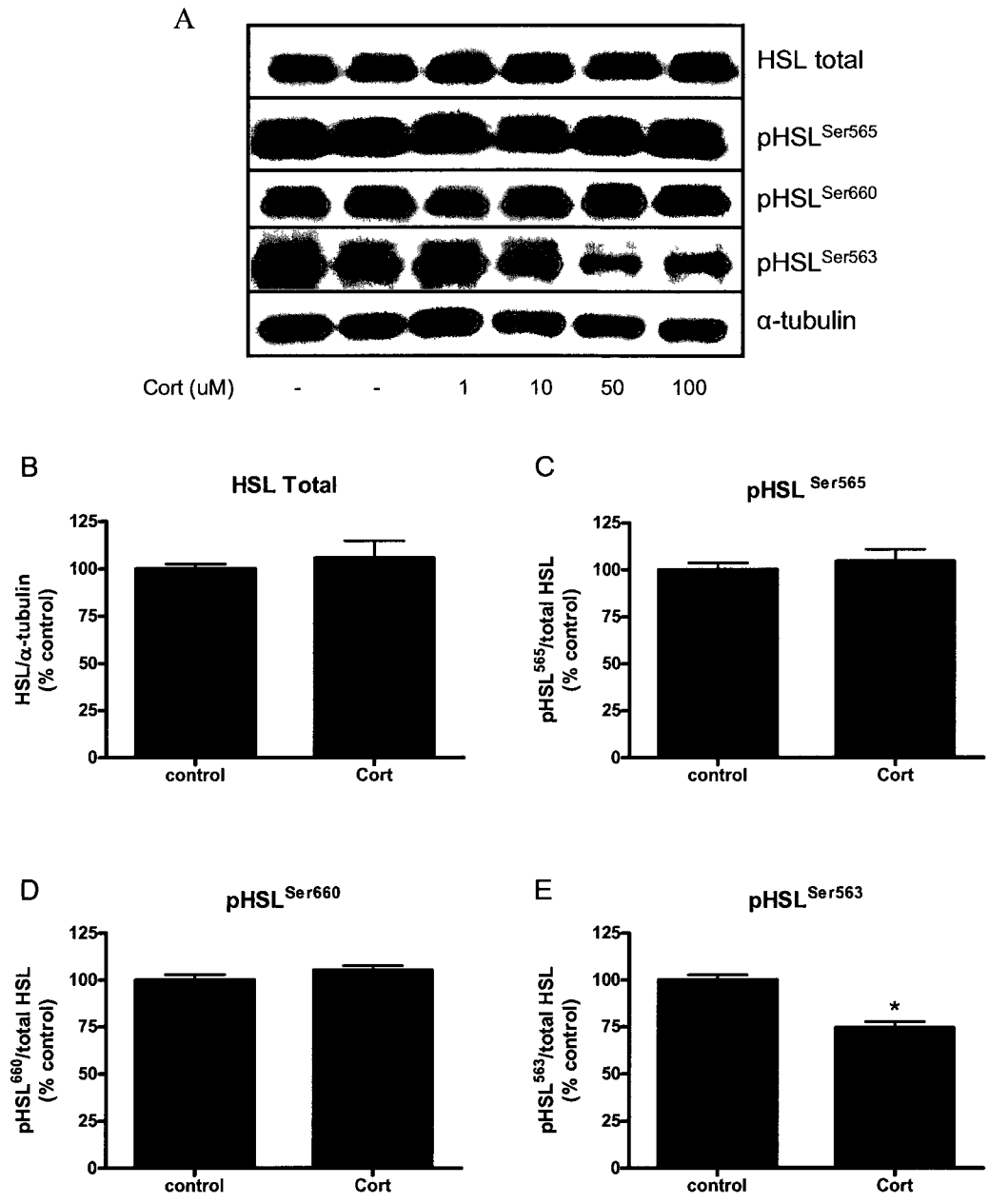


Figure 5: Cort alters HSL phosphorylation status in the basal state.
3T3-L1 adipocytes were maintained in DMEM with or without 100μM Cort for 30 minutes. Blots are representative of those used to quantify total and phophorylated HSL expression (A). Cort for 30 minutes did not produce changes in total HSL (B; $n \geq 8$), pHSL^{Ser565} (C; n=4-8) or pHSL^{Ser660} (D; n>3). There was however a significant decrease in pHSL^{Ser563} (E; n>7). *p<0.05 vs. control

