Annexin A1 Regulates Adiposity in Mice

BY

RAND AKASHEH
B.S., Jordan University of Science and Technology, 2009

THESIS

Submitted as partial fulfillment of the requirements
for the degree of Master of Science in Nutrition
in the Graduate College of the
University of Illinois at Chicago, 2012

Chicago, Illinois

Defense Committee:

Giamila Fantuzzi, Chair and Advisor
Zhenyuan Song
Rhonda Kineman

This thesis is especially dedicated to my parents, Talal Akasheh and Lucine Jweinat, from whom I learned the great value of education. Without their patience and love none of this would have been accomplished. It is also dedicated to all my family and friends who make my life meaningful.

ACKNOWLEDGEMENTS

I would like to express my deep gratitude to my thesis committee chair, Professor Giamila Fantuzzi for her constant patience, support, and brilliant guidance in conducting this study. I am extremely grateful to be a member in her lab family, and deeply appreciate having the opportunity and the great honor to work with and learn from such a leading scholar in science.

I also wish to thank my thesis committee members, Professor Rhonda Kineman and Professor Zhenyuan Song, for giving some of their precious time to review my work and provide constructive advice and valuable information.

I would also like to thank all former and present Fantuzzi lab members who have been helpful throughout the process of conducting experiments for my thesis.

At last, I am forever indebted to my family, friends, and teachers, especially those who kindly provided encouragement and advice throughout my academic life.

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LIST OF ABBREVIATIONS

11βHSD1	11-beta Hydroxy-Steroid Dehydrogenase
ACTH	Adrenocorticotropic Hormone or Corticotropin
ALXR	Lipoxin A4 Receptor
ANXA1	Annexin A1
ATGL	Adipose Triglyceride Lipase
AUC	Area Under the Curve
BW	Body Weight
CD68	Cluster of Differentiation 68
CVD	Cardiovascular Disease
DIO	Diet Induced Obesity
DXA	Dual Energy X-ray Absorptiometry
ELISA	Enzyme Linked Immunosorbent Assay
FPR2	Formyl Peptide Receptor-2
GAPDH	Glyceraldehyde-3 Phosphate Dehydrogenase
GC	Glucocorticoid(s)
GTT	Glucose Tolerance Test
HFD	High Fat Diet
HSL	Hormone Sensitive Lipase
IL-1β	Interleukin-1 β
IL-10	Interleukin-10
IL-6	Interleukin-6
ITT	Insulin Tolerance Test

IP	Intraperitoneal
KO	Knockout
LPL	Lipoprotein Lipase
LPS	Lipopolysaccharide
MCP-1	Monocyte Chemotactic Protein-1
PLA2	Phopholipase A2
PPAR-γ	Peroxisome Proliferator Activated Receptor-gamma
qPCR	Quantitative Polymerase Chain Reaction
SAT	Subcutaneous Adipose Tissue
TAG	Triacylglycerol
VAT	Visceral Adipose Tissue
WT	Wild Type

SUMMARY

Annexin A1 (ANXA1) is a glucocorticoid- (GC-) induced protein that mediates at least a part of the anti-inflammatory actions of these hormones. However, it is unknown whether ANXA1 also mediates some of the metabolic functions of GC. The role of this protein in adipose tissue metabolism and inflammation needs to be carefully investigated.

Our aim was to study the effect of ANXA1 deficiency on markers of adiposity in mice by measuring body weight (BW), body composition, and gene expression of metabolic factors in mice fed both chow and high fat diet (HFD). We also aimed at evaluating the effect of ANXA1 deficiency on gene expression of pro- and anti-inflammatory mediators in visceral adipose tissue (VAT).

Annexin A1 Knock-out (KO) mice developed significantly higher adiposity compared to while type (WT) mice. The effect of ANXA1 deficiency on adiposity was independent of food intake, as no significant difference was observed in food intake between any of the mice groups. In addition, we found a significant elevation in the gene expression levels of ANXA1 in WT-HFD mice as compared to WT-chow mice, with no changes in expression of the ANXA1 receptor, formyl peptide receptor-2 (FPR2), between the four groups of mice.

In terms of glycemic control, KO-HFD mice had significantly elevated fasting blood glucose and plasma insulin as compared to KO-chow and WT-HFD mice, while no significant difference was observed comparing WT-HFD to WT-chow mice,. Furthermore, KO mice in both diet groups developed significant insulin resistance compared to both WT groups as evaluated by insulin tolerance test (ITT). Although KO-

HFD mice showed a trend towards glucose intolerance in the glucose tolerance test (GTT), the difference to other groups of mice was not significant.

A trend towards elevated plasma levels of corticosterone in the KO mice on both diets compared to WT mice was observed, which could help explain the increased susceptibility of ANXA1 KO mice to obesity. Additionally, gene expression of 11-beta hydroxy-steroid dehydrogenase (11βHSD1) in VAT was observed in the KO-HFD mice compared to WT-HFD mice, which might have led to increased intracellular activation of GC. However, expression of the hormone sensitive lipase (HSL), adipose triglyceride lipase (ATGL) and of peroxisome proliferator activated receptor-gamma (PPAR-γ) was comparable between the two strains. Furthermore, no significant difference in VAT inflammation was found between KO and WT mice.

In conclusion, in a mouse model of DIO, we show that deletion of ANXA1 leads to metabolic alterations that switch the obesity-resistant phenotype of female BALB/c mice towards increased adiposity and insulin resistance when compared to sex-, age-, and diet-matched WT mice. Further studies are needed to replicate our findings and explain the mechanisms behind the protective roles of ANXA1 against obesity and insulin resistance.

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I. INTRODUCTION

Obesity is currently considered as a global epidemic with a rising prevalence. This pathological condition is a major risk factor for many diseases, including cardiovascular disease (CVD), type 2 diabetes, cancer, infertility, sleep apnea, depression, osteoarthritis, chronic obstructive pulmonary disease, and others (Haslam, 2007). The etiology of obesity can be genetic, environmental, or both (Haslam, 2007). Several research models have been proposed to study factors that are associated with or cause obesity.

Annexin A1 (ANXA1) is a protein induced by GC and known to mediate part of their anti-inflammatory actions (Lim & Pervaiz, 2007). While GC are by far the most potent agents to suppress inflammation and immunity, they are known to promote obesity and the metabolic syndrome, in addition to other side effects (Schacke, Docke, & Asadullah, 2002). Understanding mechanisms behind the side effects of chronic GC exposure might help in establishing recommendations that improve clinical practice. Furthermore, exploring downstream anti-inflammatory agents that mimic the action of GC can help in developing more targeted treatments with less adverse events.

GC have been studied extensively for their effects on metabolism as well as their anti-inflammatory effects (Stahn & Buttgereit, 2008), however, it is unknown whether ANXA1 mediates the metabolic alterations associated with chronic GC use. Modest evidence supports that ANXA1 plays a role in adipose tissue metabolism. We used a mouse model of DIO to compare the outcomes in BALB/c WT mice and ANXA1 KO mice. We hypothesized that ANXA1 deficiency would lead to metabolic alterations and increased inflammation in ANXA1 KO mice.

II. REVIEW OF THE LITERATURE

A. Inflammation

Activation of the immune system is a protective response that occurs in response to disease or insult. The response can be non-specific (Innate Immunity) and/or specific (Adaptive Immunity) (Roitt, Brostoff, & Male, 2001).

Inflammation is a term that describes a non-specific immune reaction through which living organisms respond to injury (ex: bacteria, trauma, wounds, allergens, toxins) and injury-induced damage (removal of dead cells and recovery). In organisms with a vascular component, inflammation involves directing fluids and plasma proteins to the site of injury. Leukocytes, triggered by the synthesis and recruitment of specialized proteins (cytokines, ex: interleukin-1, interleukin-6 (IL-6), interleukin-10 (IL-10), and tumor necrosis factor-α) and lipid derivatives (prostaglandins and leukotrienes) (DeCaterina & Basta, 2001), move from the vasculature to tissues and have the ability to induce and/or resolve the inflammatory process (Ward, 2010).

While inflammation is an essential protective mechanism, an impaired ability to resolve the process can turn an acute inflammatory response into a chronic state of constant irritation, a condition that may emerge from an imbalance in the ratio of pro- to anti-inflammatory mediators (Perretti & Dalli, 2009). Chronic inflammation has been implicated in the pathophysiology of several chronic diseases like CVD, neurodegenerative diseases, autoimmune diseases, allergies, and cancer (Ward, 2010).

B. Adipose tissue and Obesity

Adipose tissue is more than an energy storage site; it is an endocrine organ that secretes various hormones, like leptin, adiponectin, and steroid hormones (Kershaw & Flier, 2004). Additionally, it is able to produce and secrete various cytokines that are major participants in immune modulation and inflammation, like tumor necrosis factor-α and IL-6 (Fantuzzi, 2005). Anatomically, white adipose tissue is mainly located in two depots; subcutaneous adipose tissue (under the skin, SAT) and VAT (inside the peritoneal cavity). When the amount of energy consumed exceeds the amount of energy expended, this results in the expansion of the white adipose tissue due to excessive accumulation of lipids in the lipid droplets of adipocytes (Jo et al., 2009). Adipose tissue expansion mainly involves increased adipocyte size (hypertrophy) due to excess lipid production and storage through lipogenesis and decreased lipolysis, but can also result from increased adipocyte count (hyperplasia) through adipogenesis (Jo et al., 2009).

Obesity in adults is defined by the World Health Organization as having a body mass index of 30 Kg/m^2 or higher. A body mass index at the 97^{th} percentile or greater is the criterion for childhood obesity. Obesity is currently considered a global epidemic: although it was previously thought to be exclusive to high-income regions, it is currently understood that increases in the prevalence of obesity since 1980 took place also in low and middle income regions (Ahima, 2011). In 2008, the World Health Organization estimated the prevalence of obesity to affect 10% of adults all over the world (200 million men and 300 million women). Forty three million children under the age of 5 are overweight (90^{th} - 97^{th} percentile) (Ahima, 2011). According to The National Health and

Nutrition Examination Survey obesity report of 2009-2010, obesity prevalence in the United States reached 16.9% of children and adolescents, and more than 35% of the adult population (American Heart Association, 2012).

Obesity is a major risk factor for many diseases, including CVD, type 2 diabetes, cancer, infertility, sleep apnea, depression, osteoarthritis, chronic obstructive pulmonary disease, and others (Haslam, 2007). Abdominal obesity, characterized by an increase in VAT, is associated with increased risk of chronic diseases, while SAT is less detrimental, possibly because it draws lipid accumulation away from metabolically sensitive organs like the liver and the pancreas. Higher macrophage infiltration, increased inflammatory cytokine expression, and decreased adiponectin expression is observed in VAT compared to SAT (De Heredia, Gomez-Martinez, & Marcos, 2011). Moreover, obesity is associated with increased leptin expression and plasma levels. Plasma leptin has been used as a marker of adiposity, especially in rodents (Jequier, 2002).

C. Regulation of Lipid Metabolism

Lipids are mainly stored in adipose tissue in the form of triacylglycerols or triglycerides (TAG). Lipid accumulation in adipocytes revolves around two main mechanisms: the first is lipolysis, which is the process of lipid breakdown to release fatty acids and use them to liberate energy through beta-oxidation (catabolism); the second is lipogenesis, which leads to the synthesis of TAG from lipid and non-lipid precursors (anabolism). Examples of enzymes central to these processes are fatty acid synthase, lipoprotein lipase (LPL), ATGL, HSL, monoacylglycerol lipase, and many more, each having its specific function (Lampidonis, Rogdakis, Voutsinas, & Stravopodis, 2011;

Zechner et al., 2012). The imbalance between lipolysis and lipogenesis has been studied extensively for its implications in the etiology of obesity.

Various hormones regulate lipid metabolism. Insulin is an anabolic hormone that is secreted in response to feeding; it favors lipogenesis and inhibits lipolysis (Chernick & Chaikoff, 1950; Froesch, Bürgi, Bally, & Labhart, 1965), while other counter-regulatory hormones like glucagon, catecholamines, and GC are secreted during fasting and starvation to promote lipolysis and provide energy (Slavin, Ong, & Kern, 1994). An imbalance in the secretion and/or signaling of these hormones can alter metabolism. In pathological conditions like obesity and type 2 diabetes, cells become resistant to insulin, which delays glucose clearance from the blood and forces the pancreas to secrete massive amounts of insulin, favoring lipid accumulation and further exacerbating obesity along with a mild increase in blood glucose. Thereafter, when the pancreas can no longer compensate for the cells' poor response to insulin, counter regulatory hormones dominate to favor catabolism and worsen hyperglycemia (Porte, 1999).

One of the most important pathways that regulate lipolysis is the beta-adrenergic pathway (Peckett, Wright, & Riddell, 2011). Catecholamines stimulate lipolysis through binding to beta-adrenergic receptors, which stimulates cyclic Adenosine Mono-Phosphate (cAMP), leading to the activation of protein kinase A, which in turn causes the phosphorylation of perilipin and HSL; thereafter, ATGL starts TAG hydrolysis by releasing one fatty acid molecule from the glycerol backbone. Then, HSL cleaves the second fatty acid, and monoacylglycerol lipase cleaves the last to liberate glycerol and the third fatty acid. Notably, insulin exerts an anti-lipolytic effect by increasing

phosphodiesterase 3B levels, which reduces the levels of cAMP available to activate protein kinase A (Manganiello & Vaughan, 1973).

