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The Role of Angiopoietin-like 4 in Lipid Homeostasis

By

Nora Elizabeth Forbes Gray

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Committee in charge:
Professor Jen-Chywan Wang, Chair
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Abstract

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Alterations in the regulation of lipid homeostasis are major causes of metabolic diseases like obesity, insulin resistance and the metabolic syndrome. These diseases affect millions of people and therefore constitute a pressing public health concern. The mobilization of lipids is a key regulatory step in lipid homeostasis and the proteins that mobilize lipids from adipocytes to other tissues are therefore potential targets for therapeutic interventions. One such protein is angiopoietin-like 4 (Angptl4), a secreted protein induced by fasting and glucocorticoid treatment that inhibits lipoprotein lipase (LPL) and induces intracellular adipocyte lipolysis. These studies seek to characterize the role of Angptl4 in modulating lipid homeostasis.

We found that Angptl4 is involved in the lipolytic response to fasting, glucocorticoids and catecholamines and in each case it modulates intracellular cAMP levels. We further discovered that purified Angptl4 can directly increase cAMP and lipolysis in adipocytes in a dose-dependent manner. We were also able to dissociate ability of Angptl4 to inhibit LPL from its ability to induce lipolysis by determining that just the C-terminal domain, which cannot inhibit LPL, can induce adipocyte lipolysis. Initial attempts to uncover the mechanism by which Angptl4 modulates cAMP showed that soluble adenylate cyclase is important for the lipolytic response to Angptl4. Additionally, Angptl4 treatment increases activation of focal adhesion kinase (FAK) and inhibition of FAK attenuates the lipolytic response to Angptl4, suggesting the potential involvement of integrins in Angptl4 signaling in adipocytes although more research is needed to confirm this possibility.

In addition to its expression in white adipose tissue, Angptl4 levels are also high in the liver where its expression is regulated by glucocorticoids. Because mice lacking Angptl4 are protected from glucocorticoid-induced fatty liver and hyperlipidemia we wanted to investigate the lipogenic role of Angptl4 in the liver. Using stable isotope labeling and gene expression analysis we found that glucocorticoids increase the rate of triglyceride synthesis as well as *de novo lipogenesis* but this effect is blunted in mice that lack Angptl4. There was also differential hepatic expression of genes involved in lipogenesis in the mice without Angptl4. Treatment of hepatocytes with purified Angptl4 revealed that the increase in triglyceride synthesis is not a direct effect of the protein, nor are the alterations in the expression of most lipogenic genes. Two notable exceptions, however, are Agpat1 and Agpat2, the expression of which was induced by Angptl4 treatment. This is a previously undescribed role of Angptl4

and further research is necessary to understand the mechanism by which it could be modulating transcription.

A third tissue with high expression of Angptl4 is brown adipose tissue. Because of its role in lipolysis and the fact that lipolysis is critical for adaptive thermogenesis, we investigated the how Angptl4 might be involved in thermoregulation. We found that mice lacking Angptl4 maintained a consistently lower body temperature during cold exposure. We further determined that lipolysis in brown adipose tissue was impaired in these mice and the induction of thermogenic genes was compromised relative to wild types. We also found that mice without Angptl4 maintain a lower body temperature during fasting than WT mice. These results indicate a potential role of Angptl4 in thermogenesis, which could have important implications for obesity.

These studies confirm that Angptl4 is important for the regulation of lipid homeostasis. A more complete understanding of its mechanism, including the identification of a receptor that can mediate its intracellular effects, will be crucial for evaluating the potential of Angptl4 as a therapeutic target for diseases of deregulated lipid metabolism.

To my family and friends who have been there for me throughout the highs and lows of this process, especially my parents Patricia Forbes and Gary Gray whose unwavering support makes everything possible.

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Symbols and Abbreviations

11βHSD: 11-β hydroxysteroid dehydrogenase
Acc: acetyl coenzyme A carboxylase
ACTH: adrenocorticotropin hormone
Adcy: adenylate cyclase
AGF: Angiopoietin-related growth factor (or angiopoietin-like 6, Angptl6)
Agpat: 1-acylglycerol-3-phosphate O-acyltransferase
AMPK: adenosine monophosphate activated protein kinase
AngII: angiotensinogen II
Angptl: angiopoietin-like protein (or fasting-induced adipose factor, FIAF, or hepatic fibgrinogen/angiopoietin-related protein, HFARP, or PPARγ Angiopoietin-related, PGAR)
Angptl4-/-: *Angptl4* knockout mice
APO: apolipoprotein
ATP: adenosine triphosphate
BAT: brown adipose tissue
BMI: body mass index
CAD: coronary artery disease
cAMP: cyclic adenosine monophosphate
Cgi-58: abhydrolase domain containing 5 (or Abdh5)
CGL: congenital generalized lipodystrophy
Cidea: cell death-inducing DNA fragmentation factor a
CNS: central nervous system
COX: cyclooxygenase
Cpt1α: carnitine palmitoyltransferase 1 alpha (or Cpt1a)
CRH: corticotropin releasing hormone
D2O: deuterated water
db/db: a strain of mice where leptin receptor is dysfunctional
DDA: 2'5' dideoxyadenosine
DEX: dexamethasone
DEXA: dual energy X-ray absorptiometry
Dgat: diacylglycerol acyltransferase
Dio2: deiodinase, iodothyronine 2
DMSO: dimethyl sulfoxide
DNL: *de novo lipogenesis*
EL: endothelial lipase
ELISA: enzyme-linked immunosorbant assay
Elovl3: elongation of very long chain fatty acids-like 3
eNOS: endothelial nitric oxide synthase
Erk: extracellular regulated mitogen-activated protein kinase
EWAT: epididymal white adipose tissue
FA: fatty acids
FAK: focal adhesion kinase
Fas: fatty acid synthase (or Fasn)
FFA: free fatty acids
Fgf21: fibroblast growth factor 21

FOXO1: forkhead box, sub-group O
GAP: GTPase-activating protein
GBR: glucocorticoid receptor binding region
GC: glucocorticoid
GC/MS: gas chromatography/mass spectrometry
GDP: guanosine diphosphate
GEF: guanine exchange factor
Gnas: g protein alpha subunit
Gnb1: g protein beta subunit
Gpat: glycerol-3-phosphate acyltransferase
GPCR: G-protein coupled receptor
GPIHBP1: glycosylphosphatidylinositol-anchored HDL binding protein
GR: glucocorticoid receptor
GRE: glucocorticoid response element
GTP: guanosine triphosphate
hANGPTL4: human angiopoietin-like 4
HDL: high density lipoprotein
HIF1α: hypoxia-inducible factor α
HL: hepatic lipase
Hsl: hormone sensitive lipase
IBMX: 3-isobutyl-1-methylxanthine
ICV: intracerebroventricular
IL: interleukin
IWAT: inguinal white adipose tissue
KO: knockout mice
LDL: low density lipoprotein
Lpin: phosphatidate phosphatase
LPL: lipoprotein lipase
LXR: liver X receptor
MAP: mitogen activated protein
MAPK: mitogen activated protein kinase
Mek: mitogen activated protein kinase kinase
MGL: monoglyceride lipase
NE: norepinephrine
NEFA: non-esterified fatty acids
NO: nitric oxide
PAK: rac-activated kinase
PBS: phosphate buffer solution
PCSK3: proprotein convertase subtilisin/kexin type 3
PDE: phosphodiesterase
Pepck: phosphoenolpyruvate carboxykinase
PG: prostaglandin
PGC1α: peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (or Pgc1a)
PI3K: phosphoinositide-3-kinase
PKA: protein kinase A
Plin: perilipin

Pnpla2: patatin-like phospholipase domain containing 2 (or adipose triglyceride lipase, ATGL)
Ppar: peroxisome proliferator-activated receptor
Prdm16: PR domain containing 16
qPCR: quantitative polymerase chain reaction
Rac1: ras-related C3 botulinum substrate
Raf: effector of Ras
Ras: rat sarcoma
RXR: retinoid X receptor
sAC: soluble adenylate cyclase
Scd: stearoyl-coenzyme A desaturase
siRNA: small inhibitory RNA
Src: sarcoma oncogene
TG: triglyceride
Tie2: endothelium-specific receptor tyrosine kinase 2
TLC: thin layer chromatography
Ucp1: uncoupling protein 1
VCAM: vascular adhesion molecule
VLCFA: very long chain fatty acid
VLDL: very low density protein
WAT: white adipose tissue
Wnt: wingless-related mouse mammary tumor virus integration site
WT: wild-type
βAR: beta adrenergic receptor (or bAR)

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The Physiological and Metabolic Functions of Angiopoietin-like 4

INTRODUCTION

I. GLUCOCORTICOIDS

Glucocortcoids are steroid hormones that signal through a nuclear hormone receptor, the glucocorticoid receptor (GR). The GR contains both a ligand binding domain as well as a DNA binding domain[1]. The binding of ligand to the GR initiates its translocation from the cytosol to the nucleus where it can bind specific DNA sequences known as glucocorticoid response elements (GREs) to modulate the transcription of nearby genes. Glucocorticoids affect the expression of approximately 10% of human genes [2].

The major endogenous glucocorticoid in humans is cortisol, the rodent analog of which is corticosterone. Cortisol is secreted by the adrenal glands in response to adrenocorticotropin hormone (ACTH) being released by the pituitary, which is the result of corticotropin releasing hormone (CRH) secretion from the paraventricular nucleus of the hypothalamus. ACTH is regulated via negative feedback inhibition by increased levels of cortisol[3]. Circulating cortisol levels fluctuate diurnally with a peak in the early morning [4]. Glucocorticoids can be interconverted between the active cortisol and an inactive form called cortisone by the 11β hydroxysteroid dehydrogenase enzymes (11β-HSD) inside of cells. The enzyme 11β-HSD type 1 converts cortisone to cortisol while 11β –HSD type 2 catalyzes the conversion of cortisol back to the inert cortisone. Cell-type specific expression and selective activation of these 11β-HSD enzymes are essential for determining the intracellular concentration of cortisol[5].

Physiological role of glucocorticoids

Glucocorticoids play important roles in many aspects of mammalian physiology (Table 1). Glucocorticoids are essential for lung development. Mice lacking GR died after birth because of atelectasis of the lungs resulting from lack of surfactant[6]. Glucocorticoids affect many regions in central nervous system (CNS) as well, including hippocampus, amygdala and frontal lobes, and modulate distinct functions in CNS, such as feeding, emotion and memory[7, 8]. Glucocorticoids also play a critical role in immune function. They are immunosuppressive and anti-inflammatory. They inhibit prostaglandins and leukotrienes, the main products of inflammation as well as the cytokine genes for IL1-6 and 8, which reduces T cell proliferation[9]. Glucocorticoids also suppress humoral immunity by decreasing the expression of IL2 in B cells and suppressing COX1 and COX2 expression[10-12]. These functions of glucocorticoids are the main reasons for their use in treating various inflammatory and immune diseases.

Glucocorticoids also play an essential role in the regulation of intermediary metabolism. They are critical for the physiological adaptation to fasting and starvation because of their ability to modulate glucose, protein and lipid metabolism. Increased levels of glucocorticoids stimulate gluconeogenesis in the liver from amino acids and glycerol. Glucococorticoids also inhibit glucose uptake in both muscle and adipose tissue, where the hormone also induces fat breakdown[13]. The breakdown of fat generates fatty acids and glycerol, the former can be utilized by other tissues as fuel and the latter can serve as a gluceneogenic substrate in the liver. Fatty acids released from adipose tissue can also be used to generate ketone bodies in the liver which in turn are a fuel for the brain in the later stages

of food deprivation[14]. These metabolic consequences of glucocorticoids are critical for survival of mammals during fasting or starvation.

Table 1: The Effects of Glucocorticoids on Various Tissues

Tissue	Biological Effect of Glucocorticoids	Negative Side-Effects of Excess Glucocorticoids
Adipose	Lipogensis, lipolysis and glucose uptake	Obesity, insulin resistance
Liver	Gluconeogenesis, lipogenesis	Hepatic steatosis, hyperglycemia, dyslipidemia
Muscle	Glucose uptake, protein metabolism	Muscle atrophy, insulin resistance
Bone	Osteoblast differentiation, bone formation and resorption	Osteoporosis
Cardiovascular System	Acute vasoconstriction, anti-inflammation, anti-angiogensis	Dyslipidemia, hypertension, thrombosis, atherosclerosis
Immune System	Acute immunosuppression, anti-inflammation	Broad immunosuppresion, activation of latent viruses
CNS	Memory, mood, feeding behavior	Cerebral atrophy, psychosis

Pathologies caused by excess glucocorticoids.

Although many of the effects of physiological elevations glucocorticoids are absolutely essential for the important biological responses mentioned above, excess glucocorticoids (whether attained through exogenous administration or chronic endogenous elevations) have detrimental effects on a number of bodily functions. High levels of glucocorticoids can inhibit bone formation, suppress calcium absorption and delay wound healing. They can also cause muscle weakness and atrophy and increase the risk of infection[15, 16]. Excess glucocorticoids can also exert effects on the mineralcorticoid receptor and induce salt and water retention, which results in an expansion of extracellular fluid volume, hypertension, potassium depletion and metabolic alkalosis. At physiological doses the rapid breakdown of cortisol by 11β-HSD2 in target tissue of mineralcorticoids prevents this from occurring[17].

High levels of glucocorticoids can also have negative effects on lipid metabolism. Exogenous glucocorticoid treatment can precipitate either type I or type II diabetes in a person already in the process of developing it. It is believed that this steroid diabetes doesn't occur with mineralcoricoids, anabolic steroids or sex steroids because those hormones have very little effect on glucose metabolism[18].

Many of the same negative phenotypes associated with high doses of exogenous glucocorticoids can also be seen in an example of prolonged elevations of endogenous glucocorticoids, namely Cushing Syndrome. Cushing Syndrome is characterized by chronically high circulating cortisol levels, which usually result from tumors that produce either cortisol or ACTH. In 65% of Cushing patients the disease is caused by a pituitary tumor which causes increased ACTH. Adrenal tumors are the next most common in people with Cushing followed by tumors outside the hypothalamic-pituitary-adrenal axis that produce ACTH, like small cell lung cancer for example[19].

People suffering from Cushing syndrome display a unique lipid phenotype characterized by accumulation of fat in certain depots, such as around the face, at the nape of the neck and centrally, but a decrease of fat accumulation in other, mostly subcutaneous, depots such as the arms and legs. Cushing patients also suffer from hypertension and insulin resistance[2, 20].

Deleterious metabolic consequences of excess glucocorticoids.

Although the pathological phenotype associated with Cushing Syndrome clearly highlights the ability of excess glucocorticoids to modulate lipid metabolism both anabolically

and catabolically, it affects a very small percentage of the population. However, an abundance of evidence exists for direct effects of glucocorticoids on lipid metabolism and the ensuing negative consequences.

Using a stable isotope labeling technique it has been shown that in the white adipose tissue of mice treated with the synthetic glucocorticoid dexamethasone (DEX) for 4 days, there is an increase in both triglyceride (TG) synthesis and lipolysis concurrently [21]. It is known that during fasting glucocorticoids participate in the induction of adipose tissue lipolysis. There is also evidence that glucocorticoids can directly induce adipocyte lipolysis[22]. In primary rat adipocytes DEX treatment down-regulated the expression of phosphodiesterase 3B (PDE3B), the enzyme that degrades cyclic-AMP (cAMP) resulting in an elevation of intracellular cAMP and an increase in PKA activation. DEX treatment also uppregulated expression of hormone-sensitive lipase (HSL) as well as adipose triglyceride lipase (ATGL) the enzymes that catalyze the first two steps in TG hydrolysis. Consistent with the increase in cAMP content, there was also a DEX-stimulated increase in phosphorylation of HSL at Ser-563 and Ser-66, resulting in increased HSL activity, also increased [22]. The ability of glucocorticoids to directly modulate the expression of lipolytic genes including ATGL, HSL and PDE3B, as well as monoglyceride lipase (MGL) and angiopoietin-like 4 has been confirmed in other studies as well [21, 23].

The increases in circulating fatty acids resulting from enhanced lipolysis, and the ectopic lipid accumulation that can occur in both liver and muscle as a result of high levels of glucocorticoids, are highly correlated with insulin resistance[24, 25]. Additionally it's been shown that a single-dose of DEX can alter cardiac fatty acid metabolism as well as carbohydrate metabolism and can induce whole-body insulin resistance[26]. Conversely, either inhibiting 11β-HSD 1 or activating 11β-HSD 2, and thereby suppressing glucocorticoid action, improves lipid profiles and insulin sensitivity. In fact mice with adipose tissue overexpression of 11β-HSD 2 were resistant to weight gain on a high-fat diet[27] whereas those with hepatic overexpression 11β-HSD 1 induced increased hepatic lipid synthesis and steatosis[28], while whole body overexpression of 11β-HSD 1 leads to the development of complete metabolic syndrome in mice[29]. Consistent with these findings, obesity has been associated with increased adipose tissue cortisol levels[30] and clinical studies in obese humans have found elevated levels of adipose tissue 11β-HSD 1 expression and decreased 11β-HSD 2 [31].

Increasing active levels of glucocorticoids has a significant effect lipid accumulation. It has long been noted that glucocorticoids are necessary for proper feeding efficiency[32, 33]. Animals starved for 36-56 hours and then refed a high glucose diet have dramatic increases in hepatic *de novo lipogenesis* (DNL) yet these effects were impaired with adrenalectomy and restored glubycocorticoid treatment, indicating the direct participation of glucocorticoids in this lipogenic process[34, 35]. However when lipogenesis in hepatocytes was examined directly some studies have shown that there is an increase following glucococorticoid treatment [36] while others show no effect [37]. Yet it has been consistently observed that glucocorticoids and insulin act synergistically to increase triglyceride synthesis.

Mechanistically, it's been shown that glucocorticoids regulate many genes involved in TG synthesis. ChIP sequencing experiments in 3T3-L1 adipocytes identified glucocorticoid receptor binding regions in stearoyl coenzyme A desaturase (Scd) 1 *Scd1, Scd2,* glycerol 3-phosphate acyltransferase (Gpat) 3*, Gpat4,* 1-acylglycerol-3-phosphate O-acyltransferase (Agpat) 2 and phophatidate phosphatase (Lpin) 1, all components in the pathway of

triglyceride synthesis[21]. Furthermore, it's been reported that insulin and glucocorticoids both potentiate the transcription of *Scd1*[38]. Insulin and glucocorticoids together also increase lipoprotein lipase (LPL) expression[39, 40]. Since LPL is the enzyme, which breaks down circulating TG into fatty acids that can be taken up the tissue, an increase in LPL expression results in an increase in tissue lipogenesis. Thus is can be said that glucocorticoids are able to modulate the expression of triglyceride synthesis genes both in the presence and absence of insulin.

II. ANGIOPOIETIN-LIKE PROTEINS

Glucocorticoids exert their various effects on lipid metabolism through modulating the expression of primary and secondary target genes. Primary target genes are those that contain a GRE to which the GR directly binds to affect transcription whereas secondary target genes are ones whose expression is altered following glucocorticoid treatment but not through a physical interaction with the GR. Identifying novel target genes in different metabolic tissues and characterizing their physiological effects are areas of intense research in the field glucocorticoid study. One gene that has been identified as a primary GR target gene in adipose tissue as well as liver, is angiopoietin-like 4 (Angptl4)[23]. The biological functions of Angptl4 are consistent with the actions of glucocorticoids on lipid metabolism and will be discussed in detail later.

Angptl4 is one member of a seven-member family of angiopoietin-like (Angptl) proteins. Angptl proteins share structural homology with the four known angiopoietin proteins. Angiopoietins have a N terminal signal sequence for protein secretion, as well as a coiled-coil domain and a fibrinogen-like domain all of which are shared by Angptl proteins as well. The function of angiopoietins is to maintain the vascular system and hematopoietic stem cells which they do through binding of the C terminal fibrinogen-like domain with the Tie2 receptor[41]. Tie2 signaling is essential for regulating vascular remodeling and stabilization. Regulation of angiogenesis is a key role of angiopoietins. Angiopoietin-like proteins also play a role in angiogenesis mainly through regulating the migration and survival of endothelial cells. Angptl 2, 3, 5 and 7 have recently been shown to expand the hematopoietic stem cell pool in culture, however it was seen that Angptl4 lacks this ability[42-44]. Importantly, although Angptls contain the C-terminal fibrinogen-like domain necessary for these angiogenic effects, the proteins cannot bind the Tie2 receptor. Angptls remain orphans ligands but appear to have pleiotropic effects on metabolism and tumor biology in addition to their effects on vascular cells[41].

Angptl proteins are secreted and can be detected in systemic circulation as well as cell culture medium after transfection, which suggests some potential endocrine functions [44-48] however the evidence supporting this varies with each Angptl protein.

Angptl1 and Angptl2

Angptl1 is highly expressed in endocrine organs rich in blood vessels like the thyroid, pituitary and adrenal glands, as well as in heart, liver, kidney and skeletal muscle. Angptl1 appears to play an important role in intermuscular connective tissue, joint capsules and cartilage during development[49]. In contrast Angptl2 seems to play a role in inflammation and atherosclerosis[50] and has been shown to be abundantly expressed in skeletal muscle, heart, stomach, intestine, uterus and adipose tissue [45]. Angptl2 is a circadian gene and seems to be of importance in carcinogenesis and metastasis both of which it has been shown

to facilitate[51]. Angptl2 can also potentiate insulin sensitivity in murine adipocytes [52] and it has been suggested that suppressing Angptl2 could have therapeutic potential in treating obesity-related insulin resistance[53]. Human ANGPTL2 is induced by chronic hypoxia and appears to have the ability to induce an inflammatory response in blood vessels [54, 55]. Additionally elevated levels of Angptl2, both in adipose tissue as well as in circulation, have been observed in obese mice [54].

Angptl3

Angptl3 has also been shown to affect lipid metabolism. In mice Angptl3 is expressed most highly in the liver and to a much lower degree in the lung and kidney[56]. The expression of Angptl3 is regulated by multiple transcription factors. The Angptl3 promoter contains an LXR regulatory element and Angptl3 expression has been shown to induced by LXR agonists[57]. Angptl3 expression also appears to be regulated by PPARδ, the inhibitory effect of which is thought to be the result of a disruption in dimerization between LXR and RXR that impairs the ability of LXR to activate the Angptl3 gene[58]. Additionally leptin, insulin and thyroid hormone can decrease hepatic Angptl3 expression[59-61].

Angptl3 is a secreted protein and can be post-translationally modified by glycosylation or cleavage[62]. In addition to undergoing proteolytic cleavage, Angptl3 has been shown to form higher order multi-subunit complexes, which don't appear to require sulfide bond formation between molecules. Although the exact reason for this oligomerization remains unknown it has been speculated that their formation be protective against degradation [48].

Angptl3 is commonly found in circulation and its expression is decreased by both leptin and insulin, but increased in people with diabetes[63]. Angptl3 is proangiogenic, as it has been shown to induce blood vessel formation [64]. The earliest mechanistic link between Angptl3 and lipid metabolism came from the observation that KK/San mice, a strain that while obese has significantly lower plasma fatty acid and TG levels. Injection of adenoviral Angptl3 increased plasma TG, fatty acids and cholesterol in both wild type and KK/San mice[65]. An elevation in fasting levels of circulting TG was also seen in response to ANGPTL3. This was attributed to an ability to inhibit LPL and the subsequent suppression of very low density lipoprotein (VLDL) clearance[66, 67]. There is also evidence that intravenous injection of Angptl3 can increase circulating fatty acids and glycerol, indicative of increased lipolysis, and can bind to the surface of adipocytes[56].

Angptl5

No evidence of LPL binding has been seen yet for Angptl5 although it appears to play a role in lipid metabolism as well. When examining the lowest quartile of plasma TG in the Dallas Heart Study, 4% of participants had loss-of-function mutations in either ANGPTL3, ANGPTL4 or ANGPTL5[68]. Little is known about the exact function and regulation of Angptl5. There are no known orthologs for Angptl5 in other species but in humans it is predominantly expressed in the heart [69] though recently evidence has emerged suggesting that it can be expressed and secreted by mesenchymal stem cells and this secretion supports expansion of hematopoietic stem cells[70].

Angptl6/Angiopoietin-related growth factor

In contrast to Angptl5, a great deal is known about the biological role of Angptl6 (also called angiopoietin-related growth factor or AGF). Its expression, though abundant, is

restricted to liver in humans yet in mice Angptl6 expression can also be found in kidney, heart, skeletal muscle and brain [71]. Angptl6 is most closely related to Angptl2 and is secreted as evidenced by the fact that it can be detected in circulation [46]. It has been shown that Angptl6 can induce angiogenesis and arteriogenesis by activating ERK-eNOS-NO pathway in endothelial cells [72]. Also Angptl6 appears to play a role in epidermal proliferation and remodeling in mice where Angptl6 was overexpressed in keratinocytes [73]. Metabolically Angptl6 seems to be involved in resting metabolic rate, lipid profiles and insulin sensitivity[74, 75]. An increase in serum levels of Angptl6 has been found in patients with metabolic syndrome[75]. Angptl6 knock-out mice are obese and exhibit hypercholesterolaemia, elevated circulating fatty acids, and insulin resistance as well as a decrease in energy expenditure that was unrelated to food intake [55, 75]. In contrast, mice overexpressing Angptl6 are lean and have enhanced energy expenditure and are resistant to diet-induced obesity and insulin resistance[75].

Angptl7

With its discovery in 2006, Angptl7 is the most recently identified member of the Angptl family. It is highly expressed in cornea and neural tissue as well as in human melanotic melanoma and some endometrial cancers. While it's regulation has not been well characterized the Angptl7 promoter contains several T cell factor/lymphoid enhancer factor binding sites which suggest that Angptl7 may be a target of Wnt/β-catenin signaling [75]. Although recently evidence has emerged describing a role for Angptl7 in extracellular matrix formation in the eye [75], to date there are no known reports characterizing a metabolic function for Angptl7.

III. ANGIOPOIETIN-LIKE 4

Angiopoietin-like 4 (Angptl4) was discovered concurrently in 2000 by three different researchers. Kim et al. used a degenerative PCR approach to look for additional members of the angiopoietin family and discovered Angptl4 which they hamed HFARP (hepatic fibrinogen/angiopoietin-related protein[47]. Yoon et al. identified Angptl4 while doinga subtractive cloning screen looking for targets of troglitazone, the PPARγ agonist, in adipose tissue and subsequently named it PGAR (PPARγ angiopoietin-related)[47] while Kersten et al. found Angptl4 by way of a similar screen except they were looking for novel target genes of PPARα. Because they observed that its expression was regulated by fasting, Kersten et al. named the gene FIAF (fasting-induced adipose factor)[76].

Protein structure

Angptl4 is a 50kD protein that is 406 amino acids long. In mice, Angptl4 is expressed mostly highly in white and brown adipose tissue followed by liver, lung, intestine, ovary and heart.[76, 77]. In contrast, in humans ANGPTL4 appears to ubiquitously expressed with highest expression in liver, adipose, small intestine and heart[78].

Angptl4 is a secreted protein that is glycosylated and can be proteolytically modified. In vitro it is cleaved to produce an N-terminal section containing a coiled-coil domain, and a C-terminal section containing the fibrinogen-like domain [79, 80]. It was later determined that this cleavage occurs at the recognition site RRKR, is mediated by protein convertases including PCSK3 and is serum-dependent [80, 81]. In vivo both the full length Angptl4 as well as the truncated form containing just the N-terminus can be found in circulation, and it

appears in humans and mice, full-length Angptl4 is secreted mostly by adipose tissue whereas the truncated form comes from the liver[23, 82]. In mice full-length Angptl4 has been found to be physically associated with HDL, whereas truncated Angptl4 was associated with LDL, however in humans both the full-length and truncated forms were associated with HDL[83]. Circulating Angptl4 can also form higher order oligomeric structures, believed to be important for its function. These aggregate multimers are likely formed via specific cysteine residues in the N-terminal forming disulphide bridges[79].