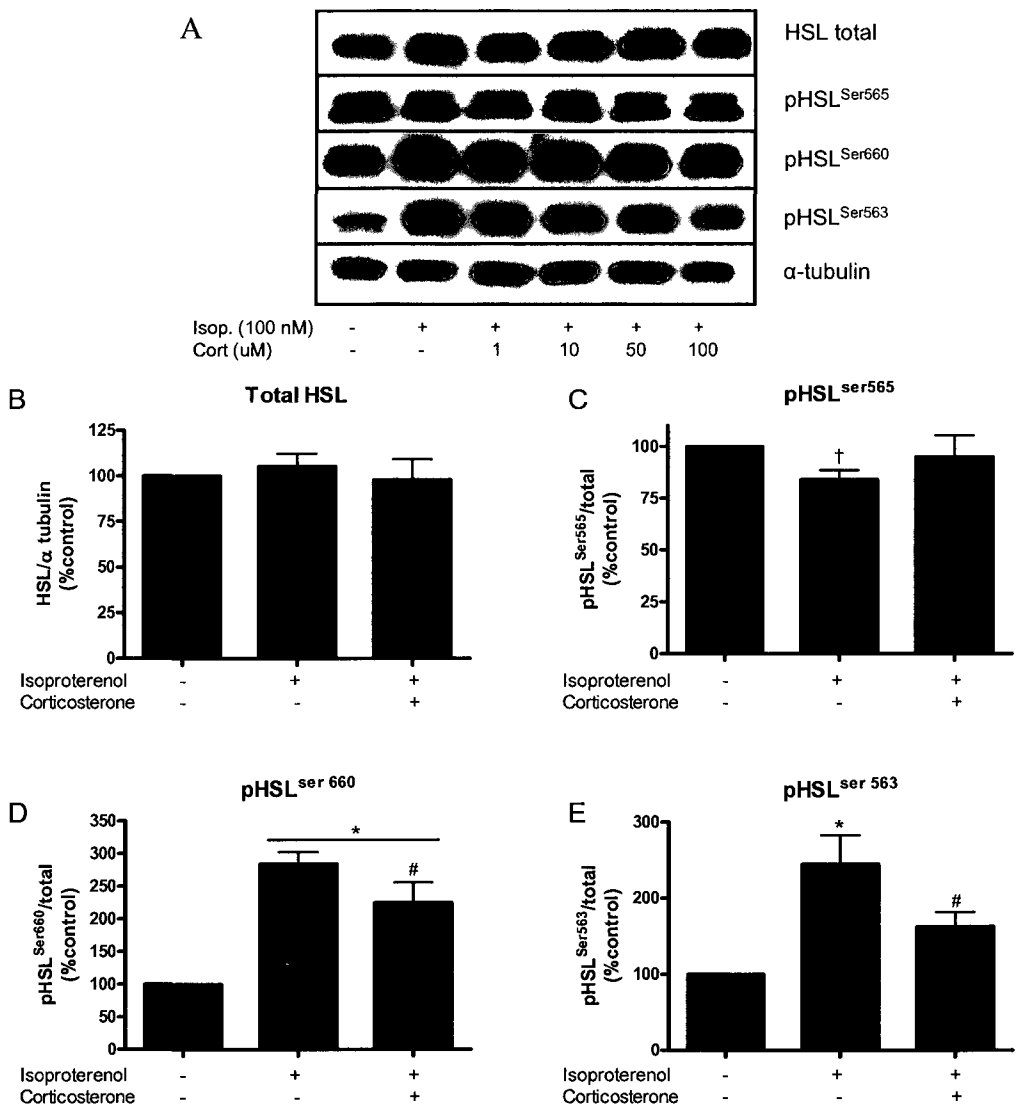


Figure 6: Cort reduces ß-adrenergic stimulatory phosphorylation of HSL
3T3-L1 adipocytes were treated with 100nM isoproterenol in KREBS Ringer buffer for 30 minutes, then 100 μM Cort or an equivalent volume of DMSO was added for an additional 30 minutes. Following treatment adipocytes were collected and immunoblotted for total HSL, $pHSL^{Ser565}$, $pHSL^{Ser660}$ and $pHSL^{Ser563}$. With ß-adrenergic stimulated lipolysis it is expected that $pHSL^{565}$ would be reduced while $pHSL^{Ser660}$ and $pHSL^{Ser563}$ would be increased. A) Blots represent those used to quantify the expression of total and phosphorylated HSL. B) Total HSL protein was not changed with treatment (n=11-14). C) As expected $pHSL^{Ser565}$ was reduced with isoproterenol treatment compared to controls, but was normalized to levels of controls with the addition of Cort (n=6-8). D) $pHSL^{Ser660}$ was increased with isoproterenol treatment, however this increase was attenuated with the addition of Cort (n=5). E) Isoproterenol increased $pHSL^{Ser563}$, and in combination with Cort phosphorylation was reduced (n=6-7). *$p<0.05$ vs. controls, #$p<0.05$ vs. isoproterenol alone, †$p<0.05$ vs control in a paired t-test

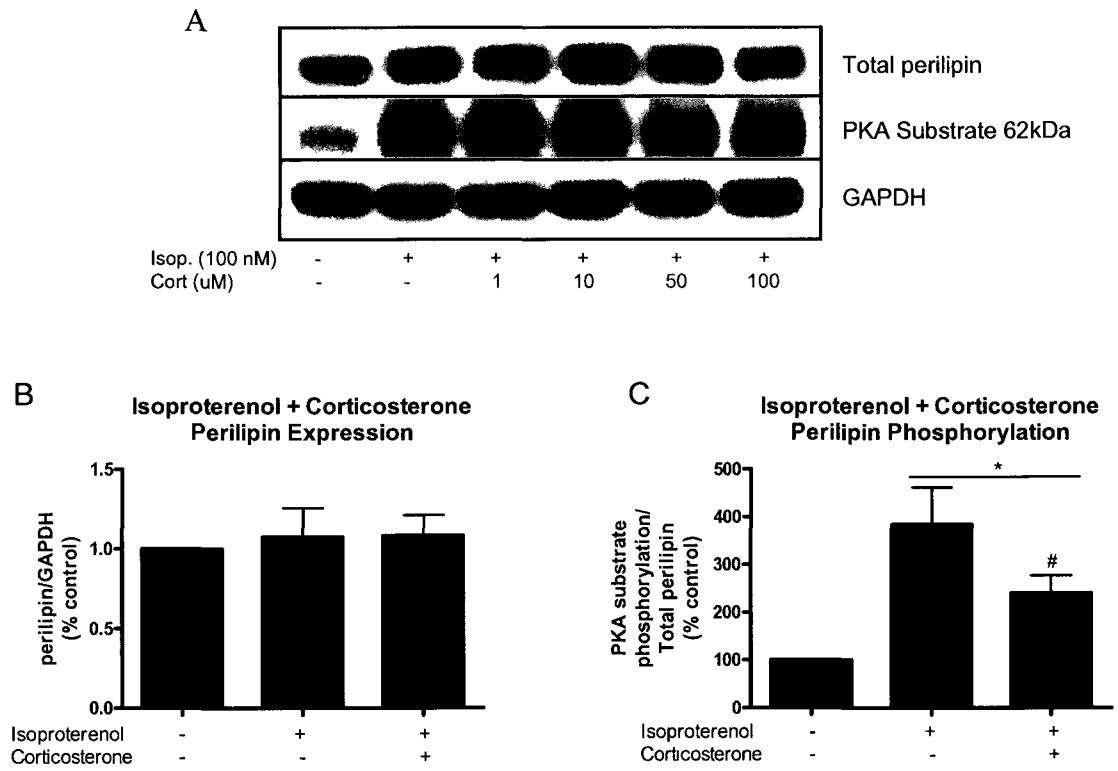


Figure 7: Cort reduces ß-adrenergic, stimulatory phosphorylation of perilipin
3T3-L1 adipocytes were treated with 100nM isoproterenol in KREBS Ringer buffer for 30 minutes, then 100 μM Cort or an equivalent volume of DMSO was added for an additional 30 minutes. Following treatment adipocytes were collected and immunoblotted for total perilipin, and phosphorlyated PKA substrate 62 kDa (representative of phosphorylated perilipin). With ß-adrenergic stimulated lipolysis it is expected that perilipin phosphorylation would be increased. A) Blots represent those used for quantification. B) Perilipin expression was unchanged with treatment (n=7-8). C) Perilipin phosphorylation was significantly decreased with the addition of Cort compared to isoproterenol alone (n=4-8). *p<0.05 vs. controls, #p<0.05 vs. isoproterenol alone