Another important enzyme in lipid metabolism is LPL. This enzyme cleaves TAG within circulating very low density lipoproteins and chylomicrons, causing the release, uptake, and storage of free fatty acids and glycerol by surrounding cells. LPL is elevated in the fed state (Oliver & Rogers, 1993).

D. Glucocorticoids

Glucocorticoids are cholesterol-derived steroid hormones that, among several other functions, play a major role in resolving inflammation. They are secreted by the adrenal cortex when the Hypothalamic-Pituitary-Adrenal axis is activated in response to physiological or psychological stress. Hypothalamic corticotropin releasing hormone is released to stimulate the secretion of Adrenocorticotropic Hormone (ACTH) by the anterior pituitary gland, which stimulates the production and secretion of active cortisol or corticosterone in humans or mice, respectively, into the blood stream to be distributed to various tissues (Rhen & Cidlowski, 2005). Inside the cytoplasm, the active hormone can be deactivated into cortisone or 11-dehydrocorticosterone in humans or mice, respectively, by the enzyme 11-beta hydroxysteroid dehydrogenase-2, a reaction that can be reversed by the enzyme 11βHSD1 (Rhen & Cidlowski, 2005).

It has been proposed that GC modulate approximately 1% of the genome (Perretti & D'Aquisto, 2009). When GC bind to their receptor, the complex translocates to the nucleus where it binds to transcription factors, therefore inhibiting the synthesis of inflammatory proteins (transrepression), while the complex can bind to GC-response

elements, which stimulates the transcription and synthesis of anti-inflammatory proteins (transactivation).

E. Glucocorticoids and Lipid Metabolism

Glucocorticoids have been studied extensively for their effects on lipid metabolism and adiposity. Along with other stress hormones such as the catecholamines epinephrine and norepinephrine, and some cases growth hormone they are considered as key regulators of energy mobilization within adipocytes (Fain & Saperstein, 1970; Fain, Dodd, & Novak, 1971). These hormones have been widely studied for their contribution to excess adiposity and altering food intake (Peckett, Wright, & Riddell, 2011).

Conflicting data have been reported on the role of GC in regulating lipid metabolism, as many studies support a prolipolytic role, while many others report the opposite.

Pro-lipolytic evidence: Through genomic effects, GC increase the expression and transcription of ATGL and HSL in differentiating and primary adipocytes (Fain & Saperstein, 1970; Slavin, Ong, & Kern, 1994; Xu et al., 2009). Recently, a more direct non-genomic effect was also shown in primary rat adipocytes, as dexamethasone (a type of GC) lead to a time- and dose-dependent increase in lipolysis, an effect that was muted when the GC receptor antagonist RU486 was added (Xu et al., 2009). In vivo, dexamethasone injections lead to increased lipolysis, evidenced by raised plasma levels of free fatty acids in fed and fasted rats. It is worth noting that increased levels of circulating free fatty acids are known to induce insulin resistance (Arner, 2002), one of the major side effects of chronic GCs exposure.

Anti-lipolytic, lipogenic, and adipogenic evidence: In vivo starvation-refeeding studies on rats reported increased de novo lipogenesis (synthesis of lipids in the liver from non-lipid sources like glucose) upon exposure to GC (Berdanier, 1989), leading to increased production of very low density lipoprotein by the liver and increased lipid delivery to adipose tissue for storage (Bagdade, Yee, Albers, & Pykalisto, 1976). Moreover, LPL is increased by GC at the pre- and post-transcriptional level (Appel & Fried, 1992; Fried, Russell, Grauso, & Brolin, 1993; Ottosson, Vikman-Adolfsson, Enerback, Olivecrona, Bjorntorp, 1994). This effect favors the accumulation of lipids in VAT rather than SAT (Fried, Bunkin, & Greenberg, 1998).

Glucocorticoids can also potentiate the action of insulin, therefore strengthening its lipogenic action (Wang et al., 2004). The clearest evidence on the pro-lipogenic effects of GC is the adipocyte hypertrophy in patients Cushing's syndrome; a condition associated with increased circulating cortisol levels (Rebuffe-Scrive, Krotkiewski, Elfverson, & Bjorntorp, 1988). In rodents exposed to GC, adipocyte hypertrophy also developed (Rebuffe-Scrive, Walsh, McEwen, & Rodin, 1992). Moreover, in the study by Xu and colleagues (2009), despite the pro-lipolytic findings, dexamethasone was not able to cause an interaction between HSL and lipid droplets, an effect that is ultimately needed for lipolysis to occur.

The adipogenic action of GC is also supported by evidence that these hormones are necessary for adipogenesis to occur, as they stimulate preadipocytes to differentiate into mature adipocytes (Bujalska, Kumar, Hewison, & Stewart, 1999; Hauner et al., 1989; Pantoja, Huff, & Yamamoto, 2008), which explains why adipose tissue expresses the 11βHSD1 enzyme that is needed to increase the availability of active corticosterone

for adipocytes to mature (Bujalska et al., 1999). A selective inhibitor to this enzyme prevents human adipogenesis in vitro (Bujalska et al., 2008). Furthermore, corticosterone pellet implantation in male rats resulted in increased VAT but not SAT, with depots containing a greater number of smaller adipocytes (Campbell, Peckett, D'souza, Hawke, & Riddell, 2011).

Stress leads to increased levels of GC, which appear to favor accumulation of adipose tissue. In a meta-analysis of longitudinal studies, psychosocial stress that causes elevations in GC levels was considered a significant risk factor for developing obesity (Van Jaarsveld, Fidler, Steptoe, Boniface, & Wardle, 2009). GC secretion is also very active in fasting states (Dallman, 1999), leading to increased levels of neuropeptide Y, which increases food intake, causes humans and even rodents to choose high energy foods, and favors energy to be stored as fat (Dallman, Pecoraro, & la Fleur, 2005; Epel, Lapidus, McEwen, & Brownell, 2001; Strack, Sebastian, Schwartz, & Dallman, 1995).

Finally, a recent *in vitro* study by Campbell and colleagues (2011) provided evidence that the effect of GC is dose-dependent; meaning that lipolysis is maximized when corticosterone concentration is at the minimal sufficient levels of 1-10 μmol/L. After exceeding this dose, lipolysis starts to gradually decline, until it gets significantly suppressed at GC concentration of above 100 μmol/L.

In conclusion, evidence on the effect of GC in modulation of lipolysis is conflicting, possibly due to differential effects of GC on lipid metabolism depending on the dose, time of exposure as well as presence of additional factors modulating metabolism, such as fasting/feeding, stress, etc.

F. Annexin A1

Annexin A1 is a GC-induced protein with potent anti-inflammatory effects. It was first isolated by Flower and Blackwell in 1979 as a protein that modulates the anti-phopholipase 2 (PLA2) effect of GC (Erasfa & Russo-Marie, 1989; Flower & Blackwell, 1979). This 37-kDa protein is the first identified member of the annexins superfamily of proteins that have the ability to bind (to annex) to phospholipids in cell membranes in a calcium-dependent manner (Blackwell et al., 1980; Crumpton & Dedman, 1990; Geisow, 1986; Geisow, Walker, Boustead, & Taylor, 1987). Annexin A1 is formed by 346 amino acids, encoded by the *Anxa1* gene located on chromosome 19q24 in mice (Horlick, Cheng, Wong, Wakeland, & Nick1991; Lim & Pervaiz, 2007; Raynal & Pollard, 1994) and chromosome 9p13-q21 in humans (Lindgren, Nilsson, Orho-Melander, Almgren, & Groop, 2001). It was originally named macrocortin (Blackwell et al., 1980), later renocortin (Rothhut, Russo-Marie, Wood, DiRosa, &Flower, 1983), lipomodulin (Hirata et al., 1981), lipocortin-1 (Di Rosa, Flower, Hirata, Parente, & Russo Marie, 1984), and Annexin A1 (Geisow, 1986).

Annexin A1 is widely expressed in cells of the endocrine system: in the median eminence of the hypothalamus (expressed by ependymal and endothelial cells), folliculostellate cells of the pituitary gland (John et al., 2004) pancreas, testes, ovaries, thyroid, and adrenals. Annexin A1 is also expressed by endothelial vascular cells and pericytes in the brain (Solito et al., 2008). Cells of the immune system like neutrophils, monocytes, and macrophages contain high levels of ANXA1 (Lim & Pervaiz, 2007). Annexin A1 is also detectable in stromal cells, thymus, placenta, lungs, bone marrow, intestine, and biological fluids (Lim & Pervaiz, 2007; Perretti & Dalli, 2009), with the

highest concentration in seminal fluid (150 µg/ml) (Christmas, Callaway, Fallon, Jones, & Haigler, 1991).

G. The Annexin A1 Receptor

In humans, ANXA1 signals through binding to FPR2, which is also known as Lipoxin A4 receptor (ALXR), that also binds serum amyloid A (a positive acute-phase protein), (John, Gavins, Buss, Cover, & Buckingham, 2008; Migeotte, Communi, & Parmentier, 2006; Perretti & D'aquisto, 2009). Human ALXR belongs to a family of G-coupled protein receptors that includes FPR1, ALXR (FRP2), and FPR3. In rodents, although the FPR receptor family is more complex, FPR2 is still considered the putative ANXA1 receptor.

H. Annexin A1 and Glucocorticoids

Annexin A1 and its receptor FPR2 are induced by GC through genomic effects (Damazo, Yona, Flower, Perreti, & Oliani, 2006; Hannon, 2003; Sawmynaden & Perretti, 2006). However, the exact underlying mechanisms are yet to be understood. Moreover, a more rapid non genetic effect of GC includes ANXA1 mobilization and secretion by target cells (Perretti & D'Acquisto, 2009), a process that is cell-specific. Glucocorticoids and ANXA1 share common effects on leukocytes and pathways related to inflammation, which has provided evidence that the action of GC is at least in part mediated by ANXA1. While GC are by far the most potent anti-inflammatory and immune suppressive drugs, many adverse outcomes are associated with their chronic use, including insulin resistance, diabetes, dyslipidemia, osteoporosis, impaired growth, and increased risk of infections (Schacke, Docke, & Asadullah, 2002). Therefore,

exploring downstream anti-inflammatory agents that mimic the action of GC can help in developing more targeted treatments with less adverse events.

I. Annexin A1 and Innate Immunity

Annexin A1 exerts potent anti-inflammatory activities. It was first identified as an anti-PLA2 protein (Erasfa & Russo-Marie, 1989; Flower & Blackwell, 1979), an enzyme that causes the release of arachidonic acid from lipids to make it available for prostaglandin production (Diaz & Arm, 2003). It is now known that ANXA1 inhibits this enzyme by direct interaction (Kim, Kim, Park, Kim, & Na, 1994). Annexin A1 also mediates the inhibitory effect of GC on the enzyme cycloxygenase-2, thus blocking this enzyme's inflammatory, pyretic, and hyperalgesic effect (Ferreira et al., 1997; Lim & Pervaiz, 2007). Annexin A1 also inhibits neutrophil trafficking to sites of inflammation, and causes detachment of neutrophils upon the activation of the mitogen activated protein kinase signaling pathway (Perretti & D'Acquisto, 2009; Perretti at al., 1996), and also induces neutrophil apoptosis (Perretti & Solito, 2004). Finally, ANXA1 also triggers macrophages to phagocytose apoptotic cells (Perretti & Flower, 2004; Parente & Solito, 2004).

J. Annexin A1 and Adaptive Immunity

A study by Goulding and colleagues (1990) found lower levels of ANXA1 mRNA and protein in T-cells as compared to myelocytes (neutrophils, basophils, eosinophils). However the expression of ANXA1 and FPR2 increases once the cells get activated, which causes the activation of the mitogen activated protein kinase pathway.

In contrast with what happens in cells of the innate immune system, treatment of murine and human T-cells with GC caused a significant reduction in ANXA1 mRNA and

protein levels in a model of rheumatoid arthritis (D'Acquisto et al., 2008). In fact, ANXA1 seems to exert activating, rather than suppressive, effects in T cells, leading to cell proliferation (D'Acquisto et al., 2007), Glucocorticoids have inhibitory effects on T cell proliferation and activation (Perretti & D'Acquisto, 2009), in contrast with the observation that ANXA1 stimulates T cell proliferation. These findings support the notion that GC suppress ANXA1 in T cells in order to counteract the activating effects of ANXA1 in these cells.

K. Annexin A1 in Metabolism and Obesity

Data on the role of ANXA1 in modulating metabolism and obesity are scant and at times controversial. Studies evaluating differentiation of murine 3T3-L1 fibroblasts into mature adipocytes indicated a gradual decline in ANXA1 mRNA and protein levels during the process when 1-methyl-3-isobutylxanthine was added as a factor to accelerate adipocyte maturation. The decline in ANXA1 levels occurred despite the continued presence of the GC dexamethasone (Wong, Nick, & Frost, 1992). This study proposed that the reduction in ANXA1, the anti-PLA2 protein, led to increased arachidonic acid release, an effect that is needed in this situation for adipocytes to differentiate. According to this hypothesis, the decline in ANXA1 levels during adipocyte differentiation would be necessary to accelerate adipogenesis. In agreement with these results, ANXA1 protein levels were significantly higher in the stromal vascular compartment as compared to adipocytes of WT C57BL6 adult male mice (Warne et al., 2006). However, in a human adipocyte cell line, ANXA1 expression and protein levels were higher in mature adipocytes compared to pre-adipocytes (Kosicka et al., 2012),

thus, the cellular source of ANXA1 in adipose tissue may be species-specific, although experiments directly comparing the murine versus human tissue would be necessary.