Regulation of expression

As evidenced by the methods of its discovery Angptl4 expression is regulated by PPARα and PPARγ. It has also been shown that Angptl4 expression can be induced by fatty acids signaling through PPARβ/δ in the heart [84]. Inflammatory prostaglandins, including PGD2, PGE2 and PGJ2 can also induce Angptl4 expression [85, 86]. Insulin and glucocorticoids have opposing regulatory effects on Angptl4 expression. Insulin suppresses Angptl4 expression in adipose tissue via PI3K/FOXO1 pathway [87] and suppression of insulin signaling has been shown to upregulate Angptl4 expression in liver and adipose tissue in diabetic mice[88]. In contrast glucocorticoids directly induce Angptl4 expression in both liver and adipose tissue [23]. There is also a report that thyroid hormone can induce Angptl4 expression in liver cells [89]. Physiological states can also modulate Angptl4 expression. In addition to being induced by fasting[76], Angptl4 expression is also induced during hypoxia [90, 91] and this regulation is thought to mediated through the transcription factor hypoxia inducible factor 1α (HIF1α)[92].

Function - Angiogenesis

Given the diversity of its regulation it is not surprising that Angptl4 has been reported to have a variety of functions. The fact that it's induced during hypoxia and that it's a downstream target of both PPARα and PPARγ suggests that Angptl4 has a role in angiogenesis however whether its net effects are pro- or anti-angiogenic remains a subject of debate. Full-length, extracellular matrix-bound Angptl4 has been shown to suppress the formation of stress fibers and focal contacts in endothelial cells block their adhesion a process that negatively affects their angiogenic capacity [93]. It was further confirmed that through an interaction between the coiled-coil domain and glycosaminoglycans Angptl4 is protected from cleavage and that is what regulates its antiangiogenic activity [81]. However in a model of renal cell carcinoma Angptl4 was seen to be proangiogenic [94].

Likely because of its effects on angiogenesis Angptl4 has been linked to the progression of various types of cancer, though again contradictory evidence exists as to whether it promotes or suppresses carcinogenesis. In vitro ANGPTL4 was shown to prevent metastasis by inhibiting vascular permeability as well as the motility and invasiveness of tumor cells[95]. However, in another model overexpression of Angptl4 was seen to promote cell proliferation and tumor growth in vitro and in vivo[86], findings that were confirmed by a second study showing that overexpressing Angptl4 significantly increased transendothelial cell migration in vitro as well as metastasis in vivo [92].

Angptl4 has also been implicated in cell migration in the context of wound healing. In 2010 Goh et al. published two reports showing that Angptl4 knockout mice have impaired keratinocyte migration leading to delayed wound reepithelialization [96]. They also were able

to demonstrate that Angptl4 is produced by keratinocytes and modulates communication between the cell and the extracellular matrix thereby regulating wound healing [97].

It has also been shown that Angptl4 is highly expressed in osteoclasts and its induction by HIF in those cells can induce resorption without affecting osteoclast viability. They further confirmed this effect by demonstrating that siRNA knockdown of HIF1α prevents this resorption but the effect can be rescued by addition of exogenous ANGPTL4 [98].

Function - Metabolism

Inhibition of LPL

Metabolically two principle biological roles have been described for Angptl4. The protein has been shown to inhibit LPL and also to induce intracellular lipolysis. LPL is a member of the same lipase family as endothelial lipase and hepatic lipase. Its expression is regulated in adipose tissue by PPARγ [99, 100] and in liver by LXR and PPARα [99, 101]. LPL is synthesized and secreted by adipocytes, macrophages and muscle cells. Active LPL is a dimer that is bound by heparin sulfate proteoglycans to the vascular endothelium[102]. Because of it's ability to bind both LPL as well as chylomicrons, glycosylphosphatidylinositol-anchored HDL binding protein (GPIHBP1) is the likely platform LPL-dependent hydrolysis of TG-rich lipoproteins [103].

In addition to transcriptional regulation activity of LPL is also highly regulated. In adipose tissue it LPL activity is highest after a meal while in skeletal muscle its activity is higher during fasting and after exercise[104]. There are several known modulators of LPL activity many of which are apolipoproteins. APOC2 and APOC5 stimulate LPL activity whereas APOC3 inhibits LPL [105].

Angptl3 and 4 have also been shown to affect LPL activity, though the mechanisms by which they do so appear to be disntict [106]. In vitro purified recombinant Angptl4 was able to inhibit both mouse LPL as well as human LPL[107]. Altered LPL activity was also observed in Angptl4 KO as well as Angptl4 transgenic mice. Transgenic mice have decreased post-heparin plasma LPL activity along with the subsequent elevation in plasma TG levels whereas Angptl4 KO mice had the opposite phenotype, hypotriglyceridemia and increased post-heparin plasma LPL activity[107].

It is known that Angptl4 suppresses LPL activity by accelerating the irreversible conversion of dimeric LPL to its inactive monomeric confirmation. It has been shown that the N-terminal coiled-coil domain transiently binds to LPL converting the catalytically active dimers into inactive monomers that are no longer bound to the endothelium [106, 108]. It was further confirmed by multiple sequence alignment analysis that a highly conserved 12 amino acid motif within the coiled-coil domain mediates the ability of Angptl4 to block the dimerization of LPL [109].

The interaction between Angptl4 and LPL is believed to be inhibited through interactions with GPIHBP1. In vitro GPIHPB1 stabilizes but doesn't activate LPL and although treatment with purified Angptl4 can potently inhibit non-stabilized LPL, it could not inhibit GPIHBP1-stabilized LPL [103]. These results were confirmed in vivo with the observation that fasting circulating TG of Angptl4/GPIHBP1 double KO mice were much lower than the fasting levels in the GPIHBP1 single KO. Treating the GPIHBP1 KO with Angptl4 antibody mimicked the phenotype seen in the double knockout[103].

Beyond its ability to inhibit LPL, many lipid-altering roles have been described for Angptl4. For instance, Angptl4 has been shown to inhibit hepatic lipase (HL) and may also

affect endothelial lipase (EL). In Angptl4 transgenic mice, as well as Angptl4 KO mice, there are marked changes in HDL-phospholipids that are consistent with an inhibitory effect of ANGPTL4 on HL or EL[110].

Increasing Angptl4 has been shown to result in elevation in plasma TG and LDL-cholesterol in mice. Both male and female mice overexpressing Angptl4 were found to be hyperlipidemic, displaying significant increases in both TG and cholesterol[107]. Consistent with this observation intravenous injection of Angptl4 protein results in a transient increase in both plasma TG as well as LDL-cholesterol [111]. Transgenic mice also showed an increase in hepatic cholesterol synthesis secondary to the inhibition of LPL and hepatic-lipase dependent hepatic cholesterol uptake[110]. Conversely, Angptl4 knockout mice have significantly lower circulating cholesterol and TG compared to wild types [107, 112].

The effects of Angptl4 on cholesterol have important implications for the development of atherosclerosis as well. ApoE/Angptl4 double knockout mice showed decreased fasting TG levels compared to ApoE single knockout mice. Also there was a 75% reduction in atherosclerotic lesion size in the double knockouts along with significantly decreased foam cell formation [113].

In addition to decreased circulating TG Angptl4 knockout mice also show diminished TG accumulation in the liver [23]. Angptl4 knockout mice also have a decreased anorectic responses to leptin, insulin and are resistant to diet-induced obesity suggesting a potential obesity promoting effect of Angptl4[114]. In contrast, adenoviral overexpression of Angptl4 in the liver leads to hepatic steatosis and hyperlipidemia [107]. When Angptl4 is overexpressed in adipose tissue and muscle there is also increased circulating TG along with higher circulating fatty acids. Interestingly, these animals have decreased body weight and 50% lower adipose tissue weight relative to wild types and these differences are independent of food intake[83]. It has also been observed that in order to get proper TG deposition in adipocytes, suppression of Angptl4 in the intestinal epithelium by conventionalization is required[115].

Angptl4 appears to alter cellular energetics as well, by affecting mitochondrial function. Adenoviral overexpression of Angptl4 in mouse liver down-regulated a number of mitochondrial proteins yet increased the activity of respiratory chain complexes II, III and IV[116]. Overexpression of Angptl4 has also been shown to result in increased expression of genes involved in fatty acid oxidation as well as uncoupling in white adipose tissue[83].

The importance of Angptl4 in the regulation of energy metabolism, particularly with regard to lipid homeostasis, has been validated in humans as well. A recent study of monozygotic twins revealed that Angptl4 expression in adipose tissue and circulation was inversely correlated with body weight [117], suggesting a role for Angptl4 in acquired obesity. Additionally, in a large populational study it was shown that sequence variation in *ANGPTL4* gene were associated with altered plasma TG levels. Polymorphisms, including G77R, E167K, G223R, R336C, W349C, G361S, R384W, were all associated with TG levels in the bottom quartile of the Dallas Heart Study, while P251T and RR71Q were associated with the uppermost quartile of TG levels. Functional follow up studies showed that all these mutations interfered with either the synthesis or secretion of the Angptl4 or its ability to inhibit LPL [68].

Perhaps the most widely studied human variant of Angptl4 is the E40K polymorphism. This variant is found in about 3% of European Americans and is associated with significantly lower circulating TG and higher HDL[118]. The E40K variant has been

studied in connection to coronary artery disease (CAD) but the results have been inconsistent. The Atherosclerosis Risk in Communities study found that people with at least one E40K variant had lower mean TG and higher HDL as well as a lower incidence of CAD[119]. However, a review of 11698 participants in three prospective studies confirmed that although E40K carriers had lower TG and higher HDL, their risk of CAD was increased compared to people without the polymorphism [120].

Effects on glucose metabolism and insulin sensitivity

Angptl4 has also been shown to alter glucose metabolism. Overexpression of Angptl4 results in glucose resistance, a phenotype that is believed to be driven by the increased fatty acids seen in those animals [121]. A more direct role of Angptl4 in glucose homeostasis was seen in db/db mice where treatment with ANGPTL4 protein normalized blood glucose levels and significantly attenuated glucose intolerance. Additionally primary hepatocytes treated with ANGPTL4 displayed decreased glucose production and enhanced insulin-mediated inhibition of gluconeogenesis[121]. Consistent with these findings, in rodent models where either type I diabetes is induced by streptozotocin or type II diabetes is induced by high-fat diet, Angptl4 expression is elevated in liver as well as white and brown adipose tissue [122]. Similarly, in obese animals, Angptl4 expression was up 2-4 fold over lean controls in visceral and subcutaneous depots. Treatment with valsartan, an AngII receptor type 1 antagonist clinically shown to reduce the incidence of type II diabetes, returned Angptl4 expression to normal levels but only in subcutaneous, but not visceral, fat[123].

There is also increasing evidence for a role of Angptl4 in insulin resistance in humans. Plasma levels of ANGPTL4 are decreased in patients with type II diabetes and do not appear to be related to BMI[82, 121]. In the Look AHEAD clinical trial it was further demonstrated that people with type II diabetes who had either an E40K or T266M polymorphism in ANGPTL4 had lower TG levels than patients without those variants [124]. The ability of insulin to suppress ANGPTL4 expression also appears to be compromised in type II diabetic subjects. In the adipose tissue of healthy, young subjects, ANGPTL4 expression decreased 3-fold during hyperinsulinemic-euglycemic clamp while no change was observed in subjects with type II diabetes[125]. In another cohort study Angptl4 concentration was positively correlated with fasting FA during oral glucose tolerance test [78]. Although its exact role remains unknown, these studies suggest that Angptl4 plays a role in insulin sensitivity and glucose metabolism.

Induction of lipolysis

The ability of Angptl4 to increase circulating FA is thought to be one of the driving factors for how it contributes to the pathogenesis of diabetes. This lipolytic role of Angptl4 has been widely reported. Intravenous injection of ANGPTL4 rapidly and significantly increased plasma FA levels[111]. Consistent with this finding, mice overexpressing Angptl4 have elevated circulating FA on a low fat diet and this increase is magnified when the animals are put on a high fat diet [83]. In contrast Angptl4 knockout have lower basal and fasting-induced FA levels. This same study also found that conditioned media taken from cells transfected with Angptl4 significantly increased lipolysis in adipocytes[126]. To date this is the most direct evidence seen for the lipolytic role of Angptl4 and the lipolytic mechanism remains elusive.

Effects on feeding

Angptl4 may also modulate feeding behavior, a process in which the AMPK signaling pathway has been implicated in the mechanism of Angptl4 action. In the hypothalamus, Angptl4 expression is increased with food intake or administration of leptin and insulin. Intracerebroventricular (ICV) administration of Angptl4 decreased food intake but increased energy expenditure resulting in weight loss [114]. Consistent with this, it was observed that Angptl4 KO mice had decreased energy expenditure and increased adiposity, although there were no changes in food intake. Angptl4 KO mice also displayed a hyperphagic response to refeeding following a fast as compared to wild types, even if the duration of the fast was as short as 1-2 hours. However, this effect could be normalized by centrally-administered Angptl4. It was determined that the effects of ICV Angptl4 administration were mediated by suppression of hypothalamic AMPK and hypothalamic AMPK activity was found to be significantly higher in Angptl4 KO mice[114]. Thus it appears that Angptl4 may participate in regulating feeding and energy metabolism.

Mechanism – N-terminus

Beyond its N terminal-mediated ability to disrupt LPL dimerization, very little is known about the mechanism by which Angptl4 exerts any of its biological effects. Much of this ambiguity is because the receptor for Angptl4 has yet to be identified. However, some signaling pathways have been identified as downstream targets of Angptl4 in certain cell types (Table 2) and it appears for several of them their activation by Angptl4 is domain specific.

In addition to a signal peptide necessary for secretion, Angptl4 has two functional domains separated by a linker region, an amino-terminal coiled-coil domain and a caboxy-terminal fibrinogen-like domain[47]. Cleavage of the two domains occurs at a canonical protein convertase cleavage site. Oligomerization of full-length Angptl4 has been observed yet after cleavage this multimer structure was only retained in the N-terminal domain while the fibrinogen-like domain dissociated into monomers. Both full-length and N-terminal segment of Angptl4 are able to inhibit LPL yet this appears to be dependent on oligomerization[127]. When a non-oligomerizing N terminal domain was overexpressed by adenovirus decreased stability of the protein was observed in the plasma and it was no longer able to increase plasma TG [79].

Table 2: Summary of pathways affected by Angptl4 in different cell types

Cell Type	Signaling Pathway	Physiological Consequence
Keratinocytes	Activates integrins β1and β5, FAK	Increased keratinocyte migration and accelerated wound healing
Endothelial cells	Inhibits Raf, Rak, Mek, Erk	Decreased angiogenesis
Endothelial cells	Inhibits VEGF and FGF	Decreased proliferation, migration and tubule formation
Endothelial cells	Activates α5β1 Integrin, Rac1/PAK, VE-cahderin and claudin-5	Increased vascular leakiness, disrupted endothelial function
Hepatocyte carcinoma cells	Activates VCAM, integrin β1	Increases migration and metastasis
Hypothalamic neurons	Inhibits AMPK activation	Decreased feeding and increased energy expenditure

While the inhibition of LPL is known to be mediated by the N-terminus of Angptl4, conflicting reports exist as to which domain mediates the effects of the protein on angiogenesis. The N-terminal domain of Angptl4 has been shown to bind heparin and dematan-sulfates and exert an antiangiogenic effect by inhibiting endothelial cell adhesion, motility and tubule-like formation [81]. However in another report, it was found that the C-terminal fibrinogen like domain potently inhibited FGF- and VEGF induced epithelial cell

proliferation, migration and tubule formation[80].

Mechanism – C-terminus

Despite the confusion regarding the exact role of each domain of Angptl4 it is suspected that the C-terminal fibrinogen-like domain is what mediates the intracellular responses to Angptl4. The C-terminus of Angptl4 blocks ERK1/2 MAP kinase and suppresses phosphorylation of Raf and MEK1/2 in endothelial cells without modulating Akt or P38 MAP kinase activity [80]. This is consistent with the activity of endogenous and pharmacological inhibitors of angiogenesis like platelet factor, prolactin and the extracellular adherence protein from *Staphylococcus aureus*, all of which exert their functions by inhibiting ERK1/2 MAPK signaling[128-130]. The inhibition of this signaling pathway by the C-terminus of Angptl4 could be responsible to the anti-angiogenic effects of the protein.

However, the effects of Angptl4 on vascular biology may be more complicated. There are also reports that link Angptl4 to vascular disruption and metastasis. It was observed in primary human microvascular endothelial cells that the C-terminus of Angptl4 binds and activates integrin α5β1, which in turn activates Rac1/PAK signaling to weaken cell-cell contacts causing vascular leakiness. There was also a subsequent association with VE-cahderin and claudin-5 leading to declustering further disrupting endothelial function [131]. The physiological significance of this disruption was observed in vivo using wild-type and Angptl4 knockout mice injected with either control or Angptl4 knockdown tumors and treated intravenously with the C-terminus of Angptl4. The animals treated with the C-terminus Angptl4 had increased vascular leakiness and enhanced lung metastasis[131].

The ability of Angptl4 to activate integrin signaling during tumor metastasis was confirmed Li et al. They found that secreted full length Angptl4 contributes to migration and metastasis of hepatocellular carcinoma cells both in vitro and in vivo by activating the vascular adhesion molecule-1 (VCAM)/integrin β1 axis [92].

Integrin signaling also appears to mediate the effects of Angptl4 on keratinocyte migration. In two different models of Angptl4 ablation (genetic and immune-neutralization) mice without Angptl4 showed delayed wound-healing due to impaired keratinocyte migration[96]. During wound healing, integrins on the cell surface act as sensors to constantly monitor the changing environment. The context in which matrix proteins are presented to the cells affects productive integrin activation. If there's a low ratio of soluble to substrate-anchored matrix the integrin-matrix protein interaction may have accelerated turnover. The C-terminal fibrinogen-like domain of Angptl4 has been found to bind to matrix proteins and delay their degradation by proteases without interfering with integrin-matrix recognition leading to an increase in integrin signaling[97].

In addition to being involved in regulating cell-matrix communication, a direct interaction between Angptl4 and integrins has also been described. Membrane extracts from wound biopsies show that the C-terminus of Angptl4 associates with integrins, specifically β1/β5 subunits, but not β3, to modulate FAK-Src-PAK1 signaling a pathway known to be important for keratinocyte migration [96].

IV. PROJECT GOALS

The major goal of this thesis is to elucidate the physiological and metabolic functions of Angptl4, especially its role in glucocorticoid-regulated lipid metabolism. The first chapter addresses the role of Angptl4 in glucocorticoid-regulated TG breakdown. Preliminary results

in this area led to an investigation into the role of Angptl4 in adipocyte lipolysis promoted by catecholamine and fasting. The second chapter addresses the role of Angptl4 in glucocorticoid-induced *de novo lipogenesis* and TG synthesis. Finally, as Angptl4 is highly expressed in brown adipose tissue, the third chapter investigates the potential role of Angptl4 in adapative thermogenesis.

Chapter I: Angiopoietin-like 4(Angptl4) is a Physiological Mediator of Intracellular Lipolysis in Murine Adipocytes

SUMMARY

Intracellular triacylglycerol (TG) hydrolysis and fatty acid release by the white adipose tissue (WAT) during a fast is stimulated by counter-regulatory factors acting in concert, although how adipocytes integrate these lipolytic inputs is unknown. We tested the role of angiopoietin-like 4 (Angptl4), a secreted protein induced by fasting or glucocorticoid treatment, in modulating intracellular adipocyte lipolysis. Glucocorticoid receptor blockade prevented fasting-induced tissue *Angptl4* expression and WAT TG hydrolysis in mice, and TG hydrolysis induced by fasts of 6- or 24 hours was greatly reduced in mice lacking Angptl4 (*Angptl4*$^{-/-}$). Glucocorticoid treatment mimicked the lipolytic effects of fasting, though with slower kinetics, and this too required Angptl4. Thus, fasting-induced WAT TG hydrolysis requires glucocorticoid action and Angptl4. Both fasting and glucocorticoid treatment also increased WAT cAMP levels and downstream phosphorylation of lipolytic enzymes. Angptl4 deficiency markedly reduced these effects, suggesting that Angptl4 may stimulate lipolysis by modulating cAMP-dependent signaling. In support of this, cAMP levels and TG hydrolysis were reduced in primary *Angptl4*$^{-/-}$ murine adipocytes treated with catecholamines, which stimulate cAMP-dependent signaling to promote lipolysis, and was restored by treatment with purified human ANGPTL4 (hANGPTL4). Remarkably, hANGPTL4 treatment alone increased cAMP levels and induced lipolysis in these cells. Pharmacologic agents revealed that Angptl4-modulation of cAMP-dependent signaling occurs upstream of adenylate cyclase and downstream of receptor activation. We show that Angptl4 is a glucocorticoid-responsive mediator of fasting-induced intracellular lipolysis and stimulates cAMP signaling in adipocytes. Such a role is relevant to diseases of aberrant lipolysis, such as insulin resistance.

INTRODUCTION

An essential step in intermediary metabolism that occurs during a physiological fast or prolonged exercise involves the organized flux of energy in the form of free fatty acids (FFAs) from the white adipose tissue (WAT) to the liver and skeletal muscle for utilization. Several counter-regulatory factors, including neurotransmitters such as catecholamines and hormones such as thyroid hormone, growth hormone, glucagon, and glucocorticoids play critical roles in regulating the flux of FFAs during fasting. However, the way in which adipocytes integrate these inputs is incompletely understood.

A net flux of FFAs out of the WAT can result when the rate at which adipocytes hydrolyze intracellular triacylglycerols (TGs) and release FFAs is greater than the rate at which they take up and esterify dietary fats. The uptake of dietary fats stored within circulating lipoproteins by adipocytes requires the action of lipoprotein lipase (Lpl) enzymes (extracellular lipolysis), while the mechanisms governing TG hydrolysis (intracellular lipolysis) by adipocytes are more complex.

One factor that is known to regulate extracellular lipolysis and that is induced by fasting is angiopoietin-like 4 (Angptl4, a.k.a fasting-induced adipose factor, FIAF), a glycoprotein secreted by the WAT and liver in response to fasting and that inhibits the action of Lpl [132, 133]. Studies using genetically altered mice confirm that Angptl4 plays a key role in fat metabolism [105, 134]. For example, transgenic mice overexpressing *Angptl4* in the WAT have increased levels of plasma TG and FFAs [83, 107], whereas the opposite is true of mice

lacking *Angptl4* (*Angptl4*$^{-/-}$) [23].

In considering how Angptl4 functions, it is intriguing to note that, in addition to inhibiting Lpl, Angptl4 also promotes the expression of WAT genes involved in TG hydrolysis and the lipolytic release of intracellular FFAs by adipocytes [83]. Therefore, it is possible that Angptl4 may modulate both extracellular and intracellular lipolysis.

Much effort has been placed on determining the mechanisms governing the induction of *Angptl4* transcription during fasting. We previously focused on exploring *Angptl4* gene regulation by glucocorticoids. Glucocorticoids act by binding to the glucocorticoid receptor (GR) and promoting its recruitment to the nucleus, where it binds to genomic response elements in order to modulate the transcription of nearby genes. Treating cultured primary hepatocytes and adipocytes with the synthetic glucocorticoid dexamethasone (DEX), we identified *Angptl4* as a direct transcriptional target of GR [23].

Glucocorticoids act to modulate intermediary metabolism in a nutritionally-dependent manner, stimulating lipogenesis and TG formation in the fed state [32, 135] and white adipose tissue (WAT) lipolysis in the fasted state [136, 137]. Glucocorticoid levels fluctuate throughout the day in accordance with this, displaying their largest peak in the morning following an overnight fast [138]. Although the involvement of glucocorticoid action in fasting-induced WAT lipolysis has been described [139, 140], the extent to which glucocorticoids regulate intracellular adipocyte lipolysis and the mechanisms by which this occurs remain to be determined.

This contrasts with the knowledge gathered from studying pathological states of chronic glucocorticoid excess, such as the Cushing syndrome, where normal dietary and diurnal regulation of systemic lipid flux gives way to a tonically enhanced flux of FFAs away from peripheral WAT depots into visceral ones and leading to the development of central obesity, hepatic steatosis, and dyslipidemia [140, 141] [2, 142].

The relationship between Angptl4 and fat metabolism is seen in humans as well as in mice. For example, a large population-based study showed that sequence variations in human ANGPTL4 are associated with reduced plasma TG levels [118], and another showed that ANGPTL4 levels in the WAT correlate with body weight in monozygotic twins [117]. Angptl4 is therefore an attractive target for studying how fasting and glucocorticoids induce WAT lipolysis.

Here we examine the role of Angptl4 in mediating intracellular lipolysis and FFA release by adipocytes in response to stimulation by fasting, glucocorticoids, and catecholamines. Using WT and *Angptl4*$^{-/-}$ mice, we demonstrate the importance of glucocorticoid action in WAT lipolysis stimulated by fasting within the physiologic range and identify Angptl4 as a key downstream effector in this process. Using primary murine adipocytes and purified human ANGPTL4, we go on to explore how Angptl4 exerts its pro-lipolytic effects, showing that Angptl4 participates in cAMP-dependent signaling and the phosphorylation of key lipolytic enzymes and represents a common regulatory point in the lipolytic cascade induced by multiple stimuli.

EXPERIMENTAL PROCEDURES

Animals. *Angptl4*$^{-/-}$ mice were provided by the laboratories of Andras Nagy (Samuel Lunenfeld Research Institute, Mount Sinai Hospital) and Jeff Gordon (Washington University)[115]. *Angptl4*$^{-/-}$ mice were generated on a mixed B6:129□Sv background. WT mice were the littermates of *Angptl4*$^{-/-}$ mice. The PCR protocols and strategy for mouse

genotyping were as described [115]. C57BL/6J mice were from Charles River Laboratories (Wilmington, MA). Glucocorticoid-treatment of mice (2-4 months old) involved the intraperitoneal injection of water-soluble dexamethasone (5 mg/kg body weight; Sigma) in PBS once between 9 and 10am, and the collection of tissues 6 or 24 hours after the injection. GR antagonism was achieved *in vivo* by providing 2-4 month-old C57BL/6J mice with water containing 0.12 mg/mL mifepristone (RU486; Sigma) dissolved in DMSO for 3 days. Control mice were given water containing only DMSO. The Office of Laboratory Animal Care at the University of California, Berkeley approved all the animal experiments (#R306-0111).

Immunoblots. The protocol for western blot was as described [23]. Proteins were detected by fluorescence imaging (LiCor Odyssey imager) using the following antibodies: β-actin (C4) mouse monoclonal IgG1 (sc-47778; Santa Cruz Biotechnology), Hsl (rabbit polyclonal, 4107S Cell Signaling), phospho–Hsl (serine 660 rabbit polyclonal; 4126S Cell Signaling), Pnpla2 (rabbit polyclonal, 2138S Cell Signaling), Plin1 (rabbit polyclonal, ab3526 AbCam), serine 492-phospho–Plin1 (serine 492 mouse monoclonal, 4855 Vala Sciences), Abhd5 (goat polyclonal, ab111984 AbCam), β_1AR (rabbit polyclonal, sc-568 Santa Cruz Biotechnology), β_2AR (rabbit polyclonal, sc-570 Santa Cruz Biotechnology), β_3AR (rabbit polyclonal, sc-50436 Santa Cruz Biotechnology), GR (provided by Keith Yamamoto, UCSF), FAK (mouse monoclonal, sc-271126 Santa Cruz Biotechnology), phosphor-FAK (serine mouse monoclonal, sc-81493 Santa Cruz Biotechnology). sAC (rabbit polyclonal, ARP47447_P050 Aviva Systems Biology), goat anti-rabbit IRDye 800CW (LiCor), and goat anti-mouse IRdye 800CW (LiCor). The optical density of the bands was quantified using Image J software (http://rsbweb.nih.gov/ij) and normalized to β-actin.