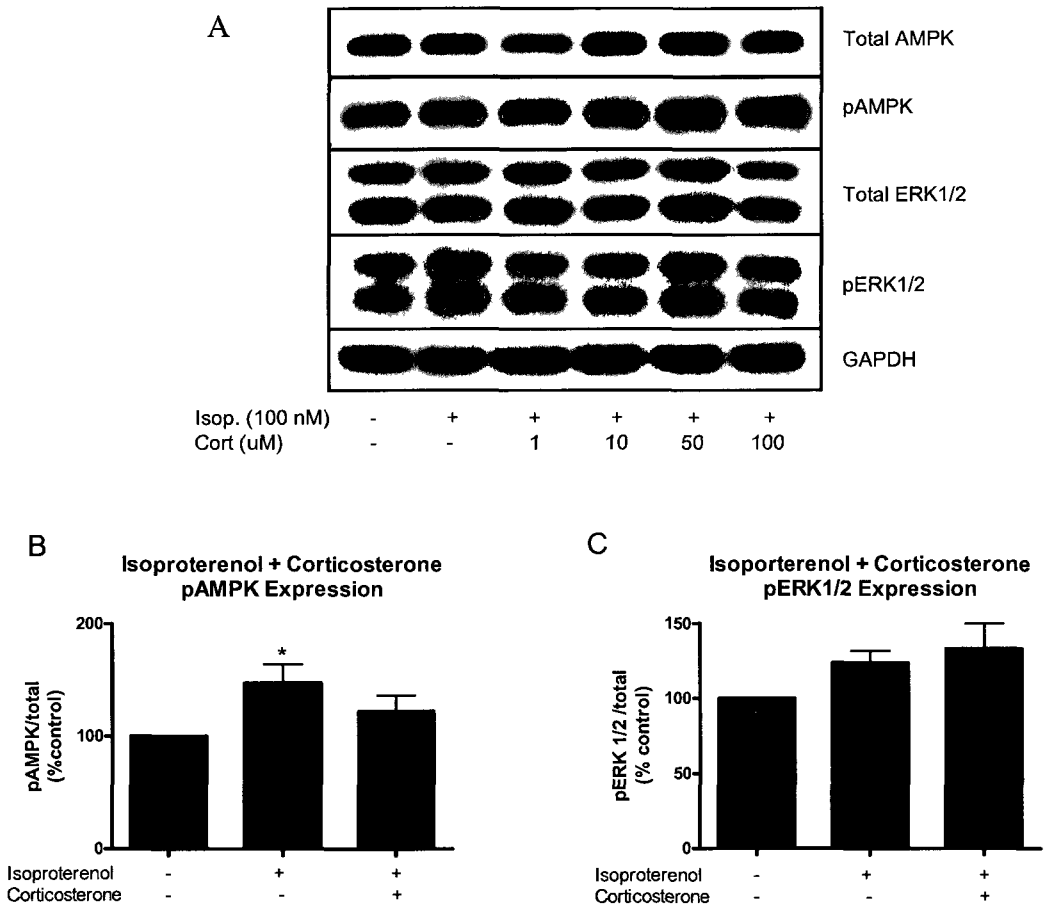


Figure 8: Cort's inhibitory effects are not mediated through AMPK or ERK
3T3-L1 adipocytes were treated with 100nM isoproterenol in KREBS Ringer buffer for 30 minutes, then 100 μM Cort or an equivalent volume of DMSO was added for an additional 30 minutes. Following treatment adipocytes were collected and immunoblotted for pAMPK and pERK1/2. Phosphorylation of AMPK is known to occur with ß-adrenergic stimulation, and acts as a negative feedback mechanism, increasing inhibitory phosphorylation of $pHSL^{Ser565}$. ERK is a member of the MAPK family and is phosphorylated with ß-adrenergic stimulation, increasing HSL activity by phosphorylating it at Ser600. A) Blots represent those used to quantify the expression of each protein. B) AMPK is phosphorylated as a result of isoprotereonol stimulation, the addition of Cort normalizes pAMPK to levels of controls (n=4-6). C) The phosphorylation status of ERK1/2 is not significantly altered with treatment, although there are trends for increases with isoproterenol and Cort treatment (p=0.07 for interaction; n=5-6). *p<0.05 vs control

SUMMARY AND FUTURE DIRECTIONS 6

Summary:

In this study, we investigate the acute effects of Cort on 3T3-L1 adipocyte lipolysis. By focusing our attention on the effects of 30 minutes of Cort exposure we were able to isolate the non-genomic effects of these stress hormones in vitro. We observed non-genomic, antilipolytic effects of Cort that oppose the well known genomic, lipolytic effects (9, 74). We believe that inhibitory effects result from reductions in PKA activity and persist as long as Cort is present, as evidenced by the fact that even after 48 hours of exposure doses greater than 10μM Cort reduce lipolysis. This would suggest that at high doses the antilipolytic effects of Cort are strong enough to overcome the genomic, lipolytic effects and contribute to adipose accumulation, which is typically charactistic of individuals with elevated cortisol levels (55). Interestingly, the synthetic GC, dexamethasone, does not display such potent antilipolytic effects with either short (30min) or longer (24h) exposure. In this and previous studies (7, 9), we have demonstrated that GCs are able to produce differential effects on adipocyte lipolysis depending on dose, type of GC used, and duration of exposure, and these differential effects may explain some of the conflicting reports within the literature. This study has thus been important not only for understanding potential mechanisms for GC induced adipose accumulation, but also in elucidating possible reasons for contradictions within the literature.

Limitations and Future Directions:

Although we have observed important changes in adipocyte lipolysis with Cort treatment, there are limitations when making inferences to the clinical understanding of individuals with elevated GC levels. By studying 3T3-L1 adipocytes, we are able to investigate effects on isolated adipocytes to specify the effects of Cort. 3T3-L1 adipocytes are differentiated from an immortal embryonic mouse fibroblast cell line, and thus are expected to act similarly to primary rodent adipocytes. It is possible, however, that there are differences between rodent and human adipocyte function. It also seems likely that adipocytes located in specific adipose depots (ie. visceral or subcutaneous) function differently. By comparing the effects of Cort on 3T3-L1 adipocytes and epididymal adipose tissue (Fig 1D), it would appear that these adipocytes act similarly, however preliminary data comparing the effects of Cort on epididymal and subcutaneous depots (Appendix Fig 10 and 11) suggests that Cort may have differential effects in each depot. More work is thus needed to clarify particular differences between sites as well as species.

Another major limitiation of this thesis and other research studies using *in vivo* or *ex vivo* techniques, is that measuring tissue levels of Cort is technically quite challenging. Although we know that 1μM of Cort treatment represents approximately the plasma levels of Cort in rodents (9, 20), measurements within the tissue are much more difficult, likely due to the fact that intracellular Cort is bound to receptors while commercially available kits are designed to measure free, unbound Cort. Elevated levels of 11βHSD-1 in the visceral adipose tissue suggest that Cort levels would be higher in this region,

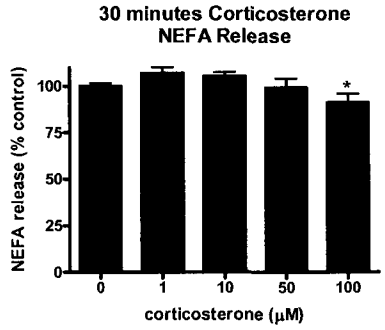
perhaps highlighting antilipolytic effects there. Given the fact that Cort has dose dependent effects on lipolysis, further work is needed to gain a better understanding of Cort levels in the adipose depots.

Finally, it will also be important to study Cort's effects *in vivo*. GCs have whole body effects that may cause lipid accumulation: GCs are known to cause changes in food intake, increase lipogenesis within the liver, elevate FA delivery and uptake into the adipose tissue, and alter adipokine release, in addition to altering lipid metabolism within the tissue itself (reviewed in (50)). Reductions in lipolysis therefore could contribute to increases in lipid accumulation, but are not likely solely responsible for the adiposity seen in individuals with GC excess. Therefore a better understanding of GC's full effects on the body and how these factors may all interact is necessary as well.

APPENDIX 7

Appendix A: Supplementary Work

A



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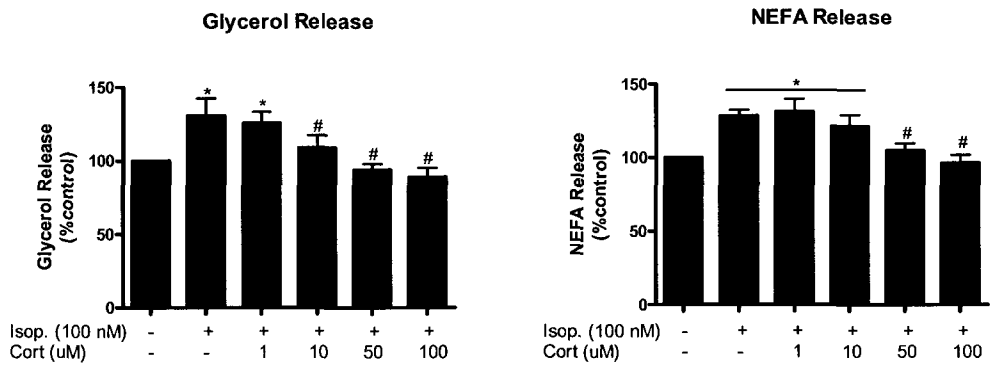


Figure 1: Cort reduces NEFA release
A) 3T3-L1 adipocytes were exposed to increasing doses of Cort in DMEM + 3.5% FBS for 30 minutes. Media was collected and NEFA content was measured. There were no significant changes to NEFA release with low doses of Cort, however at a dose of 100μM NEFA release was reduced by 11% ($n \geq 11$, $p<0.05$). B) 3T3-L1 adipocytes were treated with isoproterenol and Cort as before, however DMEM + 3.5% FBS was used in place of Krebs Ringer + BSA as the BSA contained fatty acids that altered the NEFA assay. In this media neither glycerol nor NEFA release was not increased to the same extent as in Krebs, however Cort reduces glycerol and NEFA release similarly with increasing concentrations ($n=6$, *$p<0.05$ vs control, #$p<0.05$ vs isoproterenol alone).