A significant reduction in epididymal fat pad mass, both in grams and as percentage of BW, was observed in C57BL6 ANXA1 KO mice (Warne et al., 2006). However, this did not result in any significant differences in BW between WT and ANXA1 KO mice. Additionally, no difference in mean adipocyte area was observed (Warne et al., 2006). Unexpectedly, when these fat pads were cultured, a significant suppression in catecholamine- or lipopolysaccharide- (LPS) induced lipolysis was observed in those that belong to ANXA1 KO mice when compared to WT mice. The results of the study by Warne and colleagues in 2006 show a conflicting role for ANXA1 in modulating adiposity in mice; despite suppressed lipolysis in ANXA1 KO mice, their phenotype shows reduced fat pad mass.

In humans, plasma ANXA1 levels were inversely correlated with markers of adiposity like BMI, body fat mass, waist-to-hip ratio, and leptin levels. Plasma ANXA1 was also inversely correlated with plasma C-reactive protein levels. These data suggest that reduced plasma ANXA1 might lead to the systemic inflammation that is associated with obesity (Kosicka, et al., 2012).

At the hormonal level, ANXA1 increased in vitro pancreatic secretion of insulin in rats (Hong, Won, Yoo, Auh, & Park, 2002; Ohnishi et al., 1995), but surprisingly, ANXA1 KO mice had significantly higher insulin levels as compared to WT mice (Warne et al., 2006). However, a study evaluating if ANXA1 variants and single nucleotide polymorphisms correlate with type 2 diabetes concluded that variants in ANXA1 gene

were unlikely to be associated with increased risk in developing type 2 diabetes (Lindgren et al., 2001).

In conclusion, the role that ANXA1 plays in lipid metabolism and adiposity is yet to be understood. Among other depots, VAT is the tissue most associated with systemic inflammation and metabolic syndrome, yet it has not been carefully investigated in ANXA1 KO mice. Therefore, more accurate measures can be taken to evaluate adiposity and VAT inflammation in these mice.

L. Mouse Models of DIO

Various factors are known to alter the sensitivity of mice to high fat DIO, including the age, sex, and strain of the mouse. Among various mouse strains, female BALB/c mice are known to be the most resistant to DIO and the insulin resistance that is associated with it, as opposed to male C57BL6 mice that are known to be the most sensitive to DIO (Nishikawa, Yasoshima, Doi, Nakayama, & Uetsuka, 2007). Therefore, these factors have to be considered when choosing a mouse model of DIO. As evidence shows muted lipolysis in response to catecholamines and LPS in C57BL6 ANXA1 KO mice (Warne et al., 2006), we hypothesized that ANXA1 has a pro-lipolytic effect. To test this, we chose the least obesity prone mice, female BALB/c, to prove that the deficiency of this protein would significantly alter their phenotype towards increased adiposity and insulin resistance.

III. AIMS AND HYPOTHESES

A. Rationale

Although ANXA1 is an important mediator of GC action and exerts potent anti-inflammatory activities, the role of ANXA1 in modulating adiposity and inflammatory markers in adipose tissue has not been carefully investigated.

B. Specific Aims and Hypotheses

The main objective of the study is to determine if ANXA1 deficiency results in alterations in the inflammatory response and adiposity in mice.

Specific aim 1:

To investigate the effect of ANXA1 on adiposity by comparing WT and ANXA1 KO mice

Hypothesis 1:

Because catecholamine- and LPS-induced lipolysis was suppressed in epididymal fat pad of ANXA1 KO mice, we hypothesize that ANXA1 would have a pro-lipolytic action. Hence, ANXA1 KO mice would have increased adiposity compared to WT mice.

Specific Aim 2:

To study the effect of ANXA1 on VAT inflammation by comparing WT and ANXA1 KO mice.

Hypothesis 2:

Because ANXA1 is an anti-inflammatory protein, we hypothesize that ANXA1 KO mice would have more inflammation in VAT compared to WT mice.

IV. Materials and Methods

A. Animals and Housing

This study was approved by the Animal Care and Use Committee at the University of Illinois at Chicago. Adult female (18 weeks) ANXA1 KO mice and age-matched WT BALB/c female mice were housed in the Biological Resource Building at the University of Illinois at Chicago. Breeding pairs of ANXA1 KO mice were a kind gift of Dr. Asma Nusrat (Emory University, Atlanta, GA). BALB/c WT mice were purchased from the Jackson Laboratory (Bar Harbor, ME).

B. Diet Induced Obesity (DIO) Model

Mice from each genotype were randomly assigned to either chow diet or HFD (60% of calories from fat, by Research Diets), with n = 14 - 20 in each diet group. Water and food were available *ad libitum.* Mice were weighed weekly, and food intake was measured daily over a course of 6 days. After 14 weeks of the diet, mice were anaesthetized using isoflurane; retro-orbital blood was collected in EDTA treated tubes. Mice were sacrificed by cervical dislocation.

C. Glucose Tolerance Test (GTT)

Five-hour fasting blood glucose was measured at time 0, then mice received intra-peritoneal (IP) injections of 1 gram of glucose per Kg of BW. Blood glucose was measured from the tail at time 15, 30, 60, and 90 minutes. Area under the curve (AUC) was measured for each mouse, then mean AUC were calculated per mouse group for comparison and statistical analysis.

D. Insulin Tolerance Test (ITT)

Blood glucose of fed mice was measured at time 0, then mice received IP injections of 1 unit of insulin per Kg of BW. Blood glucose was measured from the tail at time 15, 30, 60, 90, and 120 minutes. Data is reported as percentage of change in blood glucose to baseline (time 0). AUC was measured for each mouse, then mean AUC were calculated per mouse group for comparison and statistical analysis.

E. Evaluation of Adiposity

Dual-energy X-ray Absorptiometry (DXA) was used to quantify body composition of mice immediately after they were sacrificed. The whole body was included in the analysis region, except for the head and the tail.

F. Plasma Preparation

Blood collected in EDTA treated tubes was centrifuged at 3000 rpm for 15 minutes at 4°C. Supernatants were transferred into microcentrifuge tubes and centrifuged again for 5 minutes before plasma was transferred into new microcentrifuge tubes and frozen at -80°C until use.

G. Measurement of Circulating Mediators.

Enzyme Linked Immunosorbent Assay (ELISA) kits from R&D Systems were used to quantify plasma levels of leptin (sensitivity = 125 pg/ml). Insulin was measured using an ELISA kit from Alpco (sensitivity = 188 ng/mL), and corticosterone was measured using a kit from Cayman Chemicals (sensitivity: 16.4 pg/mL).

H. RNA Extraction

Total RNA was extracted using the TRIzol method. Pieces of VAT were homogenized in TRIzol with a bead-beater, then centrifuged at 12,000 rpm for 10

minutes. Supernatants were collected and transferred into microcentrifuge tubes containing 200 µL of chloroform, then centrifuged again for 15 minutes. Top clear layers of supernatants were transferred into new microcentrifuge tubes containing 500 µL of isopropanol, tubes were inverted to mix and allowed to sit at room temperature for 30 minutes. Supernatants were discarded, and 100 µL of 75% ethanol was added to wash each RNA pellet by centrifuging for 5 minutes. Ethanol was removed and pellets were air-dried for no more than 10 minutes. Then 50 µL of RNA storage solution (Qiagen) was added to dissolve the pellets. Samples were then heated at 60°C for 5 minutes.

I. RNA Quantification and Normalization

RNA concentration was quantified using a spectrophotometer (Nanodrop 2000 by ThermoScientific) at an absorbance of 260 nm. Samples were then normalized using RNAse-free water to the lowest concentration measured (200 µg/mL).

J. Reverse Transcription Quantitative Polymerase Chain Reaction (qPCR)

Total RNA was reverse-transcribed to generate complementary DNA strands (cDNA). To quantify gene expression, qPCR was performed using probes for TaqMan system to measure ANXA1, cluster of differentiation (CD68), FPR2, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), interleukin-1β (IL-1β), IL-6, IL10, Leptin, monocyte chemotactic protein-1 (MCP-1), and peroxisome proliferator-activated receptor-gamma (PPAR-γ), (Applied Biosystems). Forward and reverse primers were used for quantification of ATGL, β-Actin, 11βHSD1, and HSL (IDT-DNA Technology) fusing the SYBR Green system. Target gene expression values were normalized to a reference gene, β-Actin or GAPDH, and analyzed using the Comparative CT method:

$$\frac{X_{test}}{X_{control}} = 2^{\Delta\Delta C_T} = 2^{\left(C_{T,X} - C_{T,R}\right)_{control} - \left(C_{T,X} - C_{T,R}\right)_{test}}$$

K. Statistical Analysis

Statistical significance was defined as P-value of less than .05. Two-Way Analysis of Variance (ANOVA) and Student t-test were used to test for significance, using MedCalc software (Mariakerke, Belgium).

V. RESULTS

A. Increased Adiposity in ANXA1 KO Mice on HFD

We hypothesized that ANXA1 deficiency would cause increased adiposity in ANXA1 KO mice. Body weight, food intake, body composition, plasma leptin, and VAT gene expression were used to evaluate adiposity.

Both WT and ANXA1 KO mice on HFD showed significantly higher BW as compared to WT and KO groups on chow. No significant difference was observed when comparing WT to KO mice on chow (Figure 1A). However, the weight of ANXA1 KO mice on HFD was significantly higher compared to that of WT mice on HFD mice (Figure 1A).

In order to test if differences in food intake could explain the increased BW in ANXA1 KO mice, food was weighed daily over a course of 6 days in all four groups. No significant difference in food intake was observed between any of the groups (Figure 1B).

Quantification of body composition by DXA indicated that fat mass in grams was significantly higher in the HFD groups as compared to the chow groups, and also significantly higher in KO-HFD mice compared to WT-HFD mice (Figure 1C). When expressed as percentage of BW, fat mass was also significantly higher in the HFD groups as compared to the chow groups. Using this parameter, ANXA1 KO mice had a significantly higher percentage of body fat as compared to the WT mice in both the chow and HFD groups (Figure 1D).

Circulating levels of leptin positively correlate with adipose tissue mass (Jequier, 2002). As further confirmation of increased adiposity in ANXA1 KO mice compared to

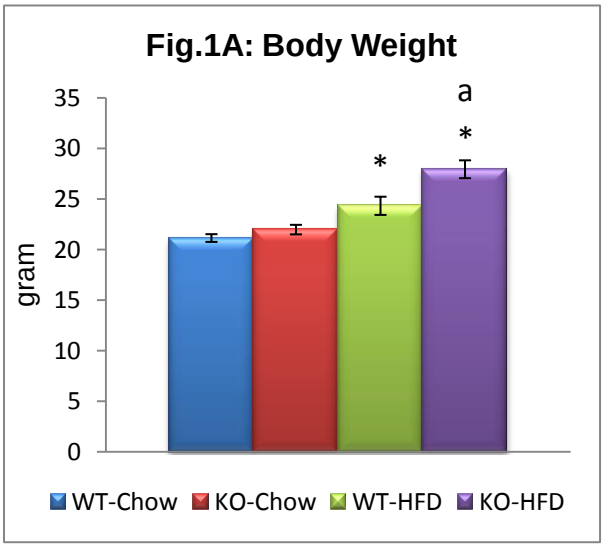
WT mice, we measured plasma leptin by ELISA (Figure 1E). As expected, mice on HFD had significantly higher plasma leptin as compared to mice on chow. In agreement with DXA results, KO-HFD mice had significantly higher plasma leptin as compared to WT-HFD mice. Additionally, KO-chow mice showed a trend towards increased leptin levels compared to WT-chow mice, but this was not statistically significant.

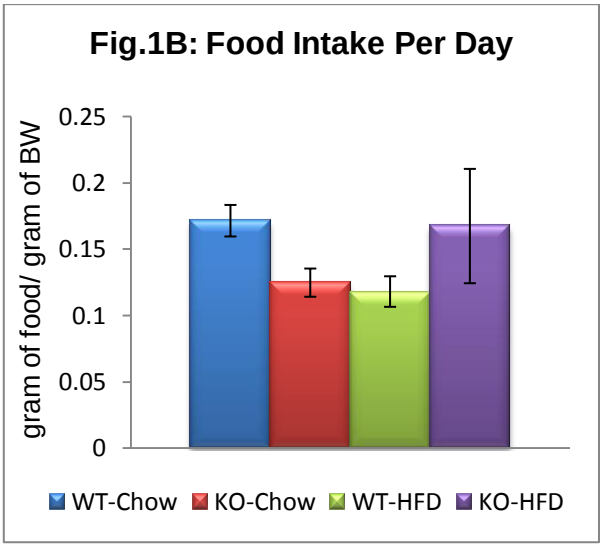
To test if increased lean mass contributed to increased BW in KO-HFD mice, data from DXA scans were used. No significant difference was observed in lean mass in grams between any of the groups (Figure 1F). However, as expected, when expressed in percentage, KO-HFD mice showed significantly lower percentage of lean mass when compared to both KO-chow and WT-HFD mice (Figure 1G), but no significant difference was observed in comparing WT-HFD to WT-chow or in comparing KO-chow to WT-chow (Figure 1G).