Lipolysis Assays. Lipolysis was measured as described [143]. Explants from freshly removed epididymal and inguinal WAT depots (~100mg) were incubated at 37°C in 500mL of Krebs-Ringer Buffer (12mM HEPES, 121mM NaCl, 4.9mM KCl, 1.2 mM $MgSO_4$ and 0.33mM $CaCl_2$) with 3% BSA and 3mM glucose. Glycerol release was determined over time using a free glycerol reagent (Sigma). Measurements were normalized to the total protein content of the explants using Bradford protein dye (BioRad).

Measurement of Corticosterone Levels. An ELISA (Enzo Life Sciences) was used to measure corticosterone levels from plasma immediately after isolation from the whole blood of mice.

WAT cAMP Measurement. Epididymal WAT was isolated, weighed and homogenized in Krebs-Ringer Buffer. Tissues homogenates were centrifuged (13,000 x g for 10 min at 4°C) and the cAMP content of the tissue supernatants was measured by ELISA (Enzo Life Sciences)[143].

Quantitative Real-Time PCR (qPCR). Total RNA was isolated from the livers and epididymal WAT of mice using Tri-reagent (Molecular Research Center Inc.). Reverse transcription was performed as described [23]. The resulting cDNA was diluted to 170µL and 3.5µL was used to perform qPCR in a 25µL reaction using EVA qPCR SuperMix Kit (Biochain) per the manufacturer's protocol. qPCR was performed on a StepOne PCR-system (Applied Biosystems) and analyzed using the delta-delta CT method as supplied by the manufacturer. Primer Sequences were as follows: *Rpl19* (*For*:AGCCTGTGACTGTCCATTCC, *Rev*:GGCAGTACCCTTCCTCTTCC; used for internal normalization), *Pnpla2* (*For*:CCAACGCCACTCACATCTAC, *Rev*:CCTCAATAATGTTGGCACCTG), *Angptl4* (*For*:GCCATTCCAATCTCAATGG, *Rev*:ATCAACAGGGTGGTAGCCTG), *Pepck* (*For*:GTCCGATCCCCGTTTATTCT,

Rev:ACCTTGGTTTTGGGGGTAAC), *Adcy3* *(For:*CCACGATGATAGCACACAGG, *Rev*:CTTGTGTTGGGGGTCACTGT), *Adcy6* *(For:*CAGCAGGGTAGTGTGTGCAG, *Rev*:TCTGCATTTGATTTTGGCCT), *Adcy8* *(For:*AGGTGCTCATCCTCCACATT, *Rev*:CTCCGCTTGGAGACAGAGAA), *Pde3B* (*For*:TCCTGAACATCTTGCCACTG, *Rev*:AGTACCGCGGAGGAAAAAGT), *Gnas* (*For*:GCAGGATCCTCATCTGCTTC, *Rev*:CTAATGGGTGACTCCGTCCA), *Gnb1* *(For:*TTTCTGGTCTGGTTTCCCAC, *Rev*:GCTGGGACTGGAACAGCC*)*. Primers were from Elim Biopharmaceuticals.

FFA Assays. Plasma was isolated from whole blood immediately after collection and a colorimetric kit (Wako) was used to measure plasma FFA levels.

Measurements of Liver TG. Livers samples were weighed and homogenized in a buffer consisting of 50 mM Tris-HCl (pH 7.4), 250 mM sucrose, and protease inhibitors. Lipids were extracted in chloroform:methanol (2:1) and separated by TLC on silica gel G-60 plates with the solvent hexane:ethyl ether:acetic acid (v:v:v 80:20:1). The TG bands were visualized by exposure to iodine, and then scraped and analyzed as described [144], with triolein (Sigma) as a standard, and expressed per tissue weight.

Body Fat Measurements. Mice that were either fasted or fed *ad-libitum* for 24 hours were anesthetized with isofluorane and scanned by dual-energy X-ray absorptiometry (DEXA) with a PixiMus2 scanner (GE Healthcare Lunar) in order to measure body fat content. Body composition was also measured prior to fasting experiments to ensure there were no basal differences between treatment groups.

Isolation of Adipocytes. WT and *Angptl4*$^{-/-}$ mice were euthanized and epididymal WAT was harvested and minced thoroughly in Krebs-Ringer buffer containing collagenase. The mixture was transferred to a conical tube and shaken at 220 rpm and 37°C for 1 hour. After digestion, the mixture was filtered through a 250mm gauze mesh and spun for 5 minutes at 200 rpm. The buffer was carefully removed using a needle and syringe, and the floating layer of adipocytes was washed three times with 10ml of Krebs-Ringer buffer and then resuspended in media containing 10% stripped FBS. Aliquots of cells were placed into microcentrifuge tubes containing media at a final volume of 500 mL for further treatments. Cells were incubated at 37°C with shaking at 220 rpm for all treatments. Agents used to treat adipocytes included isoproterenol, norepinephrine, forskolin, and 3-isobutyl-1-methylxanthine (IBMX), from Sigma, 8-bromo-cAMP (8-Br-cAMP) and 2', 5' dideoxyadenosine from Santa Cruz Biotechnology as well as KH7 and PF573228 from Tocris Bioscience.

Purification of human ANGPTL4. HEK293 cells cultured in media with 5% FBS were infected with adenovirus expressing a FLAG-tagged version of human ANGPTL4 (provided by Sara Vienberg and Ronald Kahn, Joslin Diabetes Center) for 1 hour, at which point the media was replaced. After 72 more hours, the media was collected and Angptl4 protein was purified using an anti-FLAG M2 Affinity gel (Sigma). The purified protein was then dialyzed and concentrated 10x using Slide-A-Lyzer dialyzing cassettes and concentrating solution (Thermo Scientific). Western Blot and Coomassie staining confirmed protein purity. The Affinity gel elution buffer (TBS) was also dialyzed and concentrated to serve as a control.

Site-directed Mutagenesis and Deletion. A mutagenesis kit (QuikChange Lightening) was used to make site-directed mutations and deletions in the Angptl4 gene as per the manufacturers instructions (Stratagene).

Determination of Tissue and Plasma Angptl4 Levels. Plasma and epididymal WAT were isolated from C57B/6J mice that had been fed *ad-libitum* or fasted for 24 hours. Approximately 200 mg of the WAT was homogenized in 500mL of RIPA buffer (10 mM

Tris-HCl, pH 8.0, 1mM EDTA, 150 mM NaCl, 5% glycerol, 0.1% sodium deoxycholate, 0.1% SDS, and 1% Triton X-100) supplemented with protease inhibitors. The homogenates were centrifuged (13,000 x g for 10 minutes at 4°C) and the Angptl4 content of the tissue supernatants and plasma samples were analyzed by ELISA (UCSN).

Statistics. Data are expressed as standard error of the mean (S.E.M) for each group and comparisons were analyzed by Student's *t* test.

RESULTS

Glucocorticoid action is required for fasting-induced Angptl4 expression. Fasting, a potent inducer of Angptl4 expression [145], also increases circulating glucocorticoid levels. We therefore investigated whether glucocorticoid action is required for the induction of Angptl4 by physiological fasting in mice. To do this, we provided mice with drinking water containing either the GR antagonist RU486 or DMSO (control). The mice were treated for 3 days and either fasted or allowed to feed *ad libitum* for the final 17 hours, after which liver and WAT samples were collected for measurement of *Angptl4* mRNA levels. There was no difference in hepatic *Angptl4* mRNA levels when mice fed *ad libitum* were treated with either RU486 or DMSO (Figure 1A). However fasting induced a 9.1-fold increase in *Angptl4* mRNA levels, and this was reduced by ~60% in mice treated with RU486 (Figure 1A). A similar reduction by RU486 treatment was observed for mRNA levels of the phosphoenolpyruvate carboxykinase gene (*Pepck*), a hepatic GR target known to play an important role during fasting (Figure 1A) [146, 147].

Figure 1: The role of glucocorticoids in Angptl4 expression in liver and white adipose tissue

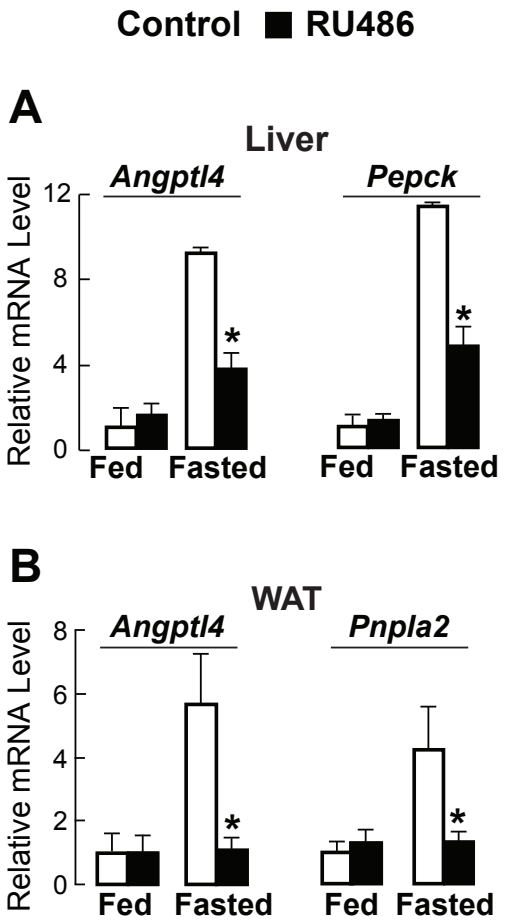

FIGURE 1: **A.** Relative mRNA levels (vs. fed controls) of hepatic *Angptl4* and *Pepck* measured by qPCR from the livers of mice treated with either RU486 (0.12mg/ml in the drinking water) or control (DMSO) for 3 days and either allowed to feed *ad libitum* or fasted for 17 hours, showing reduced levels in fasted *Angptl4*$^{-/-}$ mice (n = 8; *p<0.05 vs. fasted WT mice). **B.** As for A, except that mRNA levels were for *Angptl4* and *Pnpla2* and were measured from Epididymal WAT.

As in the livers, *Angptl4* mRNA levels in the WAT were not altered by RU486 treatment in mice fed *ad libitum*, but the increase induced by fasting in control mice (5.7-fold over fed) was abolished by RU486 treatment (Figure 1B). This reduction matched that seen for the patatin-like phospholipase domain containing 2 (Pnpla2) gene (*Pnpla2*; a.k.a adipose triglyceride lipase/ATGL), which is known to be a direct GR target gene in the WAT (Figure 1B)[148]. Together these results indicate that glucocorticoids are required for the induction of *Angptl4* by physiological fasting in the liver and WAT.

Glucocorticoid action is important for fasting-induced lipolysis. Physiological fasting also stimulates the hydrolysis and mobilization of intracellular TG by adipocytes in the WAT. To determine the importance of glucocorticoid action on WAT lipolysis during such a fast, we analyzed the blood and WAT samples collected from the control and RU486-treated mice described above. Fasting increased plasma FFA levels in control mice by 2-fold, an effect that was reduced ~30% by RU486 treatment (Figure 2A). There was also an increase in the amount of glycerol released from WAT samples explanted from mice fasted for 24 hours (2.7-fold for epididymal WAT and 2-fold for inguinal WAT), and this was similarly reduced (1.8-fold increase for epididymal WAT and 1.6-fold increase for inguinal WAT) by RU486 treatment (Figure 2B). These reductions were not a function of lowered circulating levels of endogenous corticosteroids, as fasting increased the levels of plasma corticosterone to a similar extent in both control and RU486-treated mice (Figure 2C). Together, these findings establish the physiological importance of glucocorticoid action in fasting-induced WAT TG hydrolysis and FFA release.

Figure 2: Glucocorticoid action in fasting-induced lipolysis

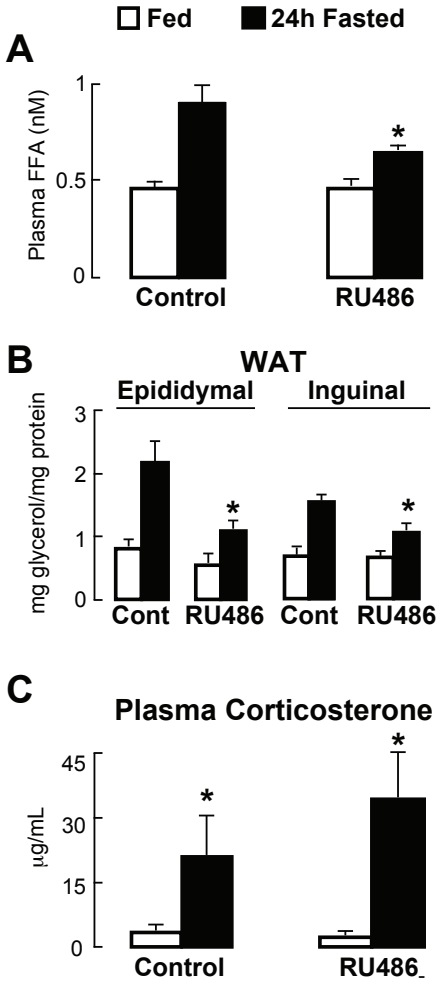

FIGURE 2: A. Plasma FFA levels from mice treated with RU486 or control (DMSO) for 3 days and either fasted or allowed to feed *ad libitum* for the final 17 hours of treatment, showing that RU486 treatment lowers fasting-induced increases in FFA levels (n = 5-6; *p<0.05 vs. fasted controls). **B.** Glycerol concentration measured from epididymal and inguinal WAT explants taken from the mice in A after a 2-hours in medium, showing an RU486-dependent reduction in glycerol release (n = 6; *p<0.05 vs. fed; **p<0.05 vs. WT fasted). Data are normalized to total protein. **C.** Plasma corticosterone levels from treated mice (n=5-6; *p<0.05 vs. fed).

Angptl4 is needed for fasting-induced WAT lipolysis. Given that fasting in mice both induces WAT *Angptl4* expression and stimulates WAT lipolysis, we wondered whether Angptl4 could be a mediator of the fasting-induced lipolytic response in the WAT. We tested this hypothesis by fasting WT and *Angptl4*$^{-/-}$ mice. Measuring the amount of glycerol released into medium over 2 hours by WAT samples explanted from WT mice that had either been fed *ad libitum* or fasted revealed that 6 hours of fasting induced a 3-fold increase in the concentration of glycerol released from epididymal WAT (Figure 3A). However, this increase was greatly reduced when the explants were from *Angptl4*$^{-/-}$ mice (Figure 3A). The increase in glycerol release from WT explants was more pronounced when the duration of fasting was prolonged to 24 hours (10.7-fold), but remained reduced (4.9-fold) when from *Angptl4*$^{-/-}$ WAT (Figure 3A). A similar relationship (~17-fold increase for WT vs. ~7.4-fold for *Angptl4*$^{-/-}$) was observed from inguinal WAT samples after a 24-hour fast

(Figure 3B) and from both depots when glycerol release was measured over 1- or 4 hours (data not shown). Thus, fasting for as little as 6 hours, a duration well within the daily physiological range, stimulates WAT lipolysis by a mechanism that is compromised in *Angptl4*$^{-/-}$ mice.

Figure 3: Angptl4 involvement in fasting-induced lipolysis

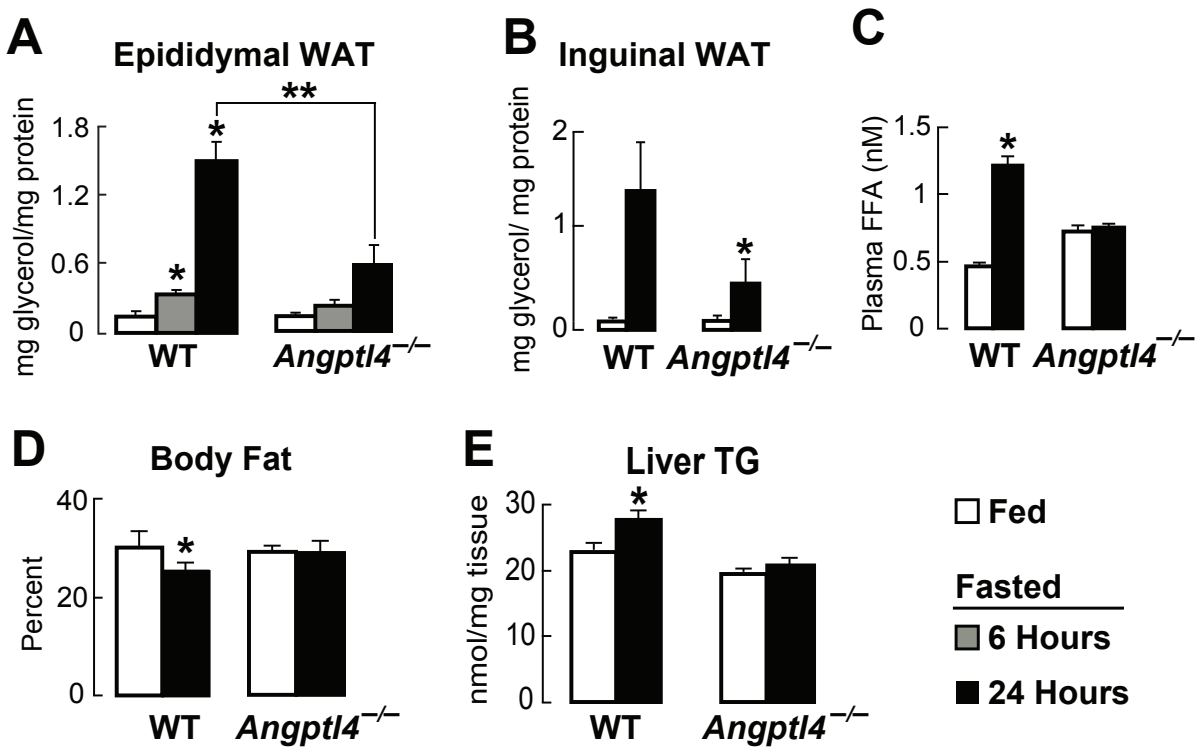

FIGURE 3: A. The concentration of glycerol released by epididymal WAT explants taken from mice that were fed *ad libitum* or fasted for 6- or 24 hours, showing decreased release from *Angptl4*$^{-/-}$ WAT (n = 6; *p<0.05 vs. fed; **p<0.0001 vs. WT 24-hour fasted). Glycerol concentrations were normalized to protein. **B.** As for A, using inguinal WAT explants (n = 6; *p<0.05 vs. WT fasted). **C.** FFA levels measured from the plasma of WT and *Angptl4*$^{-/-}$ mice that were either fed *ad libitum* or fasted for 24 hours, showing a reduction in the fasting-induced plasma FFA levels in *Angptl4*$^{-/-}$ mice (n=6; *p<0.01 vs. WT fed; **p<0.001 vs. WT fasted). **D.** Body composition as measured by DEXA, showing a lack of body fat loss induced by fasting in *Angptl4*$^{-/-}$ mice (n = 9; *p<0.05 vs. WT fed). **E.** Liver triglyceride content measured by TLC in response to a 24 -hour fast, showing a loss of fasting-induced hepatic steatosis in *Angptl4*$^{-/-}$mice (n=9; *p<0.05 vs. WT fed).

To confirm this conclusion, we also measured changes in the circulating FFA levels and body composition of WT and *Angptl4*$^{-/-}$ mice fasted for 24 hours. Fasting greatly increased circulating FFA levels in WT mice, but not in *Angptl4*$^{-/-}$ mice [Figure 3C, and as seen previously [140]]. Fasting also markedly reduced the percent body fat and increased hepatic TG content in WT mice, both of which remained relatively constant in fasted *Angptl4*$^{-/-}$ mice (Figures 3D and E). Together, these results support the concept that *Angptl4*$^{-/-}$ mice have a reduction in fasting-induced WAT lipolysis and that Angptl4 is therefore important in this process.

Angptl4 is required for normal glucocorticoid-induced WAT lipolysis. Given our findings that fasting-induced WAT lipolysis is dependent on glucocorticoid action and that glucocorticoid administration, like fasting, induces *Angptl4* expression in tissues [23], we wanted to know whether Angptl4 functions to mediate WAT lipolysis when directly

stimulated by glucocorticoids. To do this, we measured the release of glycerol into medium over 2 hours from WAT samples explanted from WT and *Angptl4*$^{-/-}$ mice that were first administered a single dose of either PBS (control) or a synthetic glucocorticoid (dexamethasone; DEX). Unlike with fasting, we could not measure a change in the release of glycerol from epididymal WAT explants taken within 6 hours of DEX treatment, regardless of genotype (Figure 4A). However, when the WAT explants were taken 24 hours after DEX treatment, we could clearly observe an increase in glycerol release from WT epididymal (Figure 4A) and inguinal (Figure 4B) WAT explants, demonstrating that WAT lipolysis stimulated solely by glucocorticoids mirrors that stimulated by fasting in mice, though with a slower kinetic onset.

In contrast, glycerol release by WAT explants taken from *Angptl4*$^{-/-}$ mice 24 hours following DEX treatment was greatly reduced (epididymal WAT; Figure 4A) to essentially absent (inguinal WAT; Figure. 4B). The differences in glycerol release between WT and *Angptl4*$^{-/-}$ WAT were also present when measured over 1- or 4 hours (data not shown) and demonstrate that Angptl4 is necessary for WAT lipolysis following a single administration of glucocorticoids in mice.

Figure 4: Angptl4 involvement in glucocorticoid-induced lipolysis

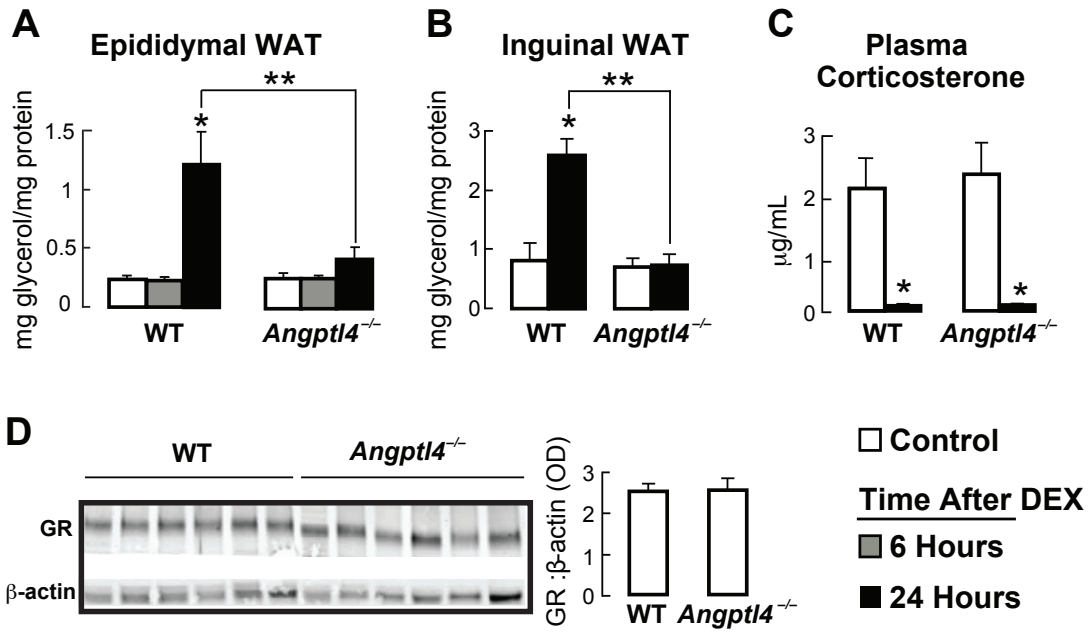

FIGURE 4: A. The concentration of glycerol released into the medium over 2 hours from epididymal WAT explants taken from control (PBS-treated) mice and from mice either 6- or 24 hours following a single 5 mg/kg intraperitoneal dose of DEX, showing a reduction in the concentration of glycerol released by *Angptl4*$^{-/-}$ WAT 24 hours after DEX treatment (n = 7-8; *p<0.05 vs. control; **p<0.05 vs. WT 24-hour DEX). **B.** As for A, using inguinal WAT explants (n=7-8; *p<0.0001 vs. WT control; **p<0.001 vs. WT 24-hour DEX). **C.** Plasma corticosterone levels, showing DEX-induced suppression across genotypes (n = 5-6; *p<0.05 vs. control). **D.** WAT GR protein abundance by immunoblot (n = 6). The image is a grouping of representative images from different areas of the same gel.

We also monitored the levels of plasma corticosterone, the primary endogenous glucocorticoid in mice, and WAT GR expression in response to DEX treatment. Plasma corticosterone levels were similar at baseline and were suppressed to a comparable degree by DEX treatment (95% and 94%, respectively) in WT and *Angptl4*$^{-/-}$ mice (Figure 4C). GR expression levels in the WAT were also similar in WT and *Angptl4*$^{-/-}$ mice (Figure 4D).

Together, these data indicate that impaired DEX-induced WAT lipolysis in *Angptl4*$^{-/-}$ mice is not due to differences in circulating levels of endogenous glucocorticoids or WAT GR expression.

Fasting and DEX treatment stimulate Angptl4-dependent cAMP signaling in the WAT. In response to acute lipolytic stimuli such as physiological fasting, cAMP levels in adipocytes rise and stimulate the activation of protein kinase A (PKA), which then phosphorylates Hsl and perilipin-1 (Plin1), two important lipolytic enzymes [149, 150]. We therefore investigated

Figure 5: The role of Angptl4 in fasting- and glucocorticoid-induced cAMP signaling

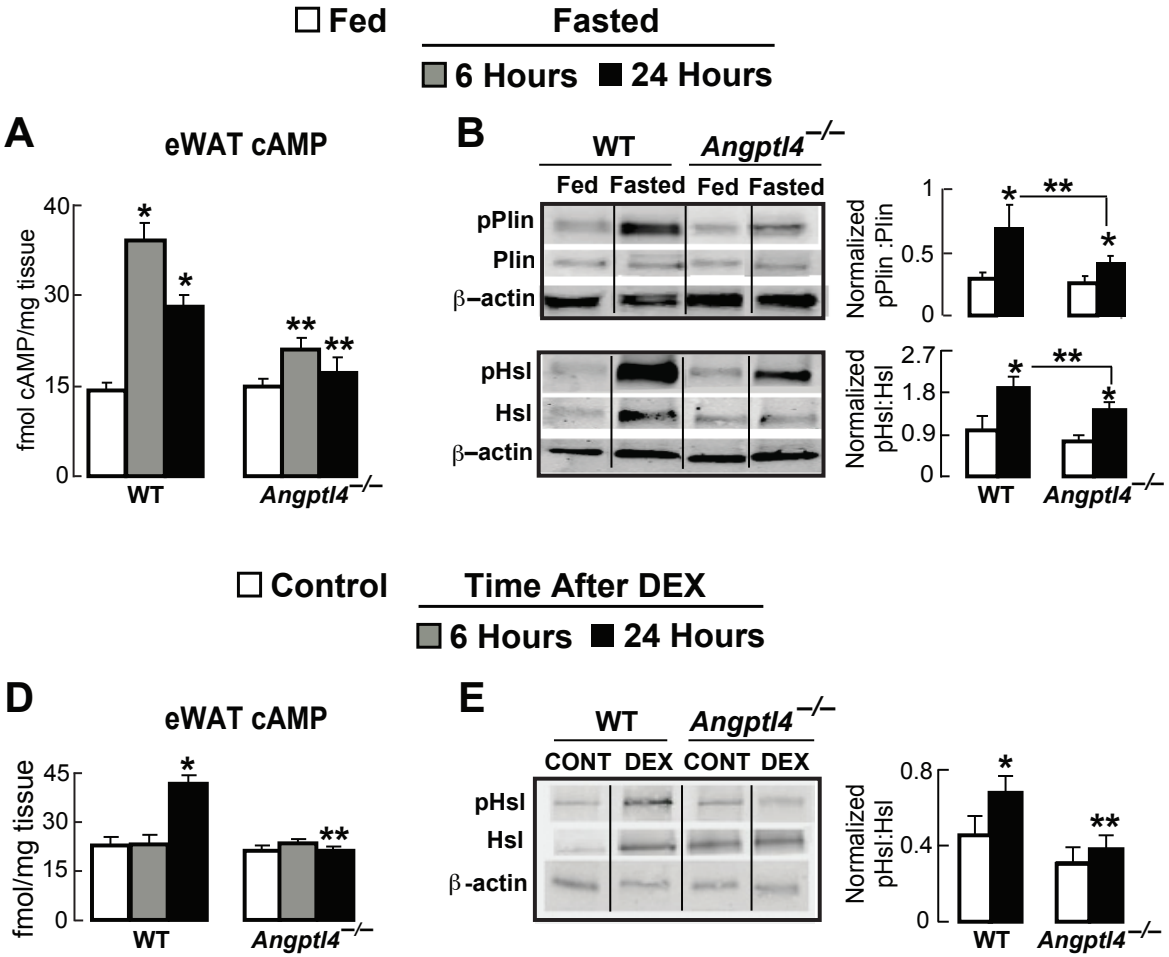

FIGURE 5: A. Cytosolic cAMP levels (normalized to sample weight) measured from epididymal WAT, showing decreased values in WAT from *Angptl4*$^{-/-}$ mice fasted for 6- and 24 hours (n = 6; *p<0.01 vs. WT fed; **p<0.05 vs. WT similarly fasted). **B.** Immunoblots probing for the levels of total Plin and pPlin as well as Hsl and pHsl in epididymal WAT explants taken from mice fed *ad libitum* or fasted for 24 hours, showing that *Angptl4*$^{-/-}$ mice have a decrease in the relative induction of pPlin and pHsl by fasting. The intensity of bands quantified from 5 separate blots per treatment condition were expressed as the ratio of pPlin to total Plin and pHsl to total Hsl and normalized to actin (*p<0.05 vs. fed; **p<0.05 vs. WT fasted). **C.** cAMP levels measured as in A from epididymal WAT taken from control (PBS-treated) mice or either 6- or 24 hours following a single intraperitoneal dose of DEX, showing that the increase in WAT cAMP levels 24 hours after DEX treatment is absent in *Angptl4*$^{-/-}$ mice (n = 4-5; * p<0.001 vs. WT control; **p<0.05 vs. WT 24 hours after DEX). **D.** Total Hsl and pHsl measured as in B from epididymal WAT 24 hours following DEX treatment, showing a reduction in the DEX-induced increase in the ratio of pHsl to total Hsl (*p<0.05 vs. WT control; **p<0.05 vs. WT DEX). Data are the mean band intensities measured from 8 separate blots per treatment condition. Each immunoblot image is a grouping of representative images from different parts of the same gel.

the role of Angptl4 in regulating cAMP levels and the activation of components of the lipolytic machinery in the WAT of fasted mice. Fasting for 6 and 24 hours increased cAMP levels by 2.8- and 1.9-fold, respectively, in the WAT of WT mice (Figure 5A). However, this was greatly reduced in the WAT of *Angptl4*$^{-/-}$ mice, where a 6-hour fast produced a relatively modest (1.5-fold) increase in WAT cAMP levels that was absent altogether when fasting was extended to 24 hours (Figure 5A).