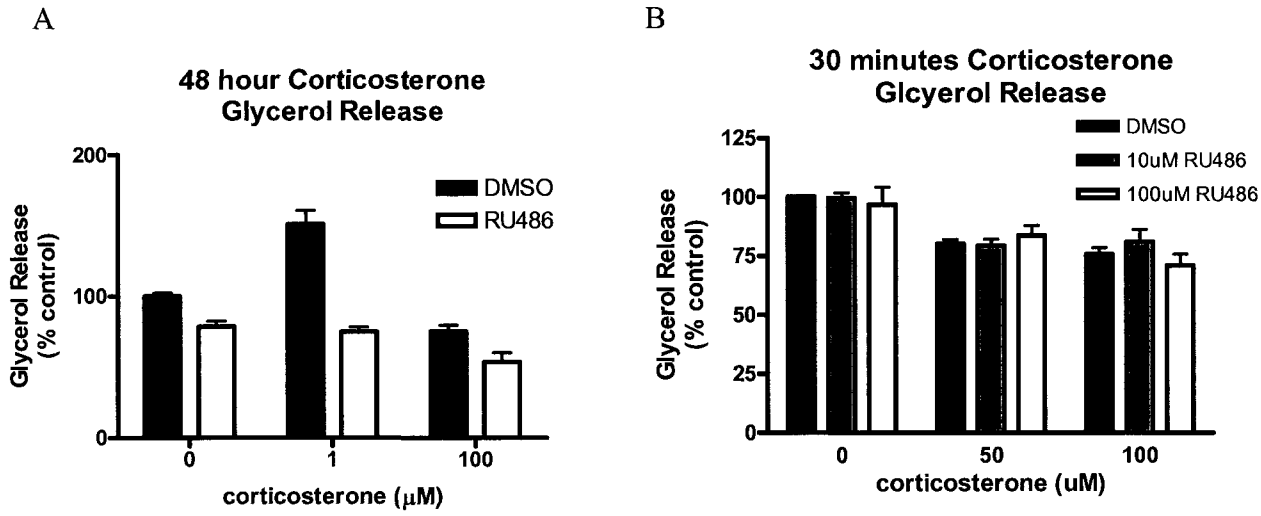


Figure 2: RU486 reduces long term genomic effects, but not acute effects of Cort
3T3-L1 adipocytes were treated with or without Cort in DMEM as before, along with either RU486, a glucocorticoid receptor (GR) or an equivalent volume of DMSO as a placebo. A) The addition of 10μM RU486 inhibited the lipolytic effects of 1 μM Cort with 48 hours of exposure (n=3) B) The acute inhibitory effects of Cort were not attenuated with the addition of 10μM or 100 μM RU486 (n=4).

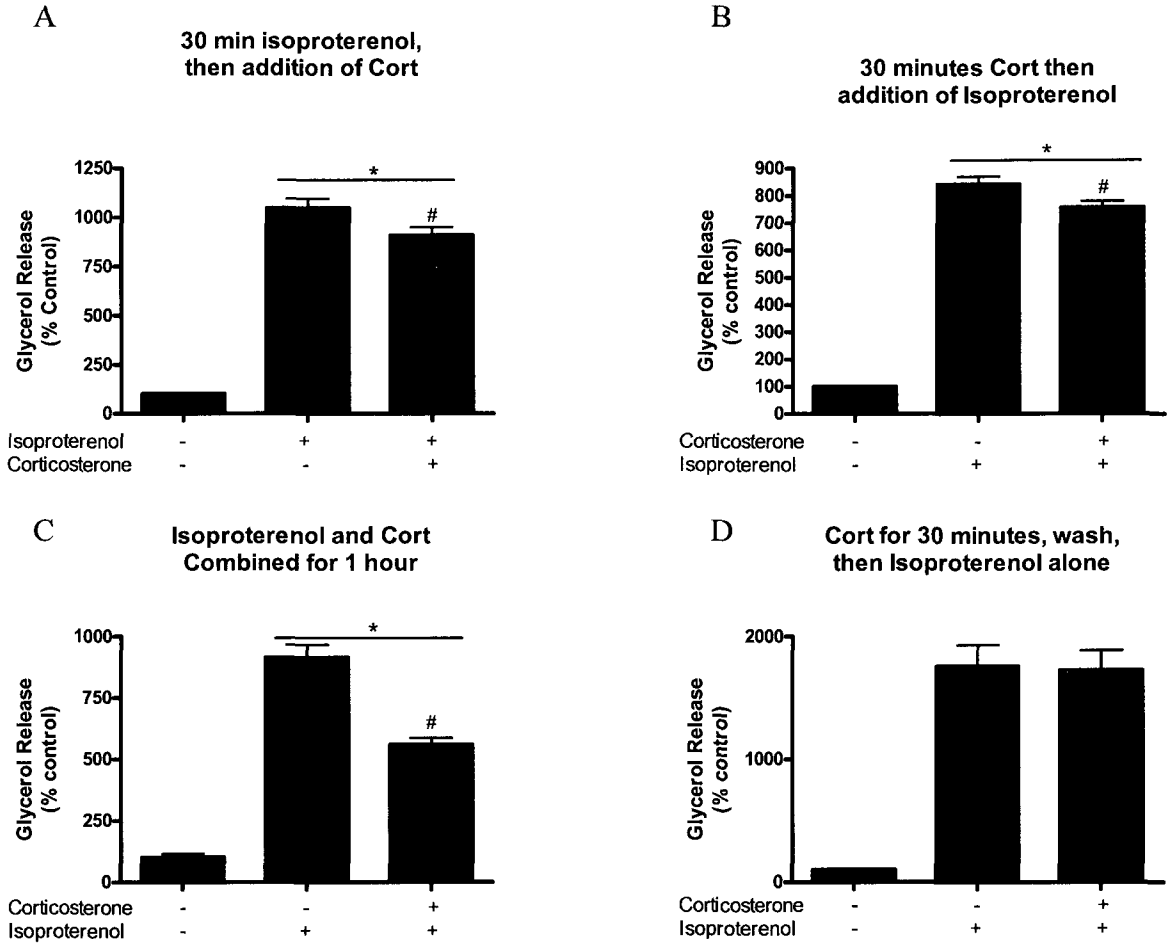


Figure 3: The presence of Cort reduces isoproterenol stimulated lipolysis regardless of order added.