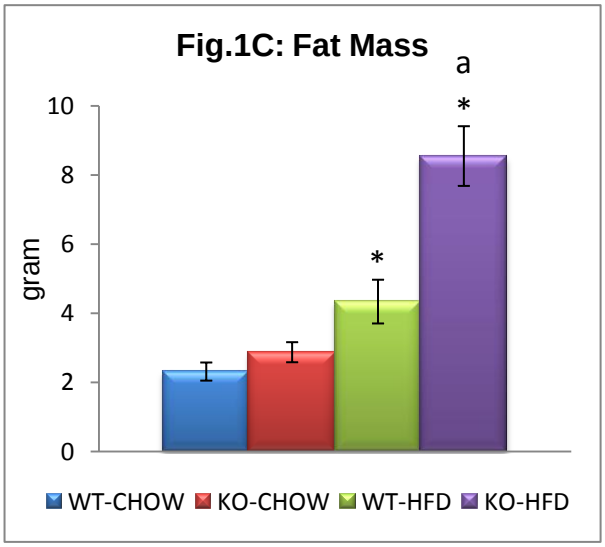
Expression of various markers of lipid metabolism and adiposity was measured in VAT of fed mice using qPCR (Figure 1H). No difference was observed for expression of PPAR-γ in VAT between any of the groups. However, a trend towards elevated PPAR-γ expression was observed in VAT of KO-HFD mice as compared to WT-HFD mice. Leptin expression was significantly elevated in the HFD mice as compared to their strain-matched mice on chow. Expression of ATGL and HSL showed no statistical significance between groups; however, a trend towards higher expression of these enzymes was observed in KO mice when compared to the WT mice.

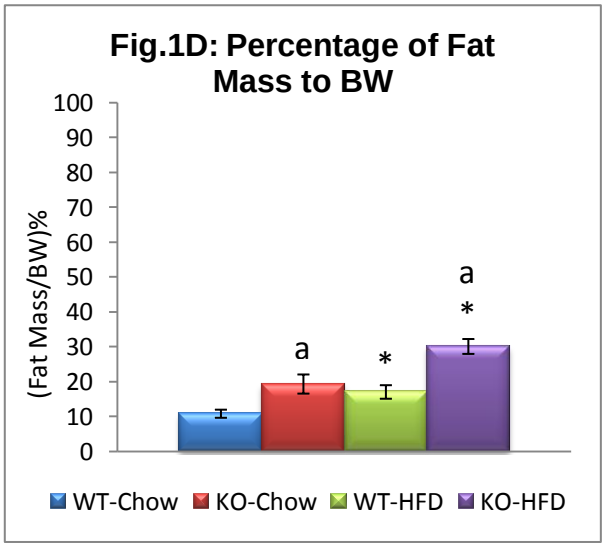
Taken together, these data indicate the presence of increased adiposity in ANXA1 KO mice compared to WT mice, particularly when mice were fed HFD. This

effect does not seem to be influenced by differences in food intake or changes in lean mass.









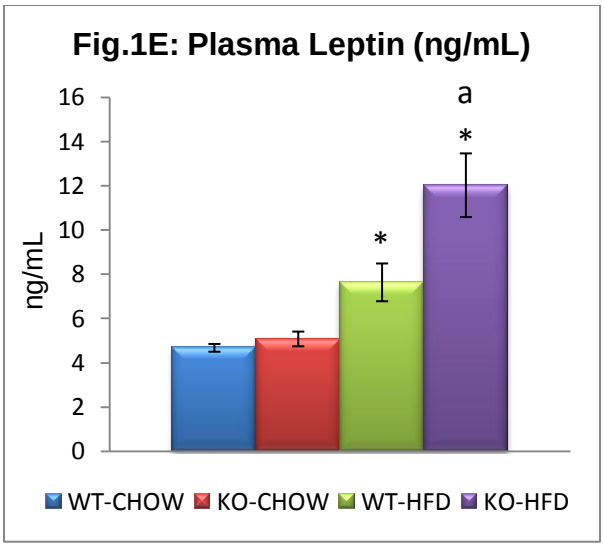


Fig.1E: Plasma Leptin (ng/mL)
ng/mL
16
14
12
10
8
6
4
2
0
a
*
*
WT-CHOW
KO-CHOW
WT-HFD
KO-HFD

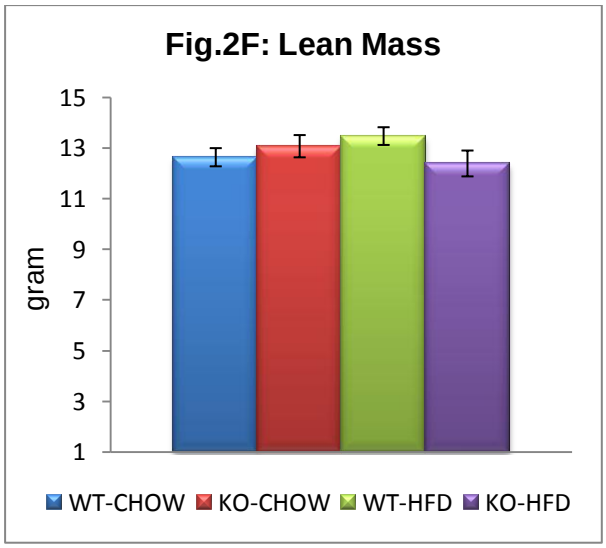


Fig.2F: Lean Mass
gram
15
13
11
9
7
5
3
1
WT-CHOW
KO-CHOW
WT-HFD
KO-HFD

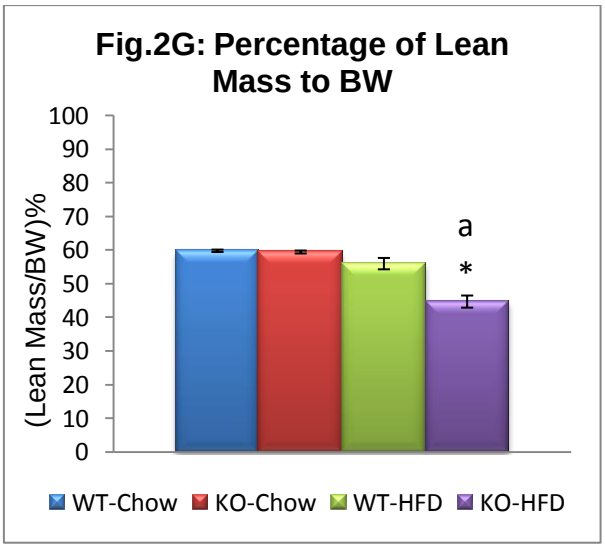


Fig.2G: Percentage of Lean Mass to BW
(Lean Mass/BW)%
100
90
80
70
60
50
40
30
20
10
0
a
*
WT-Chow
KO-Chow
WT-HFD
KO-HFD

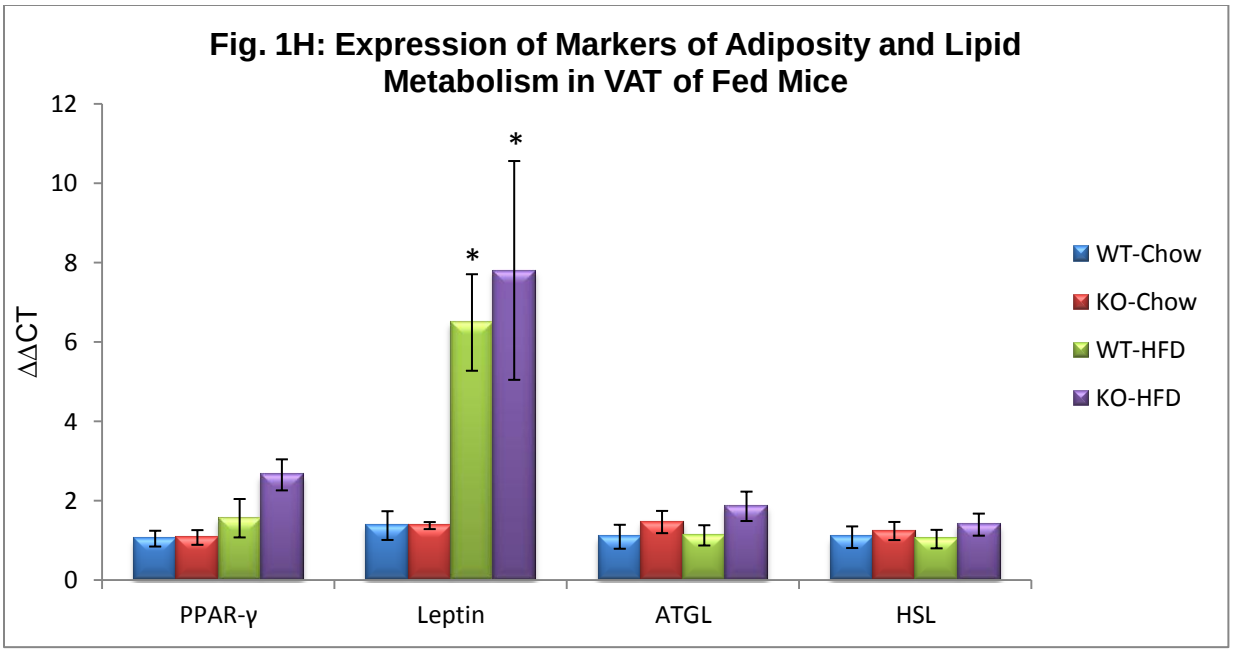


Figure 1: Measures of Adiposity and Lipid Metabolism. (A) Body weight in grams, (n = 14 - 20). (B) Average grams of food consumed in 1 day per average grams of BW in a group (n = 4 - 5 mouse per group/cage), measured for 6 days. (C) Fat mass in grams measured by DXA. (D) Percentage of fat mass to BW, (n = 14 - 19). (E) Plasma leptin measured by ELISA in ng/mL, (n = 13 - 17). (F) Lean mass in grams measured by DXA. (G) Percentage of lean mass to BW, (n = 14 - 19). (H) Relative gene expression of markers of adiposity (leptin, normalized to GAPDH), and lipid metabolism (PPAR-γ normalized to GAPDH, ATGL and HSL normalized to β-Actin), (n = 4 - 5). Data represents mean ± SE per group (n). (*P<.05: HFD group vs strain-matched chow group, aP<.05: KO group vs diet-matched WT group).

B. <u>Increased ANXA1 Gene Expression in VAT of WT Mice on HFD.</u>

Data in Figure 1 suggest that ANXA1 may have a protective role against obesity in mice. Therefore, we hypothesized that ANXA1 and its putative receptor (FPR2) would be upregulated in response to HFD feeding. Therefore, we measured mRNA expression of ANXA1 in the VAT of WT mice on chow or HFD, and FPR2 expression in the VAT of WT and KO mice on both diets. Data demonstrated a significant increase in the expression of ANXA1 in the WT-HFD group as compared to chow-fed WT mice. Data

showed no significant difference in the expression of FPR2 expression between groups, (Figure 1). We conclude that ANXA1 is upregulated in obesity. However, changes in diet or ANXA1 deficiency do not influence FPR2 expression in VAT.

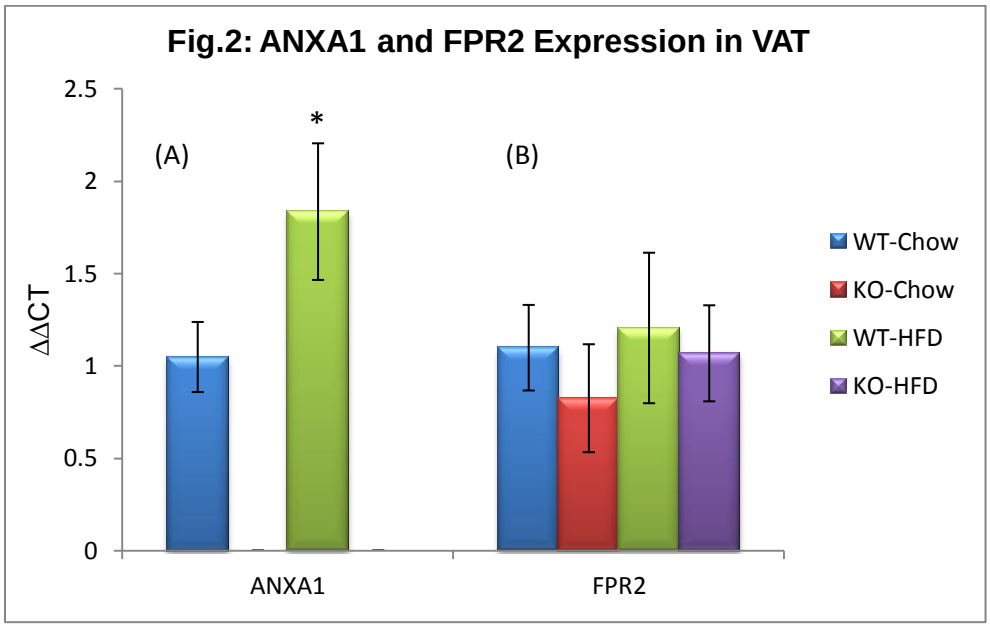


Figure 2: Relative Gene Expression of ANXA1 and FPR2 in VAT.

(A) Gene expression of ANXA1 in VAT using qPCR normalized to GAPDH. (B) Gene expression levels of FPR2 using qPCR normalized to GAPDH. Data represents mean ± SE per group (n = 4 - 5). (*P<.05: HFD group vs strain-matched chow group, aP<.05: KO group vs diet-matched WT group).