Fasting also produced a marked increase in the absolute abundance of the phosphorylated forms of both Hsl (serine 660; pHsl) [151] and Plin1 (serine 492; pPlin1) [152, 153] as well as the ratio of phosphorylated to total enzyme for each (Figures 5B). In contrast, both the absolute levels and relative abundances of pHsl and pPlin1 were lower in the WAT of fasted *Angptl4*$^{-/-}$ mice (Figures 5B), indicating that Angptl4 is needed for cAMP-dependent protein phosphorylation events that are important in the fasting-induced lipolytic response.

To determine whether DEX treatment could mirror the increase in WAT cAMP levels stimulated by fasting, we analyzed cAMP levels in the WAT of mice following a single dose of DEX. As for glycerol release from WAT explants, cAMP levels within the WAT were not altered 6 hours after an intraperitoneal dose of DEX but were markedly increased (1.8-fold) by 24 hours. Remarkably, DEX treatment was unable to increase cAMP levels in the WAT of *Angptl4*$^{-/-}$ mice, as was the case when fasting was used as the stimulus (Figure 5C). Consistent with these data, DEX treatment increased the ratio of pHsl to total Hsl by ~35% in the WAT of WT mice but did not alter this ratio in the WAT of *Angptl4*$^{-/-}$ mice (Figure 5D). Together these findings indicate that a single DEX treatment can mimic physiologic fasting to increase cAMP levels in murine WAT, albeit with a slower time-course. Moreover, as for glycerol release, the ability of DEX to increase cAMP levels in the WAT and stimulate downstream phosphorylation events is dependent on Angptl4.

Figure 6: The glucocorticoid-regulated expression of Pnpla2 and CGI-58

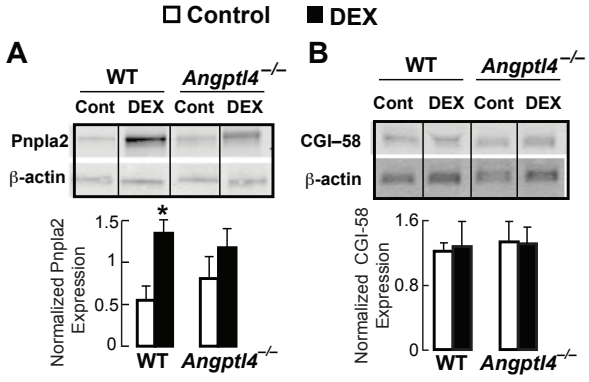

FIGURE 6: A. Immunoblots probing for the levels of Pnpla2 in epididymal WAT explants taken from 24 hours after DEX treatment, showing a significant increase in expression after DEX treatment in WT mice but not in *Angptl4*$^{-/-}$ mice. The intensity of bands quantified from 5 separate blots per treatment condition were expressed as the ratio Pnpla2 normalized to actin (*p<0.05 vs. fed; **p<0.05 vs. WT control). **B.** Expression of CGI-58 was also measured by immunoblot in WAT of the same animals. No differences in expression were observed in response to DEX treatment in either genotype. Data are the mean band intensities measured from 4 separate blots per treatment condition.

Other enzymes are involved in mobilizing TG from its storage depot within adipocyte lipid droplets during WAT lipolysis, including Pnpla2 and CGI-58 (Abhd5), both of which are implicated in diseases associated with aberrant lipolysis [154, 155]. We found that DEX treatment stimulated an increase in the overall abundance of Pnpla2 within the WAT of WT mice despite some variability in expression levels between individual mice. However, we did not see this relative increase in the epididymal WAT of DEX-treated *Angptl4*$^{-/-}$ mice (Figure 6A). On the other hand, CGI-58 protein levels were not altered by DEX treatment within the WAT of either WT or *Angptl4*$^{-/-}$ mice (Figure 6B). These data suggest that DEX may act to transcriptionally regulate the expression of some lipolytic genes, such as *Pnpla2*, but not others, such as *CGI-58*, and that this transcriptional control may be at least partially determined by the presence or absence of Angptl4. In any case, the impairment of

glucocorticoid-induced WAT lipolysis in *Angptl4*$^{-/-}$ mice was not associated with a reduction in the basal expression of either Hsl, Plin1, Pnpla2, or CGI-58 in the WAT.

Angptl4 is a mediator of catecholamine-induced lipolysis in adipocytes. Beyond glucocorticoids, catecholamines are regarded as dominant regulators of intracellular adipocyte lipolysis during fasting and do so by stimulating cAMP-dependent signaling [150, 156]. Because *Angptl4*$^{-/-}$ WAT had less lipolysis and less cAMP-dependent signaling in response to both physiological fasting and glucocorticoid treatment, we explored whether Angptl4 plays a role in catecholamine-induced lipolysis. We measured the concentration of glycerol released into the medium by primary adipocytes isolated from WT and *Angptl4*$^{-/-}$ mice during a 1-hour treatment with either 200nM isoproterenol or 1mM norepinephrine. Glycerol release by *Angptl4*$^{-/-}$ adipocytes in response to either isoproterenol or norepinephrine treatment was significantly lower than for WT adipocytes (Figure 7A), indicating that the requirement of Angptl4 for normal lipolysis in response to fasting or glucocorticoid treatment *in vivo*, is also present in cultured murine adipocytes treated with catecholamines.

We affinity-purified human angiopoietin-like 4 protein (hANGPTL4) from HEK293 cells to further explore how Angptl4 regulates the lipolytic response of adipocytes. In order to determine the concentration range over

Figure 7: The effects of Angptl4 on lipolysis

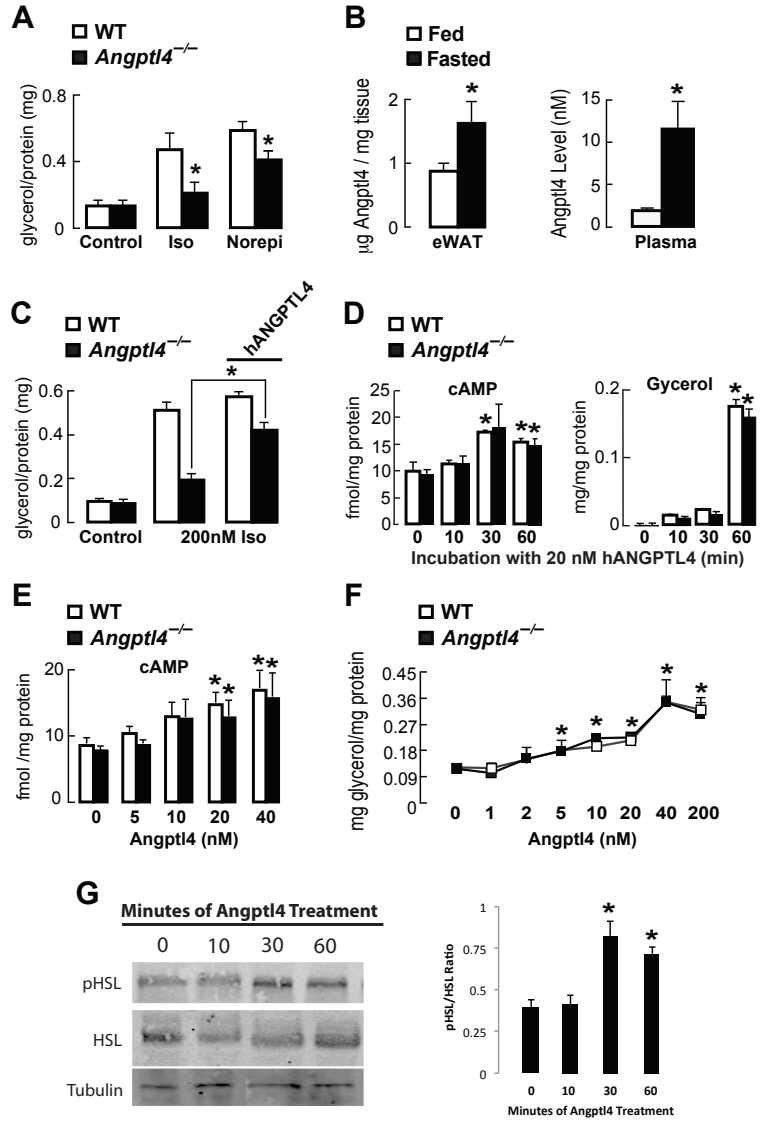

FIGURE 7: A. Glycerol concentration measured in the medium bathing primary murine adipocytes treated for 1 hour with isoproterenol (200 nM) or norepinephrine (1 mM), showing a decrease in catecholamine-stimulated glycerol release by *Angptl4*$^{-/-}$ adipocytes (n = 6; *p<0.05 vs. similarly treated WT cells). **B.** Angptl4 concentrations measured by ELISA in the epididymal WAT and plasma of mice fed *ad libitum* or fasted for 24 hours (n=5-6; *p<0.05 vs. fed). **C.** Glycerol concentration measured in the medium bathing primary murine adipocytes treated for 1 hour with isoproterenol alone or plus 20 nM hANGPTL4, showing that addition of hANGPTL4 largely rescues the impaired catecholamine-stimulated glycerol release seen in Angptl4 deficiency (n = 6; *p<0.05). **D.** Cytosolic cAMP levels and glycerol release measured from WT and *Angptl4*$^{-/-}$ adipocytes treated with 20nM hANGPTL4, showing the kinetics for each response over 60 min (n=4-5; *p<0.05 vs. time-zero). **E.** Dose-response relationship for the increase in cytosolic cAMP in WT and *Angptl4*$^{-/-}$ adipocytes stimulated by treatment with hANGPTL4 for 1 hour (n = 8; *p<0.05 vs. buffer alone). **F.** Dose-response relationship for the stimulation of glycerol release into the medium by WT and *Angptl4*$^{-/-}$ adipocytes treated with hANGPTL4 for 1 hour (n= 4; *p<0.05 *versus* buffer alone). **G.** Immunoblots probing for the levels of pHSL and HSL in isolate epididymal adipocytes treated with 20nM ANGPTL4, showing a significant increase pHSL after 30 and 60 minutes of treatment. The intensity of bands quantified from 4 separate blots per treatment condition were expressed as the ratio pHSL to HSL normalized to actin (*p<0.05 vs. 0 time point).

which treatments with hANGPTL4 should be performed, we first measured the extent to which Angptl4 levels in the WAT and plasma are elevated by physiological fasting. In both compartments, fasting for 24 hours resulted in a marked increase in Angptl4 protein concentration (Figure 7B). Although the concentration reached in the epididymal WAT (1.6 mg/mg tissue; ~32.5 nM) was notably higher than that in the plasma (11.6 nM), these values determined the range of hANGPTL4 concentrations (20 nM) used to treat primary adipocytes in subsequent studies.

We tested whether purified hANGPTL4 could rescue the impaired lipolytic response of *Angptl4*$^{-/-}$ adipocytes to catecholamine treatment. Indeed, the concentration of glycerol released into the medium in response to isoproterenol treatment by *Angptl4*$^{-/-}$ adipocytes was restored to near WT levels by the addition of 20 nM hANGPTL4 into the medium (Figure 7C), indicating that extracellular Angptl4 is a mediator of catecholamine-stimulated TG hydrolysis in adipocytes. hANGPTL4 treatment did not further enhance isoproterenol-stimulated glycerol release by WT adipocytes; however the response of these cells to isoproterenol treatment was already quite robust even without added hANGPTL4.

Extracellular hANGPTL4 increases cAMP levels and TG hydrolysis in adipocytes. We treated primary WT and *Angptl4*$^{-/-}$ adipocytes with 20 nM hANGPTL4 to determine if this alone was sufficient to increase cAMP levels and stimulate lipolysis. hANGPTL4 treatment increased cytosolic cAMP levels similarly in WT and *Angptl4*$^{-/-}$ adipocytes and did so within 30 minutes (Figure 7D). hANGPTL4 treatment also stimulated glycerol release, again to a similar degree, from WT and *Angptl4*$^{-/-}$ adipocytes (Figure 7D). However, the kinetics of hANGPTL4-stimulated glycerol release had a slower onset than that for elevation of cytosolic cAMP, increasing markedly after 60 minutes. Together these findings indicate that addition of extracellular Angptl4 alone is sufficient to increase the level of cytosolic cAMP and stimulate lipolysis in adipocytes and that, as seen in response to lipolytic stimuli *in vivo*, the time-course for glycerol release by adipocytes is slower than that needed to elevate cytosolic cAMP.

We also tested the concentration-dependence of the effect of hANGPTL4 treatment on increasing cytosolic cAMP levels and stimulating lipolysis in adipocytes. WT and *Angptl4*$^{-/-}$ adipocytes were treated with hANGPTL4 for 60 minutes at concentrations ranging up to 200 nM. Over this period, 20 nM hANPTL4 was the minimum concentration sufficient to increase cAMP levels, and did so similarly in WT and *Angptl4*$^{-/-}$ adipocytes (Figure 7E). On the other hand, glycerol release over 60 minutes was stimulated by as little as 5 nM hANGPTL4, although the concentration of glycerol released did not increase further until 40 nM hANGPTL4 was used (Figure 7F).

Figure 8: Dissociating LPL-inhibition from the lipolytic effect of Angptl4

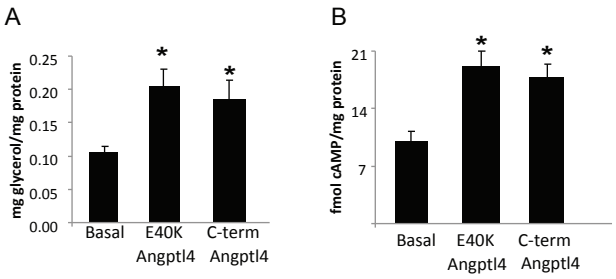

FIGURE 8: A. Glycerol concentration measured in the medium bathing primary murine adipocytes treated for 1 hour with 20nM Angptl4 containing the E40K mutation or 20nM of the C-terminal domain of Angptl4 showing an increase in glycerol release from the adipocytes with both treatment conditions (n = 6; *p<0.05 vs. control treated cells). **B.** Cytosolic cAMP levels were measured from isolated adipocytes treated as in A showing an increase in cAMP content following treatment with E40K ANGPTL4 as well as the C-terminal truncation of ANGPTL4 (n=6; *p<0.05 vs. control-treated adipocytes).

We further wanted to confirm that purified hANGPTL4 could directly modulate downstream cAMP targets, namely HSL phosphorolyation. We found that 20nM hANGPTL4 significantly increased HSL phosphorylation after 30 and 60 minutes of treatment (Figure 7G).

The N-terminal domain of Angptl4 is not required for its lipolytic effects. The single nucleotide polymorphism

E40K in the N-terminal coiled-coil domain of Angplt4 is a genetic variant found in certain human populations that results in a full-length version of the protein that no longer has the capacity to inhibit LPL. We were interested to know if the ability to inhibit LPL is required for the lipolytic effect of Angptl4. By site-directed mutagenesis we introduced an E40K mutation into hANGPTL4. Treating isolated adipocytes with this purified variant significantly increased both glycerol release (Figure 8A) and cAMP content (Figure 8B). Since the E40K mutant cannot inhibit LPL but was still able to induce lipolysis we decided to investigate if the N-terminal was necessary at all for lipolytic induction by Angptl4. We treated isolated adipocytes with a truncated form of Angptl4 where the N-terminal had been deleted leaving only the C-terminal fibrinogen like domain. This C-terminal domain increased glycerol release 2.1 fold over control (Figure 8A) and cAMP content 1.7 fold (Figure 8B), both of which were comparable to inductions seen following treatment with the E40K mutant. These results indicate that the LPL-inhibitory effect and the lipolytic effect of Angptl4 can be dissociated.

Angptl4 regulates lipolysis at a point upstream of adenylate cyclase. The cascade leading from beta-adrenergic receptor (bAR) stimulation to TG hydrolysis is well characterized, and we took advantage of established pharmacologic approaches to dissect the point within this cascade at which hANGPTL4 exerts its lipolytic effects. WT and *Angptl4*$^{-/-}$ adipocytes were treated with either isoproterenol or with one of several other agents which also stimulate lipolysis, but do so by engaging signaling steps distal to bAR activation. Interestingly, Angptl4 deficiency in adipocytes impaired glycerol release in response to treatment with isoproterenol but not forskolin, which directly activates adenylate cyclase (Figure 9A). Angptl4 deficiency likewise did not affect lipolysis stimulated by treatment of adipocytes with IBMX, which inhibits the hydrolysis of cAMP by phosphodiesterases (PDEs), or with 8-Br-cAMP, which is resistant to such hydrolysis (Figure 9A). Taken together, these data indicate that Angptl4 modulates cellular signaling at a point downstream of bAR but upstream of adenylate cyclase and more distal modulators.

Figure 9: Lipolytic impairment in Angptl4-/- mce is upstream of adenylate cyclase

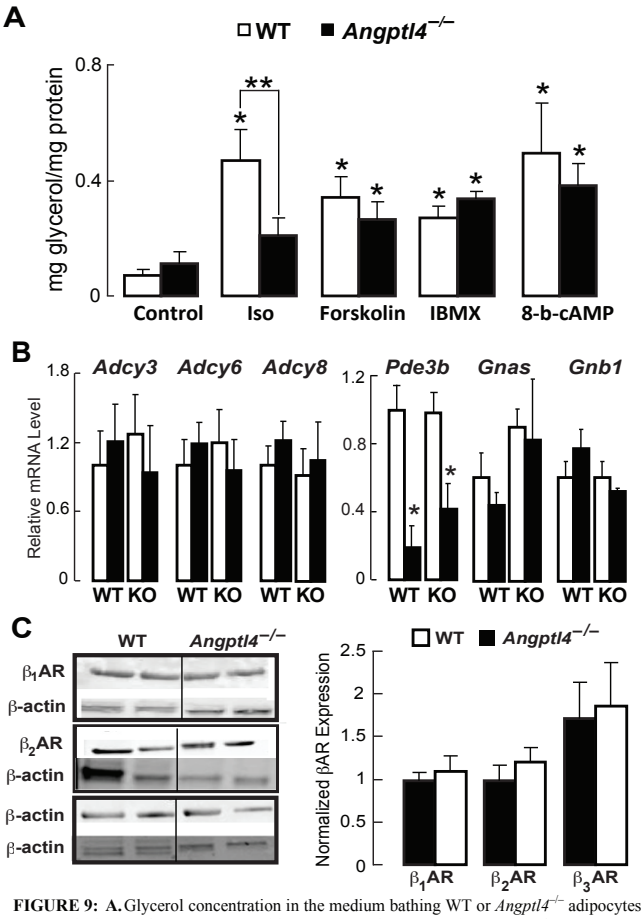

FIGURE 9: A. Glycerol concentration in the medium bathing WT or *Angptl4*$^{-/-}$ adipocytes measured after a 1-hour treatment with PBS (control), 200 nM isoproterenol (Iso), 10mM forskolin, 100 mM IBMX or 10 mM 8-b-cAMP, showing that only isoproterenol-stimulated glycerol release was decreased by Angptl4 deficiency (n=6; *p<0.05 vs. WT control, **p<0.05 vs. WT isoproterenol). **B**. mRNA levels of several genes encoding proteins involved in the cAMP-dependent signaling cascade in epididymal WAT taken from control (PBS-treated) mice and from mice 24 hours after a single intraperitoneal treatment with DEX, showing a DEX -induced reduction only in the levels of *Pde3b* but no differences between genotypes (n=12; *p<0.05 vs control). **C.** Immunoblots from epididymal WAT, showing no differences in the protein levels of b_1-, b_2-, or b_3AR between WT and *Angptl4*$^{-/-}$ mice (n=6 separate blots per group). Data are the mean band intensities measured from 6 separate blots per treatment condition.

We wanted to make sure that Angptl4 deficiency was not associated with differences in the expression of genes and proteins critical to the cAMP-dependent lipolytic cascade that would have confounded the interpretation of data obtained using pharmacologic inhibitors. The mRNA levels of genes encoding several adenylate cyclase isoforms (*Adcy3*, *Adcy6*, and *Adcy8*), PDE3B (*Pde3b*), the stimulatory G protein alpha-subunit (*Gnas*), and the G protein beta subunit (*Gnb1*) at baseline or following DEX treatment were all similar in WT and *Angptl4*$^{-/-}$ adipocytes, as was the abundance of β_1-, β_2-, and β_3AR proteins (Figures 9B-C). Therefore, the effect of Angptl4 deficiency on cAMP levels and glycerol release in adipocytes is not due to a reduction in the expression of elements critical to cAMP-dependent signaling.

Figure 10: The role of soluble adendylate cyclase in Angptl4-mediated lipolysis

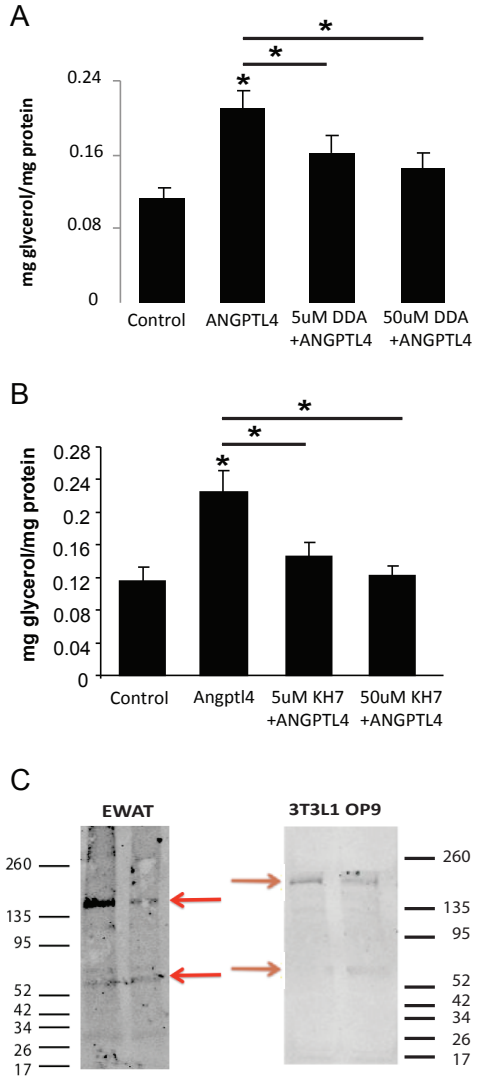

FIGURE 10: A. Glycerol concentration in the medium bathing isolated adipocytes pre-treated for 1-hour with the transmembrane adenylate cyclase inhibitor 2',5' dideoxyadenosine (DDA) and then treated with 20nM ANGPTL4 for 1-hour. The increase in glycerol release induced by ANGPTL4 treatment was significantly attenuated in cells treated with either 5uM or 50uM DDA (n=12; *p<0.05). **B.** As in A, isolated adipocytes were pre-treated for 1-hour with the soluble adenylate cyclase inhibitor KH7 and then treated with 20nM ANGPTL4 for 1-hour at which point the glycerol release into the medium was measured. The increase in glycerol release induced by ANGPTL4 treatment was significantly attenuated in cells treated with either 5uM or 50uM KH7 (n=12; *p<0.05). **C.** Immunoblots from epididymal WAT, 3T3L1 adipocytes and OP9 adipocytes confirming expression of sAC in adipocytes.

Soluble adenylate cyclase (sAC) participates the lipolytic response to Angptl4. The increase in lipolysis following Angptl4 treatment is preceded by an increase in cAMP and the canonical pathway of increasing cellular cAMP is through activating transmembrane adenylate cyclase, which converts ATP to cAMP. To evaluate how much of the lipolytic response to Angptl4 is mediated by transmembrane adenylate cyclase we pre-treated isolated adipocytes with 2'5' dideoxyadenosine (DDA), a transmembrane adenylate cyclase inhibitor, for an hour prior to treatment with Angptl4. Treatment with 5uM and 50uM DDA attenuated the Angptl4 induction of lipolysis by 43% and 50% respectively (Figure 10A). Because inhibiting transmembrane adenylate cyclase only partially abrogated the lipolytic response to Angptl4 we investigated whether soluble adenylate cyclase (sAC) might be playing a role as well. Pre-treatment of isolated adipocytes with the soluble adenylate cyclase inhibitor KH7 dramatically decreased the glycerol release in response to Angptl4. hANGPTL4 treatment increased lipolysis 1.9 fold but pre-treatment with 5uM KH7 reduced that induction to 1.2 fold and 50uM KH7 abolished it entirely (Figure 10B). These results indicate that sAC may be participating in Angptl4-induced lipolysis.

sAC is expressed in murine adipose tissue as well as mouse adipocyte cell lines. Research on sAC is fairly limited and little is known about its regulation, function or expression. Expression is believed to be highest in the testes, where sAC has been implicated

in sperm motility [157] but whether or not there is measurable expression in adipose tissue has not been previously reported. We used immunoblot to try and detect sAC in epididymal WAT of mice as well as in 3T3-L1 and OP9 adipocyte cell lines. In the adipose tissue, we were able to detect the 140kD band and a faint but visible 60kD band characteristic of full-length sAC and it's smaller cleavage product (Figure 10C). In the cell lines, we were still able to detect two bands but they ran slightly higher than what was predicted by the antibody. We speculate this is due to modification of the sAC protein in the cells and not a lack of specificity of the antibody.