To ensure that the order of isoproterenol and Cort addition did not affect lipolytic stimulation, 3T3 adipocytes were exposed to isoproterenol and Cort in Krebs Ringer Buffer + 3.5% BSA in a variety of combinations. A new batch of cells was used for these experiment; although these adipocytes responded more strongly to isoproterenol, 100 μM Cort still produced a significant reduction in glycerol release and also confirms that all 3T3-L1 adiopcytes respond similarly to Cort. Significant reductions in glycerol release occurred regardless of whether Cort was added 30 minutes after the introduction of isproterenol (A), 30 minutes prior to isoproterenol (B), or at the same time as isoproterenol (C). When adipocytes were first exposed to Cort for 30 minutes, then washed and exposed to isoproterenol alone for 30 minutes there were no reduction in glycerol release, confirming that the presence of Cort is necessary for inhibitory effects (D).

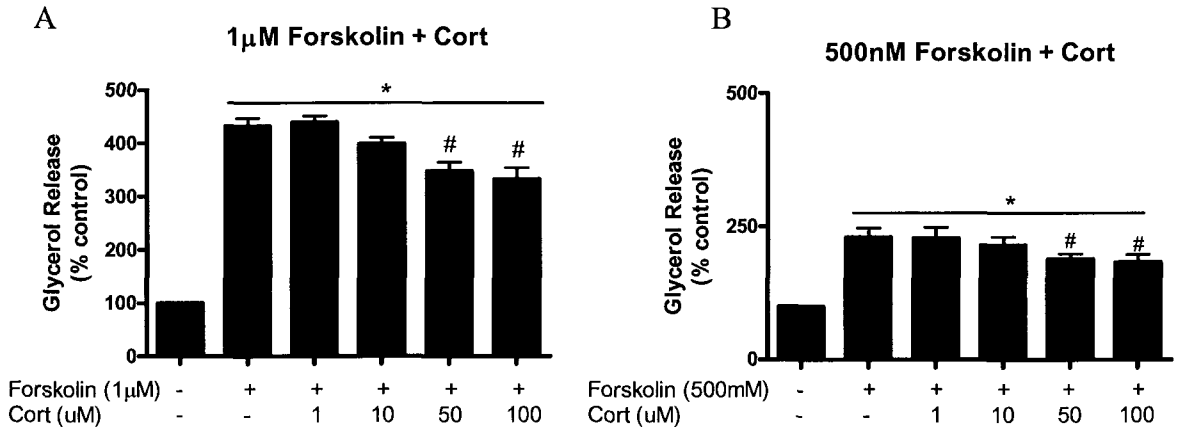


Figure 4: Cort reduces forskolin stimulated lipolysis similarly at doses of 500nM and 1 μM forskolin

To ensure that heightened lipolytic stimulation by 1 μM forskolin did not mask the effects of Cort, 3T3-L1 adipocytes were treated with 500nM Forskolin prior to the addition of Cort. Similar reductions appeared as when 1μM forskolin was used; glycerol release was significantly reduced compared to forskolin alone at doses of 50μM and 100μM Cort. *p<0.05 vs control, #p<0.05 vs forskolin alone

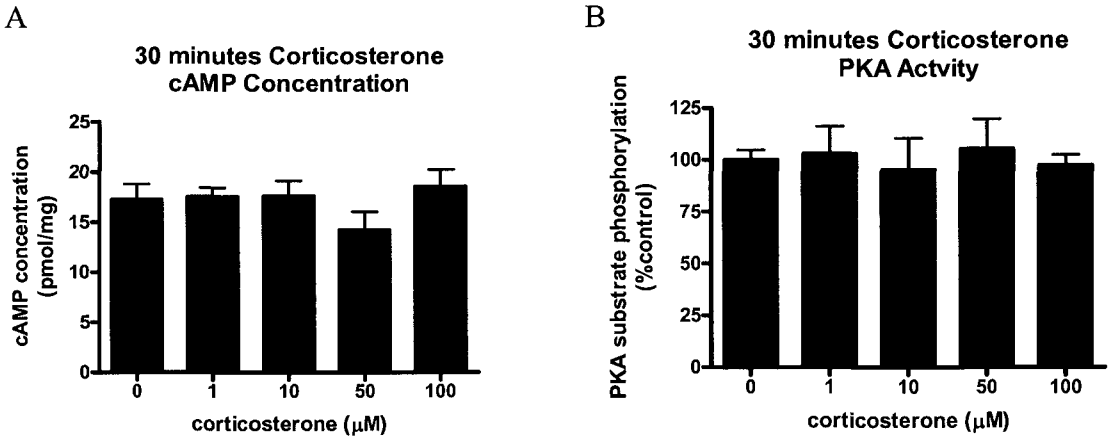
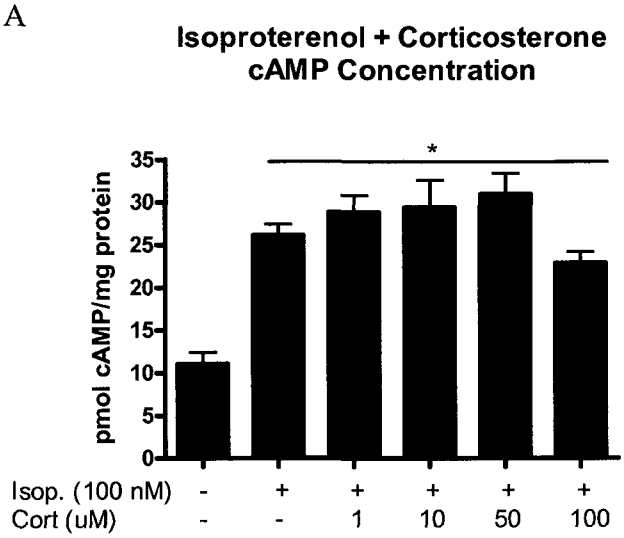


Figure 5: There are no detectable changes to cAMP concentration or PKA activity with Cort alone - full dose response

3T3-L1 adipocytes were maintained in DMEM + 3.5% BSA, with or without Cort for 30 minutes, and then collected for cAMP analysis or immunoblotting. There were no changes in cAMP concentration with Cort treatment (A; n=4-8), nor were there any changes in PKA activity (B; n=4-8).

A



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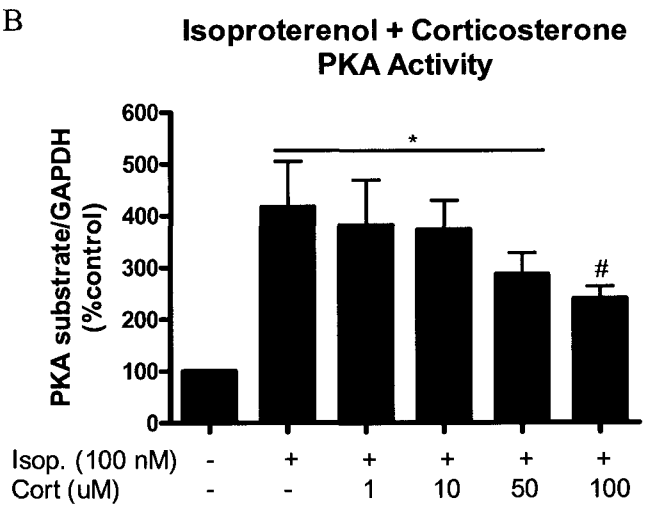

Figure 6: Corticosterone reduces stimulated lipolysis with reductions in PKA activity – full dose response