C. Altered Glycemic Control in ANXA1 KO Mice.

Because ANXA1 KO mice had increased adiposity, we hypothesized that these mice would have altered glycemic control. After 8 hours of fasting, ANXA1 KO mice on HFD had significantly higher blood glucose and plasma insulin levels compared to KO-chow mice and WT-HFD mice (Figure 3A and 3B). No significant difference was observed between WT-chow and KO-chow mice, or between WT-chow and WT-HFD mice (Figure 3A and 3B).

A glucose tolerance test was performed following a 5-hour fast and AUC was measured. The KO-HFD mice showed a trend that was not significant towards a larger AUC and higher baseline blood glucose when compared to all other groups of mice. No difference was observed between WT-chow and KO-chow mice, or between WT-chow and WT-HFD mice (Figure 3C and Table 1).

To evaluate the effect of ANXA1 deficiency on insulin sensitivity, ITT was performed in fed mice. The test demonstrated that KO-HFD mice were significantly more insulin resistant as compared to WT-HFD mice as evaluated by AUC. Mice in the KO-chow group were also insulin resistant as they had significantly higher AUC when compared to both WT-chow and WT-HFD mice. No difference was observed between the WT-chow and WT-HFD mice. KO-HFD mice had a higher AUC as compared to KO-chow, but the difference was not significant (Figure 3D, Table 1).

These data show that ANXA1 KO mice on either diet develop insulin resistance, unlike the insulin-sensitive WT mice. A trend towards glucose intolerance in the KO-HFD group was observed when compared to other groups of mice. ANXA1 KO-chow mice were also slightly insulin resistant, supporting a role of ANXA1 in glucose control and metabolism. However, the group size was small in GTT and ITT, therefore, these experiments need to be repeated in order to expand the group size and further validate the findings.

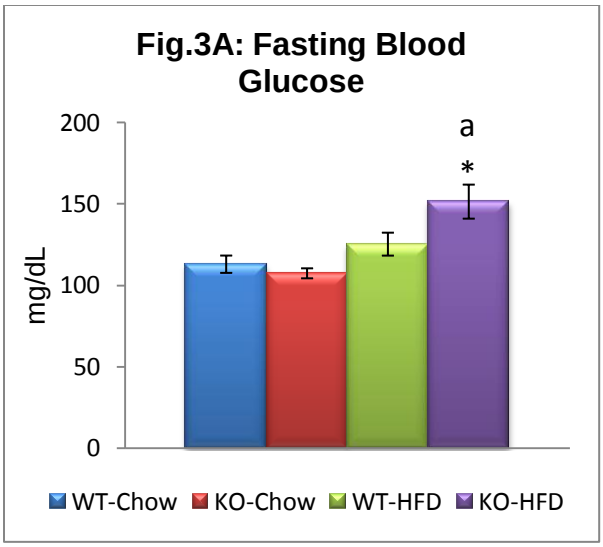


Fig.3A: Fasting Blood Glucose
200
150
100
50
0
mg/dL
a
*
WT-Chow
KO-Chow
WT-HFD
KO-HFD

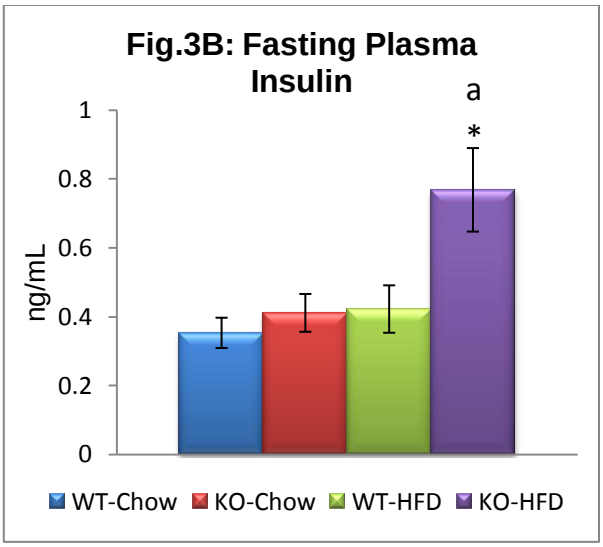


Fig.3B: Fasting Plasma Insulin
1
0.8
0.6
0.4
0.2
0
ng/mL
a
*
WT-Chow
KO-Chow
WT-HFD
KO-HFD

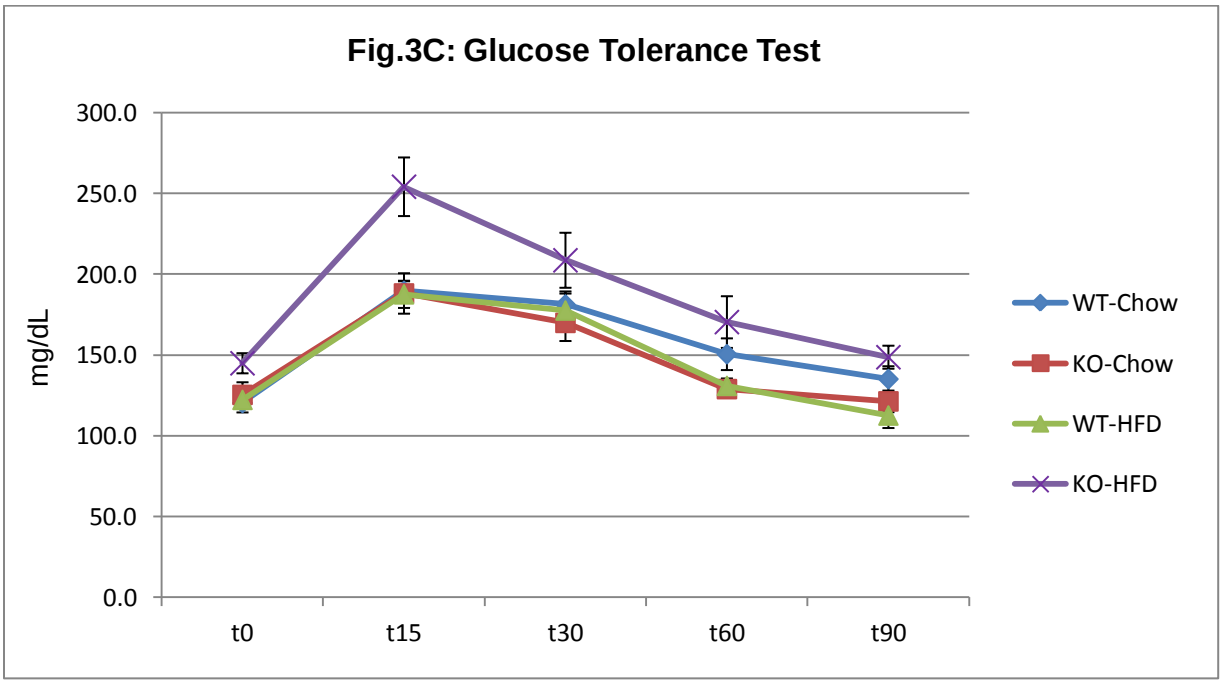


Fig.3C: Glucose Tolerance Test
300.0
250.0
200.0
150.0
100.0
50.0
0.0
mg/dL
t0
t15
t30
t60
t90
WT-Chow
KO-Chow
WT-HFD
KO-HFD

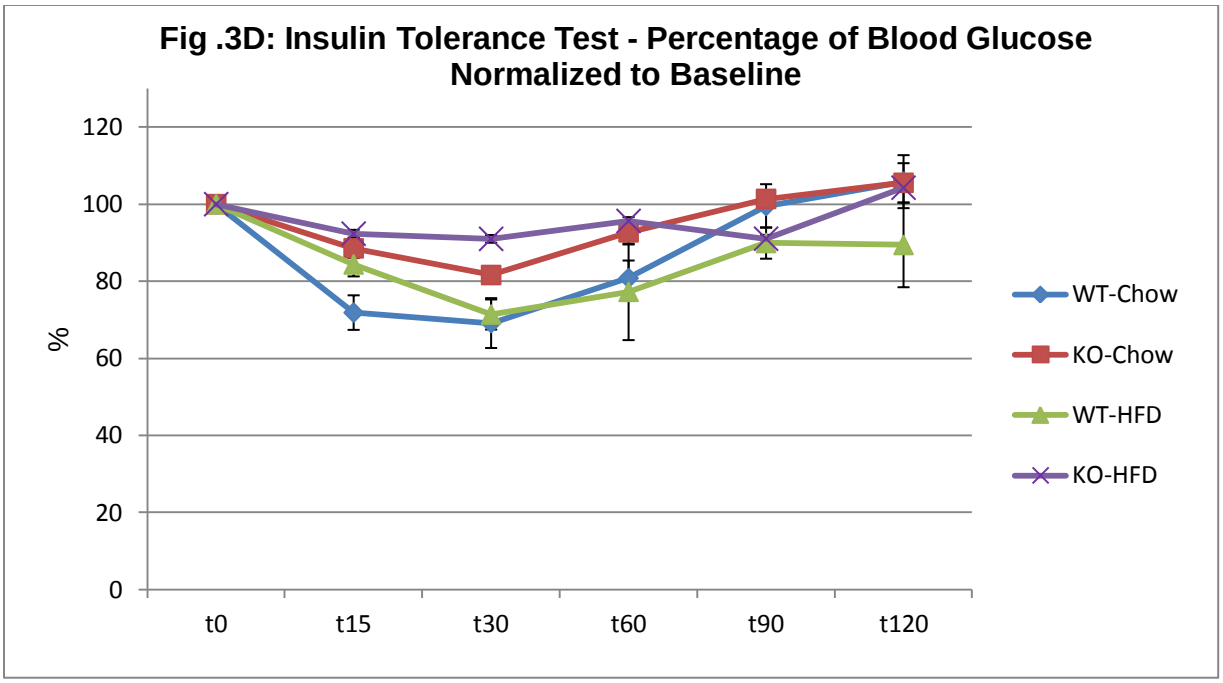


Figure 3: Measures of Glycemic Control. (A) Fasting Blood Glucose in mg/dL, (n = 9 - 16 per group). (B) Fasting plasma Insulin in ng/mL, (n = 9 - 15 per group). (C) Glucose tolerance test: changes in blood glucose following IP injections of glucose, (n = 3 - 5 per group). (D) Insulin tolerance test: changes in blood glucose expressed as percentage to baseline following IP injections of insulin, (n = 3 - 5 per group). Data represents mean ± SE per group. (*P<.05: HFD group vs strain-matched chow group, aP<.05: KO group vs diet-matched WT group).

Table 1. Area Under the Curve for GTT and ITT

	GTT - AUC	ITT - AUC
WT-chow	3541.5 ± 330.79	-2054.00 ± 138.46
KO-chow	1993.5 ± 455.68	-232.19 ± 222.85 *
WT-HFD	2340 ± 613.278	-2021.99 ± 407.12
KO-HFD	3894.38 ± 836.02	-917.29 ± 353.06 **

(*P<.05 KO-chow vs WT-chow and WT-HFD, **P<.05 KO-HFD vs WT-HFD).

D. A Trend Towards Elevated Plasma Corticosterone and 11βHSD1 Gene Expression in VAT in ANXA1 KO Mice.

Annexin A1 mediates the feedback inhibition of GC on ACTH release, an effect that was shown in vivo and in vitro (Loxley, Cowell, Flower, & Buckingham, 1993; Philip et al., 2001; Taylor, Cowell, Flower, & Buckingham, 1993; Taylor, Christian, Morris, Flower, & Buckingham, 1997). In order to explore the mechanisms leading to increased adiposity in ANXA1 KO mice, we hypothesized that ANXA1 KO mice would have elevated corticosterone levels due to lack of this negative feedback loop.

The enzyme 11βHSD1 regulates intracellular levels of corticosterone (Rhen & Cidlowski, 2005), and increased expression of this enzyme was found to promote adiposity (Masuzaki et al., 2001), therefore, we hypothesized that ANXA1 KO mice would have elevated 11βHSD1 gene expression.

Our data show a trend towards elevated corticosterone levels in the KO mice as compared to the WT mice in both diet groups; however, the difference was not statistically significant. A trend towards elevated corticosterone levels was also observed when comparing mice on HFD to mice on chow, but again no statistical significance was found (Figure 4A). Group size was small (n = 3 - 5), therefore these results will need to be confirmed.

Moreover, a trend towards elevated 11βHSD1 gene expression was observed in VAT of KO mice when compared to their diet-matched WT mice; however this was not significant. Additionally, HFD mice had a significant elevation in the 11βHSD1 expression as compared to strain-matched mice on chow (Figure 4B). These data also need to be replicated due to small group size (n = 4 - 5).