Angptl4-induced lipolysis involves activation of FAK. We were next interested in identifying other components of the lipolytic pathway induced by Angptl4. Because Angptl4 has been shown to activate FAK in keratinocytes, we thought perhaps it played a role in Angptl4 action in adipocytes as well. By immunoblot we were able to determine that phosphorylation of FAK increases after 30 and 60 minutes of Angptl4 treatment (Figure 11A). Additionally pre-treatment of isolated adipocytes with 0.1uM of the FAK inhibitor PF-573228 decreased the induction of lipolysis by Angptl4 by 44%. Increasing the dose of PF-573228 to 1uM and 100uM only increased glycerol release marginally, 46% and 50% respectively (Figure 11B). Angptl4-induced cAMP content was comparably decreased by 1uM PF-573228, down 40% (Figure 11C). These data together indicate that FAK could participate in Angptl4 lipolytic signaling.

Figure 11:The role of focal adhesion kinase in Angptl4-mediated lipolysis

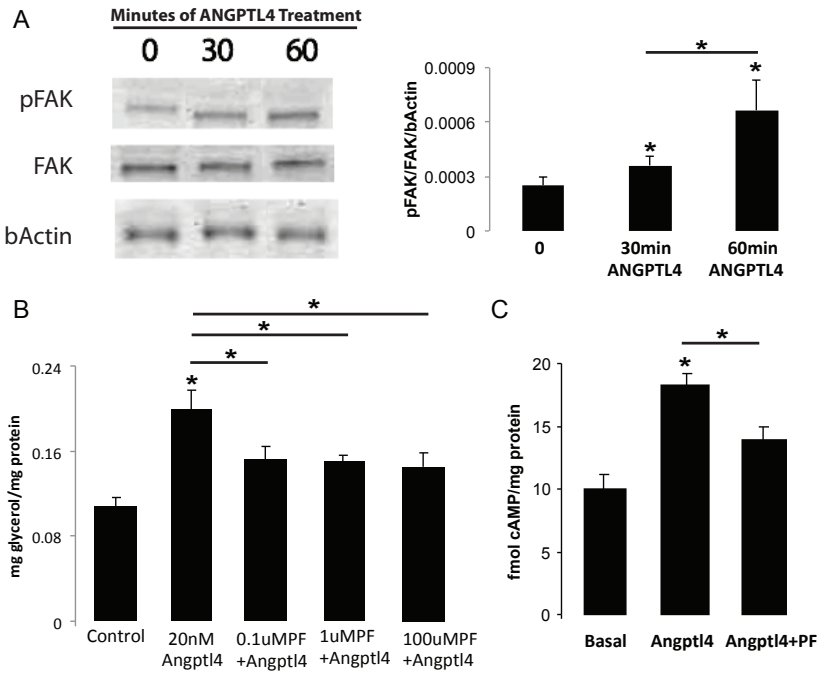

FIGURE 11: A. Immunoblot measuring total FAK and pFAK in isolated epididymal adipcoytes treated with 20nM ANGPTL4 showing increasing FAK phosphorylation after 30 and 60 minutes, with the highest levels of pFAK at 60 minutes. Data are the mean band intensities measured from 6 separate blots per treatment condition (n=6 blots per condition; *p<0.05). **B.** Glycerol concentration measured in the medium bathing primary murine adipocytes pre-treated for 1 hour with the FAK inhibitor PF-573228 (PF) and then treated with 20nM ANGPTL4 for 1 hour. ANGPTL4 treatment increased glycerol release but pre-treatment with PF diminished this increase (n=12; *p<0.05). **C.** Cytosolic cAMP levels were measured from isolated adipocytes treated as in B showing that (1uM) PF was also able to attenuate the induction in cellular cAMP caused by ANGPTL4 (n=6; *p<0.05).

DISCUSSION

Lipolysis is an essential component of the response to fasting, in which TG stores in the WAT are hydrolyzed in order to mobilize substrates for hepatic gluconeogenesis and b-oxidation in the liver and skeletal muscle. Our studies in mice reveal that, beyond inhibiting extracellular Lpl, the secreted protein Angptl4 also stimulates intracellular TG hydrolysis and FFA release by adipocytes during fasting in response to classical physiologic cues. Mice lacking Angptl4 failed to appropriately release glycerol, a marker of TG hydrolysis, in response to a physiological fast. We built on these findings by exploring the role of glucocorticoid action, which directly induces *Angptl4* transcription during fasting [23], in

WAT TG hydrolysis. We show that glucocorticoid action is a determinant of the lipolytic potential of WAT during fasting in mice and that these effects require Angptl4. In addition to stimulating TG hydrolysis, short-term fasting and glucocorticoid treatment *in vivo* and catecholamine treatment *in vitro* each also increased cAMP levels in adipocytes, and Angptl4 was necessary for the ability of each of these stimuli to do so. In exploring this role further, we found that purified human ANGPTL4, when added on its own to cultured murine adipocytes, can remarkably increase intracellular cAMP levels and rescue the lipolytic impairment produced by Angptl4 deficiency. Our studies combine to implicate Angptl4 as a common downstream mediator that integrates the acute lipolytic actions of glucocorticoids and catecholamines during fasting in adipocytes by increasing WAT cAMP levels and the phosphorylation of cAMP-dependent components of the lipolytic machinery.

Although administration of RU486, a GR antagonist, greatly reduced fasting-induced WAT TG hydrolysis and FFA release, the role of Angptl4 in this reduction may be tissue-specific. While RU486 treatment completely abolished the fasting-induced increase in *Angptl4* expression in the WAT, it only suppressed it by ~60% in the liver. These findings together suggest that Angptl4 expressed locally within the WAT may be more closely linked to TG hydrolysis than that secreted into the circulation by the liver, and that other, glucocorticoid-independent, signaling pathways likely contribute to the induction of hepatic *Angptl4* expression during fasting. Indeed, the array of signals that modulate *Angptl4* expression is likely to be diverse and tissue-specific. In the hypothalamus, for example, it was recently shown that *Angptl4* levels in mice are increased by the CNS administration of leptin, insulin, or the intake of dietary macronutrients [114]. On the other hand, both leptin and insulin may signal to repress *Angptl4* expression in peripheral tissues [77].

We next explored the temporal order in which catecholamines and glucocorticoids induce WAT TG hydrolysis during fasting. An increase in glycerol release from the WAT was initially detectable after 6 hours of fasting and was markedly ramped up by prolonging the fast to 24 hours. By contrast, 6 hours of fasting could maximally increase WAT cAMP levels, suggesting that activation of cAMP-dependent signaling likely precedes TG hydrolysis and FFA release by adipocytes. Interestingly, Angptl4 deficiency severely blunted both glycerol release from the WAT and the increase in WAT cAMP levels following fasts of either 6- or 24 hours, strongly suggesting that Angptl4 can modulate events early in the cascade leading to WAT TG hydrolysis. Indeed, we were able to validate this concept in cultured adipocytes treated with catecholamines, which stimulate cAMP-dependent lipolysis within 60 minutes. Here too, Angptl4 deficiency was associated with reduced cAMP levels and rates of glycerol release.

On the other hand, lipolysis stimulated solely by a single dose of DEX was much slower; whereas fasting could elevate WAT cAMP levels within 6 hours, a single dose of DEX alone required 24 hours to do so. Despite these temporal differences, both fasting and DEX treatment still required Angptl4 to elevate WAT cAMP levels and stimulate glycerol release. Taken together, these findings suggest that early on during fasting, the Angptl4 required for TG hydrolysis is either already present in the WAT or is induced by a mechanism independent of glucocorticoid action, which displays slower transcriptional kinetics. As fasting is prolonged, or in response to DEX treatment, the role of Angptl4 induced directly by GR activation becomes more prominent, playing an important role in maintaining WAT lipolysis.

Our *in vivo* and *in vitro* findings together allow for the construction of a model piecing together the temporal contribution of several components of fasting-induced lipolysis and the integrative role of Angptl4 in this process (Figure 12). During a physiological fast, catecholamines and other counter-regulatory defenses act on the WAT early on to increase cAMP levels leading to activation of PKA and phosphorylation Hsl and Plin1, two proteins that localize to lipid droplets and undergo specific PKA-dependent phosphorylation in order to participate in lipolysis. During this early phase of fasting (as modeled here by fasting mice for 6 hours) Angptl4 serves two roles; it inhibits Lpl to limit extracellular lipolysis and fat uptake by adipocytes, and also potentiates the actions of catecholamines by enhancing their effect on cAMP-dependent TG hydrolysis. When fasting is carried out longer (as modeled by fasting mice for 24 hours) the contribution of glucocorticoid action to WAT TG hydrolysis increases and is characterized by transcriptional effects on many genes, one of which is *Angptl4*. For both catecholamines and glucocorticoids, the ability to stimulate the release of stored TG by the WAT is linked to their capacity to increase adipocyte cAMP levels. For this, they require Angptl4.

Figure 12:Model for the role of Angptl4 in fasting- and glucocorticoid-induced lipolysis

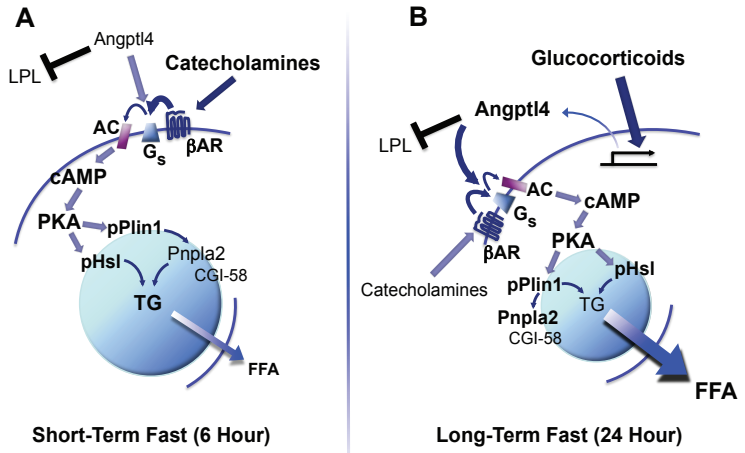

FIGURE 12: Model depicting the proposed role of Angptl4 in integrating hormonal inputs to modulate intracellular adipocyte lipolysis during short- and long-term fasting. **A.** Hormonal inputs, notably catecholamines, act early on (6-hour fast) to stimulate WAT lipolysis. Angptl4 is required for this, inhibiting extracellular Lpl activity and facilitating intracellular cAMP formation and the downstream activation of hydrolytic enzymes (pHsl) and lipid droplet proteins (pPlin1). **B.** As fasting is extended (24 hours), glucocorticoid action becomes relatively more important, regulating the transcription of several genes, including *Angptl4*. Angptl4 potentiates the lipolytic effects of catecholamines and glucocorticoids, acting upstream of adenylate cyclase.

Our next goal was to more deeply explore the role of cAMP-dependent signaling in the mechanism by which Angptl4 stimulates TG hydrolysis during fasting. We first measured the phosphorylation of Hsl at serine 660, and of Plin at serine 492, two sites of action for cAMP-dependent PKA. The relative induction of the phosphorylated forms of these two enzymes by fasting (pHsl) or DEX treatment (pHsl, pPlin1) was greatly reduced in the WAT of *Angptl4*$^{-/-}$ mice, indicating that Angptl4 modulates the PKA-dependent functional status of TG hydrolytic enzymes.

We went on to treat primary adipocytes isolated from WT or *Angptl4*$^{-/-}$ mice with norepinephrine or isoproterenol, catecholamines which rapidly raise adipocyte cAMP levels and stimulate TG hydrolysis during fasting and other stressful states [150, 156]. These studies yielded several important findings. First, the capacity of catecholamine treatment to increase glycerol release from *Angptl4*$^{-/-}$ adipocytes was greatly reduced. Remarkably, this reduction could largely be rescued by co-treatment of *Angptl4*$^{-/-}$ adipocytes with hANGPTL4. Moreover, hANGPTL4 was able to increase cAMP levels within 30 minutes when added to adipocytes, regardless of whether these were from WT or *Angptl4*$^{-/-}$ mice. These findings, when combined with those from our *in vivo* studies using WAT explants, support the concept that Angptl4 deficiency impairs intracellular WAT lipolysis by limiting its ability to increase cAMP and initiate PKA-dependent phosphorylation events in response to multiple

physiologic stimuli. Providing cultured adipocytes with hANGPTL4 can bypass this limitation, raising intracellular cAMP to similar levels in WT and *Angptl4*$^{-/-}$ adipocytes. Furthermore, stimulation with forskolin, PDE inhibitors, and cAMP analogs could also bypass the limitation on lipolysis afforded by Angptl4 deficiency, suggesting that this limitation is due to an impairment at or upstream of adenylate cyclase and downstream of receptor activation. Our limited inhibitor data also implies that sAC and FAK may be important for Angptl4 signaling in adipocytes. Angptl4 has been shown to modulate wound-healing in keratinocytes through activation of FAK [96] but this is the first instance where the involvement of sAC in mediating its actions has been suggested. Further studies are underway to investigate the exact nature of how sAC participates in Angptl4-induced lipolysis and what role if any it may have in cAMP production induced by other stimuli like, catecholamines or glucocorticoids.

Several possible mechanisms emerge when considering how Angptl4 could modulate this point in the cAMP-dependent signaling cascade. One is that it may influence the rate at which GαS cycles between the GTP and GDP-bound states. Enhanced cycling could affect the availability Ga to activate adenylate cyclase, invoking potential roles for GTPase-activating proteins (GAPs) and guanine exchange factors (GEFs) other than Gα itself. Angptl4 could also modulate the turnover rate of cAMP upon activation of adenylate cyclase, invoking the action of PDEs. We compared the WAT mRNA levels of genes encoding adenylate cyclase isoforms (*Adcy3, Adcy6, Adcy8*), PDEs (*Pde3b*), and G protein subunits (*Gnas* and *Gnb1*) and did not find differences between WT and *Angptl4*$^{-/-}$ mice at baseline or following DEX treatment. We further did not find differences in the abundance of bAR1, 2, or 3 proteins in the WAT between either genotype. However, these measurements do not exclude the possibility that WT and *Angptl4*$^{-/-}$ adipocytes have functional or regulatory differences in G proteins, adenylate cyclase, PDEs, or in receptor–G protein interaction. Determining the role of such functional differences in Angptl4 action is the subject of future studies and is of particular interest, given that catecholamines raise cAMP levels in adipocytes by activating adenylate cyclase [158, 159] while glucocorticoids may do so by inhibiting PDE3b [160]. As Angptl4 is required for the normal lipolytic response to both of these stimuli, it may act on adipocytes by altering cAMP formation, hydrolysis, or both of these.

Our findings do not preclude the possibility that pathways other than cAMP-dependent signaling may also mediate, at least in part, the effect of glucocorticoids on fasting-induced lipolysis. Beyond regulating the expression of *Angptl4*, GR activation likely modulates a network of genes to affect lipolysis. For example, glucocorticoids increase mRNA levels of *Hsl*, an effect which may be directly dependent on transcriptional regulation by GR [21]. We used chromatin immunoprecipitation along with massively parallel sequencing to identify an intronic GR binding region in murine *Hsl* that, when inserted into a reporter plasmid, could stimulate reporter activity in response to glucocorticoid treatment [21]. Glucocorticoid action also increases the expression of *Pnpla2* [148], though the mechanism(s) governing this process are unclear.

Neither do we suggest that all catecholamine-stimulated lipolysis is Angptl4-dependent. Indeed, as opposed to its effects on fasting or glucocorticoid treatment *in vivo*, Angptl4 deficiency was less able to limit increases in cAMP or glycerol release in adipocytes stimulated by treatment with norepinephrine *in vitro*, pointing to an Angptl4-independent component of catecholamine-stimulated lipolysis. Therefore, the need for Angptl4 to couple external inputs to cAMP-dependent signaling in order to stimulate TG hydrolysis in the WAT

during fasting may be bypassed, at least when lipolysis is stimulated solely by pharmacologic doses of catecholamines.

Of note, Angptl4 is secreted in both a full-length and truncated isoforms, and the full-length isoform of hANGPTL4 was used in most of our cell-based experiments. In measuring levels of Angptl4 in the plasma and the WAT of mice by ELISA, however, we are unable to distinguish between the two. Furthermore, while N-terminus of Angptl4 alone can inhibit Lpl, it was previously unknown whether truncated isoforms can modulate TG hydrolysis. Our data indicate that the LPL inhibitory effect is not necessary for Angptl4 to exert its lipolytic effect and furthermore, it seems that the C-terminal fibrinogen-like domain is sufficient for inducing cAMP and adipocyte lipolysis.

Also, levels of lipases and lipid droplet-associated proteins involved in TG hydrolysis were measured by immunoblot from lysates of whole WAT taken from mice subjected to fasting or DEX treatment, and often displayed a high degree of inter-sample variability within genotypes. For Pnpla2, this was associated with a small increase in protein levels in *Angptl4*$^{-/-}$ WAT under control (basal) conditions. The physiological importance of this increase with respect to the lack of further increase seen in response to fasting is unclear. Additionally, certain proteins interact with each other at the surface of lipid droplets during the course of TG hydrolysis; for example, Pnpla2 interacts with CGI-58. Although we measured protein levels of both Pnpla2 and CGI-58 from (total CGI-58 measured in this way was not altered by Angptl4 deficiency), further experiments are necessary to determine how Angptl4 affects the interaction between these and other components of the lipolytic machinery at the surface of lipid droplets.

In summary, we have uncovered a physiologically important role for glucocorticoids as inducers of *Angptl4* expression during a fast, both in the WAT and in the liver. Angptl4, in turn, is a key determinant of the lipolytic response of adipocytes, acting directly to increase the levels of cAMP and the phosphorylation of downstream TG hydrolytic enzymes. These findings enhance the framework for understanding intermediary metabolism and identify hormonal and WAT-specific regulators of lipolysis with relevance to conditions of insulin resistance, where lipolysis is aberrant. Given that polymorphisms in human ANGPTL4 result in dyslipidemia, the impact of these on WAT lipolysis is also worthy of investigation.

Chapter 2: The Role of Angiopoietin-like 4 in Glucocorticoid-Regulated *De Novo Lipogenesis* and Triglyceride Synthesis

SUMMARY

Angiopoietin-like 4 (Angptl4) is a glucocorticoid-stimulated gene in liver and white adipose tissue (WAT). Mice lacking Angptl4 (*Angptl4*$^{-/-}$) are protected from glucocorticoid-induced fatty liver and hyperlipidemia. However, triglyceride (TG) content in WAT is higher in *Angptl4*$^{-/-}$ mice than in wild type (WT) mice. We investigated the role of Angptl4 on the effects of glucocorticoid-regulated *de novo lipogenesis* (DNL) and TG synthesis. Using a stable isotope labeling technique we found that dexamethasone (DEX, a synthetic glucocorticoid) treatment for 4 days increased the rate of DNL and the expression of fatty acid synthase (Fas) and acetyl-CoA carboxylase 1 and 2 (Acc1, Acc2) in liver and WAT of WT mice. These inductions were diminished in *Angptl4*$^{-/-}$ mice. The rate of basal TG synthesis was lower in liver but not WAT of *Angptl4*$^{-/-}$ mice. DEX treatment increased the rate of TG synthesis in both tissues of all mice, but the absolute TG synthesis rate was lower in *Angptl4*$^{-/-}$ animals. Thus, Angptl4 plays a critical role in maintaining the normal rate of hepatic TG synthesis, and glucocorticoid-induced DNL and TG synthesis in liver and WAT. Notably, basal lipolytic turnover rates were similar in WT and *Angptl4*$^{-/-}$ WAT, however, lipolytic turnover rates after 4 days of DEX treatment were lower in *Angptl4*$^{-/-}$ WAT. Thus, the higher WAT TG accumulation in *Angptl4*$^{-/-}$ mice is mainly due to impaired lipolysis. In summary, Angptl4 plays a key role in glucocorticoid-regulated lipid metabolism in liver and WAT. Such functions are relevant in lipid disorders induced by excess glucocorticoids.

INTRODUCTION

Glucocorticoids play both catabolic and anabolic roles in the regulation of lipid metabolism. Glucocorticoids induce adipose tissue lipolysis [136, 137, 161] which generates free fatty acids (FFA) and glycerol. The former is the substrate for mitochondrial b-oxidation in various tissues, whereas the latter is a substrate for hepatic gluconeogenesis. These metabolic processes are critical for the survival of mammals during fasting and starvation. Glucocorticoids can also increase lipogenesis [32, 135] during other physiological conditions. Animals starved for a period of 36-56 hr and then fed with high glucose diet showed an increase of hepatic lipids and *de novo lipogenesis*. These effects are reduced upon adrenalectomy and restored after glucocorticoids are administered [32, 135] These results suggest that glucocorticoids play a role in the efficiency of nutrient storage as fat through potentiating lipogenesis. In addition, in liver-specific glucocorticoid receptor (GR) knockout mice, fat accumulation in liver is markedly reduced [162]. Using a stable isotope labeling technique, we previously showed that glucocorticoid treatment for 4 days increased the rate of triglyceride (TG) synthesis in the inguinal fat depot in mice [21]. Moreover, adipose TG synthesis and lipolysis exhibit coupling, partly reflecting re-esterifiation of FFA in the fat cell. The metabolic disorders caused by chronic and/or excess glucocorticoids, such as central obesity, dyslipidemia and fatty liver [140], might therefore result from either the catabolic or the anabolic effects of glucocorticoids on lipid metabolism. Despite their critical role in lipid homeostasis, the mechanisms by which glucocorticoids regulate lipid metabolism are poorly understood.

Angiopoietin-like 4 (Angptl4, a.k.a. Fasting-induced adipose factor, FIAF) is a secreted protein that inhibits lipoprotein lipase (LPL) activity and induces WAT lipolysis

[133, 134]. The role of Angptl4 in lipid homeostasis has been supported by several physiological and genetic studies. Injecting ANGPTL4 protein into mice results in an increase of circulating TG and free fatty acids (FFA) levels [111]. Adenoviral overexpression of Angptl4 in liver results in hyperlipidemia and fatty liver [121]. Moreover, transgenic mice overexpressing Angptl4 in WAT and skeletal muscle have a limited capacity to store TG and an increased plasma TG, FFA and glycerol [83]. In contrast, mice lacking Angptl4 (*Angptl4*$^{-/-}$) have decreased plasma TG levels and an increased capacity for weight gain [115, 163]. Human genomic studies also uncovered sequence variations in Angptl4 gene that are associated with loss of function and reduced plasma TG levels [118].

We have previously shown that the expression of Angptl4 is induced by glucocorticoids in both liver and WAT [164]. The role of Angptl4 in glucocorticoid-regulated lipid homeostasis is supported by the fact that mice lacking Angptl4 (*Angptl4*$^{-/-}$) are protected from excess glucocorticoid-induced fatty liver and hyperlipidemia [23]. How Angptl4 deficiency can protect animals from glucocorticoid-induced lipid disorders is unclear. Angptl4 could be involved in either glucocorticoid-stimulated adipocyte lipolysis or lipogenesis and TG synthesis. We recently found that Angptl4 plays a critical role in glucocorticoid-induced adipocyte lipolysis [165]. In this report, we used a stable isotope labeling technique to monitor and compare the rate of *de novo lipogenesis* (DNL) and TG synthesis in liver and WAT of WT and *Angptl4*$^{-/-}$ mice. Gene expression analysis was conducted to elucidate the role of Angptl4 in glucocorticoid-regulated DNL and TG synthesis. Finally, we treated mouse primary hepatocytes with purified ANGPTL4 protein to investigate its direct effect on the regulation of genes involved in DNL and TG synthesis.

EXPERIMENTAL PROCEDURES

Animals. *Angptl4*$^{-/-}$ mice were provided by the laboratories of Andras Nagy (Samuel Lunenfeld Research Institute, Mount Sinai Hospital) and Jeff Gordon (Washington University)[115]. *Angptl4*$^{-/-}$ mice were generated on a mixed B6:129Sv background. WT mice were the littermates of *Angptl4*$^{-/-}$ mice. The PCR protocols and strategy for mouse genotyping strategy were as described [115].

Four-day Glucocorticoid and Heavy Water Treatment. For liver analysis, animals (2-4months old) were treated with an intraperitoneal injection of either dexamethasone (DEX, 5mg/kg body weight) or PBS once a day for 4 days. On the fourth day a priming dose of 99.9% deuterated heavy water (D2O, Cambridge Isotope Laboratories; 0.018 mL/g body weight) was also administered by intraperitoneal injection, to bring the D2O content of body water up to ~5%. Animals then received drinking water containing 8% D2O ad libitum for the last day of the study. Twenty-four hours after the final injection of DEX and the D2O treatment, animals were sacrificed and blood and tissue samples were collected.

For adipose analysis, animals were injected with a priming dose of 99.9% D2O to bring the D2O content of body water up to ~5%. Animals then received drinking water containing 8% D2O ad libitum for 7 days. On each of the final four days animals were treated with an intraperitoneal injection of either 5mg/kg DEX or PBS. Twenty-four hours after the final injection of DEX, animals were sacrificed and blood and tissue samples were collected.

Tissue Triglyceride Quantification. Liver and adipose samples were weighed and homogenized in a buffer consisting of 50 mM Tris-HCl (pH 7.4), 250 mM sucrose, and protease inhibitors. Lipids were extracted in chloroform:methanol (2:1) and separated by TLC on silica gel G-60 plates with the solvent hexane:ethyl ether:acetic acid (v:v:v 80:20:1). The

TG bands were visualized by exposure to iodine, and then scraped and analyzed as described [144], with triolein (Sigma) as a standard, and expressed per tissue weight.

Plasma TG Measurement. Plasma TG levels were measured following a manual from a colorimetric kit (Roche Diagnostics).

Isolation of TG–glycerol and FA from Tissue. Liver and adipose samples were weighed and approximately 100mg was placed in glass tubes containing 1 ml of methanol–chloroform (2:1). Chloroform and water were then used to extract the solution. The aqueous fraction was disposed, and the remaining lipid phase was transesterified by incubation with 3 N methanolic HCl (Sigma-Aldrich, St. Louis, MO) for 60 min at 55°C. The Folch technique was employed to separate glycerol from fatty acid (FA) methyl esters. The phase containing FA was then evaporated under nitrogen gas and resuspended in heptane to be submitted to the gas chromatography-mass spectrometry (GC-MS) analysis. The aqueous phase containing glycerol was lyophilized by incubation with acetic anhydride–pyridine (2:1), thereby converting glycerol to glycerol triacetate. The samples were then evaporated under nitrogen gas and resuspended in ethyl acetate for GC/MS analysis.

Measurement of Body Water D2O Enrichment. Air was evacuated from a Teflon-capped vial containing carbon tetrachloride and concentrated bromine solution (40:1). Water, distilled from 100uL of the plasma samples, was added to capped vials containing enough calcium carbide to cover the bottom of the vial (vials were prepared and capped under argon atmosphere) producing acetylene gas. The acetylene gas was collected in a syringe and then injected into the CCl_4/Br_2 containing vial. Acetylene was allowed to react with the CCl_4/Br_2 solution for 2 hours at which point the vials were uncapped and 10uL of a solution containing 10% cyclohexane in CCL_4 was added. Then the vials were recapped and submitted to GC/MS analysis.

GC/MS Analyses of TG–glycerol, FA and Body water. For all analysis, a model 6890 GC with 5973 mass spectrometer (Agilent Technologies, Palo Alto, CA) fitted with a DB-225 fused silica column (J&W, Folsom, CA) was used. Glycerol triacetate was analyzed under chemical ionization conditions by selected ion monitoring of mass-to-charge ratios (m/z) 159–161 (representing M0–M2). FA methyl esters were analyzed as described elsewhere [166]), with selected ion monitoring of m/z 256–258 (representing M0–M2) of palmitate methyl ester. Body D2O enrichments were analyzed as tetrabromoethane by monitoring m/z 265 and 266 (representing M0 and M1) of the 79Br79Br81Br (parent minus Br-) isotopomer [166].