3T3-L1 adipocytes were treated with isoproterenol, a ß-adrenergic receptor agonist, in KREBS Ringer buffer for 30 minutes, then Cort was added for an additional 30 minutes. A) cAMP concentrations were elevated with isoproterenol treatment, but the addition of Cort did not significantly reduce cAMP concentrations (n=4-5). B) PKA activity, as measured by the phosphorylation of PKA substrates, was significantly increased with isoproterenol, but was reduced in a dose dependent fashion with the addition of Cort (n=7). *p<0.05 vs. controls, #p<0.05 vs. isoproterenol alone

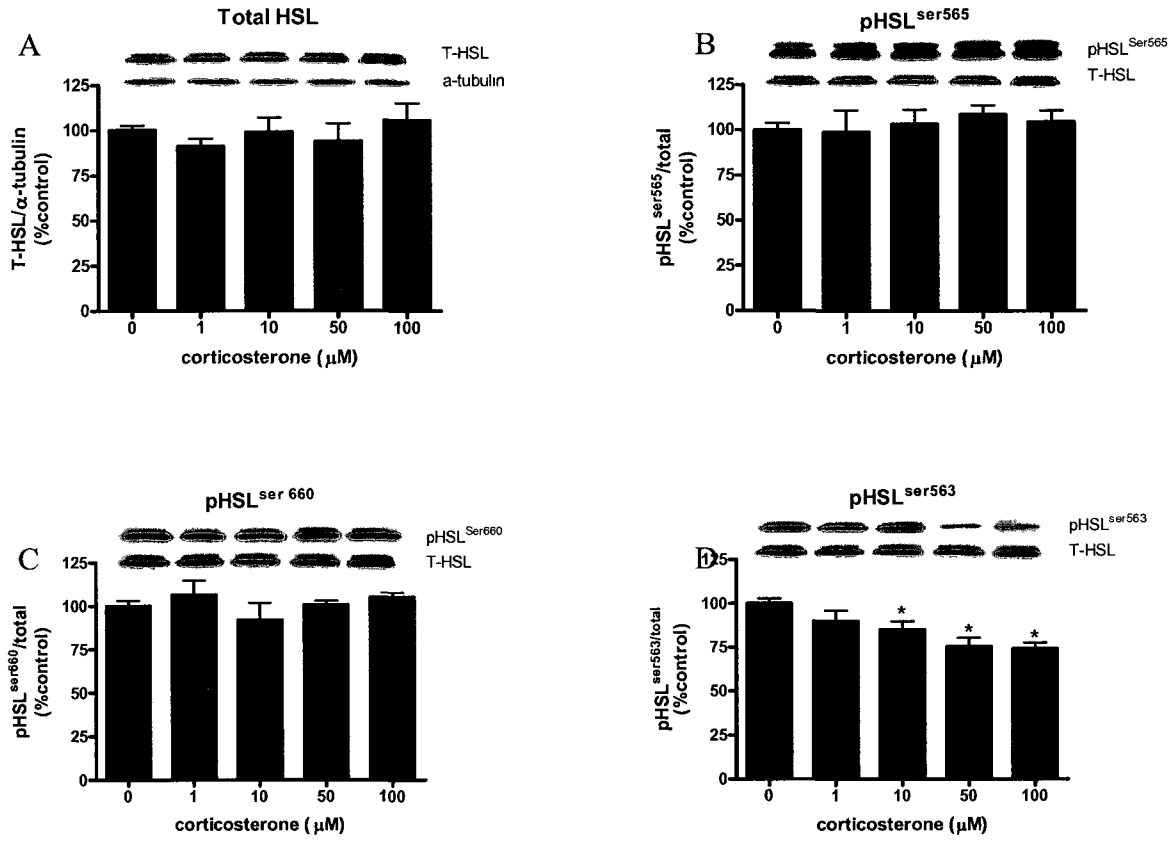

Figure 7: Cort alone alters HSL phosphorylation status – full dose response
3T3-L1 adipocytes were maintained in DMEM with or without Cort for 30 minutes, and then collected for immunoblotting. Cort for 30 minutes did not produce changes in total HSL (C; n=7-16), pHSL^{Ser565} (D; n=4-8) or pHSL^{Ser660} (E; n=3-8). There was however a significant, dose dependent decrease in pHSL^{Ser563} (F; n=7-16). *p<0.05 vs. control

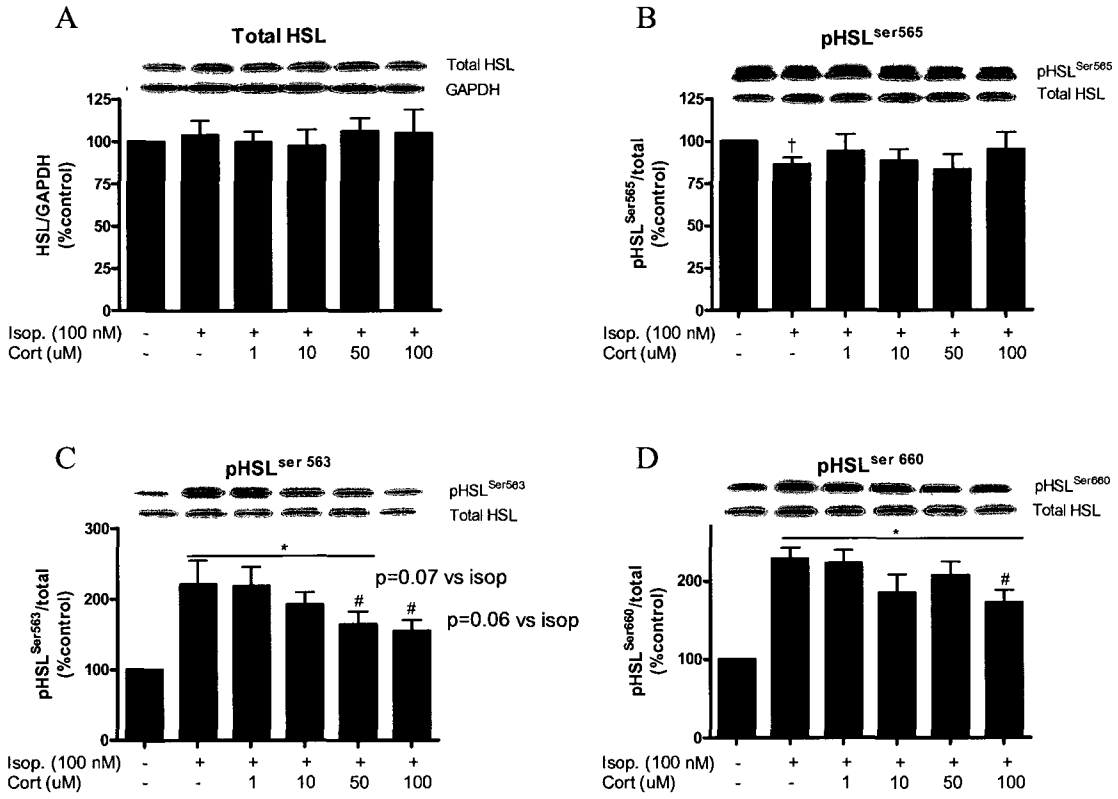

Figure 8: Cort reduces ß-adrenergic stimulatory phosphorylation of HSL – full dose response