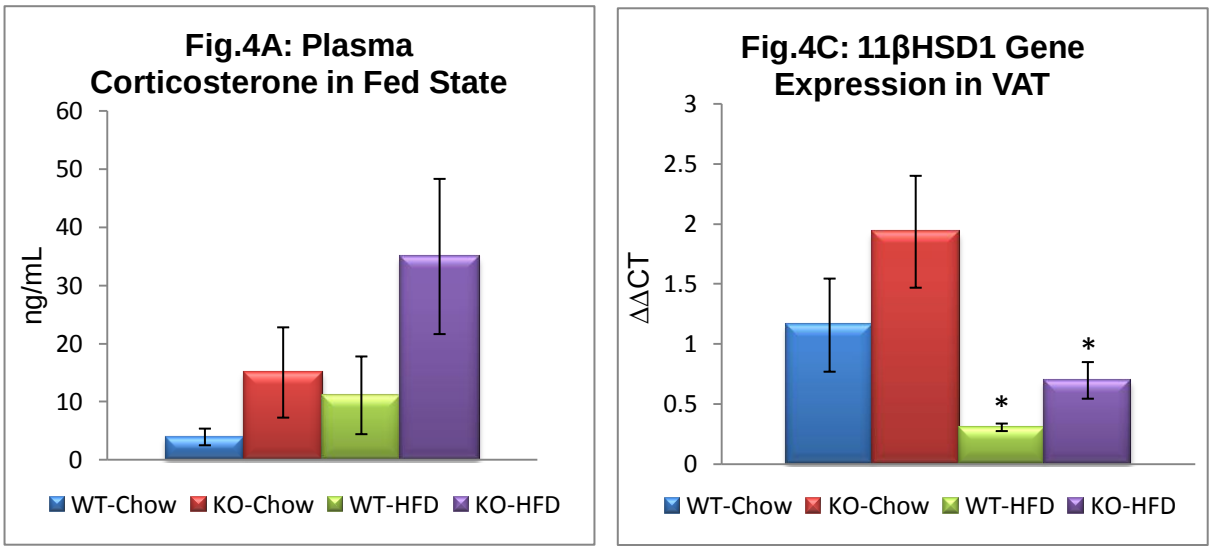


Figure 4: Plasma Corticosterone Levels and 11βHSD1 Gene Expression in VAT. (A) Plasma corticosterone levels in fed state (ng/mL), (n = 3 - 5). (B) Relative gene expression of 11βHSD1, normalized to β-Actin, (n = 4 - 5). Data represents mean ± SE per group. (*P<.05: HFD group vs strain-matched chow group, aP<.05: KO group vs diet-matched WT group).

E. No alterations in VAT inflammation in ANXA1 KO Mice.

Because ANXA1 is an anti-inflammatory protein, we hypothesized that ANXA1 KO mice would have increased inflammation in VAT as compared to the WT mice.

Gene expression levels of the pro-inflammatory markers CD68, IL-6, IL-1β, and MCP-1 were measured in VAT. The anti-inflammatory cytokine IL-10 was also measured. A significant elevation in gene expression of CD68 and MCP-1 was found in KO-HFD compared to KO-chow, and in MCP-1 expression of WT-HFD compared to WT-chow. No significant difference was observed in comparing KO mice to WT mice on either diet. However, a trend towards lower IL-10 expression was found in the KO-HFD mice when compared to the WT-HFD mice. A trend towards increased expression of IL-6 and IL-1β was also detected in the HFD mice versus the chow mice.

No difference in VAT inflammation was observed between ANXA1 KO and WT mice, which negates our hypothesis. Furthermore, unlike what we expected, VAT inflammation observed in HFD mice as compared those on chow was not significant. This could be explained by the resistance to DIO that female BALB/c mice are known to have.

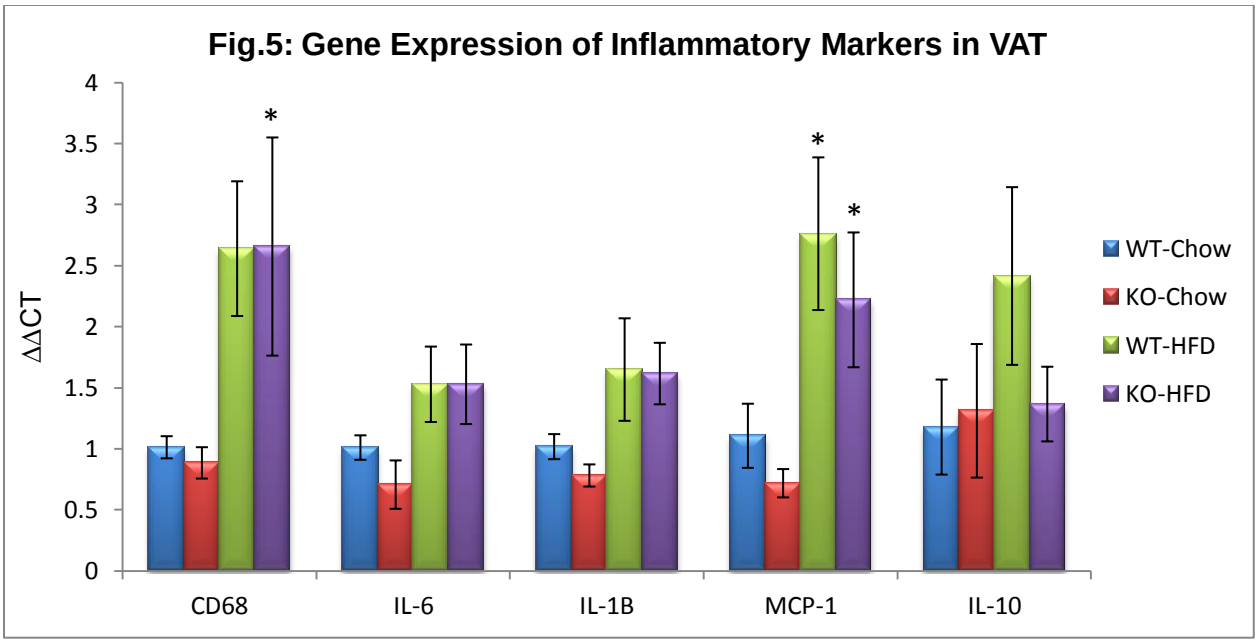


Figure 5: Markers of Inflammation in VAT. Relative gene expression of CD68 (n = 4 - 5), IL-6 (n = 3 - 5), IL-1β (n = 4 - 5), MCP-1 (n = 4 - 5), and IL-10 (n = 4 - 5) in VAT, normalized to GAPDH. Data represents mean ± SE per group (n). (*P<.05: HFD group vs strain-matched chow group, aP<.05: KO group vs diet-matched WT group).

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VI. DISCUSSION

The main objective of this study was to determine if ANXA1 plays a role in adiposity and inflammatory response in VAT. In vitro studies on epididymal fat of ANXA1 KO mice showed suppressed lipolysis in response to LPS or catecholamines when compared to WT mice (Warne et al., 2006), therefore we hypothesized that ANXA1 would have a lipolytic effect, i.e., ANXA1 KO mice would develop increased adiposity due to reduced lipolysis. Upon taking various measures of adiposity, our findings confirmed the hypothesis and data revealed a significant increase in adiposity in ANXA1 KO mice. However, these findings are in conflict with data reported by Warne and colleagues in 2006, showing decreased epididymal fat pad mass in male C57BL6 ANXA1 KO mice. Although these differences could be strain and sex specific, the size of one fat depot does not necessarily reflect the size of other depots and overall body fat mass. Therefore, a selective increase in the size of a single fat depot does not necessarily reflect increased total adiposity. The strength of our findings lies in the evaluation of total body fat composition by DXA, and inclusion of a large group size. Future studies aimed at directly determining strain- and/or sex-differences as well as the potential effect of ANXA1 in altering distribution of fat in various depots will help further clarify this apparent discrepancy.

We studied gene expression of leptin, metabolic enzymes (ATGL and HSL) and a transcription factor involved in lipid metabolism (PPAR-γ), however, no significant difference between the two strains was observed. In future studies, a larger sample size should be used. Additionally, ATGL and HSL should be measured in the fasting state, as these lipolytic hormones become more active with energy deprivation. Furthermore,

measuring the levels of phosphorylated HSL among ANXA1 KO and WT mice should be more useful in evaluating the activity of HSL and lipolysis.

Our data suggest that ANXA1 has a protective role against obesity in mice. Therefore, we hypothesized that ANXA1 and its putative receptor (FPR2) would be upregulated in response to HFD feeding in order to counteract weight gain in BALB/c mice. As expected, we found a significant elevation in ANXA1 expression in WT-HFD mice versus WT-chow mice, while no difference was observed in FPR2 expression among any of the four groups. These data are in contrast to the attenuated plasma ANXA1 levels that are observed in human obesity (Kosicka et al., 2012). However, plasma levels do not necessarily reflect gene expression, as some proteins are subject post-transcriptional modifications. This is especially true for ANXA1 which is highly regulated at the post-transcriptional and conformational level (John, et al. 2008). The human study did not evaluate ANXA1 gene expression in VAT, while we did not measure circulating levels of ANXA1. Moreover, whether ANXA1 gets sequestered inside the expanding adipose tissue in obesity, preventing its action is a question that remains to be answered. Therefore, it will be useful to evaluate both plasma and gene expression of ANXA1 in both species, in addition to evaluating the cellular source of ANXA1.

Obesity is known to induce insulin resistance (Haslam, 2007); therefore we hypothesized that the increased adiposity in ANXA1 KO mice would alter their glucose control. Our findings supported our hypothesis, as ANXA1 KO mice on HFD developed significant but mild fasting hyperglycemia and fasting hyper-insulinemia as compared to other groups. Keeping in mind the small group size used for GTT and ITT, KO mice on

both diets developed insulin resistance compared to their diet-matched groups. However, our findings contradict those in chow-fed male C57BL6 ANXA1 KO mice that developed significant hyper-insulinemia when compared to their diet-matched WT mice (Warne et al., 2006), as opposed to female BALB/c ANXA1 KO mice in our study who only showed a trend towards elevated plasma insulin. This discrepancy could be attributed to strain- and sex- specific differences. Whether the insulin resistance phenotype in ANXA1 KO mice resulted from increased adiposity or was an independent cause of ANXA1 deficiency still needs to be investigated. Moreover, insulin is an anabolic hormone that can inhibit lipolysis and promote lipogenesis and adiposity (Manganiello & Vaughan, 1973), which could explain the increased adiposity in KO mice, however, significant elevation of this hormone was only found in KO-HFD mice but not in KO-chow mice, suggesting that it was not the direct result of ANXA1 deficiency per se, but rather occurred secondary to insulin resistance and increased adiposity in KO mice. Measuring adiposity, insulin resistance, and plasma insulin levels throughout the life time of these mice can help in confirming the time sequence in which these metabolic events took place.

ANXA1 was found to mediate the acute feedback inhibition exerted by GC on corticotropin releasing hormone and ACTH secretion (Loxley, Cowell, Flower, & Buckingham, 1993; Philip et al., 2001; Taylor et al., 1993; Taylor et al., 1997). Whether the loss of the regulatory role of ANXA1 in KO mice leads to chronic elevations in corticosterone levels in mice is unknown. In attempt to explore the mechanisms through which ANXA1 deficiency leads to obesity in mice, we hypothesized that these mice would have elevated corticosterone levels, which could promote adiposity. We found a

non-significant trend of elevated plasma corticosterone in ANXA1 KO mice as compared to WT on both diets. Importantly, we cannot draw conclusions out of a single time point measurement of corticosterone, as this is subject to diurnal variations and psychological stress. Therefore, an alternate approach to use would be measuring fecal corticosterone as a more reliable method to test this hypothesis (Touma, Palme, & Sachser, 2004). Additionally, we should consider the small group size used in measuring corticosterone, which means that we have to replicate the data with a larger group size to confirm the results.

The enzyme 11βHSD1 is a regulator of intracellular levels of corticosterone. In a transgenic mouse model, over-expression of this enzyme led to increased adiposity, increased leptin, insulin resistance, and hyperlipidemia (Masuzaki et al., 2001). Furthermore, 11βHSD1 is down-regulated in A/J and C57BL6 mice in VAT and SAT in response to HFD feeding, suggesting a protective mechanism against DIO (Morton, Ramage, & Seckl, 2004). A selective inhibitor of 11βHSD1 prevents adipogenesis in a human cell line (Bujalska et al., 2008). We hypothesized that ANXA1 has an inhibitory action on this enzyme, implying that ANXA1 deficiency would lead to increased 11βHSD1 gene expression. In agreement with previous studies, we found 11βHSD1 to be significantly down-regulated in VAT of HFD-fed mice from both strains versus chow-fed mice from both strains. Furthermore, we found a trend towards elevated 11βHSD1 gene expression in ANXA1 mice when compared to their diet-matched groups, but it was not significant, possibly due to the small group size. It is also worth noting that evaluating the activity of this enzyme would be another useful measure for future studies.

Because ANXA1 is an anti-inflammatory protein, one could expect that knockouts of this gene would develop increased systemic inflammation. Surprisingly, no differences in markers of VAT inflammation were observed when comparing ANXA1 KO mice to WT mice. These results are also possibly affected by the small group size. Therefore, further measures need to be taken in order to validate our findings. Furthermore, we cannot rule out that inflammation might exist in other organs in these mice; i.e. liver. Therefore, further studies are needed in this regards.

Strengths of our study include: 1) using various measures in evaluating adiposity, including DXA; 2) data for ANXA1 KO mice on HFD are available for the first time in this study; 3) evaluation of glucose control and insulin resistance through various measures; in particular, GTT and ITT data for ANXA1 KO mice are available for the first time; 4) choosing the least obesity and insulin resistance-prone mice, female BALB/c, as a model to prove the significant effect of ANXA1 deficiency on adiposity and metabolism.