Calculation of TG–glycerol Synthesis. The incorporation of deuterium from D2O into the C–H bonds of the glycerol moiety of TG–glycerol is used to determine the all-source TG synthesis [167]. During glycolytic and glyceroneogenic reactions deuterium in cellular H2O exchanges with specific C–H bonds leading to α-glycerol phosphate, the biosynthetic precursor of TG [167]. Accordingly, TG molecules synthesized from α-glycerol phosphate during the period D2O administration will contain the 2H labeling whereas TG molecules that already existed will remain unlabeled in the glycerol moiety. The fraction of newly synthesized TG–glycerol (f) was measured as described [167]:

$$f_{TG} = EM1_{TG-glycerol} = A_1^{\infty}{}_{TG-glycerol}$$

where f is the fraction of newly synthesized TG molecules present, EM1 is the measured excess mass isotopomer abundance for M1 glycerol at time t and $A_1^{\infty}$is the asymptotic mass isotopomer abundance for M1 glycerol possible at the measured body water enrichment. The

calculation of A_1^{∞}utilizes the number (n)=4 (number of C–H bonds in glycerol that are labile and exchange with 2H2O in body water in intermediary metabolic pathways leading to α-glycerol phosphate), as shown previously to be the case under these conditions [168]. Absolute synthesis rates of liver TG were then calculated from fractional TG synthesis multiplied by liver TG mass [168]. The rate of TG turnover (net lipolysis) was also calculated, based on the absolute TG synthesis rate adjusted for the in tissue TG mass:

Absolute TG synthesis (g/day) = f_{TG} x liver TG mass (g)

Net lipolysis (g/day) = rate of newly synthesized TG (g/day) - change in adipose TG mass (g/day)

Calculation of De Novo Lipogenesis (DNL). A combinatorial model of polymerization biosynthesis, described previously [168] was used to determine the newly synthesized FA formed during the D2O labeling period (DNL). Briefly, mass isotopomer distribution analysis (MIDA) was used to determine the number (n) of hydrogen atoms in C–H bonds of FA that was derived from cellular H2O during de novo synthesis of FA, using body D2O to represent the precursor pool enrichment (p), as described previously [168]. Fractional and absolute contributions from DNL are then calculated:

$f_{DNL} = EM1_{FA} = A_1^{\infty}{}_{FA}$

Absolute DNL (g/day) = f_{DNL} × adipose TG mass(g) × fraction TG palmitate

where $A_1^{\infty}{}_{FA}$ is calculated from MIDA lookup tables (based on the calculated values of n and p in FA). The value for f_{DNL} represents the fraction of total TG palmitate in the depot derived from DNL during the labeling period, and absolute DNL represents grams of palmitate synthesized by the DNL pathway. The fraction TG palmitate was determined by flame ionization detection analysis.

The fraction of newly-synthesized TG that came from DNL was also calculated by correcting the fractional DNL by degree replacement in adipose TG over the labeling period.

DNL contribution to newly synthesized TG = f_{DNL}/f_{TG}

Primary Hepatocytes. Primary hepatocytes from B6 mice were isolated by the UCSF Liver center. Cells were cultured on 12-well collagen-coated plates and treated with ethanol or 10nM ANGPTL4. After either 5 or 22 h cells were harvested and RNA was extracted.

Purification of Human ANGPTL4. HEK293 cells cultured in media with 5% FBS were infected with adenovirus expressing a FLAG-tagged version of human ANGPTL4 (provided by Sara Vienberg and Ronald Kahn, Joslin Diabetes Center) for 1 hour, at which point the media was replaced. After 72 more hours, the media was collected and Angptl4 protein was purified using an anti-FLAG M2 Affinity gel (Sigma). The purified protein was then dialyzed and concentrated 10x using Slide-A-Lyzer dialyzing cassettes and concentrating solution (Thermo Scientific). Western Blot and Coomassie staining confirmed purity of protein. Affinity gel elution buffer (TBS) was also dialyzed and concentrated to serve as a control.

Quantification of Cellular Triglyceride Synthesis. Cellular triglyceride synthesis was

determined by incorporation of radioactive oleic acid as described before [169]. Briefly, primary hepatocytes were plated on 12-well plates at a density of 125,000 cells/well and cultured overnight in media containing 10nM ANGPTL4. After 18 hours cells were treated with 0.125 μCi/ml of [14C]oleic acid and 250 μM cold oleic acid for 1 hour. Cells were then washed twice with PBS, and lipids were extracted with hexane:isopropanol (3:2), dried down, separated by TLC and the triglycerides were quantified using a Bioscan AR-2000 instrument. The TLC plate was also exposed to film and developed providing a visual indication of the amount of triglyceride synthesized.

Quantitative Real-Time PCR (qPCR). Total RNA was either isolated from the livers and epididydmal WAT of mice using Tri-reagent (Molecular Research Center Inc.) or from cells using Nucleospin RNAII isolation kit. Reverse transcription was performed as described [23]. The resultant cDNA was diluted to 170μL and 3.5μL was used to perform qPCR in a 25μL reaction using EVA qPCR SuperMix Kit (Biochain) per the manufacturer's protocol. qPCR was performed on a StepOne PCR-system (Applied Biosystems) and analyzed using the delta-delta CT method as supplied by the manufacturer. Primer Sequences are as follows: Rpl19 F - AGCCTGTGACTGTCCATTCC, Rpl19 R – GGCAGTACCCTTCCTCTTCC; Acc1 F- CCTTTGGCCTTCTCTCTGAA, Acc1 R- ACCCCATTCATCCAAAATCA; Acc2 F – TGTTCTCGGCCTCTCTTCAC, Acc2 R – GAGGGCTGCATTGAACACAAG; Fas F – GGATCAACCTGCTCCTGAAG, Fas R – ATCAAAGGATCTGCAGGTGC; Scd1 F – GAGTACGTCTGGAGGAACAT, Scd1 R – GCCCAGAGCGCTGGTCATGT; Scd2 F – GAATGGAGGCGAGAAGTTTG, Scd2 R – GATGTTCCTCCAGACGTACT; Lpin1 F – GCTCCCGAGAGAAAGTGGTGGA, Lpin1 R – GGCTTTCCATTCTCGCAGCTCCT; Lpin2 F – GAGACTCGACCTCCACTTCG, Lpin2 R – AACACTGGACCACCTGAG; Lpin3 F – GGCTAGTAGGCCCAGGTCTT, Lpin3 R – AGCCCTTGGAGAAGGAATGT; Dgat1 F – AACACCCGGCTAAGAAGGTT, Dgat1 R – TGGCCAACGGGTTACTACTC; Dgat2 F – CTGTTGAGCCAGGTGACAGA, Dgat2 R – CGCAGCGAAAACAAGAATAA; Gpat1 F – TCATCGAGCCTCCGTCTTAT, Gpat1 R – CACCTTCATCCTCTTTTGCC; Gpat2 F – AAAGGATGGAGTTCACGTTGG, Gpat2 R – TCTCTAGCGATGTCGTGGTG; Gpat3 F – CCAGACAGCAGCCTCAAAAAC, Gpat3 R – GATAGGGACCGAACACAGATCCT; Gpat4 F – GGTATCCGAAAGCTCTACAT, Gpat4 R – CCGTTGGTGTAGGGCTTGT; Agpat1 F – TGACCCTGGAGAGAAGGAGA, Agpat1 R – CCACTGGGAGGAAATGAAAA; Agpat2 F – ATGCTGATGTCACCAAGCTG, Agpat2 R – CAGGCTCCTTAATGGCAGAG; Agpat3 F – AGCTGGGAGCTTGCTTTTCT, Agpat3 R – TGTTCACACTTGGCACATGA; Agpat4 F- CTTGAGAATCCCCACACCAT, Agpat4 R – GCGATGAACACGTAGCAGAA; Agpat5 F – TCTTGGGGAGTTTGACTGCT, Agpat5 R – GGGTTCCATACAACCAGGTG.

Western Blot. The protocol for western blot was as described [23]. Proteins were detected by fluorescence imaging (LiCor Odyssey imager) using the following antibodies: β-actin (C4) mouse monoclonal IgG1 (sc-47778; Santa Cruz Bioechnology), GR (provided by Keith Yamamoto, UCSF), Goat anti-Rabbit IRDye 800CW (LiCor), Goat anti-Mouse IRdye 800CW (LiCor). The optical density of the bands was quantified using Image J software (National Institute of Health) and normalized to β-actin.

Statistics. Data are expressed as standard error of the mean (S.E.M) for each group and comparisons were analyzed by Student's *t* test.

RESULTS

***Angptl4*$^{-/-}$ mice are protected from dexamethasone-induced hepatic steatosis and**

hyperlipidemia. WT and *Angptl4*$^{-/-}$ mice were treated with PBS or a synthetic glucocorticoid, dexamethasone (DEX), for 4 days. In *Angptl4*$^{-/-}$ mice, both plasma and liver TG levels were lower basally than those of WT mice (Figure 13A and 13B). DEX treatment increased plasma TG for approximately 33% in WT mice (Figure 13A). This effect was blunted in *Angptl4*$^{-/-}$ mice (Figure 13A). The liver TG levels were significantly elevated by DEX treatment in WT mice (Figure 13B). In *Angptl4*$^{-/-}$ mice the ability of DEX treatment to

Figure 13: The effects of glucocorticoid treatment on circulating lipids, glucocorticoid receptor and endogenous glucocorticoid levels

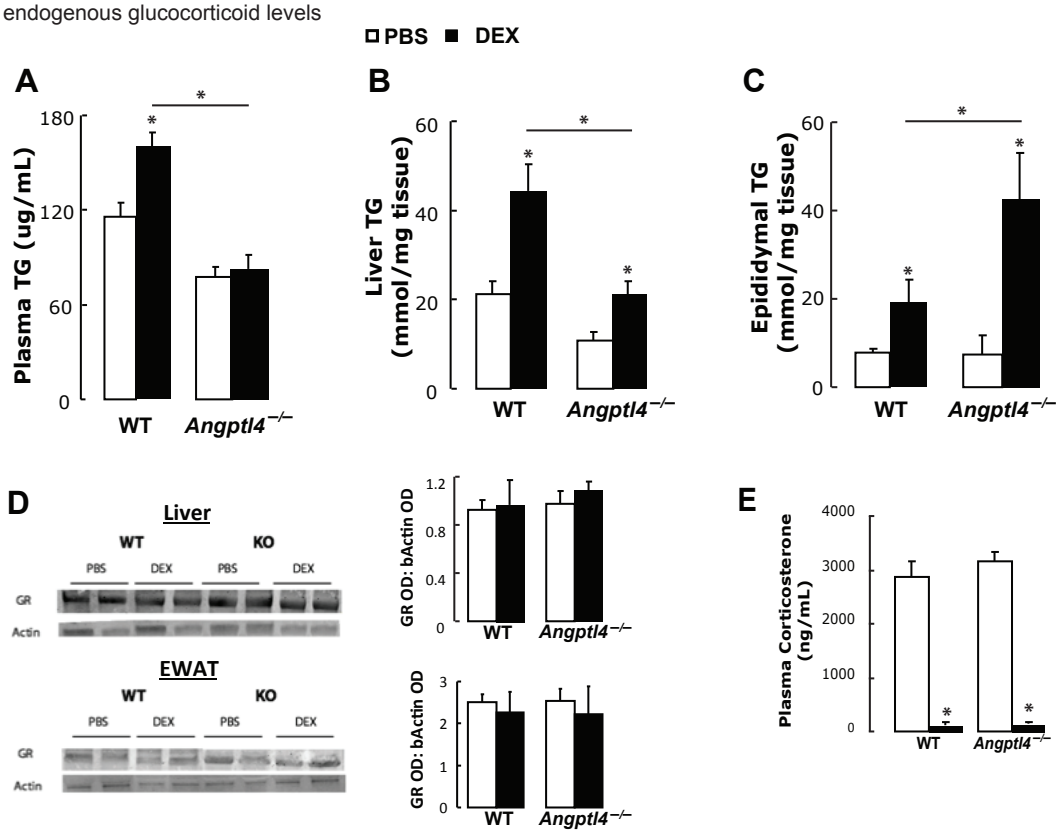

Figure 13: The effect of DEX on plasma and liver TG levels in WT and *Angptl4*$^{-/-}$ mice. A. Plasma TG levels in WT and *Angptl4*$^{-/-}$ mice treated with DEX (5mg/kg/day) or PBS for 4 days (n=7-8; *p<0.05 vs DEX-treated wild type). B. Liver TG levels in WT and *Angptl4*$^{-/-}$ mice treated with DEX (5mg/kg/day) or PBS for 4 days (n=10-11; *p<0.05 vs DEX -treated wild type). C. TG levels in epididymal adipose of WT and *Angptl4*$^{-/-}$ mice treated with DEX (5mg/kg/day) or PBS for 4 days (n=6; *p<0.05 vs DEX -treated wild type). D. GR protein expression in liver or WAT of *Angptl4*$^{-/-}$ and WT mice treated with DEX (5mg/kg/day) or PBS (graph is combination of multiple blots (n=6). E. Plasma corticoster one levels in WT and *Angptl4*$^{-/-}$ mice treated with DEX (5mg/kg/day) or PBS for 4 days (n=6).

increase liver TG levels was reduced (Figure 13B). The absolute increase of TG concentration in WT mice was approximately 2 fold higher than *Angptl4*$^{-/-}$ mice (44 nmol/mg tissue vs. 21 nmol/mg). In fact, liver TG levels in DEX-treated *Angptl4*$^{-/-}$ mice were similar to those of PBS-treated WT mice (Figure 13B). Interestingly, in contrast to liver, the levels of TG in WAT were approximately 2 times higher in DEX-treated *Angptl4*$^{-/-}$ mice than those of DEX-treated WT mice (Figure 13C). Overall, *Angptl4*$^{-/-}$ mice had lower levels of liver and plasma TG, and were protected from DEX-induced hyperlipidemia and hepatic steatosis. However, in WAT DEX treatment increased tissue TG in *Angptl4*$^{-/-}$ to a much greater extent than in WT mice.

Immunoblots showed that the expression of glucocorticoid receptor (GR) was comparable in liver and WAT of PBS- and DEX-treated WT and *Angptl4*$^{-/-}$ mice (Figure 13D). Also, plasma corticosterone levels were similar at baseline and were suppressed to a comparable degree by DEX treatment (95% and 94%, respectively) in WT and *Angptl4*$^{-/-}$ mice (Figure 13E). These indicate that the phenotypic differences observed in WT and *Angptl4*$^{-/-}$ mice were not caused by the difference in the levels of GR expression or circulating endogenous glucocorticoids.

Figure 14: The rate of glucocorticoid-induced de novo lipogenesis in liver and adipose tissue

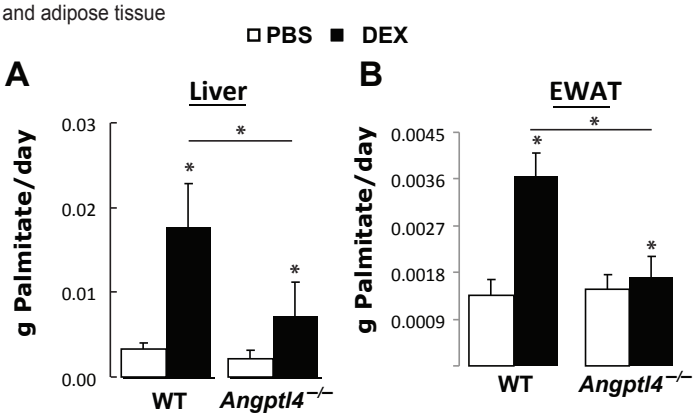

Figure 14: The effect of DEX on the absolute rate of DNL in WT and *Angptl4* $^{-/-}$ mice. A. WT and *Angptl4*$^{-/-}$ mice were treated with DEX (5mg/kg/day) or PBS for 4 days and the rate of *de novo lipogenesis* (DNL) was measured (n=5, *p<0.05 vs DEX-treated wild type). B The rate of DNL in epididymal white adipose tissue of the same mice was also measured (n=4 -6; *p<0.05 vs DEX -treated wild type).

DEX-induced DNL is impaired in *Angptl4*$^{-/-}$ mice. We used a stable isotope labeling technique to monitor the rate of DNL and TG synthesis in liver and WAT of *Angptl4*$^{-/-}$ mice. For the liver analysis, mice were treated with PBS or DEX for 4 days and labeled with heavy water for final 24 hr. At the end of treatment, fatty acids were extracted from liver and GC/MS was performed to measure the newly synthesized fatty acids. We calculated the absolute rate of DNL (g palmitate synthesized over 1 day labeling period). In PBS-treated WT mice liver the absolute rate of DNL was 0.0033 g palmitate/day (Figure 14A). DEX treatment resulted in 5.2 fold induction of absolute DNL rate (0.017 g palmitate synthesized per day, Figure 14A). In contrast, the absolute rate of DNL in PBS-treated *Angptl4*$^{-/-}$ mice liver was 0.0021 g palmitate/day (Figure 14A). DEX treatment increased the rate of DNL to 0.007 g palmitate/day (Figure 14A) in *Angptl4*$^{-/-}$ mice, however this induction was significant lower than that observed in the livers of WT mice. Overall, these results suggest that a complete response of DEX-induced hepatic DNL was depended on the presence of Angptl4.

To study the rate of DNL and TG synthesis in WAT, mice were labeled with heavy water for 7 days. They were also treated with DEX for the final 4 days. The absolute rate of DNL was 0.0014 g palmitate/day in WAT of PBS-treated WT mice (Figure 14B). DEX treatment resulted in a 2.7 fold induction of absolute DNL rate (0.0036 g palmitate synthesized per day, Figure 14B). In contrast, the absolute rate of DNL in PBS-treated *Angptl4*$^{-/-}$ mice WAT was 0.0015 g palmitate, which was not significantly increased by DEX treatment (Figure 14B). Thus, DEX-increased DNL in WAT requires

Table 3: DNL Contribution to New TG (Fractional)

	Liver		Epididymal Fat	
	PBS	DEX	PBS	DEX
WT	0.62 +/- 0.07	1.12 +/- 0.15*#	0.33 +/- 0.05	0.53 +/- 0.07*
Angptl4 -/-	0.51 +/- 0.05	0.62 +/- 0.06	0.36 +/- 0.04	0.39 +/- 0.06

**p<0.05 vs WT PBS-treated for that tissue #p<0.05 vs KO DEX-Treated*

Table 3: The contribution of newly synthesized palmitate to newly synthesized TG was calculated in liver and epididymal WAT of WT and *Angptl4*$^{-/-}$ mice.(n=6)

Angptl4.

We also calculated the newly deposited palmitate from DNL into TG and found that DEX-treatment significantly increased this fractional contribution in the liver and adipose tissue of WT but not *Angptl4*$^{-/-}$ mice (Table 3).

DEX-induced Fas and Acc1 gene expression is reduced in *Angptl4*$^{-/-}$ mice liver and WAT. Fatty acid synthase (Fas) along with Acetyl-CoA Carboxylase 1, and 2 (Acc1 and 2) are the rate-controlling enzymes in the fatty acid biosynthetic pathway. Previous studies have shown that glucocorticoids can stimulate the expression of Fas [170], Acc1 and Acc2 [171],which accounts for at least part of glucocorticoid-activated DNL. We monitored the expression of these genes in both WT and *Angptl4*$^{-/-}$ mice. We found that DEX treatment for 4 days markedly increased the expression of Fas, Acc1 and Acc2 in both liver and WAT of WT mice (Figure 15A and 15B). However, this induction was substantially reduced in *Angptl4*$^{-/-}$ mice (Figure 15A and 15B). These gene expression results were in agreement with our observation of decreased DNL in *Angptl4*$^{-/-}$ mice.

Figure 15: The expression of genes involved in de novo lipogenesis in liver and adipose tissue

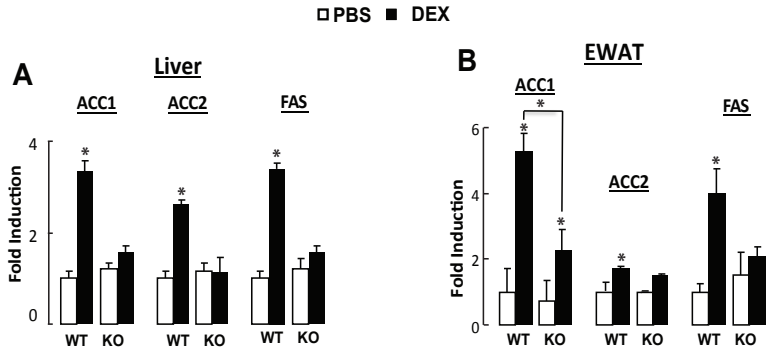

Figure 15: The effect of DEX on Acc1, Acc2 and Fas gene expression. A. Gene expression of Acc1, Acc2 and Fas was measured in the liver of WT and *Angptl4*$^{-/-}$ mice treated with DEX (5mg/kg/day) or PBS for 4 days (n=15-18; *p<0.05 vs PBS -treated WT mice). B. Gene expression of Acc1, Acc2 and Fas was measured in epididymal WAT of the same mice (n=8 -12; *p<0.05). Rpl19 gene expression was used as an internal control.

Comparing the rate of TG turnover in liver and WAT of WT and *Angptl4*$^{-/-}$ mice. We next monitored the rate of TG synthesis in WT and *Angptl4*$^{-/-}$ mice. The same treatment and labeling protocol was used as described above. At the end of treatment, TG was isolated and GC-MS was used to measure newly synthesized glycerol. We calculated the absolute TG synthesis rate (g TG synthesized per day) in PBS- or DEX-treated mice. We found that absolute hepatic TG synthesis rate was markedly lower in PBS-treated *Angptl4*$^{-/-}$ mice than those of PBS-treated WT mice (0.0058 g/day vs. 0.014 g/day, Figure 16A). DEX treatment elevated the absolute TG synthesis rate for approximately 3.3 fold in WT mice (0.047 g/day, Figure 16A). DEX also augmented the absolute TG synthesis rate for approximately 3.4 fold in *Angptl4*$^{-/-}$ mice (0.0199 g/day Figure 16A). Notably, because of a lower basal TG synthesis rate, absolute TG synthesis rate upon DEX treatment in *Angptl4*$^{-/-}$ mice was significantly lower than those of DEX-treated WT mice (0.0199 g/day vs. 0.047 g/day).

Figure 16: The rate of glucocorticoid-induced triglyceride synthesis in liver and adipose tissue

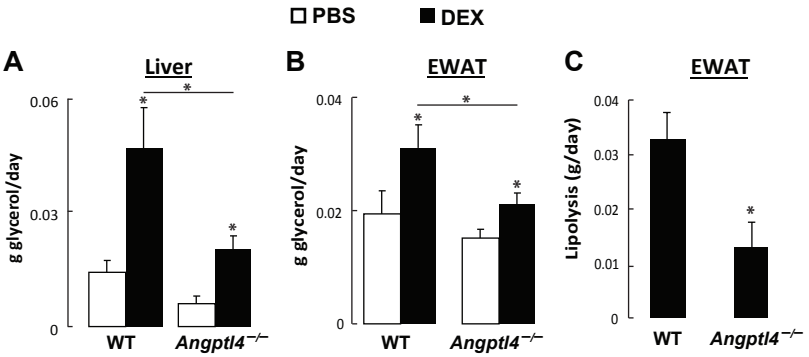

Figure 16: The effect of DEX on the absolute rate of TG turnover. A. The absolute rate of TG synthesis was measured in the liver of WT and *Angptl4*$^{-/-}$ mice treated with DEX or PBS for 4 days (5mg/kg/day) (n=5; *p<0.05 vs DEX-treated wild type animals). B. The absolute rate of TG synthesis was measured in the epididymal WAT of the same mice (5mg/kg/day) (n=10-11; *p<0.05 vs DEX-treated wild type animals). C. The rate of net lipolysis following DEX treatment was calculated in epididymal WT in the same animals (5mg/kg/day, n=6; *p<0.05).

Overall, these results indicated that Angptl4 was required for the maximal basal and DEX-induced TG synthesis rate in liver.

In WAT, the basal TG synthesis rate was similar between WT and *Angptl4*$^{-/-}$ mice (Figure 16B). DEX treatment increased TG synthesis rate in both WT and *Angptl4*$^{-/-}$ mice

Figure 17: The expression of genes involved in triglyceride synthesis in liver and adipose tissue

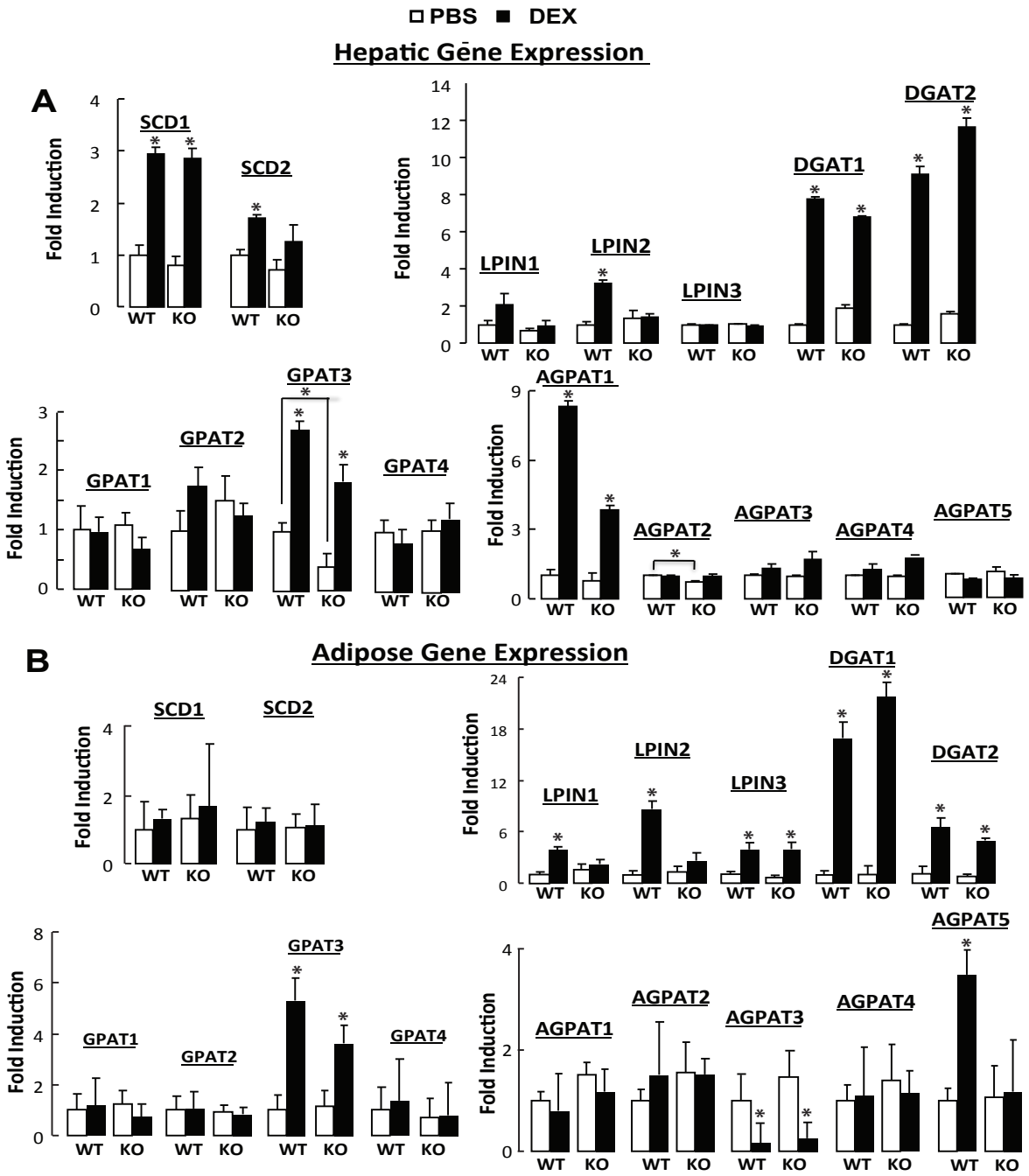

Figure 17: The effect of DEX on the expression of genes involved in TG synthesis. A. The expression of genes involved in TG synthesis was measured in the liver of WT and *Angptl4*$^{-/-}$ mice treated with DEX (5mg/kg/day) or PBS for 4 days (n=8-12; *p<0.05). B. The expression of genes involved in TG synthesis was measured in the epididymal WAT of the same mice (n=8-12; *p<0.05). (n=6-10; *p<0.05 vs control animals). Rpl19 gene expression was used as an internal control.