3T3-L1 adipocytes were treated with 100nM isoproterenol in KREBS Ringer buffer for 30 minutes, then Cort or an equivalent volume of DMSO was added for an additional 30 minutes. Following treatment adipocytes were collected and immunoblotted for total HSL, $pHSL^{Ser565}$, $pHSL^{Ser660}$ and $pHSL^{Ser563}$. With ß-adrenergic stimulated lipolysis it is expected that $pHSL^{565}$ would be reduced while $pHSL^{Ser660}$ and $pHSL^{Ser563}$ would be increased. A) Total HSL protein was not changed with treatment (n=6-8). B) As expected $pHSL^{Ser565}$ was reduced with isoproterenol treatment compared to controls, but was normalized to levels of controls with the addition of Cort (n=5-8). C) $pHSL^{Ser660}$ was increased with isoproterenol treatment, however this increase was attenuated with the addition of 100µM Cort (n=5-11). D) Isoproterenol increased $pHSL^{Ser563}$, and in combination with Cort phosphorylation was reduced in a dose dependent fashion (n=4-6). *p<0.05 vs. controls, #p<0.05 vs. isoproterenol alone, †p<0.05 vs control in a paired t-test

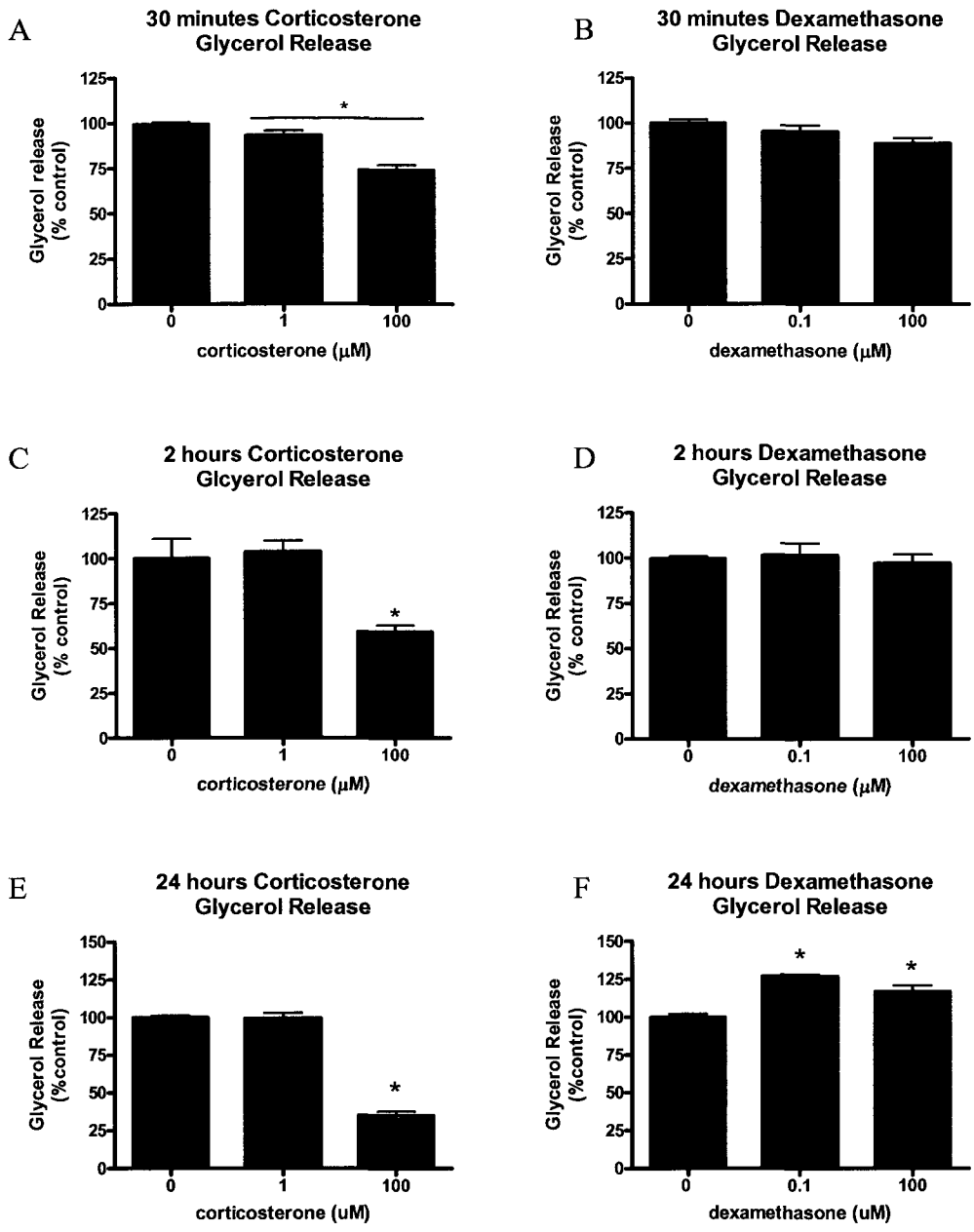

Figure 9: Dexamethasone does not produce potent antilipolytic effects
3T3-L1 adipocytes were treated with DMEM + 3.5% FBS containing Cort or dexamethasone for the given durations. A) Cort produced a dose dependent decrease in glycerol release with 30 minutes of expsosure ($n \geq 17$). B) Dexamethasone did not produce significant reductions in glycerol release after 30 minutes ($n=6$). C) Cort's antilipolytic effects persist with high doses at 2 hours ($n=4$). D) There were no changes in glycerol release with 2 hours of dexamethasone exposure ($n=3$-4). E) With 24 hours of exposure to Cort, glycerol release remains suppressed with high doses of Cort ($n=10$-12). F) Glycerol release is elevated with both low and high doses of dexamethasone after 24 hours of exposure ($n=4$). *$p<0.05$ vs. controls