Limitations of our study include: 1) imprecise method for evaluation of food intake, particularly in HFD groups; 2) WT and ANXA1 KO mice were housed under the same conditions, but in separate rooms due to quarantine regulations, which might have entailed differences in the microflora that these mice were exposed to. This could lead to differences in adiposity as shown in previous studies (Tilg & Kaser, 2011); 3) WT and ANXA1 KO mice were not littermates, which could involve epigenetic differences among these mice that can also alter their metabolic profile. Therefore, crossbreeding of WT and ANXA1 KO is needed to rule out this possibility; 4) group size was small for some of the measurements; 5) we only used one reference gene to normalize each target gene.

VI. CONCLUSIONS

While female BALB/c mice are known to be the most resistant to DIO and the insulin resistance that comes with it, we show that deficiency of a single endogenous protein, ANXA1, significantly alters the metabolic profile of this strain towards augmented susceptibility to weight gain and insulin resistance. Thus, we conclude that ANXA1 is an important regulator of adiposity and glucose metabolism in mice, an effect that seems to be independent of VAT inflammation or diet.

Our findings suggest that ANXA1 has a protective role against obesity. Further studies are needed to confirm some of the findings and explore the mechanisms through which ANXA1 regulates adiposity. Moreover, whether ANXA1 is a mediator of the lipolytic effect of GC is a question yet to be answered.

CITED LITERATURE

Ahima, R.S. (2011). Digging Deeper into Obesity. *J Clin Invest. 121*(6),2076–2079. doi: 10.1172/JCI58719

Appel, B., & Fried, S. K. (1992). Effects of insulin and dexamethasone on lipoprotein lipase in human adipose tissue. *The American Journal of Physiology, 262*(5 Pt 1), E695-9.

Arner, P. (2002), Insulin resistance in type 2 diabetes: role of fatty acids. *Diabetes Metab*. Res. Rev., 18: S5–S9. doi: 10.1002/dmrr.254

Bagdade, J. D., Yee, E., Albers, J., & Pykalisto, O. J. (1976). Glucocorticoids and triglyceride transport: Effects on triglyceride secretion rates, lipoprotein lipase, and plasma lipoproteins in the rat. *Metabolism: Clinical and Experimental, 25*(5), 533-542.

Berdanier, C. D. (1989). Role of glucocorticoids in the regulation of lipogenesis. *The FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology, 3*(10), 2179-2183.

Blackwell, G. J., Carnuccio, R., Di Rosa, M., Flower, R. J., Parente, L., & Persico, P. (1980). Macrocortin: A polypeptide causing the anti-phospholipase effect of glucocorticoids. *Nature, 287*(5778), 147-149.

Bujalska, I.J., Gathercole, L.L., Tomlinson, J.W., Darimont, C., Ermolieff, J., Fanjul, A.N., Rejto, P.A., & Stewart, P.M**.** (2008). A novel selective 11β-hydroxysteroid dehydrogenase type 1 inhibitor prevents human adipogenesis. J Endocrinol, *197*(2):297–307.

Bujalska, I. J., Kumar, S., Hewison, M., & Stewart, P. M. (1999). Differentiation of adipose stromal cells: The roles of glucocorticoids and 11beta-hydroxysteroid dehydrogenase. *Endocrinology, 140*(7), 3188-3196.

Campbell, J. E., Peckett, A. J., D'souza, A. M., Hawke, T. J., & Riddell, M. C. (2011). Adipogenic and lipolytic effects of chronic glucocorticoid exposure. *American Journal of Physiology.Cell Physiology, 300*(1), C198-209. doi:10.1152/ajpcell.00045.2010

Chernick, S. S., & Chaikoff, I. L. (1950). Insulin and hepatic utilization of glucose for lipogenesis. *Journal of Biological Chemistry*, *186*, 535-542.

Christmas, P., Callaway, J., Fallon, J., Jones, J., & Haigler, H. T. (1991). Selective secretion of annexin 1, a protein without a signal sequence, by the human prostate gland. *The Journal of Biological Chemistry, 266*(4), 2499-2507.

Crumpton, M. J., & Dedman, J. R. (1990). Protein terminology tangle. *Nature, 345*(6272), 212. doi:10.1038/345212a0

D'Acquisto, F., Merghani, A., Lecona, E., Rosignoli, G., Raza, K., Buckley, C. D., & Perretti, M. (2007). Annexin-1 modulates T-cell activation and differentiation. *Blood, 109*(3), 1095-1102. doi:10.1182/blood-2006-05-022798

D'Acquisto, F., Paschalidis, N., Raza, K., Buckley, C. D., Flower, R. J., & Perretti, M. (2008). Glucocorticoid treatment inhibits annexin-1 expression in rheumatoid arthritis CD4+ T cells. *Rheumatology (Oxford, England), 47*(5), 636-639. doi:10.1093/rheumatology/ken062

Dallman, M. F., Akana, S. F., Bhatnagar, S., Bell, M. E., Choi, S., Chu, A., & Viau, V. (1999). Starvation: Early signals, sensors, and sequelae. *Endocrinology, 140*(9), 4015-4023.

Dallman, M. F., Pecoraro, N. C., & la Fleur, S. E. (2005). Chronic stress and comfort foods: Self-medication and abdominal obesity. *Brain, Behavior, and Immunity, 19*(4), 275-280. doi:10.1016/j.bbi.2004.11.004

Damazo, A. S., Yona, S., Flower, R. J., Perretti, M., & Oliani, S. M. (2006). Spatial and temporal profiles for anti-inflammatory gene expression in leukocytes during a resolving model of peritonitis. *Journal of Immunology (Baltimore, Md.: 1950), 176*(7), 4410-4418.

De Coupade, C., Gillet, R., Bennoun, M., Briand, P., Russo-Marie, F., & Solito, E. (2000). Annexin 1 expression and phosphorylation are upregulated during liver regeneration and transformation in antithrombin III SV40 T large antigen transgenic mice. *Hepatology (Baltimore, Md.), 31*(2), 371-380. doi:10.1002/hep.510310217

DeCaterina, R., & and Basta, G. (2001). n-3 fatty acids and the inflammatory response – biological background *European Heart Journal Supplements, 3*, D42-D49. doi:10.1016/S1520-765X(01)90118-X

De Heredia, F.P., Gómez-Martínez, S., Marcos, A. (2012). Obesity, inflammation and the immune system. *Proc Nutr Soc*, *71*(2):332-8.

Diaz, B. L., & Arm, J. P. (2003). Phospholipase A(2). *Prostaglandins, Leukotrienes, and Essential Fatty Acids, 69*(2-3), 87-97.

Di Rosa, M., Flower, R. J., Hirata, F., Parente, L., & Russo-Marie, F. (1984). Anti-phospholipase proteins. *Prostaglandins*, *28*(4), 441.

Epel, E., Lapidus, R., McEwen, B., & Brownell, K. (2001). Stress may add bite to appetite in women: A laboratory study of stress-induced cortisol and eating behavior. *Psychoneuroendocrinology, 26*(1), 37-49.

Errasfa, M., & Russo-Marie, F. (1989). A purified lipocortin shares the anti-inflammatory effect of glucocorticosteroids in vivo in mice. *British Journal of Pharmacology, 97*(4), 1051-1058.

Fain, J. N., Dodd, A., & Novak, L. (1971). Enzyme regulation in gluconeogenesis and lipogenesis. relationship of protein synthesis and cyclic AMP to lipolytic action of growth hormone and glucocorticoids. *Metabolism: Clinical and Experimental, 20*(2), 109-118.

Fain, J. N., & Saperstein, R. (1970). The involvement of RNA synthesis and cyclic AMP in the activation of fat cell lipolysis by growth hormone and glucocorticoids. *Hormone and Metabolic Research = Hormon- Und Stoffwechselforschung = Hormones Et Metabolisme, 2*, Suppl 2:20-7.

Fantuzzi, G. (2005). Adipose tissue, adipokines, and inflammation. *J Allergy Clin Immunol. 115*(5), 911–920. doi: 10.1016/j.jaci.2005.02.023

Ferreira, S. H., Cunha, F. Q., Lorenzetti, B. B., Michelin, M. A., Perretti, M., Flower, R. J., & Poole, S. (2009). Role of lipocortin-1 in the anti-hyperalgesic actions of dexamethasone. *British journal of pharmacology*, *121*(5), 883-888.

Flower, R. J., & Blackwell, G. J. (1979). Anti-inflammatory steroids induce biosynthesis of a phospholipase A2 inhibitor which prevents prostaglandin generation. *Nature, 278*, 456 – 459.

Fried, S. K., Bunkin, D. A., & Greenberg, A. S. (1998). Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: Depot difference and regulation by glucocorticoid. *The Journal of Clinical Endocrinology and Metabolism, 83*(3), 847-850.

Fried, S. K., Russell, C. D., Grauso, N. L., & Brolin, R. E. (1993). Lipoprotein lipase regulation by insulin and glucocorticoid in subcutaneous and omental adipose tissues of obese women and men. *The Journal of Clinical Investigation, 92*(5), 2191-2198. doi:10.1172/JCI116821

Froesch, E. R., Bürgi, H., Bally, P., & Labhart, A. (1965). Insulin inhibition of spontaneous adipose tissue lipolysis and effects upon fructose and glucose metabolism. *Molecular pharmacology*, *1*(3), 280-296.

Geisow, M. J. (1986). Common domain structure of Ca< sup> 2+</sup> and lipid-binding proteins. *FEBS letters*, *203*(1), 99-103.

Geisow, M. J., Walker, J. H., Boustead, C., & Taylor, W. (1987). Annexins—new family of Ca 2+-regulated-phospholipid binding protein. *Bioscience reports*,*7*(4), 289-298.

Goulding, N. J., Godolphin, J. L., Sharland, P. R., Peers, S. H., Sampson, M., Maddison, P. J., & Flower, R. J. (1990). Anti-inflammatory lipocortin 1 production by

peripheral blood leucocytes in response to hydrocortisone. *Lancet, 335*(8703), 1416-1418.

Hannon, R., Croxtall, J. D., Getting, S. J., Roviezzo, F., Yona, S., Paul-Clark, M. J., & Flower, R. J. (2003). Aberrant inflammation and resistance to glucocorticoids in annexin 1-/- mouse. *The FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology, 17*(2), 253-255. doi:10.1096/fj.02-0239fje

Haslam, D. (2007), Obesity: a medical history. *Obesity Reviews*, *8*, 31–36. doi: 10.1111/j.1467-789X.2007.00314.x

Hauner, H., Entenmann, G., Wabitsch, M., Gaillard, D., Ailhaud, G., Negrel, R., & Pfeiffer, E. F. (1989). Promoting effect of glucocorticoids on the differentiation of human adipocyte precursor cells cultured in a chemically defined medium. *The Journal of Clinical Investigation, 84*(5), 1663-1670. doi:10.1172/JCI114345

Hirata, F., del Carmine, R., Nelson, C. A., Axelrod, J., Schiffmann, E., Warabi, A., and Steinberg, A. D. (1981). Presence of autoantibody for phospholipase inhibitory protein, lipomodulin, in patients with rheumatic diseases. *Proc. Natl. Acad. Sci. U. S. A., 78*(5), 3190-3194.

Hong, S.H., Won, J.H., Yoo, S.A., Auh, C.K., Park, Y.M. (2002). Effect of annexin I on insulin secretion through surface binding sites in rat pancreatic islets. *FEBS* Lett. *532*, 17-20.

Horlick, K. R., Cheng, I. C., Wong, W. T., Wakeland, E. K., & Nick, H. S. (1991). Mouse lipocortin I gene structure and chromosomal assignment: gene duplication and the origins of a gene family. *Genomics*, *10*(2), 365-374.

Jéquier, E. (2002), Leptin Signaling, Adiposity, and Energy Balance. *Annals of the New York Academy of Sciences*, *967*, 379–388. doi: 10.1111/j.1749-6632.2002.tb04293.x

Jo, J., Gavrilova, O., Pack. S., Jou, W., Mullen, S., Sumner, A.E., Cushman, S.W., Periwal, V. (2009). Hypertrophy and/or Hyperplasia: Dynamics of Adipose Tissue Growth.PLoS Comput Biol, *5*(3), e1000324.doi:10.1371/journal.pcbi.1000324

John, C. D., Christian, H. C., Morris, J. F., Flower, R. J., Solito, E., & Buckingham, J. C. (2004). Annexin 1 and the regulation of endocrine function.*Trends in Endocrinology & Metabolism*, *15*(3), 103-109.

John, C.D., Gavins, F.N., Buss, N.A., Cover, P.O., Buckingham, J.C. (2008). Annexin A1 and the formyl peptide receptor family: neuroendocrine and metabolic aspects. *Curr Opin Pharmacol*. *8*(6):765–776.

Kershaw, E.E., Flier, J.S. (2004). Adipose tissue as an endocrine organ. *J Clin Endocrinol Metab*. *89*, 2548–2556

Kim, K. M., Kim, D. K., Park, Y. M., Kim, C. K., & and Na, D. S. (1994). Annexin-I inhibits phospholipase A2 by specific interaction,not by substrate depletion. *Federation of European Biochemical Societies, 343*, 251–255.

Kosicka, A., Cunliffe, A. D., Mackenzie, R., Zariwala, M. G., Perretti, M., Flower, R. J., & Renshaw, D. (2012). Attenuation of plasma annexin A1 in human obesity. *The FASEB Journal*, 27, 000-000.

Lampidonis, A.D**.**, Rogdakis, E**.**, Voutsinas, G.E., Stravopodis, D.J. (2011). The resurgence of Hormone-Sensitive Lipase (HSL) in mammalian lipolysis. *Gene, 477*(1-2), 1-11.