(Figure 16B). However, the induction was significantly higher in WT than those of *Angptl4*$^{-/-}$ mice (0.031 g/day vs 0.021 g/day, Figure 16B). Thus, in WAT, while Angptl4 deficiency did not affect basal TG synthesis rate, the maximal rate of glucocorticoid-stimulated TG synthesis still requires Angptl4.

Using the difference between the rate of TG synthesis and the change in fat pad TG content allowed us to calculate the net DEX-induced lipolysis in adipose tissue. DEX treatment increased lipolysis to a significantly greater extent in WT mice than in *Angptl4*$^{-/-}$ animals (Figure 16C).

Some DEX-regulated genes involved in TG synthesis are differentially expressed in *Angptl4*$^{-/-}$ mice. We performed gene expression analyses to monitor the expression of genes involved in TG synthesis in WT and *Angptl4*$^{-/-}$ mice. We found that the expression of Agpat2 and Gpat3 in PBS-treated *Angptl4*$^{-/-}$ mice was significantly lower in those of PBS-treated WT mice (Figure 17A). These results could explain a lower TG synthesis rate in *Angptl4*$^{-/-}$ mice liver. DEX treatment markedly induced Scd1, Scd2, Gpat3, Agpat1, Lpin2 and Dgat1 gene expression in WT mice (Figure 17A). The induction of these genes likely accounted for an increased TG synthesis rate in DEX-treated WT mice liver. In *Angptl4*$^{-/-}$ mice, DEX treatment also markedly elevated Scd1, Gpat3, Agpat1, Dgat1 and Dgat2 gene expression, which was consistent with an increased hepatic TG synthesis in these animals.

Figure 18: The role of Angptl4 in the expression of Agpat1, Agpat2 and triglyceride synthesis

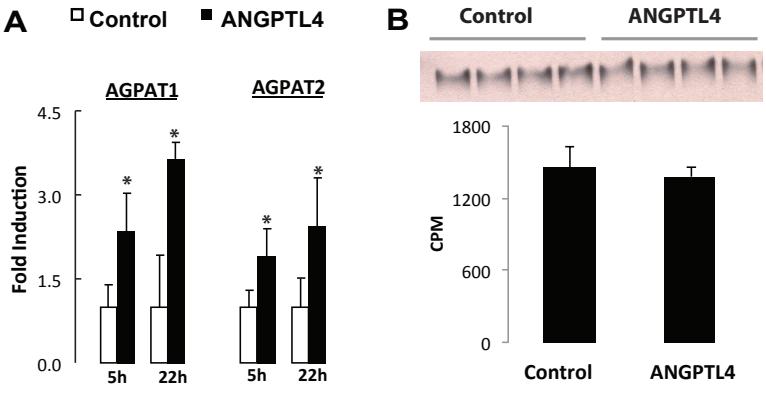

Figure 18: A. The expression of Agpat1 and Agpat2 after ANGPTL4 treatment. Primary mouse hepatocytes were treated with ANGPTL4 (10nM) for both 5 and 22 hours (n=12, *p<0.05 vs EtOH-treated hepatocytes). Rpl19 gene expression was used as an internal control. B. Primary hepatocytes were treated with 10nM Angptl4 for 18 hours and then the incorporation of radioactive C14 into triglyceride over an hour was measured.

In WAT, the basal expression levels of genes involved in TG synthesis were similar between WT and *Angptl4*$^{-/-}$ mice (Figure 17B). These results were in agreement with a similar TG synthesis rate between WT and *Angptl4*$^{-/-}$ WAT. DEX treatment induced the expression of Lpin1, Lpin 2, Gpat3, Dgat1 and Dgat2 (Figure 17B) in WAT of WT mice. In *Angptl4*$^{-/-}$ mice WAT, DEX still activated Gpat3, Dgat1 and Dgat2, but the expression of Lpin1 and Lpin2 was not affected (Figure 17B). These results were also consistent with the fact that DEX treatment elevated the rate of TG synthesis in WT and *Angptl4*$^{-/-}$ mice WAT.

ANGPTL4 increases Agpat1 and Agpat2 gene expression in mouse primary hepatocytes. Because *Angptl4*$^{-/-}$ mice liver had a lower TG synthesis rate and the expression of Agpat2 and Gpat3 than those of WT mice, we treated mouse primary hepatocytes with purified human ANGPTL4 protein to test whether it can directly regulate the expression of genes involved in TG synthesis. We found that ANGPTL4 treatment for 5 and 22 hr significantly induced Agpat1 and Agpat2 gene expression (Figure 18A). ANGPTL4 did not significantly affect the expression of Fas, Acc1, Acc2, Gpat3 and Lpin2 (Table 4). These results support the notion that ANGPTL4 plays a role in regulating the expression of certain genes that may be involved in hepatic TG synthesis.

ANGPTL4 does not directly increase TG synthesis. To investigate whether Angptl4 treatment could directly increase in hepatic TG synthesis we treated primary hepatocytes with 10nM ANGPTL4 for 10 hours and then added radiolabeled oleic acid to the cells and quantified its incorporation into TG after one hour. We found that there was no difference in the amount of newly synthesized TG between the cells treated with ANGPTL4 and the control cells (Figure 18B). This result indicates that the participation of ANGPTL4 in TG synthesis is not through a direct effect of the protein.

Table 4: Gene Expression in Primary Hepatocytes Treated with 10nM Angptl4

	5 hours	22 hours
FAS	1.18 (0.79)	1.46 (0.29)
ACC1	1.33 (0.44)	1.49 (0.52)
ACC2	1.25 (0.30)	1.39 (0.50)
GPAT3	1.15 (0.67)	1.13 (0.65)
LPIN2	1.63 (0.40)	1.18 (0.24)

Fold induction over control (p value vs control)

Table 4: The expression of other genes involved in DNL and TG synthesis were measured following ANGPTL4 (10nM) treatment. Values are fold induction over EtOH treated hepatocytes. None of the genes showed significant changes in expression in response to ANGPTL4 treatment. (n=12)

DISCUSSION

Through the use of gene expression analyses and by measuring the *in vivo* kinetics of glucocorticoid-regulated lipid metabolism, in this report we provide several novel findings. First, Angptl4 deficiency resulted in a lower rate of basal TG synthesis in liver but not WAT. ANGPTL4 protein can increase the expression of genes involved in TG synthesis, such as Agpat1 and Agpat2, in primary hepatocytes. Second, we showed that a complete response of glucocorticoid-induced DNL requires Angptl4 in vivo. Without Angptl4, the ability of glucocorticoids to activate Fas, Acc1 and Acc2 gene expression was reduced. Third, while glucocorticoids still increased TG synthesis in *Angptl4*$^{-/-}$ mice, the absolute TG synthesis rate was lower. Thus, the role of Angptl4 in glucocorticoid-stimulated hepatic DNL and TG synthesis can explain, at least in part, a lower glucocorticoid-induced liver and plasma TG levels in *Angptl4*$^{-/-}$ than those of WT mice. Finally, a maximal increase of TG synthesis by glucocorticoids also requires the presence of Angptl4.

It is interesting that Angptl4 is required for maintaining basal TG synthesis rate in liver but not WAT. Gene expression analyses were in agreement with these observations. In WAT, the expression of gene involved in TG synthesis was similar between *Angptl4*$^{-/-}$ and WT mice. In liver, however, Agapt2 and Gpat3 gene expression was markedly lower in *Angptl4*$^{-/-}$ mice. Treatment of ANGPTL4 protein on mouse primary hepatocytes for 5 and 22 hr induced the expression of Agapt1 and Agpat2 but not Gpat3. These results are intriguing, as these are the first to show ANGPTL4 directly regulates gene involved in lipid metabolism. Overexpression of Agpat1 has been shown to increase lipid synthesis in 3T3-L1 adipocytes [172, 173]. Also the role of Agpat2 in adipocyte TG synthesis has been established by studies of human patients with Agpat2 gene mutation, which causes a type of Berardinelli-Seip congenital generalized lipodystrophy (CGL) [174, 175]. It is important to note that the studies discussed above are conducted in adipocytes, and it is unclear whether the induction of these two genes in liver will also increase TG synthesis. We also examined the direct role of ANGPTL4 on TG synthesis in hepatocytes and found that there was effect. This suggests in spite of the increase in Agpat1 and Agpat2 the decreased TG synthesis observed in Angptl4$^{-/-}$ is due to secondary effects of the protein and not a direct stimulation of TG synthesis in the hepatocytes. However the role of Agapt1 and Agpat2 in hepatic TG synthesis should be further examined in future studies. How ANGPTL4 activates Agpat1 and Agpat2 gene

expression is also unclear. ANGPTL4 has been shown to modulate various signaling pathways in distinct cell types. In endothelial cells, ANGPTL4 inhibits ERK/MAPK pathway [80], whereas in the hypothalamus, ANGPTL4 inhibits AMP-activated protein kinase [114] and in keratinocytes, ANGPTL4 interacts with integrin and fibronectin and activate integrin-mediated signaling pathway, by stimulating focal adhesion kinase (FAK) [96, 97]. We have recently found that ANGPTL4 increases intracellular cAMP levels in adipocytes[165]. The signaling pathways affected by ANGPTL4 in hepatocytes, however, have not been reported. Because the regulation of Agpat1 and Agpat2 gene expression is also largely unclear, whether the signaling pathways discussed above can play a role in ANGPTL4 effect on these two genes could not be predicted at this moment.

The induction of DNL by glucocorticoids in liver is well-described. A recent study, however, showed that glucocorticoids decrease lipogenesis in adipocytes[176]. This observation is based on glucocorticoid-treated human Chub-S7 adipocytes. The expression of Fas and Acc2 gene was induced by glucocorticoids in this report. The expression of Acc1 protein was also increased, however the levels of Acc1 protein phosphorylated on serine 79 and 218, which is an inactivating phosphorylation, was elevated [176]. The increased inactive Acc1 protein may cause the reduction of lipogenesis. Notably, a study of CRH-overexpressing mice, which have chronic high corticosteron levels in circulation, observed a higher rate of DNL in WAT than those of WT mice (Rhook DJ and Harris CA, manuscript in submission). These results are in agreement with our observations. Thus, it is possible that acute and chronic glucocorticoid treatment differentially regulate WAT DNL. The short-term treatment decreases, whereas long-term treatment of glucocorticoids increases DNL.

Although the activation of Fas, Acc1 and Acc2 gene expression by glucocorticoids have been reported, the mechanism underlying these glucocorticoid effects are not completely clear. A recent report showed that human Acc1 gene promoter can be activated by glucocorticoids when it is inserted into a synthetic reporter gene [177]. Previous study also showed that glucocorticoid treatment increases the activity of a reporter gene that contains -1592 to +65 (relative to transcription start site) of human Fas gene[170]. The exact location of glucocorticoid response elements (GREs) in human Acc1 and Fas gene was not reported in these papers. Using chromatin immunoprecipitation sequencing (ChIPseq), we identified GR binding regions (GBRs) in the intronic region of mouse Acc1, Acc2, and Fas gene in 3T3-L1 mouse adipocytes [21]. Whether these GBRs can mediate glucocorticoid-induced Acc1, Acc2, and Fas gene transcription have not been tested but currently our lab is undertaking ChIP studies to determine if GR is recruited to any of these GBRs and if so we will continue with reporter assays to confirm functional GREs. How Angptl4 is involved in glucocorticoid-induced Acc1, Acc2, and Fas gene expression is unclear. Previous studies and our unpublished study showed that DEX alone can activate Fas and Acc1 gene expression in hepatocytes. Our gene expression analyses showed that ANGPTL4 protein treatment for 5 and 22 hr did not affect the expression of these three genes. These suggest that ANGPTL4 likely participates in glucocorticoid-induced Fas, Acc1 and Acc2 gene expression through indirect mechanism(s) in vivo. Angptl4 plays an important role in lipid redistribution in vivo. Angptl4 deficiency would affect the availability of lipids in plasma, which could cause the changes of levels of lipid metabolites in various tissues. Certain lipid metabolites could serve as signaling mediators to affect the ability of glucocorticoids to activate Fas and Acc1 genes. For example, polyunsaturated fatty acids have been shown to repress Fas gene expression[178]. Moreover, the change of plasma and tissue lipid levels could potentially alter the levels of various

hormones, cytokines and adipokines in plasma, which may also affect glucocorticoid response on Fas, Acc1 and Acc2 genes.

Glucocorticoids increased TG synthesis rate in both WT and *Angptl4*$^{-/-}$ mice liver. Gene expression analyses are in agreement with these observations, as we found that DEX increased several genes involved in TG synthesis in both WT and *Angptl4*$^{-/-}$ liver, though the list of genes induced were slightly different. Some of these genes, such as Scd1, Scd2, and Gpat3, have been previously shown as a potential GR primary target genes in our previous report [21]. Because of a lower basal TG synthesis rate, the absolute TG synthesis rate upon glucocorticoid treatment in *Angptl4*$^{-/-}$ was lower than those of WT mice liver. Additionally we observed that the increase in TG synthesis is not due to direct stimulation by Angptl4

In WAT, the ability of glucocorticoids to elevate absolute TG synthesis rate was reduced in *Angptl4*$^{-/-}$ mice. Gene expression analyses showed that Lpin1, Lpin2, Gapt3, Dgat1 and Dgat2 were activated by DEX in WT, whereas only the first three genes were induced by DEX in *Angptl4*$^{-/-}$ WAT. Both Lpin 1 and Lpin 2 protein are involved in TG synthesis [173, 179, 180] But, it is unclear whether the failure of DEX to stimulate Lpin1 and Lpin2 result in a lower TG synthesis rate in *Angptl4*$^{-/-}$ WAT. This possibility may be able to be tested by comparing the absolute TG synthesis rate in DEX-treated WT and mice lacking Lpin 1 and/or Lpin 2.

The observation of a lower rate of DEX-induced TG synthesis in WAT of *Angptl4*$^{-/-}$ than those of WT mice was surprising, as we found that DEX treatment resulted in higher TG levels in *Angptl4*$^{-/-}$ WAT. We recently found that glucocorticoid-induced WAT lipolysis is blunted in of *Angptl4*$^{-/-}$ mice[165]. Notably, the TG levels were similar between WT treated with PBS or DEX. This can be explained by the observation that DEX treatment induces both TG synthesis and lipolysis concurrently [21]. In *Angptl4*$^{-/-}$ mice WAT, even with a lower TG synthesis rate than WT mice, because the ability of glucocorticoids to promote WAT lipolysis was greatly impaired, TG was markedly accumulated.

In summary, in this report we highlight the importance and the complex role of Angptl4 in basal and glucocorticoid-regulated DNL and TG synthesis. Further dissecting Angptl4 function on distinct aspects of lipid metabolism should provide valuable insights into our understanding on both physiological and pathological effects of glucocorticoids on lipid homeostasis.

Chapter 3: A Role for Angptl4 in Adaptive Thermogenesis

SUMMARY

Lipolysis in adipose tissue plays a critical role in generating fatty acids that can provide energy for other tissue and also serve as substrates and activators for adaptive thermogenesis. Angiopoietin-like 4 (Angptl4) is a secreted protein that induces intracellular lipolysis. We found that Angptl4 expression in brown adipose tissue was increased by cold exposure. After 1 hour of 4° cold exposure, the body temperature of mice lacking Angptl4 (*Angptl4-/-*) became significantly lower than those of WT mice, a difference maintained throughout the 10h exposure. Cold exposure also significantly reduced the size of brown adipocytes of WT mice, however this effect was less prominent in *Angptl4-/-* mice. The lipolytic response induced by isoproternol, a β adrenergic receptor agonist, was attenuated in brown adipocytes from *Angptl4-/-*. Diminished induction of thermogenic genes was also observed in the BAT of *Angptlt4-/-* mice. Additionally, the induction of certain thermogenic genes in inguinal white adipose tissue of *Angptl4-/-* was lower than those observed in WT. The body temperature of *Angptl4-/-* animals was also found to be lower than WT mice during fasting. Together these results suggest that Angptl4 participate in thermogenesis and such a role could have relevant implications for weight gain and obesity.

INTRODUCTION

Adipose tissue plays a key role in the balance between the storage and utilization of energy. White adipose tissue (WAT) serves as a storage reserve for lipids in the form of triglyceride (TG) so that in times of energy shortage they can be hydrolyzed to generate fatty acids (FA, which can be used by other tissues. In contrast, brown adipose tissue (BAT) is critical for thermogenesis. Brown adipocytes are highly enriched with mitochondria which enables the tissue to function as a dissipator of chemical energy into heat. Uncoupling protein 1 (Ucp1) uncouples oxidative phosphorylation from ATP production by generating heat. Cold exposure activates β-adrenergic receptors in BAT through the sympathetic nervous system. This signaling induces the coactivator Pgc1α, which in turn drives the transcription of Ucp1 [181]. Pgc1α also coordinately increases mitochondrial gene expression to regulate oxidative metabolism [182]. Both Pgc1α and Ucp1 are highly expressed in brown adipose tissue.

Lipolysis is the breakdown of TG into fatty acids and glycerol and it is a process that is critical for the thermogenic capacity of BAT. Hydrolysis of lipids generates fatty acids be used for oxidation in the mitochondria and can directly activate Ucp1 [183]. β-adrenergic receptor signaling can regulate lipolysis through activation by catecholamines. Gαs-coupled receptors generate cAMP which results in the downstream phosphorylation and activation of lipolytic proteins by PKA [156]. Because fatty acids are essential for activating the genes necessary for thermogenesis as well as for providing the substrates for the process itself, properly regulated lipolysis is crucial for this adaptation. Moreover, efficient thermogenesis requires not only the fatty acids generated from BAT lipolysis but the contribution of additional fatty acids from WAT as well, a fact that is reflected in the increase in circulating non-esterified fatty acids (NEFA) during a cold exposure [184].

Thermogenesis is thought to account for approximately 10% of overall energy expenditure while adaptive thermogenesis specifically is believed to reflect changes in metabolic efficiency [185]. Indeed previous studies have shown that changes in ambient

temperature can alter energy expenditure [186]. The interdependency between thermogenesis and energy expenditure is also evidenced by the fact that thyroid hormone has been shown to interact synergistically with the sympathetic nervous system, and directly increase basal metabolic rate [187].

Angiopoietin-like 4 (Angptl4, also called fasting-induced adipose factor or FIAF) is a secreted protein that mobilizes lipids by inhibiting lipoprotein lipase and inducing WAT lipolysis. It is expressed in many tissues including liver, muscle pancreas, intestine, brain and, in rodents at least, is most highly expressed in white and brown adipose tissue [76, 77]. Hepatic Angptl4 expression has been shown to be regulated by thyroid hormone [89], an important component of the sympathetic nervous system response to cold exposure, although no links between thyroid hormone and BAT Angptl4 expression or function have been reported to date. Additionally Angptl4 has been linked to cellular energetics. Overexpression has been shown to enhance activity of respiratory chain components [116] and increase uncoupling in white adipose tissue [83].

We previously found that Angptl4 regulates lipolysis by modulating intracellular cAMP levels and has been shown to be necessary for an efficient lipolytic response to catecholamines [165]. Given its high expression in BAT and its requirement for lipolysis in WAT, along with its implications in energy expenditure, we hypothesized that Angptl4 may play a role in adaptive thermogenesis. We found that BAT and plasma levels of Angptl4 are increased upon cold exposure and using *Angptl4-/-* mice we determined that Angptl4 does, in fact, participate in adaptive thermogenesis. We also demonstrated that *Angptl4-/-* mice have altered plasma lipids during a cold exposure and impaired brown adipocyte lipolysis in response to both cold and β-adrenergic stimulation. We also found the regulation of body temperature during fasting is altered in *Angptl4-/-* mice.

METHODS

Animals. *Angptl4*$^{-/-}$ mice were provided by the laboratories of Andras Nagy (Samuel Lunenfeld Research Institute, Mount Sinai Hospital) and Jeff Gordon (Washington University)[115]. *Angptl4*$^{-/-}$ mice were generated on a mixed B6:129/Sv background. WT mice were the littermates of *Angptl4*$^{-/-}$ mice. The PCR protocols and strategy for mouse genotyping were as described [115]. C57BL/6J mice were from Charles River Laboratories (Wilmington, MA). Cold exposure experiments involved individually housing each mouse at 4 degrees for 10 hours from 7am until 5pm. Body temperature was measured every hour using a rectal thermometer (Physitemp Model BAT-12). After 10h animals were sacrificed and blood and tissue were taken for analysis. For fasting experiments mice were provided with food *ad libitum* for the first 24h and body temperature was measured at several points throughout the day. Food was removed and animals were fasted for a total of 24 hours with body temperatures being measured again after 6, 12, 18, and 24 hours. The Office of Laboratory Animal Care at the University of California, Berkeley approved all the animal experiments (#R306-0111).

Determination of Tissue and Plasma Angptl4 Levels. Plasma and epididymal WAT were isolated from C57B/6J mice that had been fed *ad-libitum* or fasted for 24 hours. Approximately 200 mg of the WAT was homogenized in 500mL of RIPA buffer (10 mM Tris-HCl, pH 8.0, 1mM EDTA, 150 mM NaCl, 5% glycerol, 0.1% sodium deoxycholate, 0.1% SDS, and 1% Triton X-100) supplemented with protease inhibitors. The homogenates

were centrifuged (13,000 x g for 10 minutes at 4°C) and the Angptl4 content of the tissue supernatants and plasma samples were analyzed by ELISA (UCSN).

Plasma TG Measurement. Plasma TG levels were measured following a manual from a colorimetric kit (Roche Diagnostics).

NEFA Assays. Plasma was isolated from whole blood immediately after collection and a colorimetric kit (Wako) was used to measure plasma NEFA levels.

Isolation of Brown Adipocytes. WT and *Angptl4*$^{-/-}$ mice were euthanized and intrascapular BAT was harvested and minced thoroughly in Krebs-Ringer buffer containing collagenase. The mixture was transferred to a conical tube and shaken at 220 rpm and 37°C for 1 hour. After digestion, the mixture was filtered through a 250mm gauze mesh and spun for 5 minutes at 200 rpm. The buffer was carefully removed using a needle and syringe, and the floating layer of adipocytes was washed three times with 10ml of Krebs-Ringer buffer and then resuspended in media containing 10% stripped FBS. Aliquots of approximately 50uL of cells were placed into microcentrifuge tubes containing media at a volume of 100 uL for further treatments. Cells were incubated at 37°C with shaking at 220 rpm for all treatments.

Lipolysis Assays. Lipolysis was measured as described [143]. Isolated adipocytes from BAT were treated with isoproterenol (1uM) and incubated in media at 37°C for 1 hour. Glycerol release was determined after one hour using a free glycerol reagent (Sigma). Measurements were normalized to the total protein content of the adipocytes using Bradford protein dye (BioRad).

Quantitative Real-Time PCR (qPCR). Total RNA was isolated from the livers and epididymal WAT of mice using Tri-reagent (Molecular Research Center Inc.). Reverse transcription was performed as described [23]. The resulting cDNA was diluted to 170µL and 3.5µL was used to perform qPCR in a 25µL reaction using EVA qPCR SuperMix Kit (Biochain) per the manufacturer's protocol. qPCR was performed on a StepOne PCR-system (Applied Biosystems) and analyzed using the delta-delta CT method as supplied by the manufacturer. Primer Sequences were as follows: Dio2 for: ccacctgaccacctttcact, Dio2 rev: tggttccggtgcttcttaac; Cidea for: gcctgcaggaacttatcagc, Cidea rev: ccatttctgtccctttcca; Elovl3 for: actggtaccaccacagcaca, Elvol3 rev: ctttctcctgcctccagatg; Ucp1 for: gccttcagatccaaggtgaa, Ucp1 rev: taagccggctgagatcttgt; Pgc1a for: atgtgtcgccttcttgctct, Pgc1a rev: atctactgcctggggacctt; Prdm16 for: gccattcatatgcgaggtct, Prdm16 rev: ccaggcgtgtaatggttctt; Cpt1a for: catgtcaagccagacgaaga, Cpt1a rev: tggtaggagagcagcacctt.

Adipocyte Size Determination. Adipocyte size was determined as previously describe [188]. Briefly intrascapular BAT were fixed in 10% buffered formalin, embedded in paraffin, cut into 5 µm-thick sections, and stained with hemotoxylin and eosin. Adipocyte size was determined with Image J software (US National Institutes of Health), measuring approximately 100 cells per sample.

Statistics. Data are expressed as standard error of the mean (S.E.M) for each group and comparisons were analyzed by Student's *t* test.

RESULTS

Angptl4 participates in adaptive thermogenesis. We've previously shown that Angptl4 is important for the lipolytic response to catecholamines in WAT [165] and is known to be highly expressed in brown adipose tissue [77]. Since lipolytic induction by catecholamines is critical for body temperature regulation during cold exposure, we therefore investigated if Angptl4 plays a role in adaptive thermogenesis. We measured Angptl4

expression in BAT as well as epididymal and inguinal WAT along with plasma levels of the protein in animals housed at room temperature and those kept at 4°C for 10h. Cold exposure significantly increased Angptl4 expression in BAT but has no effect on levels in either EWAT or IWAT (Figure 19A). Surprisingly cold exposure significantly increased circulating levels of Angptl4 but it should be noted the levels achieved following cold-exposure were only about half as high as have been reported following a fast [165].

We went on to evaluate the ability of *Angptl4-/-* mice to defend their body temperature at 4°C. WT and *Angptl4-/-* mice were maintained at 4°C for 10h and their body temperatures were measured every hour. *Angptl4-/-* mice had no trouble maintaining a stable body temperature but notably the temperature they defended was consistently about 0.5-1 degree lower than what was observed in WT animals (Figure 19B). These results indicate that while not critical for adaptive thermogenesis, Angptl4 does participate in maintaining a normal body temperature during a cold exposure.

Figure 19: Expression and role of Angptl4 during adaptive thermogenesis

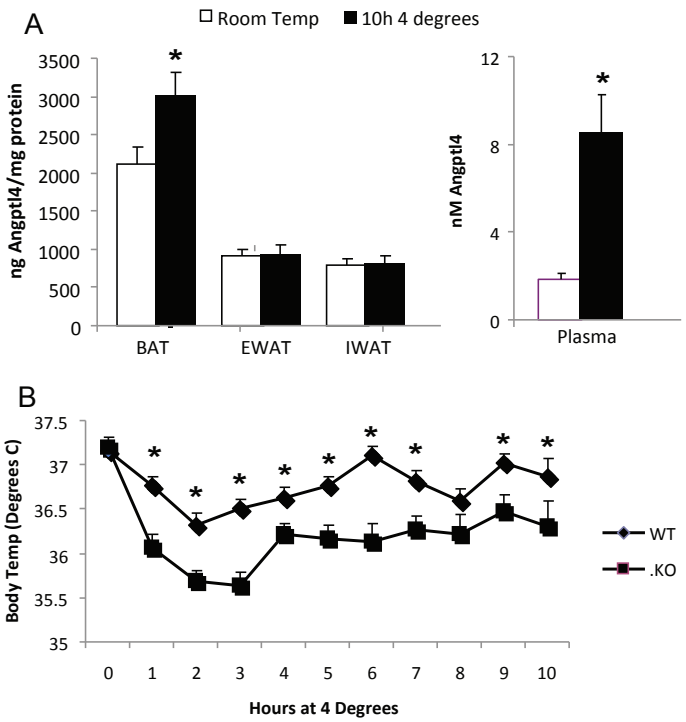

Figure 19: A. Angptl4 concentrations measured by ELISA in BAT as well as epididymal WAT (EWAT) and inguinal (WAT) and plasma of mice kept at room temperature or at 4 degrees for 10 hours (n=6; *p<0.05 vs. room temperature). **B.** Body temperature of WT and *Angptl4 -/-* mice was monitored over a 10 hour cold exposure, showing that *Angptl4-/-* mice maintained a lower body temperature over the cold exposure (n=6; *p<0.05 as compared to *Angptl4 -/- animals*).