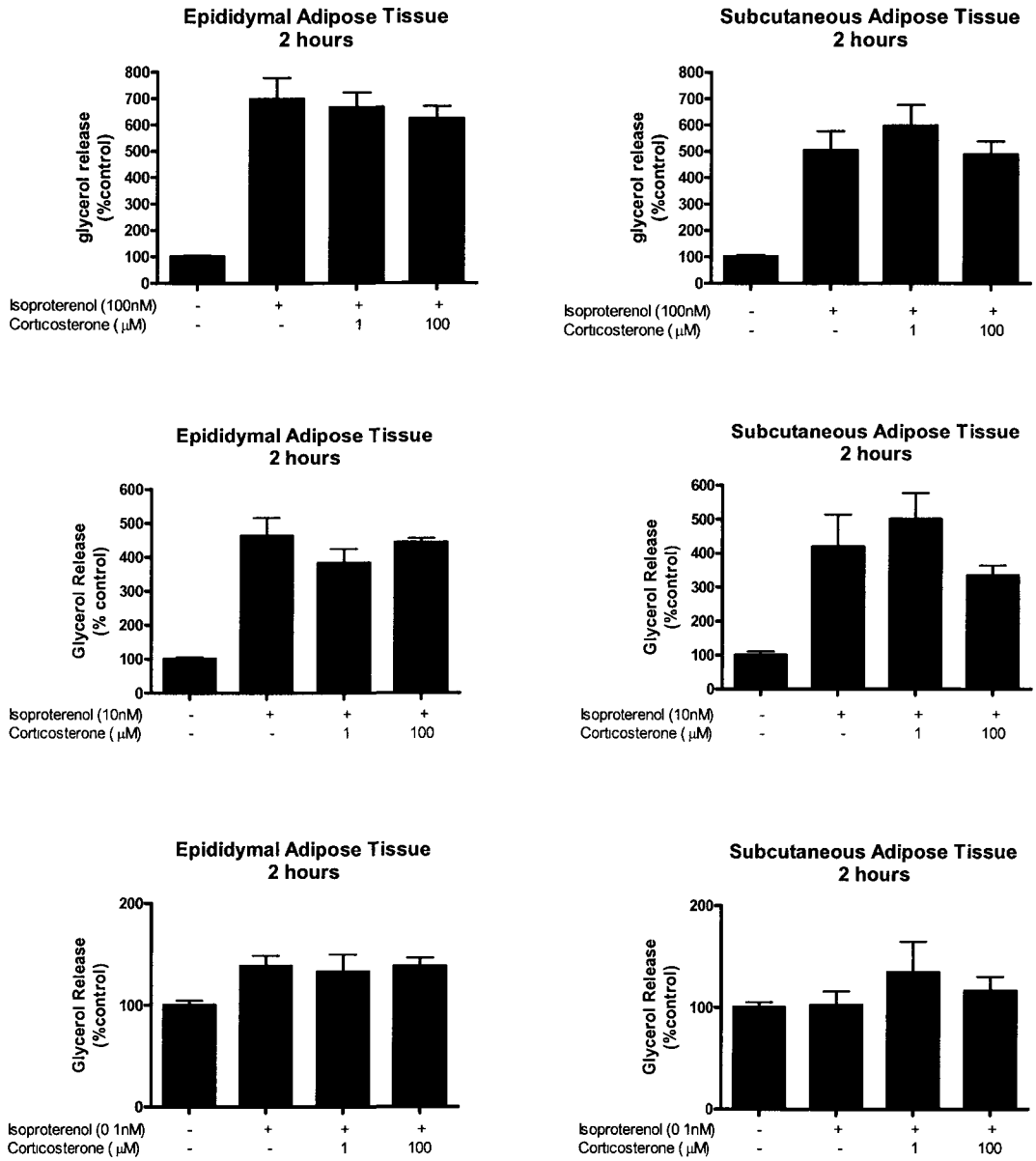

Figure 10: Cort may have differential effects on epididymal and subcutaneous adipose depots

Adipose tissue organ culture was utilized to compare epididymal and subcutaneous adipose tissues' response to isoproterenol and Cort stimulation. Tissue was minced and cultured in Medium 199, then isoproterenol and Cort were added to the media at the same time. Glycerol release was measured 2 hours following treatment. Differential concentrations of isoproterenol were used to determine the optimal amount to stimulate lipolysis and determine if Cort could have an effect. Patterns emerge that suggest that Cort may affect epididymal and subcutaneous adipose depots differently. This is a matter worth further investigation in the future. (n=2 animals for each concentration of isoproterenol)

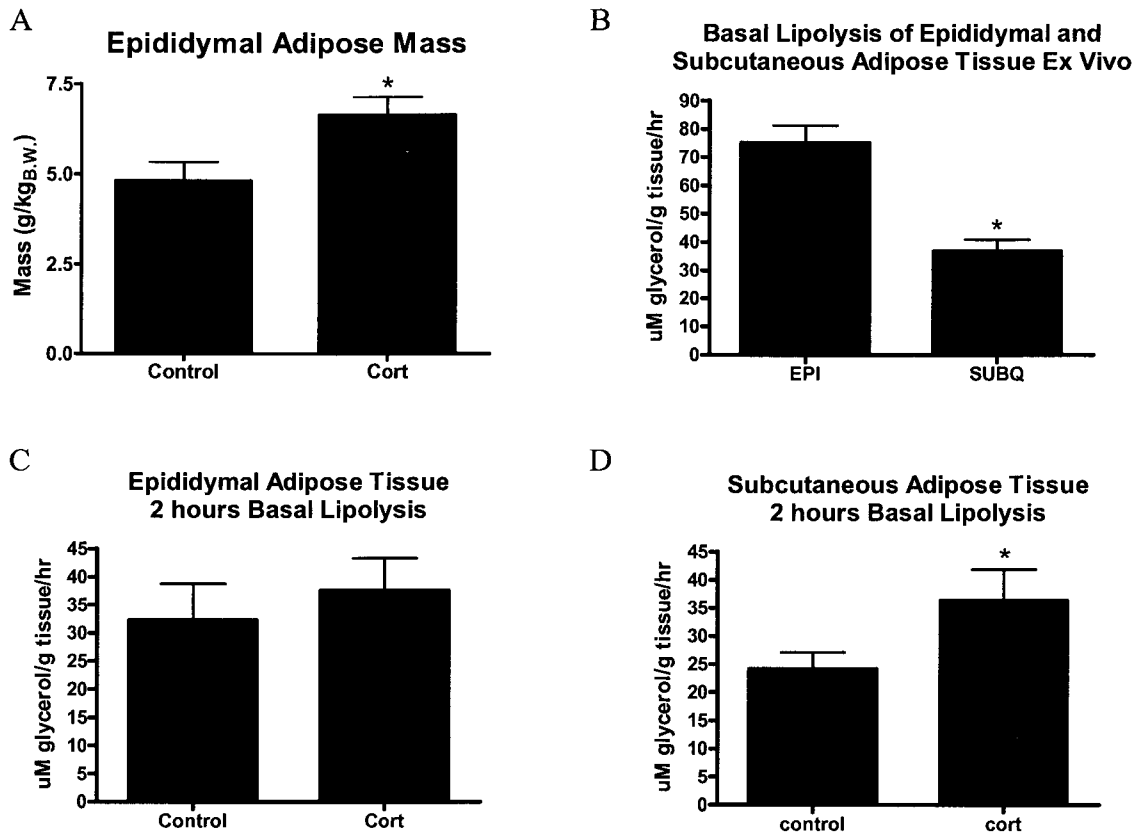

Figure 11: Subcutaneous adipose tissue from animals treated with Cort has elevated basal lipolytic rates while epididymal is unchanged.

Wax (Control) or Cort pellets were subcutaneously implanted in male Sprague-Dawley rats for 14 days to elevate plasma Cort levels. Following treatment, animals were euthanized and adipose tissue was removed from subcutaneous and epididymal depots and cultured using the adipose tissue organ culture method. 2 hours of basal lipolysis was measured in Medium 199. A) Although overall body mass was reduced in Cort treated animals, with visible peripheral wasting, relative epididymal adipose tissue mass was increased (n=7, *p<0.05). B) Subcutaneous adipose tissue had reduced levels of basal lipolysis compared to epididymal tissue, as is consistent with the literature (n=5, *p<0.001). B) There were no differences in glycerol release between the epididymal adipose tissue of animals previously treated with or without Cort (n=3-5). C) Subcutaneous adipose tissue from animals previously treated with Cort had elevated glycerol release compared to controls (n=3-5, *p=.07). This suggests that Cort may have differential effects on epididymal and subcutaneous depots and may help to explain why there is peripheral tissue wasting (due to increased lipolysis) and visceral adipose accumulation in this animal model.

Appendix B: Other Contributions

The following papers were published during the completion of this Masters thesis

Campbell J.E., **Peckett A.J.**, D'souza A.M., Hawke T.J., Riddell M.C. Adipogenic and lipolytic effects of chronic glucocorticoid exposure. Am J Physiol Cell Physiol. 2011 Jan;300(1):C198-209.

Peckett, A.J., Timmons, B.W., Riddell, M.C. The interactions of exercise, stress and inflammation on growth. In *Handbook of Growth and Growth Monitoring in Health and Disease*; Preedy, V.R., ED.; Springer Science and Business Media – **In Press**

Peckett, A.J., Wright, W.C., Riddell, M.C. The effects of glucocorticoids on adipose tissue metabolism. Metabolism. 2011 – **In Press**

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