Lim, L. H., & Pervaiz, S. (2007). Annexin 1: The new face of an old molecule. *The FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology, 21*(4), 968-975. doi:10.1096/fj.06-7464rev

Lindgren, C. M., Nilsson, A., Orho-Melander, M., Almgren, P., & Groop, L. C. (2001). Characterization of the annexin I gene and evaluation of its role in type 2 diabetes. *Diabetes*, *50*(10), 2402-2405.

Loxley, H.D., Cowell, A.M., Flower, R.J. & Buckingham, J.C. (1993). Effects of lipocortin 1 and dexamethasone on the secretion of corticotrophin releasing factors in the rat: in vitro and in vivo studies. *J. Neuroendocrinology*, *5*(1):51-61.

Manganiello, V., & Vaughan, M. (1973). An effect of Insulin on cyclic adenosine 3’:5’-monophosphate phosphodiesterase activity in fat cells. *Journal of Biological Chemistry, 248*(20), 7164-7170.

Masuzaki, H., Paterson, J., Shinyama, H., Morton, N. M., Mullins, J. J., Seckl, J. R., & Flier, J. S. (2001). A transgenic model of visceral obesity and the metabolic syndrome. *Science*, *294*(5549), 2166-2170.

Migeotte, I., Communi, D., & Parmentier, M. (2006). Formyl peptide receptors: A promiscuous subfamily of G protein-coupled receptors controlling immune responses. *Cytokine & Growth Factor Reviews, 17*(6), 501-519. doi:10.1016/j.cytogfr.2006.09.009

Morton, N. M., Ramage, L., & Seckl, J. R. (2004). Down-regulation of adipose 11β-hydroxysteroid dehydrogenase type 1 by high-fat feeding in mice: a potential adaptive mechanism counteracting metabolic disease. *Endocrinology*,*145*(6), 2707-2712.

Nishikawa, S., Yasoshima, A., Doi, K., Nakayama, H., and Uetsuka, K. (2007). Involvement of sex, strain and age factors in high fat diet-induced obesity in C57BL/6J and BALB/cA mice. *Exp Anim. 56*(4), 263-72.

Ohnishi, M., Tokuda, M., Masaki. T., Fujimura, T., Tai, Y., Itano, T., Matsui, H., Ishida, T., Konishi, R., Takahara, J. et al. (1995). Involvement of annexin-I in glucose-induced insulin secretion in rat pancreatic islets. *Endocrinology*. *136*(6), 2421-2426.

Oliver, J. D., & Rogers, M. P. (1993). Stimulation of lipoprotein lipase synthesis by refeeding, insulin and dexamethasone. *The Biochemical Journal, 292*(Pt 2)(Pt 2), 525-530.

Ottosson, M., Vikman-Adolfsson, K., Enerback, S., Olivecrona, G., & Bjorntorp, P. (1994). The effects of cortisol on the regulation of lipoprotein lipase activity in human adipose tissue. *The Journal of Clinical Endocrinology and Metabolism, 79*(3), 820-825.

Pantoja, C., Huff, J. T., & Yamamoto, K. R. (2008). Glucocorticoid signaling defines a novel commitment state during adipogenesis in vitro. *Molecular Biology of the Cell, 19*(10), 4032-4041. doi:10.1091/mbc.E08-04-0420

Parente, L., & Solito, E. (2004). Annexin 1: More than an anti-phospholipase protein. *Inflammation Research : Official Journal of the European Histamine Research Society ...[Et Al.], 53*(4), 125-132. doi:10.1007/s00011-003-1235-z

Peckett, A. J., Wright, D. C., & Riddell, M. C. (2011). The effects of glucocorticoids on adipose tissue lipid metabolism. *Metabolism: Clinical and Experimental, 60*(11), 1500-1510. doi:10.1016/j.metabol.2011.06.012

Perretti, M., Croxtall, J. D., Wheller, S. K., Goulding, N. J., Hannon, R., & Flower, R. J. (1996). Mobilizing lipocortin 1 in adherent human leukocytes downregulates their transmigration. *Nature Medicine, 2*(11), 1259-1262.

Perretti, M., & D'Acquisto, F. (2009). Annexin A1 and glucocorticoids as effectors of the resolution of inflammation. *Nature Reviews.Immunology, 9*(1), 62-70. doi:10.1038/nri2470

Perretti, M., & Dalli, J. (2009). Exploiting the annexin A1 pathway for the development of novel anti-inflammatory therapeutics. *British Journal of Pharmacology, 158*(4), 936-946. doi:10.1111/j.1476-5381.2009.00483.x

Perretti, M., & Flower, R. J. (2004). Annexin 1 and the biology of the neutrophil. *Journal of Leukocyte Biology, 76*(1), 25-29. doi:10.1189/jlb.1103552

Perretti, M., & Solito, E. (2004). Annexin 1 and neutrophil apoptosis. *Biochemical Society Transactions*, *32*, 507-510.

Philip, J.G, John, C.D, Cover, P.O, Morris, J.F, Christian, H.C, Flower, R.J, Buckingham, J.C. (2001). Opposing influences of glucocorticoids and interleukin-1 beta on the secretion of growth hormone and ACTH in the rat in vivo: role of hypothalamic annexin 1. *British Journal of Pharmacology*, *134*(4), 887–895. doi: 10.1038/sj.bjp.0704324

Porte Jr, D. (1999). Mechanisms for Hyperglycemia in the Metabolic Syndrome: The Key Role of β-Cell Dysfunction. *Annals of the New York Academy of Sciences*, *892*(1), 73-83.

Raynal, P., & Pollard, H. B. (1994). Annexins: The problem of assessing the biological role for a gene family of multifunctional calcium- and phospholipid-binding proteins. *Biochimica Et Biophysica Acta, 1197*(1), 63-93.

Rebuffe-Scrive, M., Krotkiewski, M., Elfverson, J., & Bjorntorp, P. (1988). Muscle and adipose tissue morphology and metabolism in cushing's syndrome. *The Journal of Clinical Endocrinology and Metabolism, 67*(6), 1122-1128.

Rebuffe-Scrive, M., Walsh, U. A., McEwen, B., & Rodin, J. (1992). Effect of chronic stress and exogenous glucocorticoids on regional fat distribution and metabolism. *Physiology & Behavior, 52*(3), 583-590.

Rhen, T., & Cidlowski, J. A. (2005). Antiinflammatory action of glucocorticoids--new mechanisms for old drugs. *The New England Journal of Medicine, 353*(16), 1711-1723. doi:10.1056/NEJMra050541

Roger, V.L., Go, A.S., Lloyd-Jones, D.M., Benjamin, E.J., Berry, J.D., Borden, W.B., Bravata, D.M., Dai, S., Ford, E.S., Fox, C.S., Fullerton, H.J., Gillespie, C., Hailpern, S.M., Heit, J.A., Howard VJ, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth LD, Makuc DM, Marcus, G.M., Marelli, A., Matchar, D.B., Moy, C.S., Mozaffarian, D., Mussolino, M.E., Nichol, G., Paynter, N.P., Soliman, E.Z., Sorlie, P.D., Sotoodehnia, N., Turan, T.N., Virani, S.S., Wong, N.D., Woo, D., & Turner, M.B.; on behalf of the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Heart disease and stroke statistics—2012 update: a report from the American Heart Association. *Circulation*. 2012: published online before print December 15, 2011, 10.1161/CIR.0b013e31823ac046.

Roitt, I., Brostoff, J., Male, D. (2001). *Immunology*. (6th edition). St Louis: Mosby.

Rothhut, B., Russo-Marie, F., Wood, J., DiRosa, M., & and Flower, R. J. (1983). Further characterization of the glucocorticoid induced antiphospholipase protein “renocortin”. *Biochem. Biophys. Res. Commun., 117*, 878–884.

Sawmynaden, P., & Perretti, M. (2006). Glucocorticoid upregulation of the annexin-A1 receptor in leukocytes. *Biochemical and Biophysical Research Communications, 349*(4), 1351-1355. doi:10.1016/j.bbrc.2006.08.179

Schacke, H., Docke, W. D., & Asadullah, K. (2002). Mechanisms involved in the side effects of glucocorticoids. *Pharmacology & Therapeutics, 96*(1), 23-43.

Slavin, B. G., Ong, J. M., & Kern, P. A. (1994). Hormonal regulation of hormone-sensitive lipase activity and mRNA levels in isolated rat adipocytes. *Journal of Lipid Research, 35*(9), 1535-1541.

Solito, E., McArthur, S., Christian, H., Gavins, F., Buckingham, J. C., & Gillies, G. E. (2008). Annexin A1 in the brain–undiscovered roles?. *Trends in pharmacological sciences*, *29*(3), 135-142.

Stanh, C., & Buttgereit, F. (2008). Genomic and nongenomic effects of glucocorticoids. Nat Clin Pract Rheumatol., *4*(10), 525-33. doi:10.1038/ncprheum0898

Strack, A. M., Sebastian, R. J., Schwartz, M. W., & Dallman, M. F. (1995). Glucocorticoids and insulin: Reciprocal signals for energy balance. *The American Journal of Physiology, 268*(1 Pt 2), R142-9.

Taylor, A.D., Cowell, A-M., Flower, R.J. & Buckingham, J.C. (1993). Lipocortin 1 mediates an early inhibitory action of glucocorticoids on the secretion of ACTH by the rat anteriorpituitary gland in vitro. Neuroendocrinology, *58*, 430 - 439.

Taylor, A.D, Christian, H.C., Morris, J.F., Flower, R.J., & Buckingham, J.C. (1997). An antisense oligodeoxynucleotide to lipocortin 1 reverses the inhibitory actions of dexamethasone on the release of ACTH from rat pituitary tissue in vitro. *Endocrinology*, *138*, 2909-2918.

Tilg, H., & Kaser, A. (2011). Gut microbiome, obesity, and metabolic dysfunction. *The Journal of clinical investigation*, *121*(6), 2126.

Touma, C., Palme, R., & Sachser, N. (2004). Analyzing corticosterone metabolites in fecal samples of mice: a noninvasive technique to monitor stress hormones. *Hormones and Behavior*, *45*(1), 10-22.

Van Jaarsveld, C. H., Fidler, J. A., Steptoe, A., Boniface, D., & Wardle, J. (2009). Perceived stress and weight gain in adolescence: A longitudinal analysis. *Obesity (Silver Spring, Md.), 17*(12), 2155-2161. doi:10.1038/oby.2009.183

Wang, Y., Jones Voy, B., Urs, S., Kim, S., Soltani-Bejnood, M., Quigley, N., Moustaid-Moussa, N. (2004). The human fatty acid synthase gene and de novo lipogenesis are coordinately regulated in human adipose tissue. *The Journal of Nutrition, 134*(5), 1032-1038.

Ward, P.A. (2010). Acute and Chronic Inflammation. In Serhan, C.N., Ward, P.A., & Gilroy, D.W. (Eds.), *Fundamentals of inflammation* (pp. 1-16). Cambridge: Cambridge University Press.

Warne, J.P., John, C.D., Christian, H.C., Morris, J.F., Flower, R.J., Sugden, D., Solito, E., Gillies, G.E., Buckingham, J.C. (2006). Gene deletion reveals roles for annexin A1 in the regulation of lipolysis and IL-6 release in epididymal adipose tissue. *Am. J. Physiol. Endocrinol. Metab*. *291*(6), E1264–E1273.

Wong, W.T., Nick, H.S., Frost, S.C. (1992). Regulation of annexin I in adipogenesis: cAMP-independent action of methylisobutylxanthine. *Am J Physiol*. *262*,C91–C97

Xu, C., He, J., Jiang, H., Zu, L., Zhai, W., Pu, S., & Xu, G. (2009). Direct effect of glucocorticoids on lipolysis in adipocytes. *Molecular Endocrinology (Baltimore, Md.), 23*(8), 1161-1170. doi:10.1210/me.2008-0464

Zechner, R., Zimmermann, R., Eichmann, T. O., Kohlwein, S. D., Haemmerle, G., Lass, A., & Madeo, F. (2012). FAT SIGNALS-Lipases and lipolysis in lipid metabolism and signaling. *Cell Metabolism*, *15*(3), 279-291.

VITA

NAME:	Rand T. Akasheh
EDUCATION:	B.S., Nutrition and Food Technology, Jordan University of Science and Technology, Irbid, Jordan, 2009.
TEACHING:	Jordan University of Science and Technology, Irbid, Jordan, EXPERIENCE: organized and instructed a workshop titled “Plan Your Own Diet”.
HONORS:	Chicago Consular Corps Scholarship, University of Illinois at Chicago, Chicago, Illinois, 2011
PROFESSIONAL MEMBERSHIP:	Dietetics Practice Licensure, Department of Health, Jordan American Dietetic Association Chicago Dietetic Association
ABSTRACTS:	Akasheh, R., Pini, M., Rhodes, D. H., Castellanos, K. J., Kerr, C., Cervone, C., Perretti, M., Cooper, D., Flower, R. J., Fantuzzi, G.: Annexin A1 regulates adiposity and glucose metabolism in mice. Obesity Meeting, 2012.

I have been in contact with other students sponsored by AUM who told they receive their money transfers regularly.

I am actually very stressed out. As a student living "bil ghorbeh", I am supposed to invest my time in studying rather than staying up until 2:30 am demanding my legal rights from AUM. I believe that me and my contract deserve more commitment and respect from AUM. Otherwise and legally, I do not have to show any commitment.