Figure 20: Plasma lipids following a cold exposure

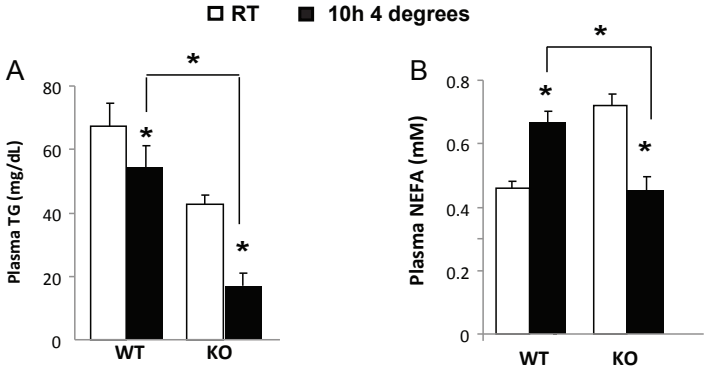

Figure 20: **A.** Plasma TG levels from WT and *Angptl4 -/-* mice kept either at room temperature or at 4 degrees for 10 hours were measured showing a decrease in TG levels upon cold exposure in both genotypes but a more profound decrease in the cold exposed *Angptl4-/- animals* (n=5; *p<0.05). **B.** Plasma non-esterified fatty acid (NEFA) levels from the same mice were measured, showing that while in WT animals cold exposure increased the levels of circulating NEFA this did not occur in *Angptl4-/-* mice (n = 5; *p<0.05).

Angptl4-/- mice have altered circulating lipids during cold exposure. Because body temperature is so tightly regulated in the body, we decided to pursue possible mechanisms behind the consistently reduced body temperature of the *Angptl4-/-* mice. Proper lipid mobilization from peripheral stores is required for efficient uptake and utilization by BAT during thermogenesis. We measured circulating TG as well as NEFA in WT and *Angptl4-/-* mice after 10h cold exposure. In both genotypes we observed a decrease in circulating TG upon cold exposure though the decrease was more dramatic in the *Angptl4-/-* (Figure 20A). This is not surprising since Angptl4 is

known to inhibit LPL and in the knockout animals this inhibition would be abolished. In contrast, cold exposure significantly increased circulating NEFA levels in the WT but not *Angptl4-/-* mice (Figure 20B). This nearly 45% increase likely reflects induced WAT lipolysis which we've previously shown to be diminished in the knockout animals [165]. In fact in the *Angptl4-/-* mice cold exposure actually decreased circulating NEFA levels by ~37% (Figure 20B). However, because the basal NEFA levels of *Angptl4-/-* mice were much higher than those of WT animals, the cold-induced decrease only translated to about 32% lower than cold-exposed WTs perhaps accounting for the body temperature phenotype observed.

Angptl4 participates in BAT lipolysis. Because adaptive thermogenesis relies on both brown and white adipose tissue lipolysis we were interested in determining if the modest decrease in body temperature in the *Angptl4-/-* mice was the result of an impairment within the BAT or if it merely reflected the diminished WAT lipolysis known to occur in those animals. We first looked at the morphology of the BAT to see if any differences in adipocyte size could be detected between cold-exposed WT and *Angptl4-/-* animals. Using paraffin embedding and hematoxylin and eosin staining we were able to determine that while basal brown adipocyte size is fairly consistent between the genotypes, it appears that the decrease in size upon cold exposure is slightly attenuated in the *Angptl4-/-* animals (Figure 21A). Cold exposure resulted in diminished adipocyte size in both genotypes, reflecting an increase in lipolysis, however WT brown adipocytes from cold-exposed animals appeared to be even smaller those from cold-exposed knockouts. Cell size was quantified across many sections for multiple animals in each condition and while cold treatment decreased WT adipocytes by ~71%, *Angptl4-/-* adipocytes were only decreased by ~54% (Figure 21A).

To more directly investigate if lipolysis is impaired in the BAT of *Angptl4-/-* animals, isolated

Figure 21: The role of Angptl4 in brown adipose tissue response to cold exposure and β adrenergic receptor agonist.

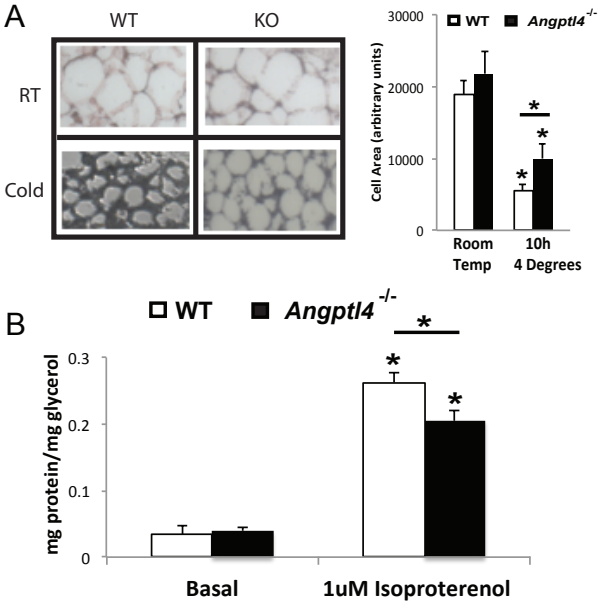

Figure 21: A. Brown adipose tissue from WT and *Angptl4-/-* mice kept at room temperature or 4 degrees for 10h was paraffin-embedded and sectioned then stained with hematoxylin and eosin. Cold exposure decreased the size of WT and *Angptl4-/-* brown adipocyte but did so to a greater extent in WTs. On the right is a quantification of cell size made from many images of multiple mice per condition (n=3 mice, 80-120 cells); *p<0.01). **B.** Glycerol concentration was measured in the medium bathing primary brown adipocytes treated for 1 hour with isoproterenol (1uM) show a decrease in catecholamine-stimulated glycerol release by *Angptl4*$^{-/-}$ adipocytes (n = 4; *p<0.05).

Table 5: Thermogenic gene expression in BAT

		WT	*Angptl4* $^{-/-}$
Dio2	Room temp	1.000	1.338
	10h 4 degrees	4.785*	3.874*
Cidea	Room Temp	1.000	1.394
	10h 4 degrees	5.801*	4.634*
Elovl3	Room Temp	1.000	1.233
	10h 4 degrees	170.862*#	112.528*
Ucp1	Room temp	1.000	1.213
	10h 4 degrees	18.003*#	8.314*
Pgc1a	Room temp	1.000	1.323
	10h 4 degrees	4.620*	4.470*
Prdm16	Room temp	1.000	1.368
	10h 4 degrees	5.442*	4.084*
Cpt1a	Room temp	1.000	1.162
	10h 4 degrees	3.609*	3.175*

Table 5: The expression of several thermogenic genes was measured by qPCR in the brown adipose tissue of WT and *Angptl4* -/- mice either at room temperature or after 10h of 4 degree cold exposure showing that although all genes were induced by cold exposure there were some that were induced to a greater degree in the WT as compared to *Angptl4* -/- mice. Values are fold induction over WT room temperature. (n=6;*p<0.05 vs WT room temp, #p<0.05 vs cold-exposed *Angptl4* -/-).

brown adipocytes were treated with the β adrenergic receptor agonist isoproterenol and glycerol (an end product of lipolysis) accumulation in the media was measured. Sixty minutes of treatment increased glycerol release 7.7 fold in WT adipocytes but only 5.2 fold in *Angptl4-/-* adipocytes (Figure 21B). This approximately 20% reduction in lipolytic response to isoproterenol suggests that Angptl4 does indeed play a role in brown adipocyte lipolysis.

Expression of cold-induced genes is altered in Angptl4-/- BAT. Adaptive thermogenesis involves coordinate regulation of the expression of multiple genes required for the process. We measured the expression of several thermogenic genes known to be regulated by cold exposure in BAT. The expression of all of the genes monitored was increased by cold exposure in both genotypes (Table 5). However, Elovl3 and Ucp1 were induced to significantly greater degree in WT BAT than in *Angptl4-/-* BAT. Upon cold exposure Elovl3 was increased about 171 fold in the WT and only 113 fold in the knockout whereas Ucp1 was induced 18 fold in the WT mice but only 8 fold in *Angptl4-/-* animals (Table 5). It is possible that the reduced induction of these two genes in the knockout mice could play a role in why they defend a lower body temperature during cold exposure.

Table 6: Thermogenic gene expression in epididymal white adipose tissue

EWAT

		WT	KO
Dio2	Room temp	1.000	0.935
	10h 4 degrees	10.980*	9.740*
Cidea	Room temp	1.000	0.959
	10h 4 degrees	24.594*	25.539*
Elovl3	Room temp	1.000	1.112
	10h 4 degrees	47.355*	46.826*
Ucp1	Room temp	1.000	1.177
	10h 4 degrees	182.165*	134.779*
Pgc1a	Room temp	1.000	0.980
	10h 4 degrees	534.411*	540.213*
Prdm16	Room temp	1.000	0.739
	10h 4 degrees	5.711*	5.133*
Cpt1a	Room temp	1.000	1.327
	10h 4 degrees	70.971*	63.797*

Table 6: The expression of several thermogenic genes was measured by qPCR in the epididymal white adipose tissue of WT and *Angptl4 -/-* mice either at room temperature or after 10h of 4 degree cold exposure. Expression of all thermogenic genes was induced by cold exposure in both genotypes.

Induction of thermogenic genes in WAT is increased upon cold exposure to a greater extent in the inguinal depot of WT mice. During prolonged cold exposure thermogenic genes, normally only expressed at high levels in BAT, are upregulated in WAT depots [175, 189]. We checked the expression of the thermogenic genes in the epididymal and inguinal depots of *Angptl4-/-* mice following a 10h cold-exposure. In the epididymal fat all of the genes evaluated were induced to a similar degree between WT and *Angptl4-/-* mice upon cold-exposure (Table 6). However in the inguinal depot while all genes were induced by the cold in *Angptl4-/-* mice, the levels achieved were about 30-60% as high as were observed in WT inguinal WAT (Table 7). The only expcetion was the gene Cidea, which was induced to a comparable degree in both WT and *Angptl4-/-* mice.

Table 7: Thermogenic gene expression in inguinal white adipose tissue

IWAT

		WT	KO
Dio2	Room temp	1.000	0.953
	10h 4 degrees	47.747*#	14.151*
Cidea	Room temp	1.000	1.270
	10h 4 degrees	24.192*	19.535*
Elovl3	Room temp	1.000	0.938
	10h 4 degrees	42.953*#	26.532*
Ucp1	Room temp	1.000	0.879
	10h 4 degrees	40.120*#	11.888*
Pgc1a	Room temp	1.000	1.072
	10h 4 degrees	39.791*#	20.017*
Prdm16	Room temp	1.000	1.485
	10h 4 degrees	8.561*#	4.870*
Cpt1a	Room temp	1.000	0.831
	10h 4 degrees	23.699*#	7.900*

Table 7: The expression of several thermogenic genes in the inguinal white adipose tissue of WT and *Angptl4 -/-* mice either at room temperature or after 10h of 4 degree cold exposure. Expression of most genes was significantly more induced by cold in WT WAT than in that of *Angptl4-/-* mice. Values are fold induction over WT room temperature. (n=6;*p<0.05 vs WT room temp, #p<0.05 vs cold-exposed *Angptl4 -/-*).

Fasting body temperature is reduced in Angptl4-/- mice. During a fast body temperature declines [190]. Because our data suggest that Angptl4 plays a role in thermogenesis in response

to cold exposure, we next decided to investigate if it was involved in body temperature regulation during fasting. We measured body temperature at multiple time points over a 24h fast and found that *Angptl4-/-* had consistently lower temperatures than did their WT counterparts (Figure 22). Interestingly, the difference between the body temperatures of fasting WT and *Angptl4-/-* mice was 0.5-1°, consistent with what we observed during the cold exposure. These data indicate dysregulated fuel generation and energy expenditure. During a fast, adipose tissue provides the fatty acids that can be oxidized for energy. Fasting-induced lipolysis is known to be diminished *Angptl4-/-* mice [165] which may result insufficient substrate to meet the energy requirements of other tissues perhaps causing a decrease in body temperature, indicative of a compensatory reduction in whole body energy expenditure.

Figure 22

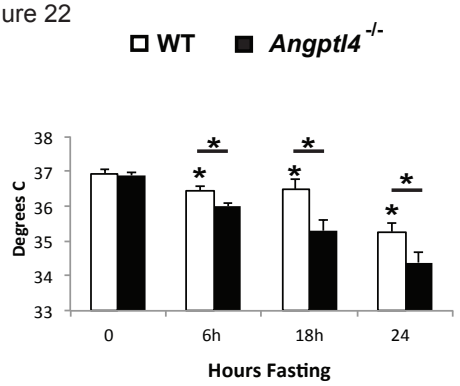

Figure 22: Body temperature of WT and *Angptl4-/-* was measured over the course a fast showing that the temperature of *Angptl4-/-* mice decreased more than WT temperature during fasting. (n=4-6; *p<0.05 vs WT fasted for the same amount of time).

DISCUSSION

In this study, we found that Angptl4 plays a significant role in adaptive thermogenesis. *Angptl4-/-* mice had a consistently lower body temperature during cold exposure than did WT animals. We found that the expression of Angptl4 was increased in BAT and in circulation upon cold exposure. Little is known about the regulation of Angptl4 by cold exposure. A recent report found that cardiac Angptl4 mRNA was decreased after cold exposure in mice on a high fat diet [191]. While these results are not in line with our own findings it's important to note that Angptl4 mRNA levels in BAT were not measured in that study and tissue specific gene regulation is not uncommon. In our experiments, we found that mRNA levels of Angptl4 in BAT were increased upon cold exposure (data not shown) and we confirmed these changes at the protein levels by ELISA, which we feel gives a more accurate portrayal of the tissue microenvironment.

Angptl4 is a target gene of PPARα and PPARγ [77, 190] both of which are highly expressed in BAT and activated by fatty acids [77]. Activation of these PPARs by the increased FA generated by cold-induced lipolysis in BAT could explain the increased Angptl4 expression in that tissue. In fact, our histology shows that adipocyte cell size in *Angptl4-/-* mice is decreased to a lesser extent following cold exposure than WT, indicating less lipolysis. Additionally, experiments directly quantifying lipolysis after treatment with the βAR agonist isoproterenol confirm that *Angptl4-/-* brown adipocytes do in fact have decreased lipolytic response.

A phenotype with a similar body temperature response to cold exposure to what we observed in *Angptl4-/-* mice was also recently seen in *Fgf21-/-* mice, whose body temperature remained approximately 0.5° lower than wild type during cold exposure [189]. In that case the diminished thermogenic response was attributed to a decreased browning of WAT as evidenced by lack of pockets of Ucp1 positive cells normally observed in cold-exposed WT adipose tissue along with diminished induction of thermogenic genes in WAT of the *Fgf21* knockout [189]. We have done some preliminary hematoxylin and eosin staining and the

only obvious morphological alteration we observed in WAT was that the size decrease in cold exposed adipocytes was attenuated in *Angptl4-/-* mice (data not shown). This finding is consistent with our previous report that *Angptl4-/-* mice have deficient white adipose tissue lipolysis [165]. We could not ascertain any noticeable browning of white fat for either genotype. However, it is possible that more extensive immunostaining, for example for Ucp1 positive cells, could uncover some differences we failed to observe. We did however see some differential induction of thermogenic genes in WAT between the genotypes. With the exception of Cidea, all of the other thermogenic genes we tested were induced by cold exposure 40-70% less in *Angptl4-/-* inguinal WAT than in WT cold exposed WAT. These differences are very similar to those observed for many of the same genes in cold-exposed *Fgf21-/-* animals which were also about 25-55% lower than cold-induced expression in WT [189].

Notably we only detected genotype specific changes in cold-induced gene expression in the inguinal WAT of *Angptl4-/-* mice but not epididydmal WAT. It is possible to speculate that perhaps the gene expression changes in the inguinal depot accompany more relevant phenotypic alterations with regard to thermogenic capacity as it has been shown that during cold exposure a greater accumulation of brown-like cells can be found in inguinal WAT than in epididymal [192]. However in our experiment, whether or not the gene expression changes we observed are sufficient to indicate browning of WAT remains to be seen, as does the role of Angptl4 in that process. Induction of thermogenic genes by direct administration of Angptl4, either systemically or to isolated adipocytes, would provide more convincing evidence for this explanation. At this point however it is premature to conclude that increased browning of WAT accounts for thermogenesis seen in *Angptl4-/-*.

Our data indicated that following 10h cold exposure the induction of the thermogeneic genes Ucp1 and Elovl3 was diminished in the BAT of *Angptl4-/-* mice. The critical role of Ucp1 in adaptive thermogenesis has been well described. Because Ucp1 is the enzyme that actually performs the dissipation of chemical energy into heat it is essential for thermogenic response to cold exposure. Mice completely lacking Ucp1 are extremely cold sensitive [193]. However our data indicate that in *Angptl4-/-* mice Ucp1 is still induced by cold exposure just not to the same level observed in the WT animals. How much the degree of expression affects thermogenesis hasn't been as exhaustively investigated. Animals that are heterozygous Ucp1 ablation appear to have a normal response to cold [193] but rigorous studies investigating the threshold for Ucp1 expression for proper thermogenesis have not been reported. Future experiments in our knockouts to overexpress Ucp1 in BAT could answer the question of if a certain minimum level of expression is required to maintain normal body temperature during cold exposure.

Since Ucp1 is activated by fatty acids [183] it is also possible that the diminished lipolytic response in *Angptl4-/-* brown adipocytes resulted in decreased activation of Ucp1. We observed a 22% decrease in the lipolytic response to isoproterenol in isolated brown adipocytes from *Angptl4-/-* mice relative to WT brown adipocytes. Isoproterenol is a synthetic β adrenergic receptor agonist so presumably *Angptl4-/-* brown adipocytes also have an impaired response to other sympathetic nervous system signals that activate β adrenergic receptor, although this remains to be confirmed experimentally. In addition to activating Ucp1 fatty acids also serve as substrates for mitochondrial oxidation and therefore decreased fatty acid availability could limit the substrates available for uncoupling. Again it's difficult to speculate if a reduction BAT lipolysis could result in the temperature differences in

Angptl4-/ animals but as white adipose tissue lipolysis is also known to impaired in these animals [165] and because we failed to observe the cold-induced increase in circulating NEFA it is likely that the availability of fatty acids was diminished in *Angptl4-/-* BAT. A future experiment using a WAT tissue specific knockout of *Angptl4-/-* could help dissociate the contribution of WAT lipolysis to thermogenesis

It has been shown that Elovl3 expression is regulated by norepinephrine (NE) [194] and by glucocorticoids in BAT [195]. Our previous data show that in WAT the lipolytic response to both glucocorticoids and NE is impaired. If *Angptl4-/-* mice cannot fully respond to these stimuli in WAT perhaps their response is compromised in BAT as well which could potentially account for the decrease in expression observed. Future experiments are necessary to determine if glucocorticoid or NE gene regulation is impaired in *Angptl4-/-* BAT.

Whether or not this diminished Elovl3 expression in response to cold could be driving the phenotype we observe in the *Angptl4-/-* animals remains to be explored as well. Elovl3 is a fatty acid elongase involved in the synthesis of C20-C24 saturated and monounsaturated VLCFAs that is expressed principally in liver and brown adipose tissue [196]. Elovl3 has also been implicated in BAT recruitment [195]. This combined with its high expression in BAT and the fact that this expression is strongly induced by cold exposure [197] is why Elovl3 is believed to play a role in thermogenesis, athough the nature of this role has yet to be described. It is possible therefore that the diminished induction of Elovl3 during cold exposure could account for the temperature difference between WT and *Angptl4-/-* mice but further experiments are required to confidently make the connection.

Interestingly we also observed an impairment in the ability of *Angptl4-/-* mice to regulate their body temperature during a fast. While the temperature of WT mice dropped 1.6° by the end of a 24h fast, the body temperature of *Angptl4-/-* mice fell almost 2.5°. A similar, but more dramatic phenotype, has been reported in *PPARα-/-* mice fasted for 24h. The body temperature of these animals decreased more than 9° [190] which is far greater than the difference observed here. Angptl4 is a PPARα target gene so it's not surprising that the phenotype we observed is consistent but weaker than what is seen with *PPARα-/-* mice. What could account for this increased susceptibility to hypothermia during a fast remains to be explored, but it is likely that the role Angptl4 is playing in thermoregulation during fasting is occurring in a tissue other than brown fat as we have seen that Angptl4 expression in BAT does not increase during fasting (data not shown). In the *PPARα-/-* mice plasma ketone bodies and glucose levels were much lower in fasted *PPARα-/-* mice than fasted WTs leading to the conclusion that the fasting-induced hypothermia was because of the lack of fuel available for energy generation. This could be the case in the *Angptl4-/-* mice as well. In our experiments we've observed that KO mice do in fact have significantly lower fasting glucose levels but the decrease is only about 22% compared to fasted WT levels (data not shown), while in *PPARα-/-* mice glucose levels are decreased by more that 50% [190]. Yet the same mechanism could still be at work in our model, just to a lesser degree. Measuring fasting ketone bodies will be an important future experiment to definitively link the decrease in fasting body temperature to defective hepatic lipid metabolism.

The implication of Angptl4 in thermoregulation in distinct physiological situations, fasting and cold exposure, raises the question that thermoregulation could be more generally misregulated in *Angptl4-/-* mice. Future experiments are planned to measure levels of thyroid hormone and evaluate the response of *Angptl4-/-* mice to its administration.

Overall our experiments confirm that Angptl4 participates in adaptive thermogenesis, likely because of its promotion of lipolysis in BAT and WAT. Further research pinpointing the exact nature of the involvement of Angptl4 in these processes could provide insights into the role of Angptl4 in weight gain and its potential as a therapeutic target for obesity.

Conclusions and Future Directions

This project was designed to address how Angptl4 regulates lipid homeostasis. Because the net physiological effects of Angptl4, to mobilize lipids into circulation, are consistent with lipid modulating effects of glucocorticoids, and the fact that Angptl4 is a primary glucocorticoid target gene, we hypothesized that Angptl4 plays an essential role in lipolysis in response to elevated glucocorticoids whether exogenously administered or endogenously elevated during fasting. We used a mouse model in which Angptl4 has been genetically deleted to test this hypothesis. We found that Angptl4 is required for a complete lipolytic response to glucocorticoids and fasting, when circulating glucocorticoids are high. Intriguingly, Angptl4 appears to be required for a maximal catecholamine response on lipolysis as well. We went further to begin initial characterizations of the mechanism by which Angptl4 exerts its lipolytic effects and by using purified human ANGPTL4 we showed that the protein can directly induce lipolysis in murine adipocytes by modulating intracellular cAMP levels.

We also determined that the LPL-inhibitory activity of Angptl4, mediated by the N-terminal coiled-coil domain, is not necessary for its lipolytic activity, which seems to be the result of the C-terminus alone. This dissociation between the LPL-inhibition and the lipolytic effects of Angptl4 has potentially important implications. If adipose tissue lipolysis could be increased without a concurrent increase in circulating TG, overexpression of the C-terminus of Angptl4 could potentially protect against genetic- or diet-induced obesity and may have effects on insulin sensitivity as well. Future experiments to investigate this possibility in rodents would provide useful information as to whether it could be a promising therapeutic approach.

The second component of this thesis investigated the role of Angptl4 in lipogenesis. We found that Angptl4 participates in the glucocorticoid-induced increase in the rates of TG synthesis and *de novo lipogenesis* in liver and WAT. Consistent with these observations, glucocorticoid-induced expression of certain lipogenic genes was significantly compromised in *Angptl4-/-* mice. However, Angptl4 did not stimulate lipogenesis in liver directly. Future studies should further address the mechanism of Angptl4 action in glucocorticoid-regulated lipogenesis.

Because of its expression in brown adipose tissue as well as its ability to induce lipolysis, in the third part of this thesis the role of Angptl4 in adaptive thermogenesis was examined. We found that the body temperature of *Angptl4-/-* mice was consistently lower than WT, decreased by 0.5-1° during cold exposure. We also examined gene expression profiles and cell morphology in BAT and determined that lipolysis was diminished in BAT of *Angptl4-/-* mice and the cold-induced increases in Ucp1 and Elovl3 were attenuated in these animals as well. Notably, *Angptl4-/-* mice also had lower body temperature than WT mice during a prolonged fast. Overall, it appears that Angptl4 is involved in the regulation of adaptive thermogenesis.

One of the central conclusions that can be drawn from these studies is that extracellular Angptl4 treatment can affect intracellular lipolysis. This suggests the presence of an adipocyte cell-surface receptor that mediates the effects of Angptl4. However, no such receptor has been identified. This is an essential future direction for understanding the effects of Angptl4 and perhaps identifying therapeutic possibilities. In keratinocytes, Angptl4 has been shown to bind integrins [96] and our preliminary data, showing FAK phosphorylation is

increased upon Angptl4 treatment, suggests that integrins may be involved in Angptl4 action in adipocytes as well. Experiments are currently underway using integrin antibodies to see if blocking particular integrins can mitigate or abolish the lipolytic effects of Angptl4.

Given its ability to modulate cAMP another possible receptor type for Angptl4 would be a G-protein coupled receptor (GPCR). Utilizing specific GPCR class inhibitors could provide information to help narrow the receptor search. Similarly, site-directed mutagenesis in the C-terminus could reveal regions or residues required for Angptl4 effects, which could generate clues about possible receptor types as well.

Identification of a receptor and downstream signaling processes for Angptl4 is critical, as it would greatly facilitate the testing of potential Angptl4-modulating approaches as a therapeutic strategy. There is pre-clinical evidence to suggest that altering Angptl4 levels may be beneficial. Angptl4 knockout mice are protected from some negative metabolic consequences like GC-induced hepatic steastosis and hyperlipidemia [23]. Additionally, our findings about the role of Angptl4 in lipolysis suggest that blocking Angptl4 could reduce the aberrant lipolysis and subsequent lipotoxicity in metabolic tissues that are serious issues in insulin resistant individuals. These studies suggest that interfering with Angptl4 action in metabolic tissues could be beneficial.

However, there is also data to support that increased Angptl4 expression could have positive effects. Angptl4 deficient mice become obese faster than normal mice [114], likely due to the lack of inhibition on Lpl and reduced lipolysis in WAT. However, it's also possible that Angptl4 can reduce adiposity by exerting its role in thermogenesis. Even if the contribution of Angptl4 to thermogenesis is relatively modest, slight energy imbalances occurring daily can have a profound effect on weight gain and obesity [198]. Yet, administering full length Angptl4 is not ideal, as it will cause hyperlipidemia because of its inhibition of Lpl. Perhaps, administering the C-terminus of Angptl4 could be sufficient for reducing adiposity, as such a protein can still induce adipocyte lipolysis without affecting LPL. But this raises questions about whether this could have consequences for insulin sensitivity because of the elevated free fatty acids in circulation.

This project provides several novel insights that contribute to the growing understanding of the role Angptl4 plays in many important physiological processes. Clearly much more research is needed to fully understand the mechanism of its action and to identify any potential therapeutic targets that may exist.

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